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Genomic Analysis of Signaling by Vitamin D3 and its Analogs in Head and Neck SCC25

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Preface

The following excerpt is taken from the guidelines concerning thesis preparation, faculty of graduate studies and research, McGill University, and applies to this thesis.

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must be bound as an integral part of the thesis.

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Abstract

The hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) is well known for its role in regulating calcium homeostasis. However, many studies have shown $1,25(OH)_2D_3$ and its analogue EB1089 have broad potential as anticancer agents due to their antiproliferative and pro-differentiating activities. Here, we examined the effects of $1,25(OH)_2D_3$ and EB1089 on proliferation and target gene regulation of human head and neck squamous cell carcinoma (SCC) lines SCC4, SCC9, SCC15 and SCC25 and mouse AT84 SCC cells. A range of sensitivities to $1,25(OH)_2D_3$ and EB1089 was observed, from complete G0/G1 arrest of SCC25 cells to only 50% inhibition of SCC9 cell growth. We have analyzed the molecular mechanisms underlying the antiproliferative effects of EB1089 on SCC25 cells by screening over 10,000 genes on cDNA and oligonucleotide arrays. These studies have identified ~200 novel target genes of $1,25(OH)_2D_3$/EB1089, many of which are essential for normal DNA repair, are markers of cellular differentiation, or are key cell cycle regulators. One of these is the growth arrest and DNA damage (GADD45α) gene. Induction of the GADD45α gene and its encoded protein in EB1089-treated cells was confirmed by northern and western blotting, respectively. Moreover, while expression of proliferating cell nuclear antigen (PCNA) was reduced in EB1089-treated cells, coimmunoprecipitation studies revealed increased association between GADD45α and PCNA in treated cells, consistent with the capacity of GADD45α to stimulate DNA repair. Our data also show that amphiregulin, a member of the epidermal growth factor family, is a $1,25(OH)_2D_3$ target gene, and suggest that its induction may contribute to the growth inhibitory effects of $1,25(OH)_2D_3$. In addition, we show that $1,25(OH)_2D_3$ analogues induce expression of the cyclin-dependent

kinase inhibitor $p27^{KIP1}$ in different cell types by inhibiting expression of subunits of the SCF^{SKP2} ubiquitin ligase that target $p27^{KIP1}$ for proteasomal degradation, and thus reducing turnover of $p27^{KIP1}$ protein.

These studies 1) suggest that differences in action of the EB1089 and $1,25(OH)_2D_3$ arise more from their relative sensitivities to metabolism and than from differing effects on VDR function, 2) substantially expand the number of known D3 target genes in cancer cells 3) enhance our understanding of the molecular mechanism controlling the antiproliferative effects of $1,25(OH)_2D_3$ analogs in cancer 4) provide a number of marker genes that will be useful in assessing the sensitivity of tumor samples to the antiproliferative effects of $1,25(OH)_2D_3$.

Résumé

La 1,25-dihydroxyvitamine D3 ($1,25(OH)_2D_3$), forme hormonale de la vitamine D_3, est connue pour son rôle dans la régulation de l'homéostasie du calcium. Plusieurs études ont cependant montré que la $1,25(OH)_2D_3$ et son analogue EB1089 ont un grand potentiel en tant qu'agents anticancéreux, lequel est attribuable à leur action inhibitrice sur la prolifération et activatrice sur la différenciation cellulaire. Dans le cadre de nos études, les effets de $1,25(OH)_2D_3$ et d'EB1089 sur la régulation de différents gènes de prolifération et de gènes ciblés ont été examinés dans les lignées tumorales « human head and neck squamous cell carcinoma (SCC) » SCC4, SCC9, SCC15 et SCC25 et dans la lignée cellulaire de souris AT84 SCC. D'importantes différences de sensibilité à la $1,25(OH)_2D_3$ et la EB1089 ont été notées, passant de l'arrêt complet en G0/G1 des cellules SCC25 à l'inhibition de seulement 50% de la croissance des cellules SCC9. Nous avons de plus analysé les mécanismes moléculaires associés aux effets anti-prolifératifs d'EB1089 sur les cellules SCC25 en examinant plus de 10 000 gènes à l'aide de « DNA microarrays » et de « DNA CHIPs ». Ces études ont permis d'identifier environ 200 gènes ciblés par la $1,25(OH)_2D_3$/EB1089, parmi lesquels un fort nombre sont impliqués dans la régulation du cycle cellulaire, la réparation normale d'ADN ou sont identifiés comme marqueurs de la différentiation cellulaire. Un des gènes identifiés ayant fait l'objet d'études plus approfondies est le « growth arrest and DNA damage 45 α » (GADD45α). L'activation de la transcription de l'ARNm codant pour ce gène et l'augmentation de la synthèse de la protéine résultante suite au traitement à la EB1089 ont été confirmés par immunobuvardage de type « Northern » et « Western », respectivement. Par ailleurs, même si l'expression de la proteine « proliferating cell

nuclear antigen » (PCNA) est diminuée dans les cellules traitées à la EB1089, les études de coimmunoprécipitation indiquent une augmentation de l'association entre GADD45α et PCNA suite au traitement, ce qui est en accord avec la capacité de GADD45α à stimuler la réparation d'ADN. Nos données montrent également que l'amphiréguline, un membre de la famille des facteurs de croissance épidermaux, est un gène ciblé par la $1,25(OH)_2D_3$ et suggèrent que son induction peut contribuer aux effets inhibiteurs sur la croissance cellulaire de la $1,25(OH)_2D_3$. Nos résultats suggèrent également que les analogues de $1,25(OH)_2D_3$ augmentent l'expression de la protéine inhibitrice des kinases cyclines-dépendantes $p27^{KIP1}$ dans différents types cellulaires et ce, par réduction du « turnover », en empêchant l'expression des sous-unités de l'ubiquitine ligase SCF^{SKP2}, lesquelles lient $p27^{KIP1}$ et la dirigent normalement vers le protéasome.

Dans l'ensemble, ces études suggèrent que les différences dans l'action de la $1,25(OH)_2\ D_3$ et de EB1089 résultent plutôt de leur différence de sensibilité au métabolisme et que des effets différents sur la fonction des récepteurs à la $1,25(OH)_2D_3$ 2) augmentent sensiblement le nombre de gènes cibles connus de $1,25(OH)_2D_3$ dans les cellules cancéreuses, 3) améliorent notre compréhension du mécanisme moléculaire contrôlant les effets anti-prolifératifs d'analogues de $1,25(OH)_2D_3$ dans le cancer 4) et fournissent un nombre de gènes marqueurs utiles dans l'évaluation de la sensibilité aux effets anti-prolifératifs de la $1,25(OH)_2D_3$ dans les tumeurs.

Contribution to Publications

The work described in Chapter 2, Chapter 3, Chapter 4 and Chapter 5 of this thesis has been published in the following journals:

Chapter 2 Akutsu, N.*, **Lin, R.*,** Bastien, Y., Bestawros, A., Enepekides, D.J., Black, M.J. and White, J.H. (2001) Regulation of Gene Expression by 1α,25-dihydroxyvitamin D_3 and its Analog EB1089 under Growth Inhibitory Conditions in Squamous Carcinoma Cells. *Mol. Endocrinol.* 15, 1127-39. ***Co-first authors**

Chapter 3 Lin, R., Nagai, Y., Sladek, R., Bastien, Y., Ho, J., Petrecca, K., Sotiropoulou, G., Diamandis, E.P., Hudson, T., and White J.H. (2002) Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 signaling on cell proliferation, differentiation and immune system regulation. *Mol. Endocrinol.* 16, 1243-56.

Chapter 4: Akutsu, N., Bastien, Y. **Lin, R.,** Mader, S. and White, J.H. (2001) Amphiregulin is a vitamin D target gene in squamous cell and breast carcinoma. *Biochem. Biophys. Res. Comm.* 281, 1051-56.

Chapter 5: Lin, R.*, Wang, T.T.*, and White, J.H. (2003) Inhibition of F-box protein $p45^{SKP2}$ expression and stabilization of cyclin-dependent kinase inhibitor $p27^{KIP1}$ in vitamin D analogue-treated cancer cells. *Endocrinology 144: 749-753.* ***Co-first authors**

The work presented in chapter 2, 3, 4 and 5 is my own except for the contribution of my co-authors as follow:

Chapter 2:

- Naotake Akutsu performed the initial cDNA microarray from CLONTECH. He also helped with the assessment of the VDR function in SCC lines as well as the transfections.

- Yolande Bastien performed the flow cytometric Analysis and the TUNEL Assay. She also helped with the proliferation of SCC lines in culture.
- A. Bestawros helped grow the SCC25 cells in culture.

Chapter 3:

- Yoshihiko Nagai helped with the clustering analysis and Data analysis
- Robert Sladek performed the screening of the Affymetrix HuGene FL human gene oligonucleotide microarrays
- Joanne Ho helped with the Immunofluorescence analysis in Figure 3.4
- K. Petrecca performed the montage of figure 4
- G. Sotiropoulou and E.P. Diamandis provided the primary antibodies for protease M and cystatin M in Figure 3.4

Chapter 4:

- Naotake Akutsu performed the growth curves in figure 4.1 and performed the experiments in figure 4.2 and performed the timepoint experiment in figure 4.3A.
- Yolande Bastien helped with the growth of the SCC25 in culture and growth curves.

Chapter 5:

- Tian-Tian Wang helped and performed with the Western blots in figure 5.2 and figure 5.3.

Abbreviations

AF	Activation Function
AR	Androgen Receptor
ARC	acitvator-recruited cofactor
BSA	Bovine Serum Albumin
CBP	CREB Binding Protein
CDK	Cyclin Dependent Kinases
CDKI	Cyclin Dependent Kinase Inhibitors
CHX	Cycloheximide
CREB	cAMP response element binding protein
DBD	DNA Binding Domain
DMEM	Dulbecco's Modified Eagle's Minimal Essential Medium
DRIP	Vitamin D receptor-interacting protein
ECaC	Calcium Channel
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ERE	Estrogen Response Element
GADD45	Growth and Arrest DNA Damage 45
GR	Glucocorticoid Receptor
HDAC	Histone Deacetylase
HNSCC	Head and Neck Squamous Cell Carcinoma
IGF	Insulin Growth Factor
IL	Interleukin
kDa	KiloDaltons
LBD	Ligand Binding Domain
NcoR	Nuclear Receptor Corepressor
MAP	Mitogen-Activated protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

pRb	Phosphorylated-Retinoblastoma
PKA	Protein Kinase A
PPAR	Peroxisome Proliferator Activator Receptor
PR	Progesterone Receptor
PTH	Parathyroid Hormone
PTHr	Parathyroid Hormone Receptor
Rb	Retinoblastoma
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
RXR	Retinoid X Receptor
SCC	Squamous Cell Carcinoma
SDS-PAGE	Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis
SMRT	Silencing Mediator of Retinoid and Thyroid Hormone Receptors
SPC	Second Primary Carcinoma
SRC-1	Steroid Receptor coactivator-1
TAF	TATA-binding protein-associated factors
TGF	Transforming Growth Factor
Th1	T-helper cell 1
Th2	T-helper cell 2
TIF-2	Tanscriptional Intermediary factor -2
TR	Thyroid Receptor
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element

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Chapter 4: Amphiregulin is a Vitamin D3 target gene in Squamous Cell and Breast Carcinoma.

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CHAPTER 1

General Introduction

1 Vitamin D_3

1.1 Brief History of Vitamin D_3

Vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is not considered a true vitamin because it can be synthesized in the skin of most vertebrates exposed to UVB light of wavelength 290-315nm. However, in the absence of UV exposure, $1,25(OH)_2D_3$ must be provided in a continuous supply as part of the diet. Deficiency of $1,25(OH)_2D_3$ metabolites in the body results in the failure to control calcium homeostasis, causing osteoporosis, osteomalacia and rickets.

Rickets, a $1,25(OH)_2D_3$-deficient disease of, appears to have been a problem in ancient times. Dr. Daniel Whistler wrote the first scientific descriptions of rickets in 1645 and by Professor Francis Glisson in 1650. During the Industrial Revolution, rickets became a health problem in northern Europe, England and the United States when many people lived in urban areas with air pollution and little sunlight. Rickets is characterized by softened bones (leading to deformity) as well as muscle spasms and seizures. Throughout the nineteenth century, sporadic reports of cures for rickets surfaced, but with little effect.

In the 1890s, scientists began to search for specific foods that could prevent rickets because, at the time, scurvy and beriberi could be prevented by having certain foods (such as citrus fruits, which contain vitamin C, and whole grain rice, which contains vitamin B_1) to the diet. In 1919, Sir Edward Mellanby, working with dogs, raised exclusively indoors (in the absence of sunlight), devised a diet that allowed him to establish that the bone disease, rickets was caused by a deficiency of a trace component

present in the diet. He further showed that rickets could be prevented by the addition of cod liver oil to the feed. He postulated that the nutritional factor preventing rickets was vitamin A since cod liver oil was known to contain vitamin A.

Shortly thereafter E.V. McCollum and associates observed that by bubbling oxygen through a preparation of the "fat-soluble vitamin" they were able to distinguish between vitamin A (which was inactivated) and $1\alpha,25(OH)_2D_3$ (which retained activity). In 1923, Goldblatt and Soames discovered that food that was irradiated and fed to rats could cure rickets; food that was not irradiated could not cure rickets. In 1925, Hess and Weinstock demonstrated that a factor with anti-rachitic activity was produced in the skin upon UV irradiation. Both groups demonstrated that the anti-rachitic agent was in the lipid fraction. They postulated that a pro-vitamin D_3 existed that could be converted to vitamin D_3 by UV light absorption. Much more work ultimately demonstrated that the anti-rachitic activity resulted from the irradiation of 7-dehydrocholesterol.

The isolation and characterization of $1,25(OH)_2D_3$ was later possible. In 1932, the structure of vitamin D_2 was simultaneously determined by Windaus in Germany, who named it vitamin D_2, and by Askew in England, who named it ergocalciferol. In 1936, Windaus identified the structure of $1,25(OH)_2D_3$ found in cod liver oil, $1,25(OH)_2D_3$. Thus, the naturally occurring vitamin is $1,25(OH)_2D_3$, or cholecalciferol. The structure of $1,25(OH)_2D_3$ was determined to be that of a steroid or, more correctly, a seco-steroid. On the other hand, the relationship between its structure and its mode of action was not realized for an additional 30 years.

1.2 $1,25(OH)_2D_3$ Structures

$1,25(OH)_2D_3$ was originally classified as a nutrient because it was discovered around the time of discovery of other micronutrients and that cod liver oil was found to have an anti-rachitic effect in infants. Further research progressed to show that exposure to sunlight or ultraviolet light also could prevent and cure rickets. It was then clear that the effects of $1,25(OH)_2D_3$ resulted from the synthesis of a hormone derived from a steroid precursor whose synthesis was partially dependent on the action of UV light. So the classification of $1,25(OH)_2D_3$ as a vitamin is a misnomer as it is actually a steroid hormone.

Although there are more than 10 substances belonging to the group of steroid compounds (Collins and Norman 2001) classified as vitamin D, it is generally used as a generic term for 2 molecules:

1) Ergocalciferol (vitamin D_2) is a much rarer form of vitamin D, and is provided by UV irradiation of the fungal steroid ergosterol. However, since fungi do not readily grow in sunlight, it is rare to find this form of vitamin D in nature, but it is the form generally used for fortification and some supplements.
2) Cholecalciferol ($1,25(OH)_2D_3$), which is formed in the skin of animals and humans through the action of UV light on the skin followed by a thermo reaction.

$1,25(OH)_2D_3$ (Fig. 1.1) is structurally related to the classic steroid hormones, which include the androgens, estrogens, progestins, glucocorticoids and mineralcorticoids. However, in contrast to the classic steroid hormones, the B-ring of the

ring structure has undergone fission by breakage of the 9, 10-carbon bond and $1,25(OH)_2D_3$ is consequently designated as a seco-steroid. In addition, $1,25(OH)_2D_3$

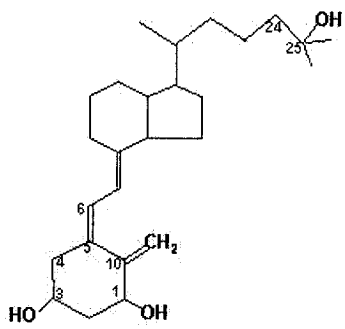


Figure 1.1: $1,25(OH)_2D_3$ structure

contains a side chain and is conformationally more flexible than the classic steroid hormones (Norman et al., 1999).

$1,25(OH)_2D_3$ is named according to the revised rules of the International Union of Pure and Applied Chemists (IUPAC). Since $1,25(OH)_2D_3$ is derived from a steroid, the structure retains its numbering from the parent compound cholesterol. Asymmetric centers are designated by using the R, S notation; the configuration of the double bonds are notated E for "entgegen" or trans, and Z for "zuzammen" or cis. Thus the official name of vitamin D_3 is 9,10-seco(5Z,7E)-5,7,10(19)cholestatriene-3Beta-ol, and the official name of vitamin D_2 is 9,10-seco(5Z,7E)-5,7,10(19), 22-ergostatetraene-3Beta-ol.

1.3 $1,25(OH)_2D_3$ deficiency diseases

$1,25(OH)_2D_3$ deficiency results in the bone diseases known as rickets in children and osteomalacia in adults.

a) Rickets is characterized by lower limb deformities including transverse metaphyseal bands, metaphyseal fraying and osteopenia (Narchi et al., 2001) and a few become critically ill with profound metabolic disturbances. There has been a recent upsurge in the number of cases of rickets presenting in industrialized countries (Narchi et al., 2001 ; Nozza and Rodda, 2001 ; Welch et al., 2000 ; Majid Molla et al., 2000 ; Shah et al., 2000), so although rickets has been considered a public health problem of the past, it is again emerging as a public health issue.

b) Osteomalacia is defined by an accumulation of unmineralized osteoid on the surface of bone. $1,25(OH)_2D_3$ deficiency also leads to secondary hyperthyroidism, which causes mobilization of more calcium from the bone and worsening of the bone loss (Holick et al., 1998).

1.4 Physiological Functions

The classic actions of $1,25(OH)_2D_3$ involve establishment and maintenance of the calcium homeostasis in the body. $1,25(OH)_2D_3$ mediates these effects primarily by regulating calcium absorption from the intestine, calcium excretion from the kidneys, and calcium resorption and mobilization from the skeleton (Holick 2001).

1.4.1 Overview of Calcium homeostasis: $1,25(OH)_2D_3$ and PTH

In calcium homeostasis, $1,25(OH)_2D_3$ works in conjunction with parathyroid hormone (PTH) to produce its regulatory effects on the plasma levels of ionized calcium and phosphate (Jones et al., 1998). The physiological loop starts by sensing calcium with the calcium receptor of the parathyroid gland (Brown et al., 1998). When the level of

ionized calcium in plasma falls, PTH is secreted by the parathyroid gland and stimulates the tightly regulated renal enzyme $25(OH)D_3$-1-α-hydroxylase in order to make more $1,25(OH)_2D_3$ from the large circulating pull of $25(OH)D_3$. The resulting increase in $1,25(OH)_2D_3$ (with the rise in PTH) causes an increase in calcium transport within the intestine, bone, and kidney. All these events raise plasma calcium levels back to normal, which in turn is sensed by the calcium receptor of the parathyroid gland.

In addition, to its action of increasing serum ionized-calcium concentrations, $1,25(OH)_2D_3$ is also recognized by the vitamin D receptor (VDR) that is present in chief cells in the parathyroid glands. The $1,25(OH)_2D_3$-bound VDR decreases the expression of the PTH gene, thereby decreasing the production and secretion of PTH (Naveh-Many and Silver, 1990).

1.4.1.1 Kidney and Intestine

The major biological function of $1,25(OH)_2D_3$ is to maintain calcium homeostasis by increasing the efficiency of the small intestine to absorb dietary calcium. VDRs are found in the nuclei throughout the small intestine, with the highest concentration in the duodenum. $1,25(OH)_2D_3$ directly affects the entry of calcium through the plasma membrane into the intestinal absorptive cell, enhances the movement of calcium through the cytoplasm, and transfers the calcium across the basolateral membrane into the circulation (Reichel et al., 1989; Norman et al., 1982; Wasserman et al., 1984).

Substantial evidence has now demonstrated that one mechanism by which $1,25(OH)_2D_3$ exerts its action in the intestine and in the kidneys is by acting with specific high affinity calcium binding proteins, the calbindins, which in turn act to facilitate and

regulate transcellular calcium transport. Two major classes of calbindin have been identified, the calbindin-D9k and the calbindin-D28k and both forms have been shown to be transcriptionally regulated by $1,25(OH)_2D_3$ (Darwish and DeLuca, 1992; Gill and Christakos, 1993; Varghese et al., 1988; Dupret et al., 1987). The extrusion across the basolateral membrane is facilitated by a Na-Ca exchanger and/or a Ca-ATPase (PCMA), which are shown to be induced by $1,25(OH)_2D_3$ (Hoenderop et al., 2000a; Hoenderop et al., 2000b). Moreover, the uptake of calcium from the intestinal lumen to the blood is facilitated by a calcium channel (ECaC), which has been shown to be induced at the mRNA level by $1,25(OH)_2D_3$ (Hoenderop et al., 1999a; Hoenderop et al., 1999b;Vennekens et al., 2000). ECaC is the prime target for hormonal control (or limiting step) of active Ca++ flux from the intestinal lumen or urine space to the blood compartment.

1.4.1.2 Bone formation and remodeling

The major biological function of $1,25(OH)_2D_3$ on bone is to enhance the mobilization of calcium stores at a time when dietary calcium is inadequate to maintain blood calcium in normal range. In the skeleton, $1,25(OH)_2D_3$ regulates both formation and resorption of bone by interacting with the osteoblasts and the osteoclasts, respectively. Proliferation and differentiation of the bone forming osteoblasts are directly stimulated by $1,25(OH)_2D_3$, while the effect of $1,25(OH)_2D_3$ on formation and activity of the bone-resorbing osteoclast is believed to be mediated indirectly via the osteoblasts (Pannabecker et al., 1995). Mature osteoblasts in the bone express the VDR for $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ increases the expression of alkaline phosphatase,

osteopontin, and osteocalcins, as well as a variety of cytokines in these cells (Gallagher & Riggs 1990, Jones *et al.* 1996, Goltzman *et al.* 2001). Although $1,25(OH)_2D_3$ has long been recognized as important for bone mineralization, there is little evidence that $1,25(OH)_2D_3$ actively participates in this procedure (DeLuca, 1988). Instead, $1,25(OH)_2D_3$ promotes the mineralization of osteoid laid down by osteoblasts, by maintaining the extracellular calcium and phosphorus concentrations within the normal range, which results in deposition of calcium hydroxyapatite into the bone matrix (DeLuca, 1988).

The importance of the calbindins in the transportation of the calcium in the bone is not clear. However, several studies have indicated that calbindins play an essential role in the process of calcification of the chondrocyte and during mineralization, processes which are both influenced by $1,25(OH)_2D_3$ (Balmain et al., 1989; Balmain et al., 1986a; Balmain et al., 1986b).

1.4.2 Cell Growth and Differentiation

$1,25(OH)_2D_3$ has been shown to control the growth of cells maintained in culture (Gross et al., 1986; Eisman et al., 1980a; Eisman et al., 1980b; Itin el al., 1994; Haugen et al., 1996; Wu et al., 1997). In vivo, $1,25(OH)_2D_3$ -deficient animals have growth rates that are considerably less than those of normal littermates, therefore, suggesting that $1,25(OH)_2D_3$ is responsible for the regulation of growth. However, it is interesting to note that the administration of a high-calcium diet that is sufficient to restore mineralization is associated with nearly normal growth.

$1,25(OH)_2D_3$ seems to have multiple and diverse actions (Freedman, 1999), often cell-specific, including effects on cell cycle arrest, apoptosis, and angiogenesis. (See section 3.3 of this chapter for more details)

1.4.3 Modulation of Immune System Function

In addition, $1,25(OH)_2D_3$ had also been shown to exert potent regulatory effects on growth and differentiation in a variety of cells, which are not directly involved in the calcium metabolism. Tumor cells that posses a vitamin D receptor (VDR), when exposed to $1,25(OH)_2D_3$, decrease their proliferative activity and may also terminally differentiate (Miyaura et al., 1985). For example, cells from the promyelocytic leukemic cell line HL-60, when exposed to physiologic amounts of $1,25(OH)_2D_3$ are induced to differentiate into macrophages (Tanaka et al., 1982; Brown et al., 1999). Of great interest is that, whereas resting T- and B-lymphocytes do not possess VDR, these cells express the VDR and become responsive to $1,25(OH)_2D_3$ when they are activated via their specific antigens (Provvedine et al., 1983). Activated T-lymphocytes respond to $1,25(OH)_2D_3$ by decreasing the production of interleukin-2 (IL-2). $1,25(OH)_2D_3$ has also been reported to inhibit DNA synthesis and immunoglobulin production in stimulated B-lymphocytes (Holick, 1995). Peripheral mononuclear cells have a VDR, and, when exposed to $1,25(OH)_2D_3$ in vitro, they are induced to become macrophages.

There is evidence indicating inhibition of a key cytokine in the immune system, interleukin-12 (IL-12), by vitamin D3. This monocyte-produced substance is the major determinant of the direction in which the immune system will be activated. IL-12 stimulates the development of CD-4 T-helper-1 (Th1) cells and inhibits the development

of CD-4 Th2 lymphocytes (Trembleau et al., 1995). Th1 participate in cell-mediated immunity (essential for controlling such intracellular pathogens). Th2 provide help for B cells and, in so doing, are essential for antibody-mediated immunity. Antibodies are needed to control extracellular pathogens. Th1 lymphocytes mainly secrete IL-2 and Interferon gamma (IFN-gamma) and are considered the most important cells in graft rejection and autoimmunity. Th2 cells secrete IL-4, IL-5, IL-10 and are considered to be regulator cells. The observation of clear inhibition of IL-12 expression by $1,25(OH)_2D_3$ in different systems is essential for understanding the observed effects of these substances in vitro on T cell proliferation and cytokine production and in vivo on graft survival and autoimmunity prevention (Mathieu et al., 1994; Casteels et al., 1995; Mathieu et al., 1997; Johnsson et al., 1996; Larsson et al., 1997).

1.5 $1,25(OH)_2D_3$ metabolism

1.5.1 Source of $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ can be obtained from the diet and by action of sunlight on the skin. Two thirds of the $1,25(OH)_2D_3$ content of the human body is synthesized from the precursor molecule 7-dehydrocholesterol in the skin by the action of sunlight and one third is obtained from the diet. Only a few food sources such as fish oils, egg yolks, and liver contain significant amounts of vitamin D_2 and $1,25(OH)_2D_3$. Although more than 10 substances belong to a group of steroid compounds that exhibit $1,25(OH)_2D_3$ activity, the commonly available forms are $1,25(OH)_2D_3$ (cholecalciferol) and vitamin D_2 (ergocalciferol). Vitamin D_2 is synthesized by irradiating precursors found in plants and

yeast. This is the form of the vitamin commonly added to milk. However, many foods are now fortified with vitamin, and minimum daily requirements are easily met.

1.5.1.1 Sunlight

The skin is the organ responsible for the production of $1,25(OH)_2D_3$. During exposure to sunlight, 7-dehydrocholesterol (7-DHC, pro-vitamin D_3), the immediate precursor of cholesterol, absorbs solar radiation with energies between 290 and 315 nm [ultraviolet B (UVB)], which, in turn, causes the transformation of 7-DHC to previtamin D_3 (Holick, 1994). Once formed, previtamin D_3 undergoes a thermally induced isomerization over a period of few hours and is transformed into vitamin D_3. Vitamin D_3 is translocated from the skin into the circulation, where it is bound to the vitamin-D-binding protein (DBP) (Holick, 1994).

This is the best source $1,25(OH)_2D_3$, as the body automatically regulates how much $1,25(OH)_2D_3$ it makes from sunlight, and there is the added benefit of controlling cholesterol. Since $1,25(OH)_2D_3$ precursors require cholesterol for conversion into the hormone-like vitamin, without adequate sun exposure $1,25(OH)_2D_3$ precursors can turn into cholesterol instead of the vitamin. It is estimated that for each 5% of skin surface exposed, approximately 435 IU of $1,25(OH)_2D_3$ can be manufactured. All it takes is about 10 to 15 minutes of sunlight on your hands, face, and/or arms several times a week, depending on your location, the time of year, the darkness of your skin, and your age (Jones et al. 1998; Brown 1999; Malloy & Feldman 1999). The process requires that the oil on the skin remain intact for a while after exposure to the sun.

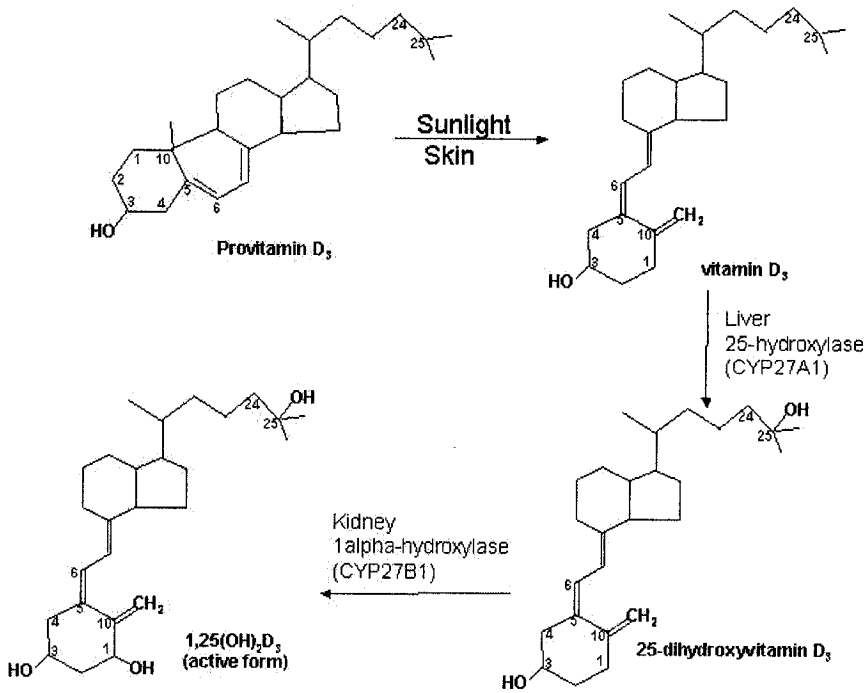


Figure 1.2: $1,25(OH)_2D_3$ metabolic pathways

As summarized in the table below, the concept of the existence of the $1,25(OH)_2D_3$ endocrine system is now firmly established. The elements of the $1,25(OH)_2D_3$ endocrine system include the following (Fig. 1.2):

(a) In the skin, photo-conversion of 7-dehydrocholesterol to vitamin D_3 or dietary intake of $1\alpha,25(OH)_2D_3$.

(b) Metabolism of $1,25(OH)_2D_3$ by the liver to $25(OH)D_3$, which is the major form of vitamin D circulating in the blood.

(c) Conversion by the kidney and other peripheral organs of $25(OH)D_3$ [functioning as an endocrine gland] to produce the two principal dihydroxylated metabolites, namely $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$.

(d) Systemic transport of the dihydroxylated metabolites $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ to distal target organs.

(e) Binding of the dihydroxylated metabolites, particularly $1,25(OH)_2D_3$, to a nuclear receptor at the target organs followed by the subsequent generation of appropriate biological responses.

1.5.2 Vitamin D_3 25-hydroxylase

The first step in the metabolic activation of $1,25(OH)_2D_3$ is hydroxylation of carbon 25. This reaction occurs primarily in the liver, although other tissues including keratinocytes, pancreas, intestine, and kidney have been reported to catalyze 25-hydroxylation of vitamin D_3 (Zehnder et al., 2001). The contribution of the extra-hepatic sources to the circulating levels of 25-hydroxyvitamin D [$25(OH)D_3$] is uncertain. So Vitamin D_3 serves as a substrate for the vitamin D3 25-hydroxylase enzyme that is present in the microsomes and mitochondria of hepatocytes (Bhattacharyya and Deluca, 1974a; Bhattacharyya and Deluca, 1974b; Madhok and Deluca, 1979; Madhok et al., 1978; Yoon and DeLuca, 1980). This enzyme metabolizes vitamin D3 to $25(OH)D_3$. The microsomal enzyme is feedback inhibited by the product, namely $25(OH)D_3$, and has a lower Km for the substrate than the mitochondrial enzyme. The mitochondrial vitamin D_3 25-hydroxylase (CYP27A1) has a higher Km for vitamin D3 and is not feedback regulated by the product, $25(OH)D_3$. Therefore, the administration of large amounts of vitamin D_3 results in a substantial increase in $25(OH)D_3$ concentrations in the blood. The cytochrome P450-containing vitamin D_3 25-hydroxylase enzyme is a multi-subunit enzyme NADP/NADPH flavoprotein, that uses molecular oxygen to convert vitamin D_3

to $25(OH)D_3$ (19-day half-life) (Madhok and Deluca, 1979; Madhok et al., 1978; Yoon and DeLuca, 1980). The production of $25(OH)D_3$ is virtually uncontrolled and seems to correlate with substrate availability rather than with physiological needs. Thus, measuring circulating $25(OH)D_3$ levels provides a useful indicator for the actual vitamin D_3 status in the body (Holick, 1995).

1.5.3 25-hydroxyvitamin D_3 1 alpha-hydroxylase 24-hydroxylase

The second step in vitamin D_3 bio-activation, the formation of $1,25(OH)_2D_3$ from $25(OH)D_3$ occurs, under physiological conditions, mainly in the kidney (Fraser and Kodicek, 1970). The renal enzyme responsible for producing $1,25(OH)_2D_3$, $25(OH)D_3$-1-α-hydroxylase (CYP27B1), is located in the inner mitochondrial membrane and is a cytochrome P-450 monooxygenase requiring molecular oxygen and reduced ferredoxin (Ghazarian et al., 1974). In recent years, many reports have demonstrated that the kidney is not unique in its ability to convert $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$. Numerous cells and tissues (keratinocytes) (Lehmann et al., 1998) express 1-α-hydroxylase in vitro. Renal synthesis of $1,25(OH)_2D_3$ is a strictly regulated process that responds to the physiological needs for calcium and phosphate. Several signaling pathways have been suggested to be involved in this regulation, including those controlled by estrogens, prolactin, calcitonin and glucocorticoids, but the two most important physiological regulators are $1,25(OH)_2D_3$ itself and parathyroid hormone (PTH) (Henry et al.,1992; Murayama et al., 1999). The active hormone stays in blood circulation for about 7 hours. As a fat soluble molecule $1,25(OH)_2D_3$ penetrates easily the plasma membrane of its target cells, where it is catabolized (Dusso *et al.* 1991).

1.5.4 25-hydroxyvitamin D_3 24-hydroxylase

$1,25(OH)_2D_3$ compounds are catabolized (Fig. 1.3) primarily by oxidation of the side chain. The 24-hydroxylation of $1,25(OH)_2D_3$ is the first catabolic step in the elimination of active hormone leading to the formation of 1,24,25-trihydroxyvitamin D_3, which is 10 times less potent than $1,25(OH)_2D_3$ (Okuda *et al.* 1995). Further oxidative reactions of 1,24,25-trihydroxyvitamin D_3 lead to progressive loss of biological activity and finally to the production of water-soluble calcitroic acid, which is excreted in urine (Makin *et al.* 1989).

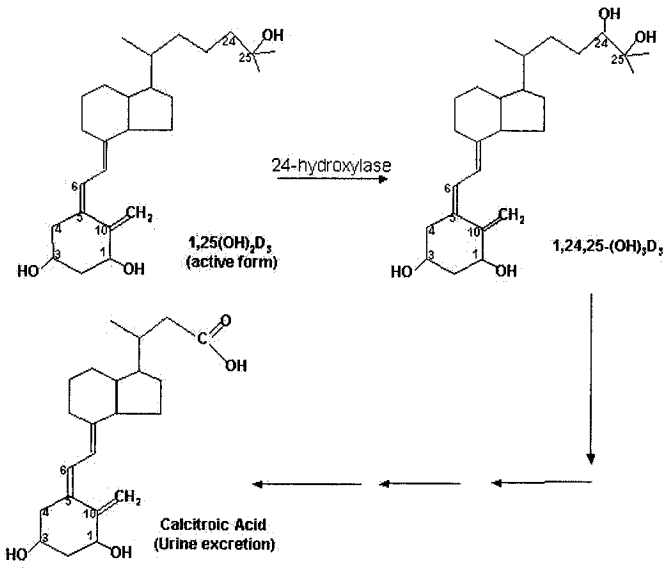


Figure 1.3: Vitamin D_3 catabolic pathways

1.5.5 $1,25(OH)_2D_3$ regulation

The high potency of $1,25(OH)_2D_3$ in elevating serum calcium and phosphate levels requires its circulating levels (~75 pM) to be tightly regulated. $1,25(OH)_2D_3$ acts via a negative feedback mechanism which has been suggested to take place at the transcriptional level and which eventually lead to suppression of the CYP27B1-activity

(Takeyama et al., 1997). In addition, $1,25(OH)_2D_3$ is able to stimulate 24-hydroxylase (CYP24), which is another mitochondrial cytochrome P-450 requiring molecular oxygen and reduced ferredoxin (Burgos-Trinidad et al., 1986; Knutson and DeLuca, 1974). By increasing the level of renal CYP24, it will catalyzes the conversion of $1,25(OH)_2D_3$ to the biologically inactive calcitroic acid along the major catabolic pathway of $1,25(OH)_2D_3$ (Kahlen and Carlberg, 1994; Zierold et al., 1994; Ohyama et al., 1994). PTH, on the other hand, stimulates the activity of the CYP27B1 and down-regulates the CYP24 activity by interfering with both the protein kinase A (PKA) and the cAMP signaling pathways (Murayama et al., 1999; Brenza et al., 1998; Armbrecht et al., 1998). Moreover, the level of PTH is further regulated by a direct action of $1\alpha,25(OH)_2D_3$ on the PTH gene in a negative manner (Mackey et al., 1996; Demay et al., 1992; Liu et al., 1996). Thus, together $1,25(OH)_2D_3$ and PTH form a complex regulatory mechanism which serves to maintain the level of calcium and phosphate within a narrow range.

2 $1,25(OH)_2D_3$ signaling pathways

2.1 Genomic actions of $1,25(OH)_2D_3$

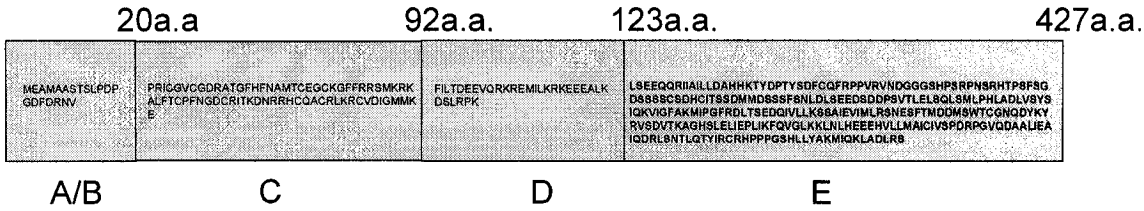
The regulation of gene transcription by $1,25(OH)_2D_3$ is known to be mediated by its interaction with a nuclear receptor protein, termed the VDR. Classically the main role of $1,25(OH)_2D_3$ is the regulation of calcium and phosphorous concentrations in serum via actions in bone, parathyroid gland, kidney and intestine that are conceived as classical target organs for $1,25(OH)_2D_3$. In addition, $1,25(OH)_2D_3$ is able to generate several other biological responses (non-classical actions of $1,25(OH)_2D_3$) that are not related to the

control of mineral homeostasis. Today there are over 30 non-classical target tissues for $1,25(OH)_2D_3$ (Bouillon *et al.* 1995).

2.1.1 Vitamin D_3 receptor: the nuclear receptor superfamily

Nuclear hormone receptors (NHRs) are grouped into a large superfamily and are thought to be evolutionarily derived from a common ancestor. Evolutionary analysis of the receptors has led to a subdivision in six different subfamilies (Laudet, 1997). One large family is formed by thyroid hormone receptors (TRs), retinoic acid receptors (RARs), vitamin D_3 receptors (VDRs) and peroxisome proliferators-activated receptors (PPARs) as well as different orphan receptors. The second subfamily contains the retinoid X receptors (RXRs) together with chicken ovalbumin upstream stimulators (COUPs), hepatocyte nuclear factor 4 (HNF4), testis receptors (TR2) and receptors involved in eye development (TLX and PNR). RXRs bind 9-cis-retinoic acid and play an important role in nuclear receptor signaling, as they are partners for different receptors that bind as heterodimers to DNA. The third family is formed by the steroid receptors and the highly related orphan receptors estrogen-related receptors (ERRs). The fourth, fifth and sixth subfamilies contain the orphan receptors NGF-induced clone B (NGFI-B), fushi tarazu factor 1/steroidogenic factor 1 (FTZ-F1/SF-1), and germ cell nuclear factor (GCNF), respectively (Laudet, 1997).

Based on sequence alignment, the structure of nuclear receptors are fairly similar consisting of six functional domains, A through F (Fawell et al., 1989; Krust et al., 1986; Kumar et al., 1987) (Fig. 1.4). The N-terminal A/B domain is the most variable



(Amino acid sequence is boxed in)

Figure 1.4: Schematic representation of the human nuclear VDR

among nuclear receptors. It houses a hormone independent transactivation domain, activation function 1 (AF-1). Adjacent to this domain is the C domain that contains the highly conserved DNA binding domain (DBD). The DBD consists of two zinc finger motifs that allow for specific recognition of hormone response elements. The C-terminal E domain houses both a ligand-binding domain (LBD) and a hormone-dependent transactivation domain, activation function 2 (AF-2). The D domain or so-called hinge region occupies the region between the DBD and the LBD. This domain has been implicated in interaction with co-repressors (Jackson et al., 1997). The function of the F domain is not clear; however one group suggested that it may be involved in tamoxifen-induced agonist activity by the ER (Montano et al., 1995). Many members of the nuclear receptor family form homo- and heterodimers, and amino acid sequences important for dimerization are contained within the DBD and LBD.

The VDR gene is mapped to chromosome 12q14 (Labuda *et al.* 1992) and contains eight exons that are invariably translated (exons 2-9) and six that are alternatively spliced (1a-1f). There are two potential translation start codons situated in exons 2, which result in a protein of either 424 or 427 amino acids. The VDR mRNA in human is a 4.8 kb whereas VDR is a 60 kDa protein. Expression of the gene is modulated by hormones and a wide range of stimuli including estrogens, cytokines, growth factors and peptide. The mRNA level is also influenced by the cell cycle and the differentiation state of the cell (Miyamoto et al., 1997).

2.1.1.1 Structure of VDR

The VDR, a member of the nuclear receptor superfamily, was first discovered in chicken intestine, and subsequently in a wide variety of cells and tissues. It has also been demonstrated in a broad range of tumors and malignant cell types. A full coding sequence for the human VDR was determined in 1988 (Baker et al., 1988). Structure and functions studies of VDR by deletion mutation analysis and amino acid sequence comparison with other nuclear receptor have led to the identification of four domains in VDR, designated A/B, C, D and E (Issa et al., 1998). Unlike some other nuclear hormone receptors, VDR does not have a C-terminal F domain at all (Jones *et al.* 1998). The domain Structure of human VDR and several function activities within these domains are presented in Fig. 1.4.

The aminoterminal A/B domain of the human VDR is short (21 amino acids) compared to 88-421 amino acids in other human nuclear receptors (Baker et al., 1988; Issa et al., 1998). The A/B domain of VDR is short compared with other members of family. The A/B region of many receptors contains an autonomous transcriptional activation function that contributes to constitutive ligand independent receptor activation (Aranda & Pascual 2001). However, VDR has no known AF-1 (Sone et al., 1991). The removal of the A/B domain from VDR does not appear to affect the ligand binding, DNA binding or transactivation properties of VDR (Issa et al. 1998).

The DNA-binding domain (domain C) of VDR is located in amino acid residues 24-90 of the human VDR (McDonnell et al., 1989; Sone et al., 1991). The three dimensional structure of several NHR DBDs is known, including TR (Rastinejad et al. 1995), RXR, RAR (Rastinejad et al. 2000, Fraydoon et al. 2000), glucocorticoid receptor

(Lusi et al. 1991, Baumann et al. 1993), estrogen receptor (Schwabe et al. 1993) and VDR (Shaffer & Gewirth 2002). The overall architecture of all known DBD core structures is very similar, suggesting that the same general structural features are present through the NHR superfamily (Khorasanizadeh & Rastinejad 2001). The core of DBD is composed of two zinc-finger motifs, each containing four highly conserved cysteine molecules which coordinate the binding of the zinc atom. Zinc atoms and cysteine residues are necessary for maintaining a three dimensional structure whose core is composed of two helices (helix I and II). Helix I is critical for the specific binding to the major groove of HRE on a DNA and helix II is involved in receptor dimerization (Khorasanizadeh & Rastinejad 2001). The C-terminal extension of DBD also takes part in receptor dimerization and contributes to response element binding affinity and specificity by sterically blocking a receptor dimer assembly on incorrectly spaced HREs. In contrast to the core DBD, whose structure is conserved in all NHRs, the structure of each C-terminal extension has a more unique character (Shaffer & Gewirth 2002). It is also suggested that the C-terminal extension of VDR DBD (amino acids 76-102) has an additional role in the nuclear accumulation of VDR (Luo *et al.* 1994).

Domain D (hinge domain) of VDR is located in amino acids 90-125 and links the DNA-binding and ligand-binding domains in a highly flexible fashion to allow for simultaneous receptor dimerization and DNA binding (McDonnell et al., 1989; Chawla et al., 2001). Domain D of VDR might also be involved in specific DNA contact (Miyamoto et al., 2001). In addition, the hinge domain contains a nuclear localization signal (Michigami et al., 1999).

The ligand-binding domain (domain E) is located in residues 125-427 of the human VDR. Several LBDs structures of NHRs have been solved, including RXR (Bourguet *et al.* 1995), RAR (Renaud *et al.* 1995), TR (Wagner *et al.* 1995), peroxisome proliferator-activated receptor (Nolte *et al.* 1998), estrogen receptor (Pike *et al.* 1999), progesterone receptor (Williams & Sigler 1998), androgen receptor (Sack *et al.* 2001) and VDR (Rochel *et al.* 2000). Despite the rather large differences in amino acid sequence composition all the known structures fold in a similar manner. The inner surface of these LBDs forms a cavity for highly specific ligand binding, whereas the outer surface serves as an interface for the interaction with other proteins. Helices 3, 5, and 12 of the LBD form an interface for the interaction with co-activator proteins. The comparison of the apo (hormone free)-structure of RXRα (Bourguet et al., 1995) with the holo (hormone bound)-structure of the closely related RA receptor (RAR)γ (Renaud et al., 1995) suggested that ligand binding induces a conformational change within the LBD that resulted in a changed orientation of helix 12. This helix contains the activation function 2 (AF-2) domain, which is exposed to co-activator, like in a closing mouse-trap. In this way, the ligand-induced conformation change of the LBD generate a transcriptionally active receptor by disrupting the binding of co-repressors and generating surfaces that allow interaction with co-activators and finally results in the onset of transactivation (Darimont *et al.* 1998, Nolte *et al.* 1998, Shiau *et al.* 1998). Ligands having antagonist properties bind into the same ligand binding pocket as agonist ligands, but in a way that prevents the correct assembly of helix 12. The wrong location of the helix 12 abolishes the binding of co-activators leading to inactivation of transcription (Brzozowski *et al.* 1997, Shiau *et al.* 1998, Pike *et al.* 1999, Bourguet *et al.* 2000). In

addition, there are regions in domain E that are needed for heterodimerization with RXR (Nakajima et al., 1994; Haussler et al., 1995; Jin et al., 1996; Gampe et al., 2000).

2.1.1.2 Regulation of VDR abundance

The regulation of VDR abundance plays an important role in determining the magnitude of the target cell response to $1,25(OH)_2D_3$. The amount of intracellular VDR in a target cell is regulated by $1,25(OH)_2D_3$ or other VDR ligands (homologous regulation) and by other hormonal and physiological signals (heterologous regulation). The regulation is species-, tissue- and cell-type-specific and is altered during development and aging and in disease states (Christakos et al., 1996; Issa et al., 1998). The state of differentiation of the cells, which is closely related to the rate of proliferation, may also be involved in the regulation of VDR abundance (Krishnan and Feldman 1997). The mechanism of regulation may involve transcriptional, post-transcriptional (alteration of mRNA stability) or post-translational effects (alteration in receptor half-life).

Ligands of VDR have been reported to up-regulate VDR abundance in pig kidney cells and human skin fibroblasts (Costa et al., 1985; Li et al., 1999), rat and human osteosarcoma cells (Arbour et al., 1993; Mahonen and Maenpaa 1994) and human colon cancer cells (Zhao and Feldman 1993). However, some reports suggest that VDR is not regulated or even down regulated by its ligands (Kizaki et al., 1991; Hulla et al., 1995; Song 1996). The mechanism of homologous up-regulation involves stabilization of VDR protein with no change (Wiese et al., 1992; Arbour et al., 1993; van den Bemd et al., 1996) or increase in VDR mRNA abundance (Costa and Feldman 1986; Mahonen and

Maenpaa 1994). It has been suggested that $1\alpha,25(OH)_2D_3$ up-regulate VDR in human skin by blocking ubiquitin/proteasome-mediated degradation without altering VDR mRNA levels (Li et al., 1999).

The heterologous regulators of VDR abundance include hormones such as glucocorticoids, estrogens, androgens, retinoids, growth factors, activators of specific intracellular second messenger systems (protein kinase A, protein kinase C, and intracellular calcium), and other growth and developmental signals (Escaleira et al., 1993; Krishnan and Feldman 1997). The effect of glucocorticoids on intestinal VDR abundance appears to be species-specific; up-regulation occurs in the rat and dog, down-regulation in the mouse and no change in the chickens (Krishnan and Feldman 1997). The capacity of PTH to activate multiple second messenger systems might provide a mechanism for tissue-, cell- and differentiation state-specific variations in VDR regulation (Krishnan and Feldman 1997). The mechanisms of heterologous regulation are complex, but the outcome is to sensitize (up-regulation) or de-sensitize (down-regulation) the target cells to the actions of vitamin D.

2.1.2 The mechanisms of vitamin D_3 receptor action

In general, binding of ligand to the nuclear receptor initiates a series of events which results in activation or repression of the target genes. The main reactions in this cascade are the activation of the nuclear receptor by its cognate ligand, and dimerization and binding of the activated receptor to specific hormone response elements is the DNA, resulting in negative or positive effects of transcription of the target genes (for review see Aranda and Pascual 2001).

2.1.2.1 Heterodimerization

Transcriptional activation of target genes by $1,25(OH)_2D_3$ involves a sequence of events centered around the VDR. The affinity of monomeric VDR to specific DNA target sequences is not sufficient for the formation of a stable protein-DNA complex and thus formation of heterodimeric complexes with a second partner receptor is required for efficient DNA binding (Carlberg and Polly, 1998). VDR preferentially forms heterodimeric complexes with RXR, which is a nuclear receptor for 9-cis retinoic acid (Levin et al., 1992; Carlberg et al., 1993). Mutagenesis of the VDR has demonstrated that the E1 region (overlapping H3-H4) and helix H10 are required for high-affinity binding to RXR (Haussler et al., 1998; Rochel et al., 2000). Binding of the VDR-RXR heterodimer to DNA is enhanced by vitamin D, whereas 9-cis retinoic acid inhibits this association, this possibly being related to the role of this retinoid in facilitating RXR homodimer formation (MacDonald et al., 1993; Kimmel-Jehan et al., 1997; Lemon et al., 1997; Jensen et al., 1998; Thompson et al., 1998). However, some reports indicate that 9-cis retinoic acid might be necessary for heterodimer formation (Carlberg et al., 1993; Dong and Noy 1998). Therefore, there might be hormonal crosstalk between vitamin A and $1,25(OH)_2D_3$ in controlling VDR-mediated transcriptional regulation. In addition, phosphorylation of RXR by mitogen-activated protein kinase (MAPK) has been shown to inhibit $1\alpha,25(OH)_2D_3$ signaling (Solomon et al., 1999).

2.1.2.2 VDREs and Target Genes

$1,25(OH)_2D_3$ induces a wide array of biological responses, some resulting in an upregulation of specific mRNAs and others that downregulate protein expression.

Stimulatory or inhibitory actions may depend on tissue specificity or on the state of cellular differentiation. Once heterodimeric VDR-RXR complex is formed, it modulates transcription by binding to specific DNA elements in the promoter regions of vitamin D responsive genes termed VDREs. Although there is considerable variation between natural VDREs, a consensus positive VDRE can be defined as a direct repeat (DR) of two half-elements of the sequence PuG(G/T)TCA, separated by three intervening nucleotides (DR3) (Umesono et al., 1991). In addition, inverted palindromes (toes pointing out) with nine intervening nucleotides (IP9-type) have been reported (Schrader et al., 1995; Carlberg and Polly 1998; Toell et al., 2000). Recently, VDREs defined as everted repeats with 6bp spacing (ER6), with the same symmetry as IR9 elements, have been described in genes encoding members of the cytochrome P450 family (Thummel et al., 2001; Drocourt et al., 2002; Thompson et al., 2002). It is clear that additional aspects of the VDRE region determine whether the $1,25(OH)_2D_3$ action on a target gene is stimulatory or inhibitory. The complexity in understanding how $1,25(OH)_2D_3$ responsive genes are regulated *in vivo* is highlighted by the different VDRE structure that exist as well as the multiple possibilities for co-regulator interactions.

2.1.2.3 Co-modulator Interactions

A large number of proteins, known as co-regulators, co-repressors and co-activators, have been found to interact with nuclear receptors and are required for gene transcription (Haussler et al., 1998; McKenna et al., 1999; Rachez and Freedman, 2000; Whitfield et al., 1999). In early reports, it has been suggested that VDR might not interact with co-repressors and act as a transcriptional silencer (Horlein et al., 1995, Chen et

al.,1996), whereas later reports show the opposite (Dwived et al., 1998; Tagami et al., 1998; Polly et al., 2000).

The co-regulatory proteins recruited to nuclear receptors, influenced by conformational changes in the receptor-ligand complex may contribute to the specificity of transcriptional regulation (Robyr et al., 2000). Specific interactions with the VDR have been shown for a number of proteins. The binding of $1\alpha,25(OH)_2D_3$ to VDR induces a conformational change within a ligand binding domain of VDR. The helix 12 (AF-2 domain) in the extreme C-terminus of the VDR functions like a lid closing the hydrophobic ligand binding pocket (Moras and Gronemeyer 1998). This conformational change facilitates the interaction of the AF-2 domain with co-activator proteins, such as activator-recruited cofactor/ vitamin D_3 receptor-interacting protein (ARC/DRIP) complexes and members of the p160 family steroid receptor co-activator 1 (SRC-1), transcription intermediary factor 2 (TIF2), and receptor-associated co-activator 3 (RAC3) (Horlein et al., 1995; Chen and Li 1998; Rachez et al., 1999; Dressel et al., 1999; Polly et al., 2000). The VDR co-activator interaction further facilitates recruitment of other factors to form a larger complex which modulates the chromatin structure and initiates transcription (Spencer et al., 1997).

The VDR has also been shown to interact with basal transcription factor TFIIB, a protein associated with basal transcriptional machinery (Jurutka et al., 2000; Blanco et al., 1995; MacDonald et al., 1995; Jurutka et al., 1997; Masuyama et al., 1997). TFIIB interaction does not involve the AF-2 domain (Jurutka et al., 1997). It has been shown to bind to the N-terminal portion of the receptor (Jurutka et al., 2000; Masuyama et al., 1997)

Deacetylation of histones leads to chromatin compaction and transcriptional repression. Some of the co-activator proteins are chromatin-remodeling factors or posses histone acetylase activity, whereas others may interact directly with the basic transcriptional machinery (Aranda and Pascual, 2001). Transcription factors and co-activators bridge contact between the VDRE-bound receptors, histone acetylases and RNA polymerase II (Issa et al., 1998). Recruitment of co-activator complexes to the target promoter leads to chromatin decondensation, allowing for binding and assembly of the transcription machinery and finally initiation for transcription (Aranda and Pascual, 2001).

2.1.2.4 Transactivation of Target Genes

In the bloodstream, $1,25(OH)_2D_3$ circulates mostly bound to vitamin D-binding protein (BDP) in equilibrium with small amount of unbound or free hormone. Upon ligand binding, VDR distribution may change. Unoccupied VDR exists in equilibrium between the cytosol and the nucleus, the minor or substantial (Berger et al., 1988; Clemens et al., 1988; Milde et al., 1989; Barsony et al., 1997) portion of VDR being in the cytosol. Upon ligand binding, VDR is relocated to the nucleus (Barsony et al., 1990; Balsony et al., 1997). In the ligand-bound state VDR is predominantly a nuclear protein. VDR, like most steroid hormone receptors, is phosphorylated hormone-dependently (Brown and DeLuca, 1990). The function of VDR phosphorylation is poorly understood, although it might represent a means for signals form the cell membrane to modulate nuclear hormone receptor function in response to growth factor stimulation (Issa et al., 1998).

In the absence of hormone, VDR exhibits a low affinity for RXR or is in the cytoplasm. Upon $1,25(OH)_2D_3$ binding, the VDR translocates to the nucleus. RXR proteins heterodimerize and form a high-affinity complex with VDR that requires the ability to recognize and bind with high affinity with VDREs through their cognate DBDs. From crystal structure studies of the VDR LBD and models based on other steroid receptors, the VDR undergoes a major conformational change upon $1,25(OH)_2D_3$ binding. The repositioning of helix H12 forms high-affinity protein surfaces capable of interacting with specific co-modulator proteins required for transactivation. The VDR-RXR heterodimer attracts co-activator protein such as SRC-1. SRC-1, through its intrinsic HAT activity, derepresses chromatin so that nucleosomes are rearranged and naked DNA is accessible. TATA-binding protein-associated factors (TAFs) are also recruited to target TATA/TBP-binding sites. Other proteins, including DRIPs and TFIIB, serve to stabilize the pre-initiation complex. Transcription is then initiated by RNA polymerase II. For stimulatory actions, mRNAs transcription is increased and the specifically induced mRNAs are translated into proteins that carry out the biological effects of the hormone. For inhibitory actions, mRNA transcription is suppressed, thereby reducing the expression level of selected proteins.

Transcriptional repression by nuclear hormone receptors occurs through a number of mechanisms (Issa et al., 1998). Inhibition of transcription factor function may be one mechanism by which VDR exerts transcriptional repression (Alroy et al., 1995). VDR may also inhibit transcriptional function of TR, RAR and growth hormone receptor (GR) by competing for dimerization with RXR or possibly squelching co-activators (Garcia-Villalba et al., 1996; Yen et al., 1996; Polly et al., 1997).

2.2 Non-genomic actions of $1,25(OH)_2D_3$

Steroid hormones can also have responses which occur within few minutes after addition of a hormone and are therefore too rapid to involve changes in gene expression. These rapid actions are mediated by mechanisms other than nuclear receptors. The non-genomic pathway involves regulation of voltage-gated calcium channels, opening of chloride channels, modulation of PKC activity and activation of mitogen-activated protein kinases (MAP kinases), which eventually lead to the onset of rapid biological responses (seconds to minutes), including inhibition of cell proliferation and stimulation of cell differentiation (Chen et al., 1999; Zanello and Norman, 1997; Capiati et al., 2000; Song et al., 1998). Recent studies have indicated that the generation of these rapid responses is mediated via a putative membrane receptor with ligand binding properties different from those of the nuclear VDR (Baran et al., 1994; Nemere et al., 1998; Kato et al., 1998; Sitrin et al. 1999). However, the existence of such a receptor is still controversial and further experiments are still needed. In addition, as an alternative to a specific membrane receptor for $1,25(OH)_2D_3$, it has been suggested that membrane-associated annexin II might serve as a receptor for $1,25(OH)_2D_3$-mediated rapid responses or that extracellular biding sites for the VDR-ligand complex may exist (Baran et al., 2000). There is also evidence that rapid responses are able to modulate the genomic pathway of $1,25(OH)_2D_3$ actions via phosphorylation of nuclear VDR (Norman et al. 2001). Receptor phosphorylation could increase the affinity of VDR to co-activator complexes and thus enhance gene activation (Barletta et al. 2002).

3 $1,25(OH)_2D_3$ and cancer

During the closing decades of the 20th century, it was demonstrated in experiments using cell lines that had been cultured from human malignant tumors, that $1,25(OH)_2D_3$ induces differentiation, thereby inhibiting the cells from proliferating. The effect was observed in several frequently diagnosed malignancies, including breast cancer, colon cancer, and prostate cancer.

The first link to link $1,25(OH)_2D_3$ with breast cancer in humans were J.A. Eisman and co-workers in 1979, who showed that certain of the cells are furnished with specific, high affinity receptors for this hormones (Eisman et al., 1979). The first to demonstrate that $1,25(OH)_2D_3$ and its receptor in combination are antiproliferative at the cellular level and can inhibit tumor growth were K.W. Colston and colleagues in 1981, using cells from a malignant melanoma (Colston et al., 1981). In the same year, E. Abe and colleagues showed that the hormone suppressed the proliferation of leukemic cells in mice (Abe et al., 1981).

The nuclear VDR has been demonstrated in almost every cell type in the body. Epithelial tissues such as kidney, liver, adrenal, thyroid, bladder, cells of the gastrointestinal tract, prostate and breast cells have all been shown to possess VDR. Moreover, pituitary, parathyroid, pancreatic, bone, muscle and skin cells as well as some activated cells of the immune system also express high affinity receptors for $1,25(OH)_2D_3$ (Haussler et al., 1998; Hannah and Norman, 1994;Zanello and Norman, 1997). Interestingly, many cancer cells derived from these tissues also possess VDR and have thus retained the ability to respond to the growth regulating effects of $1,25(OH)_2D_3$ (Hansen et al., 2000; van den Bend et al., 2000). In fact, it has been shown that a high

expression of VDR is associated with a high degree of tumor cell differentiation and a favorable prognosis in colon cancer patients (Vandewalle et al., 1994; Evans et al., 1998). Also, in breast cancer patients a significantly longer disease free survival has been observed in patients with VDR positive tumors as opposed to patients with VDR negative tumors (Eisman et al., 1986; Colston et al., 1989; Berger et al., 1991).

3.1 Epidemiological evidence

The correlation between $1,25(OH)_2D_3$ and cancer is further supported by epidemiological investigations showing an inverse relationship between $1,25(OH)_2D_3$ deficiency and cancer. As mentioned above, the $1,25(OH)_2D_3$ status in the body is mainly dependent on the exposure to UV light, but also dietary intake of $1,25(OH)_2D_3$ provides a small amount of the daily requirement. Skin cancer and internal cancers (and overall cancer death rates) were found to be inversely related to distance from the equator. Enhanced sunlight exposure has since been associated with lower prostate, breast and colon cancer death rates, while the historical geographic distribution of rickets parallels that for these cancer deaths (Garland and Garland, 1980; Garland et al., 1990; Gorham et al., 1990; Hanchette and Schwartz, 1992; Emerson and Weiss, 1992; Garland et al., 1999). Furthermore, certain risk factors for prostate cancer, including advanced age and African-American ethnicity, are associated with reduced $1\alpha,25(OH)_2D_3$ levels (Schwartz and Hulka, 1990). Morever, an inverse relationship between dietary intake of $1\alpha,25(OH)_2D_3$ and cancer has been observed in colorectal and breast cancer (Garland et al., 1991; Newmark, 1994), and a high intake of $1\alpha,25(OH)_2D_3$ from fish oils in the diet of among Japanese men has been

shown to be associated with a lower incidence of prostate cancer (Haenzel and Kurihara, 1968).

3.2 $1,25(OH)_2D_3$ and cancer therapy

The clinical use of $1,25(OH)_2D_3$ for treating advanced hyperproliferative diseases such as breast cancer and leukemia has not yet appeared to be promising because of recurrence of disease and complications resulting from hypercalcemic activity of the $1,25(OH)_2D_3$ compounds (Holick, 1995; Koeffler et al., 1985). However, the potent antiproliferative activity of $1,25(OH)_2D_3$ and its analogue calcipotriene (Dovonex), with its attendant prodifferentiating properties, had been effectively used for the treatment of the nonmalignant hyperproliferative skin disorder psoriasis (Holick, 1994; Smith et al., 1988; Kragballe et al., 1998). Taken together, these observations strongly indicate that $1,25(OH)_2D_3$ is a potentially useful agent in the treatment or prevention of various cancer types.

3.3 Anti-proliferative Effects of $1,25(OH)_2D_3$

VDR are expressed in many normal and malignant cell types, indicating a wide array of previously unrecognized potential targets for $1,25(OH)_2D_3$ (Walters, 1992). $1,25(OH)_2D_3$ seems to have multiple diverse actions often cell-specific (Freedman, 1999). In selected settings, $1,25(OH)_2D_3$ acts to inhibit cell proliferation (Walters, 1992; Manolagas et al., 1994). In others, $1,25(OH)_2D_3$ may stimulate cell proliferation (Baier et al., 1994; Itin et al., 1994). Also in a number of systems, $1,25(OH)_2D_3$ affects cells by promoting cellular differentiation (Walters, 1992; Bikle, 1992; Manolagas et al., 1994).

The hormonal modulation of intracellular calcium and the regulation of expression of nuclear oncogenes have been raised as possible mechanisms for these antiproliferative and differentiating effects of $1,25(OH)_2D_3$ (Manolagas et al., 1994; Studzinski et al., 1993). Additional mechanisms are postulated to include inhibition of the passage of cells through the cell cycle, regulation of paracrine growth factors, stimulation of terminal differentiation, and induction of apoptosis (Feldman et al., 2000; Welsh, 1997; Tong et al., 1999)

Whether induction of cell differentiation by $1,25(OH)_2D_3$ compounds is a consequence of blockage of cell cycle progression or vice versa is still a matter of debate. Moreover, it has been questioned whether it is possible to dissociate between the two processes. A few studies on $1,25(OH)_2D_3$ effects in the HL-60 line have indicated that the anti-proliferative and the differentiating effects of the compounds are uncoupled (Wang et al., 1996; Albanell et al., 1996; Studzinski et al., 1997). However, the consensus view emerging from the studies in most other cell systems is that inhibition of cell growth is accompanied by a stimulation of cell differentiation.

3.3.1 Cell-cycle regulators

In many cancer cells, vitamin, the antiproliferative actions of $1,25(OH)_2D_3$ appear to be mediated by the induction of arrest of cell cycle progression at G0/G1 phase (Fioravanti et al., 1998). This G1 arrest is associated with the inhibition of a novel growth-regulating gene, IEX-1, the upregulation of the cyclin-dependent kinase inhbitors p21WAF1 and p27KIP1, decreased cyclin-dependent kinase activity, hypophosphorylation of the retinoblastoma protein, and repressed E2F transcriptional

activity in several cell types. This effect may in part be VDR-dependent (Liu et al., 1996; Campbell et al., 1997; Reitsma et al., 1983; Veenstra et al., 1997; Munker et al., 1996; Rots et al., 1999; Kobayashi et al., 1998; Hengst and Reed, 1996; Wang et al., 1996). TGFβ1 induction may also play a role in the induction of these genes resulting in G1 arrest (Segaert et al., 1997).

The effect of $1,25(OH)_2D_3$ on proliferation in most cancer cells is centered on the G1/S checkpoint of the cell cycle. Proliferating cells progress through the cell cycle, which comprises the G0/G1 phase (most differentiated, non-dividing cells), the S phase (new DNA is synthesized), the G2 phase and the M phase (mitosis). The cell cycle is punctuated with inherent blocks that are overcome by the transient formation of cyclin-cyclin-dependent kinase (CDK) complexes (reviewed by Sherr, 1993; Hunter and Pines, 1994; Morgan, 1995). When a cell is in G0/G1 phase, the G1/S checkpoint is blocked by the non-phosphorylated form of the retinoblastoma (Rb), which binds and inactivates transcription E2F (essential for the DNA synthesis in the S phase and upregulation of proteins required for G0-S progression) (Matsushime et al., 1992; Koff et al., 1992; Helin, 1998; Nevins, 1998; Zhang et al., 1999). Phosphorylation of Rb by CDK4, 6, and 2 removes the inhibitory effect and liberates E2F. The CDKs are sequentially activated by combination with cyclins, cyclins D and E for CDK4/6 and CDK2, respectively (Helin, 1998; Nevins, 1998; Zhang et al., 1999). CDK activity is regulated by association with its activating partner, physical interaction with CDK inhibitors, and by both positive and negative regulatory phosphorylations (Morgan, 1995; Sherr and Roberts, 1995). CDK inhibitors (CDKI) include first the INK4 family; p15INK4B, p16INK4A, p18INK4C, and p19INK4D, which bind and inactivate the major G1 phase kinases,

CDK4 and CDK6, by forming inactive dimeric complexes (Serrano et al., 1993; Hannon and Beach, 1994; Guan et al., 1994). Another family of CDKIs includes p21CIP1/WAF1, p27KIP1, and p57KIP2, which inhibit a broader range of CDKs, including CDK1, -2, -4 and –6 (Polyak et al., 1994; Hall et al., 1995; Harper et al., 1995). The cyclins are activated transcriptionally by growth factors.

There is now abundant of evidence that $1,25(OH)_2D_3$ has an anti-proliferative effect on the G1/S checkpoint and may produce complete arrest of the cell cycle at that point. The two CKIs p21 and p27 appear to be the main mediators of the $1,25(OH)_2D_3$ - induced Go/G1-phase block. In 1996, Liu et al. identified a VDRE in the gene encoding p21. Their results suggested that vitamin D3 was able to directly stimulate the transcriptional activity of the p21 (Liu et al., 1996). Additional studies have later demonstrated an up-regulation of p21 at both mRNA and protein levels in most cancer cell types in response to treatment with $1,25(OH)_2D_3$ (Review in: Hansen et al., 2000; Carlberg and Polly, 1998; Chen et al., 1999; Zanello and Norman, 1997;Capiati et al., 2000; Song et al., 1998; Baran et al., 1994; Nemere et al., 1998; Kato et al., 1998; Baran et al., 2000; Holick, 1995; van den Bemd et al., 2000). p21 binds and inhibits all the G1 and S phase cyclin-CDK complexes including cyclin D-CDK4, cyclin E-CDK2 and cyclin A-CDK2 (Harper et al., 1993; El-Deiry et al., 1993; Xiong et al., 1993). The effect of the $1,25(OH)_2D_3$ compounds on the p21 expression has been shown to be independent of p53 tumor suppressor status which further supports the assumption that $1,25(OH)_2D_3$ directly regulates the p21 gene (Liu et al., 1996; Hansen et al., 2001; Jiang et al., 1994).

No VDRE has yet been identified for p27, but an up-regulation of both p27 mRNA and protein levels has been observed in a variety of different cancer cell types

after treatment with vitamin D (Wu et al., 1997; Seol et al., 2000; Park et al., 2000; Liu et al., 1996; Campbell et al., 1997; Wang et al., 1997). In one study, the induction of p27 appears to be under translational regulation (Hengst and Reed, 1996). Accumulation of p27 inactivates G1 cyclin-CDK complexes and causes cell cycle arrest at G1, which is reversed by antisense p27 overexpression (Rivard et al., 1996). The effects of $1,25(OH)_2D_3$ on p27 RNA appear to vary with cell type. Some studies have reported unchanged levels of p27 RNA in human MCF-7 cells with vitamin D3 (Wu et al., 1997; Jensen et al., 2001; Hansen et al., 2001) but another reported an increase (Verlinden et al., 1998).

As expected, the induction of p21 and p27 in response to treatment of $1,25(OH)_2D_3$ is accompanied by an up-regulation of the hypo-phosphorylated form of Rb and a down-regulation of the hyper-phosphorylated form of Rb (Simboli-Campbell et al., 1997; Jensen et al., 2001). In addition, the levels of a number of CDKs and cyclins as well as their complex formation had been show to be modulated by $1,25(OH)_2D_3$. These include Cdk2, Cdk4, Cdk6, cyclin D1, cyclin A and cyclin E, which are all crucial in regulating progression through the different phases of the cell cycle (Wu et al., 1997; Park et al., 2000; Zhang et al., 1996; Bartek et al., 1996; Wang et al., 1997; Jensen et al., 2001; Verlinden et al., 1998). Other important cell growth regulators, such as c-fos and c-myc proto-oncogenes have also been suggested to be involved in the growth inhibitory effect induced by $1,25(OH)_2D_3$ in cancer cells (Jensen et al., 2001; Reitsma et al., 1983; Karmali et al., 1989; Caligo et al., 1996; Bar-Shavit et al., 1986; Simpson et al., 1987; Okano et al., 1999). Both c-fos and c-myc have both been shown to have a VDRE, which demonstrate that $1,25(OH)_2D_3$ compounds may regulate directly their transcriptional

activities (Candeliere et al., 1996; Reitsma et al., 1983; Simpson et al., 1987; Pan and Simpson, 1999).

3.3.2 Apoptosis and growth factors.

The mechanisms by which $1,25(OH)_2D_3$ controls cellular growth involve, at least in part, the regulation of growth factors. The growth arrest in keratinocytes and osteoblast (Itin el al., 1994; Haugen et al., 1996; Wu et al., 1997) is associated with an increase in the secretion of $TGF\beta_2$ in these cells. The mechanism by which $1,25(OH)_2D_3$ increases $TGF\beta_2$ synthesis involves an increase in the transcription rate of the $TGF\beta_2$ gene. VDRs bind to Smad proteins and the Smad3 is capable of upregulating responses of $1,25(OH)_2D_3$ -dependent genes (Yanagi et al., 1999; Yanagisawa et al., 1999).

Cell death by apoptosis occurs as a part of a natural regulatory process in the body, serving to establish and maintain a proper control of the cellular turnover. Cancer is often associated with cells that fail to undergo apoptosis. This leads to the survival of aberrant cells, which would normally die, and, in turn, to malignant outgrowth. $1,25(OH)_2D_3$ mediated apoptosis is associated with generation of reactive oxygen species and mitochondrial disruption (Narvaez and Welsh, 2001). Many of the signals that elicit apoptosis converge on the mitochondria, which respond to pro-apoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis (Green and Reed, 1998). The ability of $1,25(OH)_2D_3$ to induce apoptosis has been investigated in vivo and in several cell types in vitro. For example, apoptosis was detected in prostate sections of EB1089-treated rats, an effect associated with increased of IGF-binding proteins-2,-3,-4, and –5 and of IGF-I mRNA (Nickerson and Huynh, 1999). EB1089 also promotes breast

cancer cell apoptosis in vitro and thereby enhances the sensitivity of these cells to radiation (Sundaram and Gewirtz, 1999). Recently, there is evidence suggesting that $1,25(OH)_2D_3$ induces apoptosis in colon and breast cancer cells via a mechanism independent of p53 (Mathiasen et al., 1999; Diaz et al., 2000). In contrast, accumulating evidence points to the bcl-2 family of apoptosis regulatory proteins as playing a critical role in $1,25(OH)_2D_3$ induced apoptosis. In myeloma cells, breast, colon and prostate cell lines, a down-regulation of the two anti-apoptotic members of this family, bcl-2 and bcl-X_L and an up-regulation of the pro-apoptotic, bax and bak, proteins have been demonstrated after treatment with $1,25(OH)_2D_3$ (Simboli-Campbell et al., 1997; Campbell et al., 1997; Park et al., 2000; Danielsson et al., 1997; James et al., 1998; Blutt et al., 2000a; Blutt et al., 2000b; Diaz et al., 2000).

4 $1,25(OH)_2D_3$ Analogue EB1089

The primary dose-limiting toxicity associated with acute or long-term administration of excessive amounts of $1,25(OH)_2D_3$ is altered calcium metabolism (Norman, 1995). In humans, $1,25(OH)_2D_3$ stimulates an increase in calcium absorption from intestines and bones thatresults in elevated blood calcium levels. At pharmacological doses, the most common adverse effects are hypercalcemia (A higher-than-normal level of calcium in the blood), soft tissue calcification and nephrocalcinosis (deposition of calcium in the substance of the kidney) also occur. Such effects limited dose escalation in Phase II clinical trials of $1,25(OH)_2D_3$ for chemotherapy of advanced prostatic cancer despite some evidence of efficacy (Konety et al., 1999; Miller, 1998). In preclinical toxicity studies, the primary manifestations of the vitamin's toxicity were hypercalcemia, weight

loss, and tissue calcification (Kelloff et al., 1995). Unfortunately, these calcemic effects have been noted at doses required to achieve chemopreventive efficacy. Therefore much effort has been directed to identify new analogues with a chemopreventive effect to the natural $1,25(OH)_2D_3$ but with decreased risk of inducing calcemic adverse effects.

At the moment, one of the most promising synthetic analogues is Seocalcitol (EB1089, Leo Pharmaceutical Products) (Fig. 1.5), which is characterized by an altered side chain

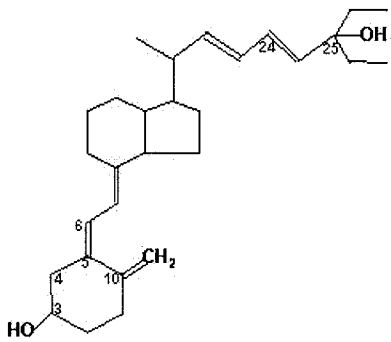


Figure 1.5: Structure of $1,25(OH)_2D_3$ analogue EB1089

structure featuring 26,27-dimethyl groups and two double bonds. A considerable number of in vitro studies have been carried out with EB1089 and the results clearly show that EB1089 is more potent than $1,25(OH)_2D_3$ with respect to regulation of cancer cell growth and differentiation. However, the effect of EB1089 on the calcium metabolism in vivo is approximately 50% weaker than that of $1,25(OH)_2D_3$, while the biological half-life is similar to that of $1\alpha,25(OH)_2D_3$ (Hansen et al., 2000; Simboli-Campbell et al., 1997;Mathiasen et al., 1993; Nayeri et al., 1995; James et al., 1997; Quack et al., 1998; Kissmeyer et al., 1997). Together, these characteristics make EB1089 a better candidate

than $1,25(OH)_2D_3$ for in vivo use and point to EB1089 as a potential useful analogue for systemic use in the clinical treatment of cancer.

5 Head and Neck Squamous Cell Carcinoma

An estimated 42,800 cases of head and neck squamous cell carcinoma (SCC) occurred in the U.S. in 1992, with 11,600 deaths. Worldwide, more than 500,000 cases are predicted annually (Vokes et al., 1993; Khuri et al., 2000; Khuri et al., 2001). The long-term prognosis of patients with advanced head and neck cancer has been poor. Early stage stage (I and II) head and neck SCC can be successfully treated with surgery and/or radiation therapy (Vokes et al., 1993). However, there remains a 3-7% annual rate of development of second primary carcinomas (SPC). More advanced SCC is treated by chemotherapy, often with a combination of cisplatin and 5-fluorouracil (Khuri et al., 2000; Hollander et al., 1999; Khuri et al., 2000; Hong et al., 1990). Tobacco and alcohol are the primary risk factors predisposing to both initial SCC and SPC. The high rate of SPC prompted a search for chemopreventative agents to suppress carcinogenesis (Vokes et al., 1993; Khuri et al., 2000; Hong et al., 1990). Retinoids (vitamin A metabolites) inhibits growth and stimulate differentiation of SCC (Hong et al., 1990; Lippman et al., 1987; Lippman & Hong, 1994; Benner et al., 1994; Shalinsky et al., 1997; Shalinsky et al., 1995; Liaudet-Coopman et al., 1997). SPCs were significantly reduced in clinical trials of high doses of 13-cis-retinoic acid (13-cis RA; isotretinoin) (Lippman et al., 1994). However, significant side effects including cheilitis (inflammation of the lips), dermatitis (inflammation of the skin), conjunctivitis (Inflammation of the conjunctiva, the membrane on the inner part of the eyelids and the membrane covering the white of the

eye), and hypertriglyceridemia (too much triglyceride in the blood) affected patient compliance. The undesirable effects of retinoids led to a search for other potentially less toxic agents that exhibit antiproliferative activity in head and neck SCC.

6 Gene Arrays

Until recently only a limited number of genes were accessible to gene expression profiling, as northern blot, RT-PCR, and ribonuclease protection assays are designed for single genes or small groups of genes at a time. During the course of human genome project, comprehensive cDNA libraries became available allowing the development of techniques for massive parallel expression profiling. Two types of microarrays emerged either using oligonucleotides directly synthesized on a chip surface (Affymetrix) or array generated by depositing cDNA-PCR products (Research genetics & Clontech) on membrane or glass slides. Therefore, high-density microarrays allowed genome-wide screening programs for identification of target genes or expression profiles in disease and cancer.

Binding of target sequences of DNA or RNA can be detected by a variety of methods, including radiolabels (such as ^{32}P and ^{33}P linked to dCTP) and red and green fluorescent dyes linked to the nucleotides (such as Cye-3 or Cye-5 to dUTP). Fluorescence offers considerable advantages over radioisotopes. Fluorescence measurements are convenient, immediate, quantitative and very flexible. Now, there is a wide range of available dyes with precisely known excitation and emission spectra. Laser-based instruments allow precise, rapid and reproducible data collection and

analysis. The computer-driven laser-scanning microscope measures and records the fluorescence intensity at each and every spot on the chip.

The use of different Fluorescent tags also allows the gene expression in two different samples to be compared directly. If each sample is labeled with a dye of different emission wavelength, and the samples are mixed and applied to the chip, the ratio of the intensity of fluorescence on each spot is the ratio of expression of each gene in the two samples. Thus, for sample, the expression of each and every gene in a treated and untreated cancer cell with vitamin D3 can be compared directly.

7 Summary and Rationale

$1,25(OH)_2D_3$ is an important regulator of calcium homeostasis and bone metabolism via actions in intestine, kidney, bone and parathyroid glands. $1,25(OH)_2D_3$ exerts its effect via the nuclear VDR that belongs to the nuclear hormone receptor family, leading to gene regulation mediating various biological responses. Within the last two decades, the receptor has been shown to be present not only in classical target tissues such as bone, kidney and intestine but also in many other non-classical tissues, e.g., in the immune system (T-and B- cells, macrophages and monocytes), in the reproductive system (uterus, testis, ovary, prostate, placenta and mammary glands), in the endocrine system (pancreas, pituitary, thyroid, adrenal cortex), in muscles (skeletal, smooth and heart muscles), in brain, skin and liver.

Besides, the almost universal presence of VDRs, some cell types (e.g. keratinocytes, monocytes, bone, placenta) are capable of metabolizing $25(OH)D_3$ to vitamin D3 by the enzyme 1alpha-hydroxylase. The combined presence of $25(OH)D_3$-

1alpha-hydroxylase , as well as the VDR in several tissues introduced the idea of a paracrine role for $1,25(OH)_2D_3$ (Bouillon et al., 1995). Moreover, it has been demonstrated that $1,25(OH)_2D_3$ can induce differentiation and inhibit proliferation of normal and malignant cells (Bikle and Pillai, 1993; Abe et al., 1981).

In addition to the treatment of bone disorders with $1,25(OH)_2D_3$, these newly discovered functions of $1,25(OH)_2D_3$ open new therapeutic applications as an immune regulator (e.g. for the treatment of autoimmune diseases or prevention of graft rejection), inhibitor of cell proliferation (e.g. psoriasis) and inducer of cell differentiation (cancer). To achieve growth inhibition or cell differentiation, supraphysiological doses of $1,25(OH)_2D_3$ are needed, causing calcemic side effects (hypercalciuria, hypercalcaemia, increase bone resorption). Therefore, new analogues of $1,25(OH)_2D_3$ are being developed such as EB1089 in order to dissociate the antiproliferative and prodifferentiating effects from calcemic and bone metabolism effects. Although the exact mechanism of dissociation is not completely understood, the use of microarray technology can help us better understand the mechanisms involved.

7.1 Objectives

1: To investigate the effects of $1,25(OH)_2D_3$ and its analogue EB1089 on the growth of HNSCC cells.

2: To elucidate the possible mechanisms responsible for the anti-cancer effects of $1,25(OH)_2D_3$ and its analogue EB1089 in vitro by using the microarray technology.

3: To study the potential of EB1089 as a chemoprevention of SCC.

CHAPTER 2

Regulation of Gene Expression by 1α,25-dihydroxyvitamin D_3 and its Analogue EB1089 under Growth Inhibitory Conditions in Squamous Carcinoma Cells

Preface

This chapter of my thesis is based on the following masnuscript:

Akutsu, N.*, **Lin, R.***, Bastien, Y., Bestawros, A., Enepekides, D.J., Black, M.J. and White, J.H. (2001) Regulation of Gene Expression by 1α,25-dihydroxyvitamin D_3 and its Analogue EB1089 under Growth Inhibitory Conditions in Squamous Carcinoma Cells. *Mol. Endocrinol.* 15, 1127-39. ***Co-first authors**

Abstract

Analogues of 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) inhibit growth in vitro and in vivo of cells derived from a variety of tumors. Here, we examined the effects of $1,25(OH)_2D_3$ and its analogue EB1089 on proliferation and target gene regulation of human head and neck squamous cell carcinoma (SCC) lines SCC4, SCC9, SCC15 and SCC25. A range of sensitivities to $1,25(OH)_2D_3$ and EB1089 was observed, from complete G0/G1 arrest of SCC25 cells to only 50% inhibition of SCC9 cell growth. All lines expressed similar levels of vitamin D3 receptor (VDR) mRNA and protein, and no significant variation was observed in $1,25(OH)_2D_3$-dependent induction of the endogenous 24-hydroxylase gene, or of a transiently transfected $1,25(OH)_2D_3$-sensitive reporter gene. The antiproliferative effects of $1,25(OH)_2D_3$ and EB1089 in SCC25 cells were analyzed by screening over 4,500 genes on two cDNA microarrays, yielding 38 upregulated targets, including adhesion molecules, growth factors, kinases and transcription factors. Genes encoding factors implicated in cell-cycle regulation were induced, including the growth arrest and DNA damage gene, gadd45α, and the serum- and glucocorticoid-inducible kinase gene, sgk. Induction of GADD45α protein in EB1089-treated cells was confirmed by western blotting. Moreover, while expression of proliferating cell nuclear antigen (PCNA) was reduced in EB1089-treated cells, coimmunoprecipitation studies revealed increased association between GADD45α and PCNA in treated cells, consistent with the capacity of GADD45α to stimulate DNA repair. While $1,25(OH)_2D_3$ and EB1089 modestly induced transcripts encoding the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, no changes in protein levels were observed, indicating that $p21^{WAF1/CIP1}$ induction does not contribute to the

antiproliferative effects of $1,25(OH)_2D_3$ and EB1089 in SCC cells. Finally, in partially resistant SCC9 cells, there was extensive loss of target gene regulation (10 of 10 genes tested), indicating that resistance arises from widespread loss of $1,25(OH)_2D_3$-dependent gene regulation in the presence of normal levels of functional VDRs.

Introduction

The active form of vitamin D3, 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) modulates gene expression by binding to the vitamin D_3 receptor (VDR), which is a member of the nuclear receptor family of transcriptional regulators. $1,25(OH)_2D_3$-bound VDR heterodimerizes with retinoid X receptors (RXRs) and binds to specific DNA sequences in target genes known as vitamin D_3 response elements (VDREs) (Mangelsdorf et al., 1995; Haussler et al., 1998). Apart from its well-characterized role in calcium homeostasis (Jones et al., 1998), $1,25(OH)_2D_3$ also inhibits growth and stimulates differentiation of cancer cells derived from a variety of tissues, including breast, prostate, colon, lung, endometrium, hematopoietic cells, and oral cavity (Liu et al., 1996; Zhuang and Bernstein, 1998; Hershberger et al., 1999; Campbell and Koeffler, 1997; Colston et al., 1992; VanWeelden et al., 1998; Hansen et al., 2000). A side chain analogue of $1,25(OH)_2D_3$, EB1089, caused apoptotic regression of MCF-7 breast carcinoma xenografts in nude mice (VanWeelden et al., 1998), and animal studies and early clinical testing have shown that therapeutic doses of EB1089 can be tolerated without inducing hypercalcemia (Hansen et al., 2000).

Analogues $1,25(OH)_2D_3$ are potential candidates for chemoprevention of squamous cell carcinomas (SCC) of the oral cavity, where formation of second primary carcinomas after surgical removal of tumors is a major concern (Vokes et al., 1993; Hong et al., 1994). Retinoids, such as 13-cis retinoic acid (13-cis RA; isotretinoin) have been used clinically in SCC chemoprevention (Hong et al., 1990). 13-cis RA functions by binding to retinoic acid receptors (RARs), which, like the VDR, are nuclear receptors and function as heterodimers with RXRs (1). However, SCC progression is associated with

reduced expression of RARs, particularly RARs β and γ, loss of retinoid-regulated differentiation markers, and resistance to the antiproliferative effects of retinoids (Hu et al., 1991;Crowe, 1998;Crowe, 1998; Lotan, 1994; Oridate et al., 1996; Lotan, 1997; McGregor et al., 1997).

Here, we have examined the effect of $1,25(OH)_2D_3$ and EB1089 on proliferation and target gene regulation of four human SCC lines, SCC4, SCC9, SCC15 and SCC25, which were derived from floor of mouth/base of tongue lesions (Hu et al., 1991). SCC25 cells express near normal levels of RARs β and γ, and retain retinoid regulation of keratin-19 (K-19) gene expression, whereas SCC4, SCC9 and SCC15 cells express reduced levels of RARγ, no RARβ, and have lost regulated K-19 expression (Hu et al., 1991). The SCC lines display differing sensitivities to $1,25(OH)_2D_3$ and EB1089. SCC25 cell growth was completely blocked by $1,25(OH)_2D_3$ and EB1089, while the other lines were partially resistant. We have identified 38 $1,25(OH)_2D_3$ target genes in SCC25 cells, which encode several components of signal transduction pathways. Our results indicate that the antiproliferative effects of $1,25(OH)_2D_3$ and its analogues are mediated by multiple downstream components. Moreover, resistance to $1,25(OH)_2D_3$ in SCC9 cells was accompanied by widespread loss of target gene regulation in spite of normal levels of functional VDRs.

Materials and Methods

Plasmids and reagents

The VDRE3-hsp68-lacZ reporter contains three VDREs (Ferrara et al., 1994), inserted upstream of minimal hsp68 promoter in the plasmid p610AZ (Kothary et al., 1994). The plasmid tk-LUC, contains a truncated Herpes Simplex Virus thymidine kinase promoter inserted upstream of a promoterless luciferase reporter gene in pXP1 (Nordeen, 1998). 1,25-dihydroxyvitamin D3 and EB1089 were kindly supplied by Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark). 13-cis RA was purchased from ICN. All hormones were dissolved in dimethylsulfoxide (DMSO), and stock solutions were stored in the dark at –20°C.

Tissue Culture.

The SCC lines, SCC4, SCC9, SCC15 and SCC25, obtained from the American Type Cultures Collection (ATCC, Rockville, MD), were cultured under recommended conditions. Effects of $1,25(OH)_2D_3$, EB1089, and 13-cis RA on cell growth were analyzed by seeding cells in 6-well plates at 15,000 cells/well in 2ml of culture medium containing charcoal-stripped serum. Media were changed after 24h to charcoal-stripped medium containing vehicle or ligand as indicated. Media were changed every 48h and fresh ligand added. Cells were harvested by washing with 2ml of phosphate buffered saline and incubation with 0.7ml of 0.25% trypsin-EDTA. Cell numbers were determined using a hemacytometer. Four grid sections were counted for each well and the results were averaged. All treatments were performed in triplicate.

Transient transfections.

SCC cells were grown to 60% confluency in 6-well plates in charcoal stripped medium, washed with 2ml of Opti-MEM I reduced serum media (GIBCO BRL, Burlington, Ontario), and cultured in 1ml of Opti-MEM I. Cells were transfected with 500ng of VDRE3-LacZ reporter plasmid, and 500ng of tk-LUC internal control using Lipofectin (GIBCO BRL, Burlington, Ontario) according to the manufacturer's protocol. After 18h media were replaced with charcoal stripped medium containing ligands as indicated. Cells were lysed 24h later using lysis buffer (Promega, Madison, WI) and β–galactosidase assays were performed as described (Tora et al., 1989). Transfections were performed in triplicate and standardized using the Luciferase Assay System with reporter lysis buffer (Promega, Madison, WI).

RNA isolation and Northern blotting.

Cells were grown in 100-mm dishes. Media were replaced with charcoal stripped medium containing ligand as indicated. Total RNA was extracted with TRIZOL (GIBCO BRL, Burlington, Ontario). PolyA+ RNAs were isolated using an Oligotex mRNA Kit (QIAGEN, Valencia, CA). One μg of polyA+ RNA was separated on a 1.0% agarose gel containing 6.3% formaldehyde, 20mM MOPS (pH 7.0), 15mM sodium acetate, and 1 mM EDTA. Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham, Baie d'Urfe, Quebec), which then was soaked in 3xSSC and 0.1% SDS at 50°C, and pre-hybridized at 42°C in 50mM phosphate buffer pH 6.5, 50% formamide, 5x SSC, 10% Denhardt's solution containing 250μg/ml sheared, denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled

cDNA probes. Membranes were washed 4 times in 2xSSC and 0.2% SDS for 5 min, 3 times in 0.1xSSC and 0.2% SDS for 30min at 50°C, dried, and autoradiographed. All blots were performed at least 3 times with independent preparations of RNA.

Reverse transcription-polymerase chain reaction (RT-PCR).

Ten micrograms of total RNA were subjected to oligo dT priming first strand cDNA synthesis by SuperScript II (Life Technologies, Burlington, Ontario). Twenty μl aliquots were diluted 5-fold with water. For RT-PCR analysis of p53 and p27 ^{kip1} mRNA, expression 1μl of RT reactions was analyzed by PCR amplification as follows: 30 s denaturation at 94°C, 45 s elongation at 72°C, and 30 s annealing starting at 60°C, down 1°C per cycle to 55°C, and continuing 20 cycles amplification (94°C for 30 s, 57.5°C for 30 s, 72°C for 45 s). Complementary DNAs for p53 and p27 ^{kip1} were amplified using 5' primer 5'-CAAGTCTGTGACTTGCACGTA-3' and 3' primer 5'-TTCTTGCGGAGATTCTCTTCC-3' for p53, and 5' primer 5'-CCGGAATTCATGTCAAACGTGCGAGTGTCT-3' and 3' primer 5'-CCGGAATTCTTACGTTTGACGTCTTCTGAGGC-3' for $p27^{kip1}$. For β-actin, 1 μl of RT reaction was subjected to 18 cycles amplification (95°C for 30 s, 56°C for 1min, 72°C for 25 s) using 5' primer 5'-GCTGTGCTATCCCTGTACGC-3' and 3' primer 5'-CCAATGGTGATGACCTGGC-3'. All of the above reactions were performed in 25 μL of 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-Cl (pH 9.0) using 2.5U of Taq DNA polymerase (Pharmacia, Baie d'Urfe, Quebec). PCR reactions were loaded on 2% agarose gel, transferred for Southern blotting to a nylon membrane (Hybond N+, Amersham, Baie d'Urfe, Quebec), and fixed by UV cross linker. The membrane was

soaked in 3x SSC and 0.1% SDS at 50°C, and pre-hybridized at 42°C in 50 mM-phosphate buffer pH 6.5, 5x SSC, 10% Denhardt's solution containing 250 μg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P end-labeled oligonucleotides 5'-CTACAAGCAGTCACAGCACAT-3' for p53, 5'-CTAACTCTGAGGACACGCATT-3' for $p27^{kip1}$, and 5'-CGAGAAGCTGTGCTACGTCG-3' for β-actin. Following hybridization, the membrane was washed four times in 2x SSC and 0.2% SDS for 5 min, three times in 0.1x SSC and 0.2% SDS for 30 min at 50°C, dried, and autoradiographed. All experiments were repeated at least three times.

Immunoprecipitation and western blotting.

After incubation with ligands, cells were washed twice with PBS and harvested by scraping in 1ml of PBS, and centrifuged at 4°C. The pellet was resuspended in 500μl of ice-cold lysis buffer (10mM Tris-HCl pH.8.0, 60mM KCl, 1mM EDTA, 1mM DTT, 0.5% NP40) containing protease inhibitor cocktail (Boehringer, Mannheim, Germany), incubated on ice for 10min. Lysates were centrifuged at 4°C (14,000rpm, 10min), and supernatants were recovered. For $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ immunoprecipitations, protein extracts (200μg) were immunoprecipitated at 4°C overnight with 3μg of F-5 and F-8 anti-$p21^{WAF1/CIP1}$ and -$p27^{KIP1}$ monoclonal antibodies, respectively (Santa Cruz Biotechnology Inc., Santa Cruz, CA) using 30μl of 50% slurry protein S-Sepharose (Amersham, Baie d'Urfe, Quebec). Beads were centrifuged, and pellets washed four times each with lysis buffer, and boiled for 3min in 2xSDS-polyacrylamide gel loading buffer. Immunoprecipitates were resolved on 20% SDS-polyacrylamide gels and analyzed by

western blotting with the same antibodies. Immunopreciptations of GADD45α and PCNA were performed with anti-GADD45α antibody 4T-27 or with anti-PCNA antibody PC-10 (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Immunoprecipitates were harvested, processed for western blotting as above and probed with anti-GADD45 antibody (H-165) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with anti-PCNA (PC-10) (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Western analysis of VDR expression was performed with 30μg of total cell protein resolved on a 15% SDS-polyacrylamide gel. VDRs were probed with 800ng of a rabbit polyclonal anti-VDR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Proteins were detected by enhanced chemiluminescence (ECL; NEN Life Scientific Products, Inc., Boston, MA).

Flow cytometry and TUNEL assays.

SCC25 cells treated with 100nM EB1089 or DMSO for 72h were harvested with 0.25% trypsin-EDTA, fixed with 70% ethanol for 1h at 4°C, treated with 200μg/ml RNase A for 30 min, stained with 5μg/ml propidium iodide for DNA, and analyzed for cell cycle status by flow cytometry (Becton Dickinson, Franklin Lakes, NJ). Experiments were repeated 3 times. TUNEL assays were performed using an Apoptag kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, after incubation with vehicle or ligand, cells were fixed for 15min in 1% paraformaldehyde, washed twice with PBS and stored in 70% ethanol at –20°C. Cells (100μl) were then incubated for 30min at 37°C with terminal deoxynucleotidyl transferase and digoxigenin-dUTP. After 2 washes with 0.1% Triton X-100 in PBS, cells were incubated with fluorescein-conjugated

antidigoxigenin antibody for 30min at room temperature. Following two washes with 0.1% Triton X-100 in PBS cells were treated with RNase A and processed for flow cytometry as above

Array screening.

SCC25 were cells treated for 24h with DMSO or EB1089 (100nM). Atlas™ cDNA Expression Arrays containing 588 genes (CLONTECH, Palo Alto, CA) were screened with 100ng of polyA+ RNA. GF211 Named Human Genes arrays containing over 4,000 genes (Research Genetics Inc., Huntsville, Alabama) were probed with 1μg of total RNA. Probe preparation and array screening were carried out according to manufacturers' instructions. Duplicate Atlas arrays were screened twice each with probe derived from control or treated cells and arrays were visualized by autoradiography. Genes that appeared reproducibly regulated were studied by Northern analysis. GF211 filters were probed three times each with probe derived from control cells and EB1089-treated cells, and visualized by phosphorimaging. Relative expression levels were compared using Pathways software (Research Genetics Inc.). Genes that were upregulated at least 1.5-fold in nine sets of cross comparisons were retained. Of these, 10 were further analyzed by Northern analysis using cDNA probes from Research Genetics Inc.

Results

Effect of $1,25(OH)_2D_3$ and EB1089 on growth of SCC lines.

The growth inhibitory effects of $1,25(OH)_2D_3$, EB1089 and 13-cis RA were evaluated in human lines SCC4, SCC9, SCC15, and SCC25, derived from squamous cell carcinomas of the oral cavity. The four lines displayed different sensitivities to $1,25(OH)_2D_3$ or EB1089 (Fig. 2.1). Over 10 days, SCC25 cell growth was completely inhibited by 100 nM $1,25(OH)_2D_3$, and 10-100 nM EB1089 (Figs. 2.1A and B), while SCC4, SCC9 and SCC15 cells displayed partial resistance to both compounds (Figs. 2.1D, E, G, H, J, K). Similarly, SCC25 cell growth was strongly inhibited by 13-cis RA (100nM; Fig. 2.1C), whereas growth of SCC4, SCC9 and SCC15 cells was partially resistant (Figs. 2.1F, I, L). Flow cytometric analysis showed that treatment of SCC25 cells with 100nM EB1089 for 72h reduced the number of cells in S phase by 2.5-fold and significantly increased the percentage in G0/G1 (Fig. 2.2A). No evidence for DNA fragmentation was observed by TUNEL assays under these conditions or over extended periods (Fig. 2.2B).

Resistance to $1,25(OH)_2D_3$ in SCC4, SCC9 and SCC15 cells is not accompanied by loss of expression of functional VDR.

Given that resistant to 13-cis RA correlated with lost or reduced expression of RARs β and γ, respectively (Hu et al., 1991), it was of interest to examine the levels of functional VDR in SCC cells. Northern and western blots showed that VDR transcript and protein levels were essentially identical in all four lines (Figs. 2.3A and B). Similarly, no evidence was found for loss of expression of the two major retinoid X receptors

Figure 2.1

Dose-dependent effects of 1,25(OH)$_2$D$_3$, EB1089, and 13-cis RA on proliferation of SCC lines in culture.

SCC lines were treated with 1, 10 or 100nM 1,25(OH)$_2$D$_3$ (A, D, G, J), 0.1, 1, 10 or 100nM EB1089 (B, E, H, K), and 1, 10 or 100nM 13-cis RA (C, F, I, L). Media was changed and fresh ligand added every 2 days over the 10-day period of the experiment. Each point represents the result obtained from triplicate wells (see Methods for details).

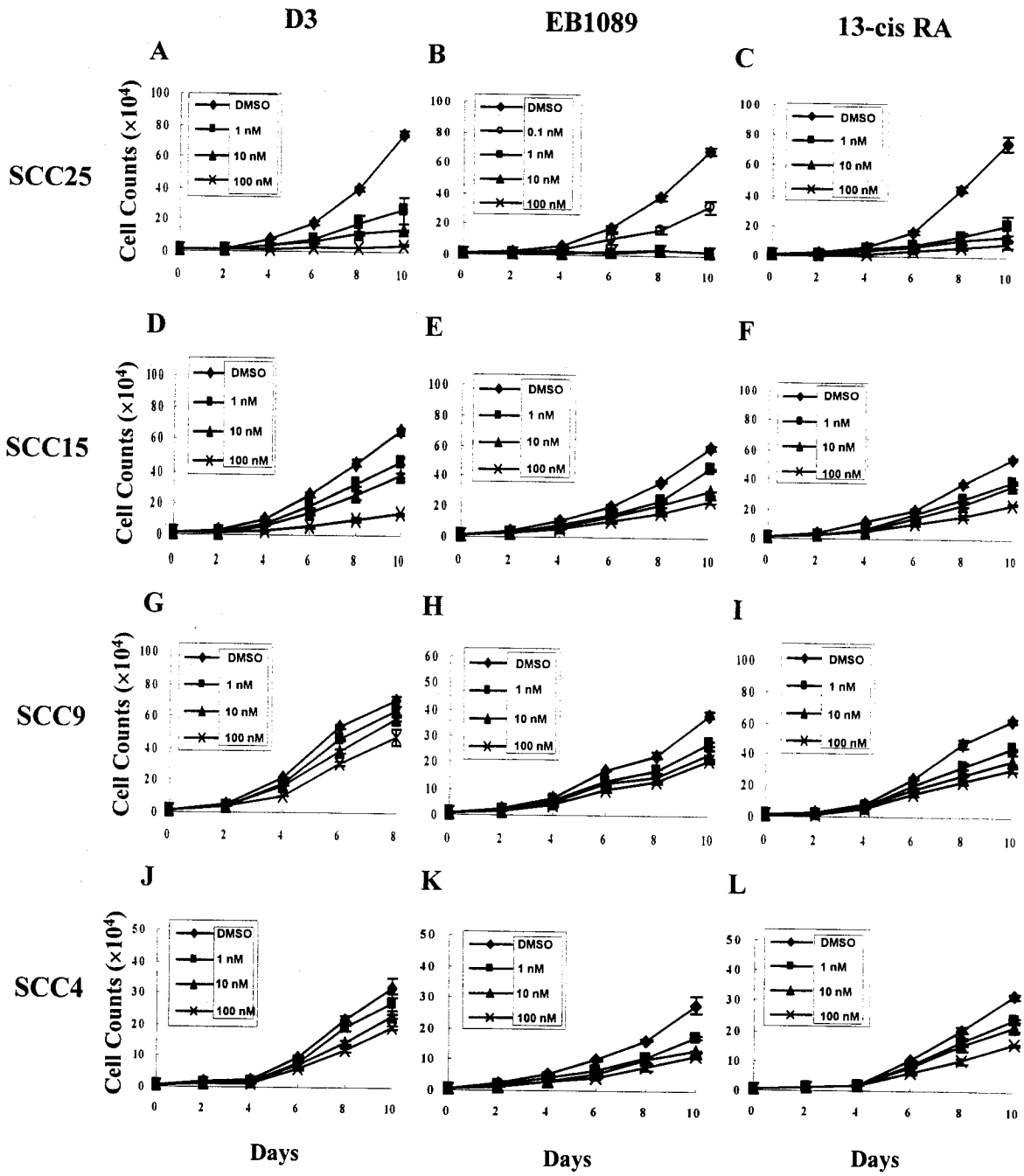
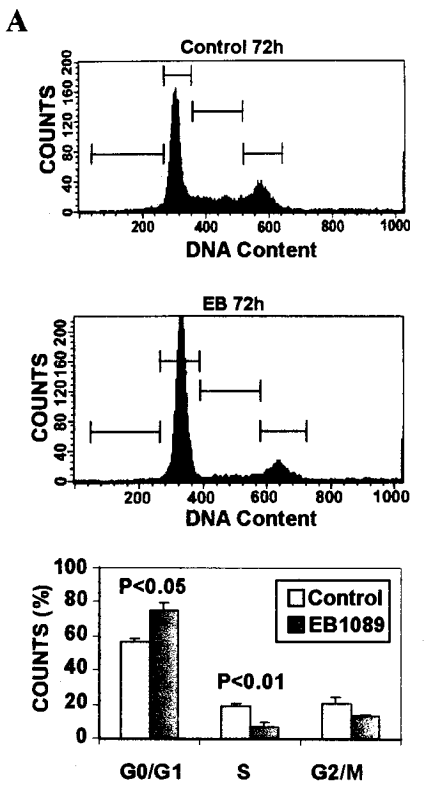
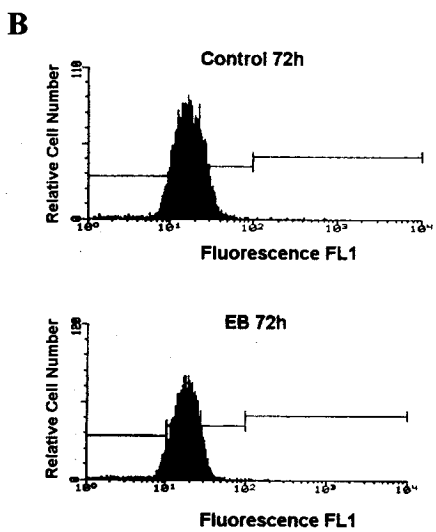

D3
EB1089
13-cis RA
A
B
C
D
E
F
G
H
I
J
K
L
SCC25
SCC15
SCC9
SCC4
Cell Counts (×10⁴)
Days
DMSO
0.1 nM
1 nM
10 nM
100 nM

Figure 2.2

Flow cytometric analysis and TUNEL assay of control or EB1089-treated SCC25 cells.

A. SCC25 cells were treated with vehicle (SCC25 cont) or EB1089 (SCC25 EB) for 72h. A histogram of fractions of cells in G0/G1, S, or G2 from 3 independent experiments is presented. Statistical significance was determined using a student t test. B. Representative histograms of 3 experiments assessing 3'OH end-labeling characteristic of apoptotic cells (TUNEL assay). Control cells and cells treated for 72h with 100nM EB1089 display minimal DNA fragmentation.


A
Control 72h
COUNTS
0 40 80 120 160 200
200 400 600 800 1000
DNA Content
EB 72h
COUNTS
0 40 80 120 160 200
200 400 600 800 1000
DNA Content
COUNTS (%)
100 80 60 40 20 0
P<0.05
P<0.01
Control
EB1089
G0/G1
S
G2/M


B
Control 72h
Relative Cell Number
Fluorescence FL1
EB 72h
Relative Cell Number
Fluorescence FL1

expressed in SCC, RXRα and RXRβ (data not shown). VDR function was tested by transient transfection of a $1,25(OH)_2D_3$-sensitive reporter-promoter plasmid containing a bacterial lacZ gene under control of a synthetic promoter containing three VDREs (Ferrara et al., 1994). High levels of $1,25(OH)_2D_3$-inducible β–galactosidase activity were detected in all cell extracts (Fig. 2.4A), suggesting that the lines expressed similar levels of functional VDRs. Both $1,25(OH)_2D_3$ and EB1089 induced similar levels of expression of the endogenous 24-hydroxylase (24-OHase) gene (Fig. 2.4B), whose promoter contains VDREs (Zou et al., 1997). Moreover, EB1089 induced 24-OHase expression with essentially identical potencies in $1,25(OH)_2D_3$-sensitive SCC25 cells and the partially resistant lines SCC4 and SCC9 (Fig. 2.4C). Taken together, the results of Fig.s 2.3 and 2.4 suggest that resistance to $1,25(OH)_2D_3$ does not arise through loss of expression of functional VDRs.

Effects of $1,25(OH)_2D_3$ and EB1089 on cell cycle regulators in SCC25 cells.

We were interested in analyzing the mechanisms underlying the antiproliferative effects of $1,25(OH)_2D_3$ and EB1089 in SCC25 cells. Previous work has shown that $1,25(OH)_2D_3$ rapidly (4h) and strongly stimulated expression of the cyclin-dependent kinase inhibitor genes $p21^{waf1/cip1}$ and $p27^{kip1}$ in myeloid leukemia cells under conditions where it induced differentiation and inhibited cell growth (Liu et al., 1996; Munker et al., 1996). However, the magnitude of the effect of $1,25(OH)_2D_3$ on $p21^{waf1/cip1}$ expression varies widely in different cell lines (Liu et al., 1996; Hershberger et al., 1999; Munker et al., 1996; Zhuang and Bernstein, 1998; Kawa et al., 1997). We found that $1,25(OH)_2D_3$- or EB1089-dependent induction of $p21^{waf1/cip1}$ transcripts in SCC25 cells was gradual and

Figure 2.3

Expression of VDR transcripts and protein in SCC lines.

A. Northern analyses are presented of transcripts encoding the VDR in SCC lines, along with GAPDH controls. B. Western blots are presented of VDR and β-actin protein levels in SCC lines.

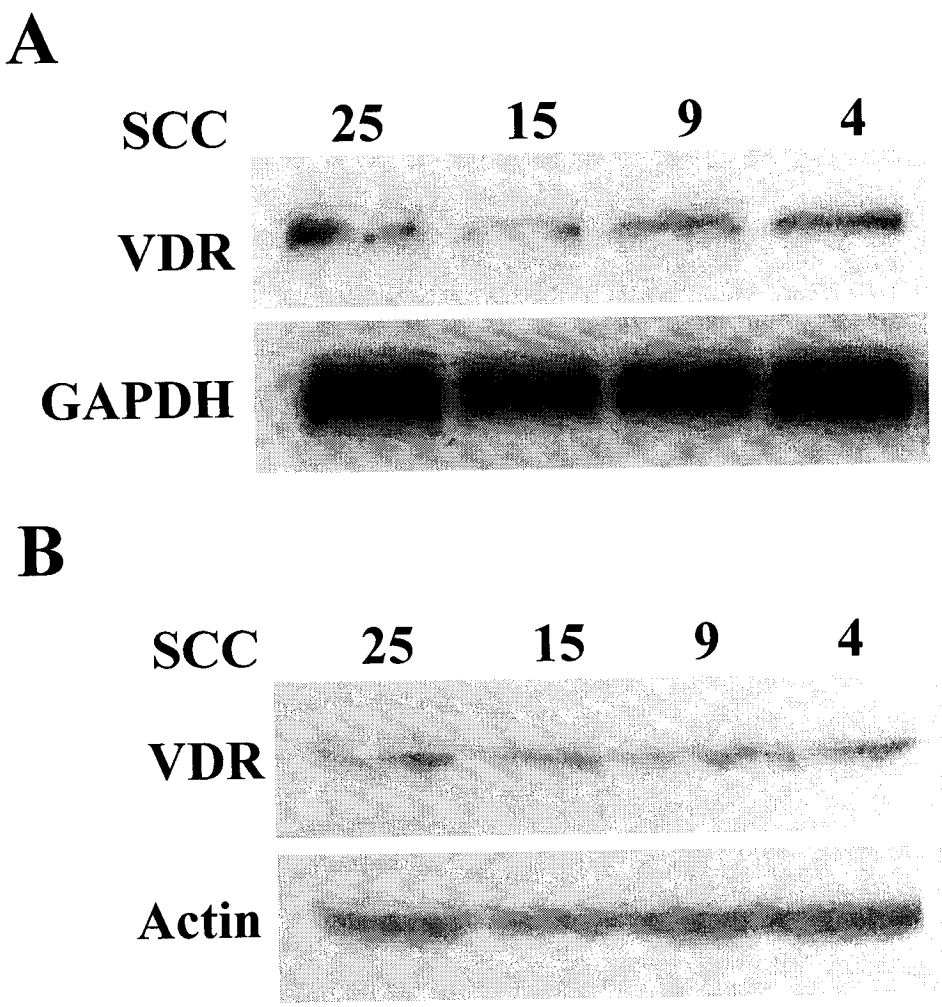

A
SCC
25
15
9
4
VDR
GAPDH
B
SCC
25
15
9
4
VDR
Actin

Figure 2.4

Assessment of VDR function in SCC lines.

A. Cells were transfected with the $1,25(OH)_2D_3$ -sensitive reporter plasmid VDRE3-hsp68-lacZ and treated with vehicle or 10nM $1,25(OH)_2D_3$ for 24h (see Materials and Methods for details). Data are presented as fold induction of lacZ expression observed in the presence of $1,25(OH)_2D_3$. B. Induction of endogenous 24-OHase gene expression by $1,25(OH)_2D_3$ or EB1089 in SCC lines. Northern blots of total RNA extracted from cells treated for 24h with vehicle (-), $1,25(OH)_2D_3$ -, or EB1089-treated cells are presented. C. Dose-dependence of 24-hydroxylase induction in $1,25(OH)_2D_3$ -sensitive SCC25 cells and partially resistant SCC4 and SCC9 cells. Northern blots of 24-OHase and β-actin controls are presented above, along with the normalized results of densitometric scanning of the 24-OHase blots below.

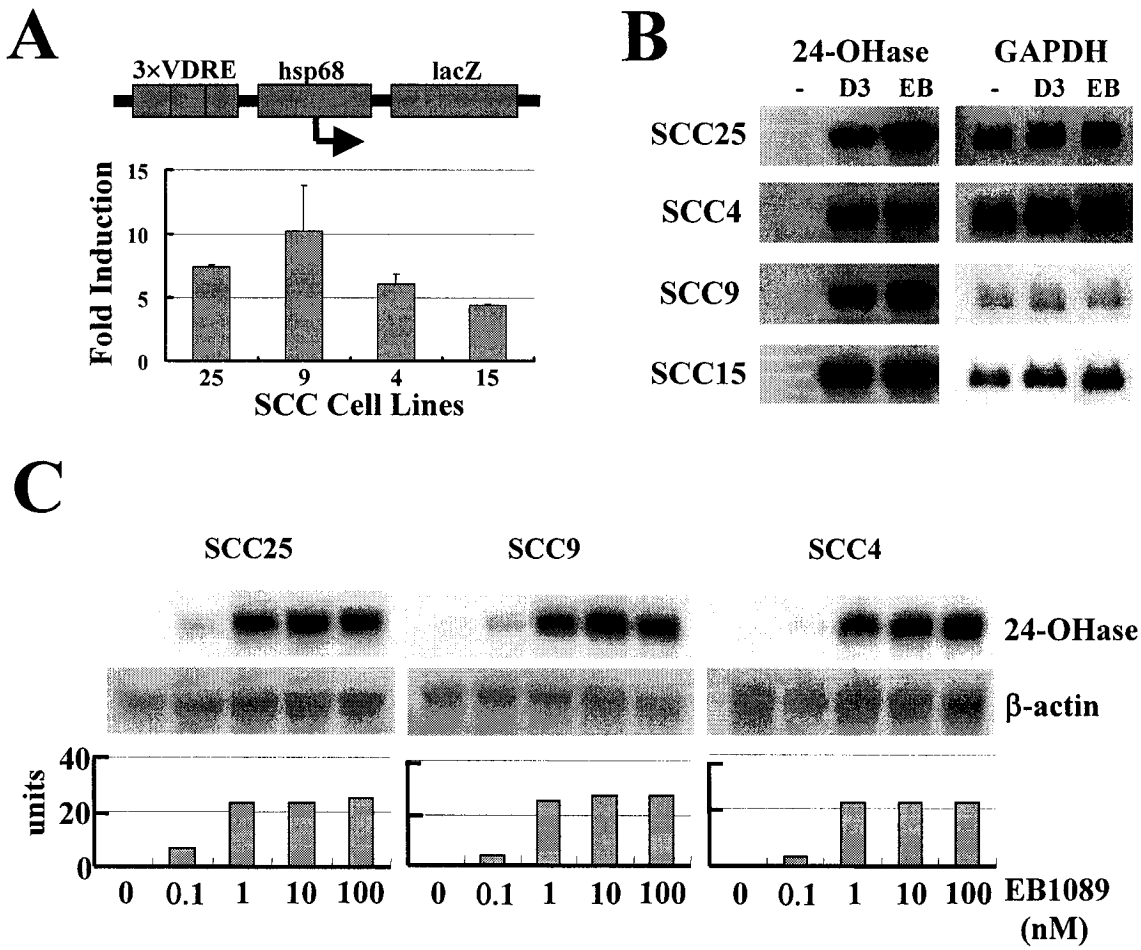
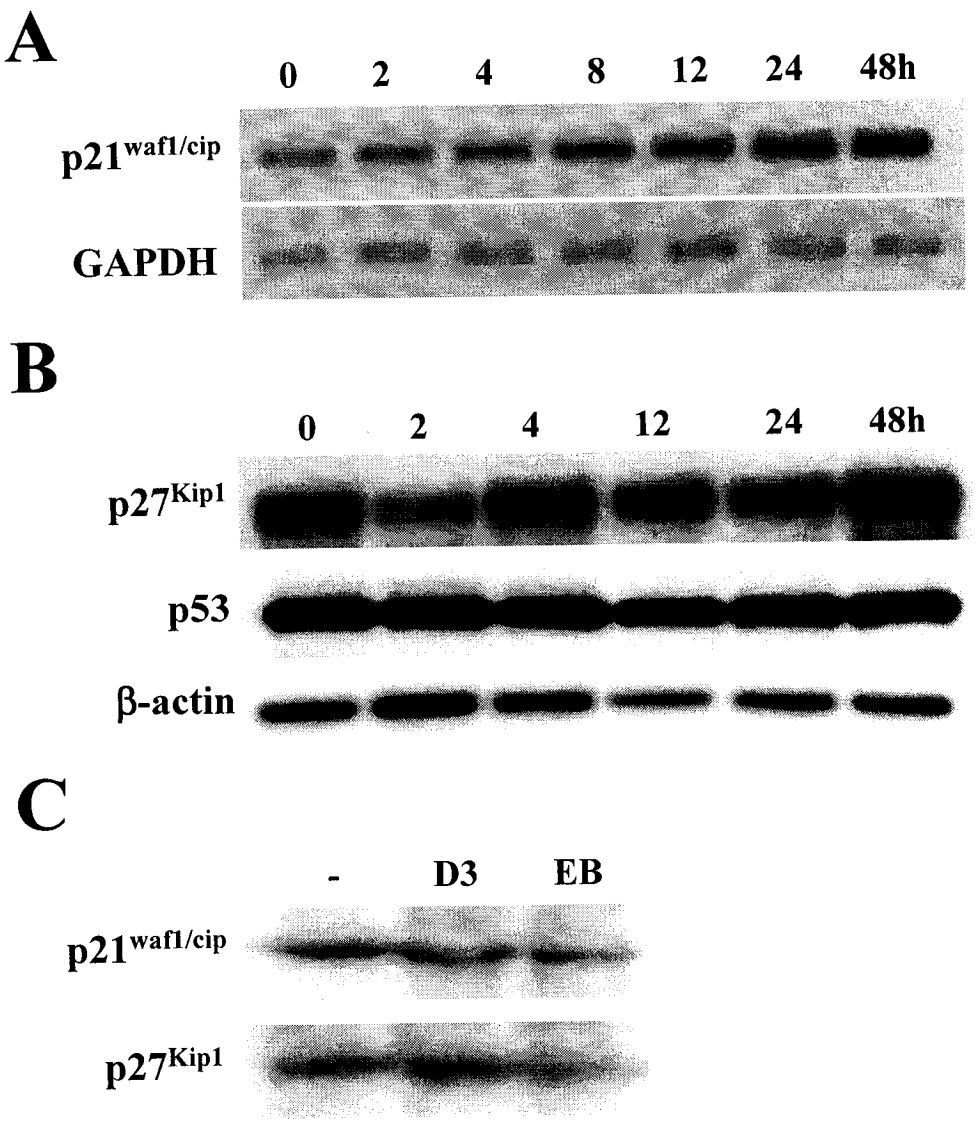

A
3×VDRE
hsp68
lacZ
Fold Induction
15
10
5
0
25
9
4
15
SCC Cell Lines
B
24-OHase
GAPDH
-
D3
EB
-
D3
EB
SCC25
SCC4
SCC9
SCC15
C
SCC25
SCC9
SCC4
24-OHase
β-actin
units
40
20
0
0
0.1
1
10
100
0
0.1
1
10
100
0
0.1
1
10
100
EB1089
(nM)

Figure 2.5

EB1089-inducible expression of p21waf1/cip1, but not p27kip1 or p53 in SCC25 cells.

A. The effect of EB1089 on expression in SCC25 cells of p21waf1/cip1and a GAPDH control were analyzed by was analyzed by Northern blotting of 20mg of total RNA from cells treated with 10nM $1,25(OH)_2D_3$ for the times indicated. B. The effect of EB1089 on expression in SCC25 cells of p27kip1 and p53 along with a β-actin control was analyzed by RT/PCR. Amplified products were probed with 32P-labeled internal oligonucleotides as detailed in Materials and Methods. C. Western blotting of immunoprecipitates of p21WAF1/CIP1 and p27KIP1 from SCC25 cells treated for 48h with vehicle (-), or 100nM $1,25(OH)_2D_3$ (D3) or EB1089 (EB).


A
0
2
4
8
12
24
48h
p21waf1/cip
GAPDH
B
0
2
4
12
24
48h
p27Kip1
p53
β-actin
C
-
D3
EB
p21waf1/cip
p27Kip1

modest (Fig 2.5A and data not shown), whereas no effect was observed on expression of $p27^{kip1}$ or p53 mRNA levels (Fig. 2.5B). The modest effect of $1,25(OH)_2D_3$ and EB1089 on $p21^{waf1/cip1}$ mRNA levels did not give rise to significant changes in $p21^{waf1/cip1}$ protein, however. In addition, no effect of $1,25(OH)_2D_3$ or EB1089 was observed on $p27^{kip1}$ proteins levels (Fig. 2.5C).

Identification of target genes of $1,25(OH)_2D_3$ and EB1089 by screening of cDNA microarrays.

We screened cDNA microarrays for novel target genes of $1,25(OH)_2D_3$ and EB1089 in SCC25 cells to identify factors mediating their antiproliferative effects. Over 4,500 genes on two different gene arrays (Clontech, Atlas array, 588 genes; Research Genetics Named Genes filter, 4,000+ genes) were screened with probes derived from vehicle-treated cells or cells treated with EB1089 for 24h. Previous work has shown that there is considerable variation in gene expression levels associated with screening gene arrays (Audic and Claverie, 1997;Schuchhardt et al., 1997; Lee et al., 2000; Bebbarth et al., 2000). Arrays were therefore screened multiple times and only reproducibly regulated genes were retained. Two rounds of screening of Atlas arrays yielded 10 candidate genes, of which 6 were revealed by Northern blotting to be regulated by $1,25(OH)_2D_3$ and EB1089 (Fig. 2.6A, and data not shown; Table 2.1). In addition to $p21^{waf1/cip1}$ (not shown), these included novel target genes amphiregulin, a member of the epidermal growth factor family, the transcription factor fos-related antigen-1 (fra-1), the growth arrest and DNA damage (gadd45α) gene, and integrin α7B. We also found that the vascular endothelial growth factor (VEGF), which has been shown to be a $1,25(OH)_2D_3$

Figure 2.6

Northern analysis of EB1089-regulated target gene expression.

A. Northern analyses of target genes identified using a Clontech Atlas array. Transcripts expressed in SCC25 cells encoding amphiregulin (amphireg.), GADD45a, FRA-1, integrin α7B and VEGF are shown. Cells were treated with vehicle (left lane) or 10nM EB1089 (right lane) for 24h, and 1 mg of poly A+ RNA was loaded on each lane. B. Northern analyses, performed as in A, of target genes identified using a Research Genetics gene filter, as follows: GAP SH3 BP, GAP SH3 binding protein; STAT3; UVRAG, ultraviolet resistance-associated gene; calmodulin; ERM BPP50, ezrin-radixin-meoisin binding phosphorprotein-50; ARP3, actin-related protein 3; OTK27; RAB-1A, ras-related protein 1A; SGK, serum- and glucocorticoid-inducible kinase; Retinobl BP3, retinoblastoma binding protein 3. C. Northern analysis of target gene regulation in SCC9 cells. Cells were treated and blots were performed as in A. Note that GAPDH controls were performed for blots in A-C and showed no significant variations (not shown).

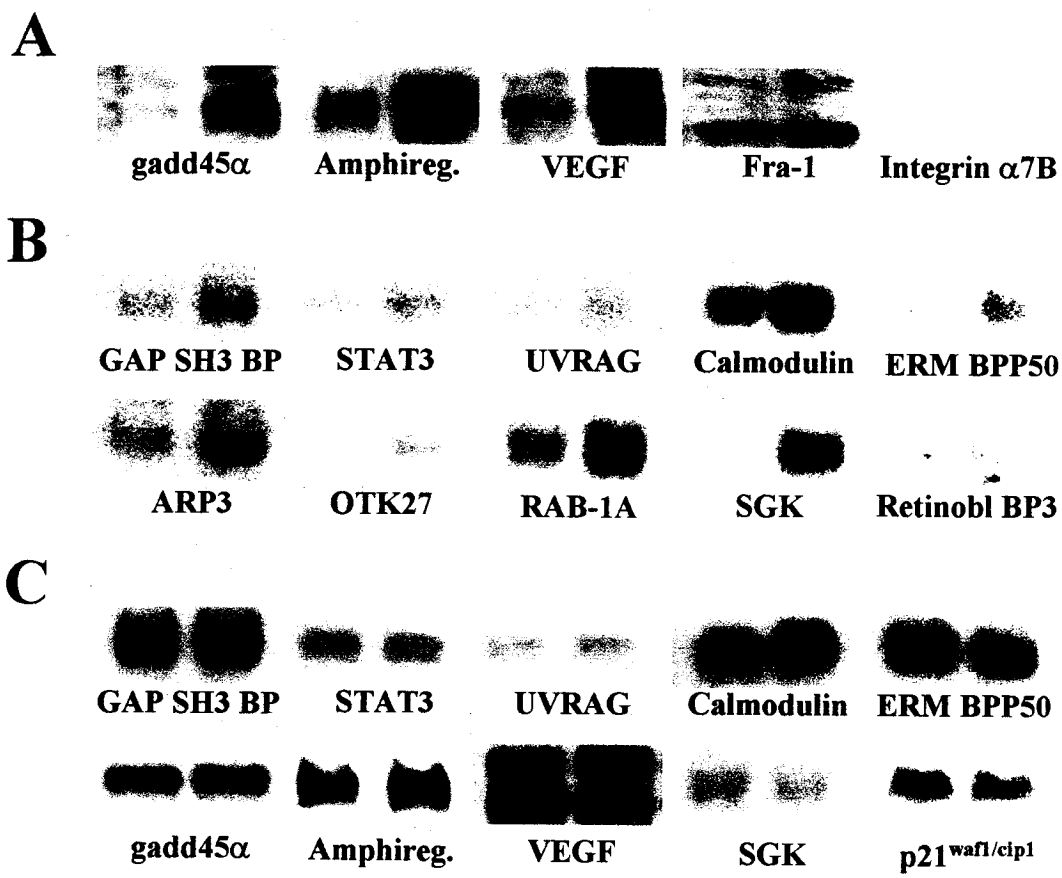

A
gadd45α
Amphireg.
VEGF
Fra-1
Integrin α7B
B
GAP SH3 BP
STAT3
UVRAG
Calmodulin
ERM BPP50
ARP3
OTK27
RAB-1A
SGK
Retinobl BP3
C
GAP SH3 BP
STAT3
UVRAG
Calmodulin
ERM BPP50
gadd45α
Amphireg.
VEGF
SGK
p21wafl/cipl

Table 2.1

Summary of target genes identified using cDNA microarrays.

EB1089-dependent regulation of genes listed in italics has been verified by Northern blotting. RG, Research Genetics Gene Filter; C, Clontech Atlas Array.

Table 1. Summary of target genes identified using cDNA microarrays.

Function	Gene	Array	Fold Induction (-/+ S.E.M.)	RG cDNA id	UniGene Accession No.
Cell Adhesion/ Extracellular Matrix	*Integrin α7B*	C	NA	NA	AF032108
	GALECTIN-2	RG	2.75 (0.77)	1472743	AA872397
	Ninjurin1	RG	2.45 (0.49)	744917	AA625806
Growth Factors/ Receptors	*Amphiregulin*	C	NA	NA	M30704
	VEGFα	C	NA	NA	AF022375
	Macrophage stimulating 1 (hepatocyte growth factor-like)	RG	2.62 (0.72)	72395	T51539
	Platelet-derived growth factor receptor, beta polypeptide	RG	2.35 (0.58)	40643	R56211
Cytoskeletal Proteins	*Ezrin-radixin-moesin binding phosphoprotein-50*	RG	3.93 (1.70)	773286	AA425299
	Actin-related protein (ARP3)	RG	3.05 (0.90)	593251	AA164562
	Sarcospan-2 (SPN2)	RG	2.78 (0.60)	1049330	AA620859
	Keratin 4	RG	1.95 (0.17)	1035889	AA629189
Signal Transduction	*GADD45α*	C	NA	NA	HUMGADD45
	Calmodulin	RG	5.16 (2.50)	321389	W44860
	Ras-related Protein (RAB-1A)	RG	3.95 (1.76)	293715	N69689
	GAP SH3 Binding Protein	RG	3.21 (1.21)	564756	AA129537
	SGK	RG	3.05 (0.90)	840776	AA486082
	Protein kinase (MLK-3)	RG	2.72 (0.91)	146868	R80779
	Retinoblastoma Binding Protein	RG	2.48 (0.51)	768260	AA424950
	Aplysia ras-related homolog 12	RG	2.50 (0.74)	897158	AA676955
	LDL-receptor related protein	RG	1.98 (0.28)	810551	AA64566
	Melanoma Differentiation-Associated gene (MDA-7)	RG	1.60 (0.25)	712049	AA281635
Transcription Factors/ Nuclear Proteins	*Fra-1*	C	NA	NA	HSFRA1M
	OTK27	RG	3.44 (1.20)	950709	AA608583
	Activating Protein 4 (AP-4)	RG	3.28 (1.04)	713839	AA284693
	STAT 3 (acute response factor)	RG	2.95 (1.02)	725746	AA399410
	IL-4 Stat	RG	2.65 (0.74)	85541	T72202
	Cytokine inducible nuclear protein	RG	2.44 (0.55)	840683	AA488072
	GCN5-like 1	RG	1.81 (0.20)	230218	H94857
Other	SCP-1	RG	3.48 (1.76)	1031799	AA609655
	HsPex13p	RG	3.28 (1.24)	128783	R16849
	T245 protein (T245)	RG	2.92 (0.81)	252382	H87106
	IAI.3B	RG	2.86 (0.59)	882511	AA676470
	Enigma gene	RG	2.67 (0.57)	502682	AA127096
	H.sapiens PAP	RG	2.54 (0.52)	511066	AA100296
	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	RG	2.52 (0.50)	811927	AA454668
	PM5 protein	RG	2.50 (0.56)	884673	AA629923
	UV Radiation Resistance Associated Gene	RG	2.48 (0.53)	823901	AA490501

EB1089-dependent regulation of genes listed in italics has been verified by Northern blotting. RG, Research Genetics Gene Filter; C, Clontech Atlas Array.

target gene in osteoblast-like cells (Wang et al., 1996; Wang et al., 1997), was regulated by EB1089 in SCC25 cells.

Initial analysis of Research Genetics gene filters screened with duplicate preparations of probe from vehicle-treated cells revealed a substantial number of differentially expressed genes (data not shown), which likely corresponded to random fluctuations in gene expression observed in expression profiling (Audic and Claverie, 1997;Schuchhardt et al., 1997; Lee et al., 2000; Bebbarth et al., 2000). Therefore, filters were screened three times each with probe from independent preparations of vehicle- and EB1089-treated cells, generating nine sets of cross comparisons. Genes that were reproducibly regulated at least 1.5-fold in all comparisons were conserved. This yielded 32 additional upregulated genes representing several different classes of proteins (Table 2.1). Screening under these conditions did not reveal any reproducibly downregulated genes. Upregulated genes included calmodulin, which has previously been shown to be a $1,25(OH)_2D_3$ target gene (Drittanti et al., 1990). Northern blotting, used to further test expression of 10 of these genes, revealed EB1089-stimulated expression in all cases (Fig. 2.6B), indicating that the data in Table 2.1 is highly reliable. Most of the genes retained from phosphorimager analysis of the Research Genetics arrays were upregulated 2 to 4-fold (Table 2.1). This range of induction agrees well with that of upregulated targets identified in a similar screen of thyroid hormone regulated genes (Papathanasiou et al., 1991).

Broad but selective loss of target gene expressionin $1,25(OH)_2D_3$-resistant SCC lines.

Given the resistance of SCC9 cells to the inhibitory effects of $1,25(OH)_2D_3$ and EB1089, we analyzed the regulation of target genes in these cells. Remarkably, in spite of

apparently normal induction of 24-OHase expression (Fig. 2.4), regulation of all of the target genes tested in SCC9 cells was either lost, or in the cases of calmodulin and GAP SH3 binding protein, attenuated (Fig. 2.6C). These results provide a strong correlation between increased resistance to the antiproliferative effects of $1,25(OH)_2D_3$, and a broad but selective loss of $1,25(OH)_2D_3$ target gene regulation in the presence of apparently normal levels of functional VDR.

EB1089 treatment induces expression of GADD45α protein and enhances formation of GADD45α-proliferating cell nuclear antigen (PCNA) complexes.

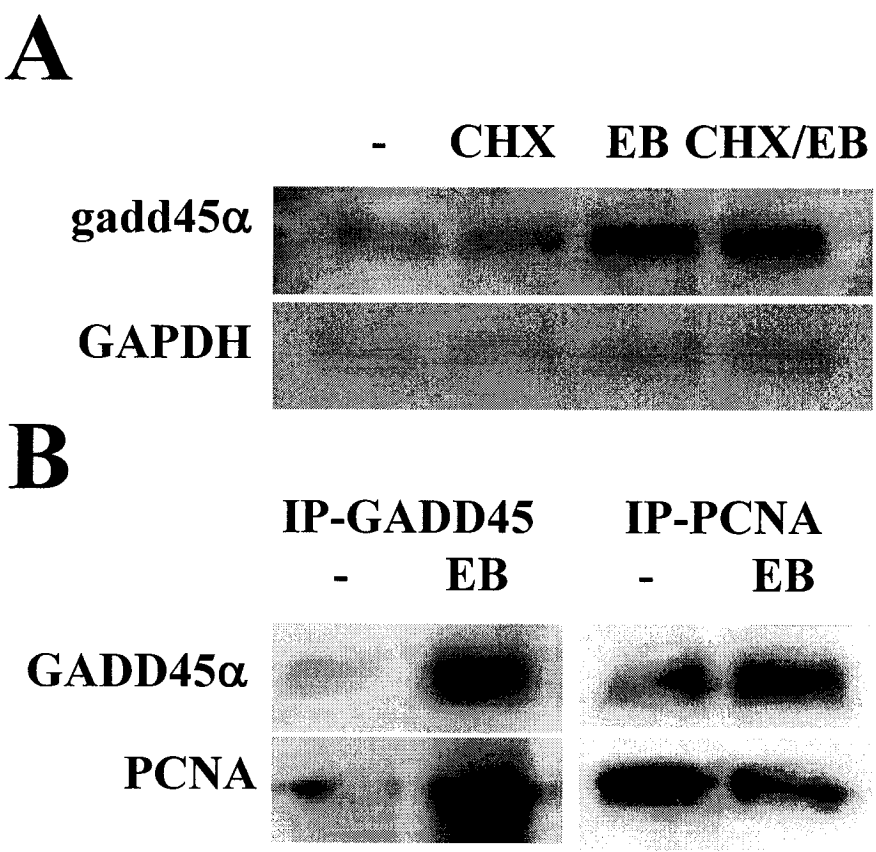
One of the more intriguing genes identified from the array screening presented above was gadd45α (Fig. 2.6, Table 2.1). Gadd45α is a p53 target gene induced by a variety of agents that damage DNA and arrest cell growth (Papathanasiou et al., 1991; Zhan et al., 1994; Hollander et al., 1999; Smith et al., 1994), and overexpression of GADD45α inhibits cell proliferation (Zhan et al., 1994). Ablation of the gadd45α gene provided evidence that GADD45α functions to maintain global genomic stability (Hollander et al., 1999). Peak expression of GADD45α occurs in G1. DNA repair is enhanced at the G1/S checkpoint and several studies have suggested that GADD45α enhances DNA repair, at least in part, through its interaction with proliferating cell nuclear antigen PCNA (Smith et al., 1994; Chen et al., 1995; Hall et al., 1995).

Induction of gadd45α mRNA by EB1089 was only partially blocked by protein synthesis inhibitor cycloheximide (Fig. 2.7A, and data not shown), indicating that the effect of EB1089 is at least partially direct. In related studies, we found no effect of cycloheximide on induction of gadd45α transcripts by EB1089 in the mouse SCC line

Figure 2.7

Induction of gadd45a expression and enhanced formation of GADD45a-PCNA complexes in EB1089-treated SCC25 cells.

A. The effect of 200nM cycloheximide (CHX) on induction of gadd45a expression by 100nM EB1089 (EB) was analyzed by Northern blotting. SCC25 cells were treated for 48h with vehicle (-), cycloheximide or EB1089 as indicated. (B) Induction GADD45a protein and association of GADD45a with PCNA was assessed by reciprocal coimmunoprecipitation of extracts of SCC25 cells treated for 48h with 100nM EB1089 (EB) using either anti-GADD45a antibody (left-hand panel) or anti-PCNA antibody (right-hand panel) followed by western blotting (see materials and Methods for details).


A
- CHX EB CHX/EB
gadd45α
GAPDH
B
IP-GADD45
- EB
IP-PCNA
- EB
GADD45α
PCNA

AT-84 (Prudencio et al., 2001). Immunoprecipitations from control and treated SCC25 cells revealed that EB1089 induced expression of GADD45α protein (Fig. 2.7B), consistent with its effects on gadd45α mRNA levels. Previous studies have demonstrated that γ and UV irradiation induce GADD45α and enhance its interaction with PCNA (Smith et al., 1994; Chen et al., 1995). It was therefore of interest to determine whether a similar interaction was induced by EB1089, which is not a DNA damaging agent. While EB1089 treatment of SCC25 cells consistently reduced expression of PCNA protein (Fig. 2.7B and data not shown), reciprocal coimmunoprecipitations revealed an increased association between PCNA and GADD45α in EB1089-treated cells (Fig. 2.7B). Thus, 1α,25$(OH)_2D_3$ analogue EB1089 induces expression of GADD45α, leading to increased formation of GADD45α-PCNA complexes. Taken together, our results suggest that induction of GADD45α contributes to the growth inhibitory effects of 1,25$(OH)_2D_3$ and EB1089 in SCC25 cells.

Discussion

The results presented above show that $1,25(OH)_2D_3$ and EB1089 were as or more potent, respectively, than 13-cis RA in inhibiting growth of SCC25 cells in culture. SCC4, SCC9 and SCC15 cells were partially resistant to 13-cis RA and to $1,25(OH)_2D_3$ and EB1089 (Fig. 2.1), raising the possibility that a common underlying mechanism of resistance. Expression of RARs β and γ is lost or reduced, respectively, in SCC4, SCC9 and SCC15 cells (Hu et al., 1991). However, no evidence was found for loss of VDR expression or function in these lines. No substantial differences were observed in induction endogenous 24-hydroxylase gene expression, whose transcription is controlled by a VDRE-containing promoter (Zou et al., 1997), or of a transiently transfected VDRE3-hsp68/lacZ reporter plasmid. This is consistent with other findings suggesting that VDR levels vary little among SCC lines, including SCC4 (Ratnam et al.,1996; Xie and Bikle, 1998). Our results showed that VDRs expressed in all four lines studied retained the capacity to activate transcription from VDRE-containing promoters. We have also characterized a mouse SCC line, AT-84, which is highly sensitive to $1,25(OH)_2D_3$ and EB1089 but resistant to the growth inhibitory effects of retinoids (Akutsu et al., 2001), showing that resistance to $1,25(OH)_2D_3$ and retinoids is not necessarily coupled.

Several results suggest that many factors contribute to the growth inhibitory effects of $1,25(OH)_2D_3$ in a cell-specific manner. Transcripts encoding the cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ were strongly and rapidly upregulated by $1,25(OH)_2D_3$ in myeloid leukemia cells, and forced expression of $p21^{waf1/cip1}$ induced myeloid cell

differentiation (Liu et al., 1996; Munker et al., 1996). Moreover, a VDRE that functioned in U937 cells was identified in the p21 promoter (Liu et al., 1996). However, the effect of $1,25(OH)_2D_3$ on $p21^{waf1/cip1}$ and $p27^{kip1}$ expression is highly cell-specific. The induction of $p21^{waf1/cip1}$ mRNA by EB1089 in SCC25 cells was gradual and modest, but no effect was observed on protein levels (Fig. 2.5). $1,25(OH)_2D_3$ treatment modestly increased $p21^{waf1/cip1}$ protein in LNCaP prostate cancer cells (Zhuang and Bernstein, 1998). However, no significant effect on transcript levels and no $1,25(OH)_2D_3$-dependent induction of the $p21^{waf1/cip1}$ promoter was observed in gene transfer experiments in LNCaP cells (Zhuang and Bernstein, 1998). Hershberger et al (Hershberge et al., 1999) found that $1,25(OH)_2D_3$ repressed $p21^{waf1/cip1}$ expression in the mouse SCCVII/SF line, and we have observed a similar repression of $p21^{waf1/cip1}$ transcripts and protein in mouse SCC line AT-84 (Prudencio et al., 2001).

The lack of induction of cyclin-dependent kinase inhibitors in $1,25(OH)_2D_3$- or EB1089-treated SCC25 cells led us to screen gene arrays to identify other regulated genes in SCC25 cells. A total of 38 target genes, including $p21^{waf1/cip1}$, were identified in two screens of more than 4,500 genes (Table 2.1). The 32 targets identified on the Research Genetics filter were retained after 9 sets of cross comparisons of data derived from screening with probe derived from vehicle- and EB1089-treated SCC25 cells, using a minimum induction of 1.5-fold as a cut-off. We confirmed that 10 of 10 candidates analyzed by Northern blotting showed $1,25(OH)_2D_3$-regulated expression (Fig. 2.6), indicating that the data obtained from the array screening is highly reliable. Most genes were upregulated 2 to 4-fold, a range in good agreement with that of upregulated targets identified in a similar screen of thyroid hormone regulated genes (Papathanasiou et al.,

1991), and generally more modest than the levels of gene regulation observed by forced overexpression of the tumor suppressor genes BRCA1 (Harkin et al., 1999) and WT1 (Lee et al., 1999).

The genes identified in this study encode several different classes of proteins, many of which are components of different signal transduction pathways. They include cell adhesion proteins (e.g. galectin-2, integrin α7B), growth factors (e.g. amphiregulin, VEGF), cytoskeletal proteins (e.g. actin-related protein 3), protein kinases (e.g. serum- and glucocorticoid-regulated kinase, sgk), other intracellular signaling molecules, and transcription factors (AP-4, STAT-3, FRA-1). Some of the genes identified here have been implicated in regulation of the cell cycle and growth arrest. One example is serum- and glucocorticoid-inducible kinase, SGK, which is shuttled between the nucleus and the cytoplasm during the cell cycle. Its forced retention in either compartment suppressed serum-induced growth and DNA synthesis in mammary tumor cells (Buse et al., 1999). We also found that $1,25(OH)_2D_3$ and EB1089 induced expression of gadd45α, which like $p21^{WAF1/CIP1}$ is a p53 target gene. However, neither compound affected p53 expression in SCC25 cells. A similar induction of GADD45α expression by $1,25(OH)_2D_3$ and EB1089 was observed in vitro and in vivo in the murine SCC line AT-84, under conditions where expression of p53 was unaffected, and $p21^{WAF1/CIP1}$ was repressed. In contrast, DNA damaging agents induced p53, $p21^{WAF1/CIP1}$ and GADD45α in AT-84 cells (Prudencio et al., 2001). Taken together, these results suggest that $1,25(OH)_2D_3$- and EB1089-dependent induction of gadd45α occurs by a p53-independent mechanism.

Consistent with its effects on gadd45α mRNA, EB1089 treatment of SCC25 cells enhanced expression of GADD45α protein and stimulated formation of GADD45α-

PCNA complexes. Previous studies have shown that DNA damaging agents, such as γ or UV irradiation, induce formation of GADD45α/PCNA complexes (Smith et al., 1994; Chen et al., 1995). Induction of GADD45α/PCNA complexes by EB1089, which is a growth inhibitor, but not a DNA damaging agent, indicates that increased DNA damage is not necessary to induce complex formation.

PCNA function is required for DNA replication in S phase, and for DNA repair through its association with polymerases δ and ε (Shivji et al., 1992). Association of GADD45α with PCNA is considered to divert PCNA from sites of DNA replication to sites of DNA repair. GADD45α modifies DNA accessibility on damaged chromatin and can stimulate DNA repair in vitro (Smith et al., 1994; Smith et al., 2000; Carrier et al., 1999). In addition, DNA damaging agents induce changes in the nuclear distribution of PCNA (Hall et al., 1993). It should be noted, however, PCNA also interacts with a number of other regulatory proteins, including $p21^{WAF1/CIP1}$ (Waga et al., 1994), at sites that overlap those recognized by GADD45α (Chen et al., 1995). The relative roles and importance of interactions of $p21^{WAF1/CIP1}$ and GADD45α with PCNA remain to be fully elucidated. Nonetheless, the induction of GADD45α expression and its central role in enhancing DNA repair, suggest that treatment of SCC cells with $1,25(OH)_2D_3$ or EB1089 would provide a genoprotective effect. This would be an important characteristic of a potential chemopreventive agent.

The observation that $1,25(OH)_2D_3$-induced expression of VEGF in SCC cells was surprising given that increased VEGF levels are associated with tumor vascularization and tumor progression (Benjamin et al., 1999). Elevated VEGF levels have been correlated with a higher rate of disease recurrence and a shorter disease free interval in

SCC of the oral cavity (Eisma et al., 1997). These results highlight the complexity of cellular responses to growth regulators such as $1,25(OH)_2D_3$ and its analogues, where a combination of regulatory signals is induced under conditions where the overall effect of $1,25(OH)_2D_3$ is growth inhibitory. It should also be noted that $1,25(OH)_2D_3$-regulated expression of VEGF is highly cell-specific. We did not observe any induction of VEGF expression in MCF-7 and MBA-MD231 breast cancer or LNCaP prostate cancer cells (data not shown), whereas others have shown that VEGF expression is regulated by $1,25(OH)_2D_3$ in osteoblast-like cells (Wang et al., 1996; Wang et al., 1997)

The partial resistance of SCC9 cells to the growth inhibitory effects of $1,25(OH)_2D_3$ correlated with broad deregulation of target gene expression (10 of 10 genes tested). It is unlikely that loss of regulation arises through repressed expression due to target gene methylation, since transcripts of all genes refractory to $1,25(OH)_2D_3$ were detected in vehicle-treated SCC9 cells (Fig. 2.6). It is possible that $1,25(OH)_2D_3$-dependent induction of these genes requires synergism of the VDR with other transcription factor(s) or downstream regulators, whose function is defective in SCC9 cells. Such factors would not be required for regulated expression of the endogenous 24-hydroxylase gene or the synthetic VDRE3-hsp68 promoter. One possible candidate is AP1, whose function is enhanced by $1,25(OH)_2D_3$ signaling (Takeshita et al., 1998; Chen et al., 1999; Johansen et al., 2000). However, this enhancement apparently requires, at least in part, upregulation of expression of AP1 components, particularly c-jun. We have also found here that $1,25(OH)_2D_3$ modestly upregulates fra-1 mRNA levels. This suggests that if loss of induced AP1 activity contributes to deregulation of $1,25(OH)_2D_3$ target gene expression in resistant SCC lines, it may not be a primary defect. It should also be noted

that we have tested the effect of cycloheximide on the six target genes identified on the Atlas array, $p21^{waf1/cip1}$, amphiregulin, VEGF, fra-1, gadd45α and integrin α7B, and found in each case there was no effect on $1,25(OH)_2D_3$-stimulated expression (Fig. 2.7, and data not shown). Therefore, in these instances, the stimulatory effect of $1,25(OH)_2D_3$ did not require protein synthesis. Moreover, with the exceptions of integrin α7B and fra-1, which were not tested, regulation of all of these genes was lost in SCC9 cells (Fig. 2.6).

Conclusion

Our studies have shown that $1,25(OH)_2D_3$ analogues can be potent inhibitors of SCC proliferation, and control the expression of several regulators of cell proliferation. However, partial resistance to $1,25(OH)_2D_3$ can arise even in the presence of apparently normal levels of functional VDR. Resistance arises from a broad, but selective loss in $1,25(OH)_2D_3$-regulated gene expression in the presence of normal levels of functional VDRs.

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CHAPTER 3

Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analogue EB1089 signaling on cell proliferation, differentiation and immune system regulation.

Preface

This chapter of my thesis is based on the following manuscript:

Lin, R., Nagai, Y., Sladek, R., Bastien, Y., Ho, J., Petrecca, K., Sotiropoulou, G., Diamandis, E.P., Hudson, T., and White J.H. (2002) Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 signaling on cell proliferation, differentiation and immune system regulation. *Mol. Endocrinol.* 16, 1243-56.

We previously found that proliferating human SCC25 HNSCC cells were arrested in G0/G1 by treatment with nanomolar concentrations of EB1089 (Akutsu et al., 2001a). To determine the molecular events underlying growth arrest, and to assess its potential as a chemopreventive agent, we analyzed the effects of EB1089 treatment on gene expression using Affymetrix HuGene FL oligonucleotide microarrays. SCC25 cells were treated for 0, 1, 2, 6, 12, 24 and 48h with EB1089.

Abstract

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] is key mediator of calcium homeostasis, and is a component of the complex homeostatic system of the skin. $1,25(OH)_2D_3$ regulates cellular differentiation and proliferation, and has broad potential as an anticancer agent. Oligonucleotide microarrays were used to assess profiles of target gene regulation at several points over a 48h period by the low calcemic $1,25(OH)_2D_3$ analogue EB1089 in human SCC25 head and neck squamous carcinoma cells. 152 targets were identified, composed 89 up- and 63 downregulated genes distributed in multiple profiles of regulation. Results are consistent with EB1089 driving SCC25 cells towards a less malignant phenotype, similar to that of basal keratinocytes. Targets identified control inter- and intracellular signaling, G protein-coupled receptor function, intracellular redox balance, cell adhesion and extracellular matrix composition, cell cycle progression, steroid metabolism, and >20 genes modulating immune system function. The data indicate that EB1089 performs three key functions of a cancer chemoprevention agent; it is antiproliferative, it induces cellular differentiation, and has potential genoprotective effects. While no evidence was found for gene-specific differences in efficacy of $1,25(OH)_2D_3$ and EB1089, gene regulation by $1,25(OH)_2D_3$ was generally more transient. Treatment of cells with $1,25(OH)_2D_3$ and the cytochrome P450 inhibitor ketoconazole produced profiles of regulation essentially identical to those observed with EB1089 alone, indicating that the more sustained regulation by EB1089 was due to its resistance to inactivation by induced 24-hydroxylase activity. This suggests that differences in action of the two compounds arise more from their relative sensitivities to metabolism and than from differing effects on VDR function.

Introduction

Naturally occurring vitamin D_3 is found in a limited number of dietary sources (e.g. cod liver oil, oily fish), and is produced through the action of ultraviolet light on 7-dehydrocholesterol in the skin (Holick, 2001). Vitamin D3 is one of several factors produced by the complex homeostatic system in the skin, which, as a protective barrier and environmental sensor, is intimately connected to the body's immune and neuroendocrine functions (Slominski and Wortsman, 2000). Vitamin D3 is 25-hydroxylated in the liver and converted into its active 1α,25-dihydroxy form [$1,25(OH)_2D_3$] in the kidney and several peripheral organs, including skin (Slominski and Wortsman, 2000; Jones et al., 1998). $1,25(OH)_2D_3$ signals through its cognate nuclear vitamin D receptor, which is a direct regulator of gene transcription. Signal transduction by $1,25(OH)_2D_3$ has a broad range of physiological effects (Slominski and Wortsman, 2000; Jones et al., 1998). Primarily, $1,25(OH)_2D_3$ controls calcium transport in the intestinal epithelia, and modulates bone resorption. However, it has widespread effects on cellular proliferation and differentiation. $1,25(OH)_2D_3$ stimulated differentiation of the OB 17 preadipocyte cell line (Dace et al., 1997), and induced immature basal layer skin cells to differentiate into keratinocytes (Hosomi et al., 1983). Hematopoietic cell lines can be induced to differentiate along the macrophage/monocyte pathway (Botling et al., 1996; Iwata et al., 1996; Nakajima et al., 1996). $1,25(OH)_2D_3$ inhibits proliferation of cells in several models of cancer, including myeloid leukemia, melanoma and carcinomas of the breast, prostate, colon and head and neck (Jones et al., 1998).

It is unlikely that regulation of a single gene provides the key to the growth inhibitory properties of $1,25(OH)_2D_3$ and its analogues. Expression of genes encoding the cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ was strongly but transiently induced by $1,25(OH)_2D_3$ in myeloid leukemia cells, and forced expression of $p21^{waf1/cip1}$ induced myeloid cell differentiation (Liu et al., 1996; Munker et al., 1996). However, the effect of $1,25(OH)_2D_3$ on $p21^{waf1/cip1}$ expression varies widely in different cell types. While $1,25(OH)_2D_3$ treatment modestly increased $p21^{waf1/cip1}$ protein levels in LNCaP prostate cancer cells, no effect was observed on $p21^{waf1/cip1}$ mRNA or the $p21^{waf1/cip1}$ promoter in these cells (Zhuang and Bernstein, 1998). Moreover, Hershberger et al (Hershberger et al., 1999) and ourselves (Prudencio et al., 2001) found that $1,25(OH)_2D_3$ repressed $p21^{waf1/cip1}$ expression in mouse head and neck SCC lines. The effect of $1,25(OH)_2D_3$ on $p27^{kip1}$ expression is generally more consistent. Rapid and transient induction of $p27^{kip1}$ transcripts is accompanied by substantially delayed and more sustained increase in $p27^{kip1}$ protein (Munker et al., 1996, Muto et al., 1999), suggesting that additional mechanisms may control its expression.

The limiting factor for use of $1,25(OH)_2D_3$ in cancer therapy has been hypercalcemia. However, many potent analogues have been developed with reduced calcemic effects (Bouillon et al., 1995; Guyton et al., 2001). One such analogue, EB1089, contains a side chain modified to render it less susceptible to catabolic degradation (Hansen et al., 1997; Hansen et al., 2000). *In vivo* studies of prostate and breast carcinomas using EB1089 dosages up to 1.0 μg/kg/day showed no clinically significant hypercalcemia (Koshizuka et al., 1999; Lokeshwar et al., 1999). Our previous experiments with a mouse model of head and neck squamous carcinoma (HNSCC) showed that an EB1089 dose of 0.25

μg/kg/day reduced tumor growth by up to 80% in the absence of hypercalcemia (Prudencio et al., 2001).

We are interested in investigating the potential chemopreventive effects of $1,25(OH)_2D_3$ analogues using HNSCC as a model. Early stage HNSCC can be successfully treated with surgery and/or radiation therapy. However, primary tumors are often associated with areas of dysplastic epithelia, which lead to the development of second primary carcinomas (SPC) at an annual rate of 3-7%. Thus, it is important to identify chemopreventive agents in head and neck SCC. Accumulating epidemiological evidence suggests that $1,25(OH)_2D_3$ analogues may have widespread chemopreventive effects (Guyton et al., 2001). Preclinical studies with models of colon (Guyton et al., 2001; Hershberger et al., 1999), cheek pouch (Rubin and Levij, 1973), gastrointestinal (Kawaura et al., 1998), and skin carcinogenesis (Wood et al., 1983; Chida et al., 1985) have also provided evidence for chemoprevention. We found that $1,25(OH)_2D_3$ and EB1089 induced the expression of the growth arrest and DNA damage (gadd45α) gene in human and mouse HNSCC lines in vitro and in tumors by an apparently p53-independent mechanism (Prudencio et al., 2001; Akutsu et al., 2001). GADD45α is required for normal DNA repair, and maintenance of global genomic stability (Hollander et al., 1999). This strongly suggests that $1,25(OH)_2D_3$ and its analogues can act as a genoprotective agents. Induction of DNA repair mechanisms may represent a feedback response to the stimulation of cutaneous vitamin D synthesis by ultraviolet light.

Here, we have used oligonucleotide microarrays to perform large scale profiling of the effects of EB1089 and $1,25(OH)_2D_3$ on gene expression in human HNSCC cells at several times over a 48h period. Nuclear receptor signaling is ideally suited for

microarray analysis, as ligand-bound receptors bind to promoter regions and directly regulate the expression of most of their target genes. These studies provide numerous insights into the effects of $1,25(OH)_2D_3$ and its analogues on cell proliferation, differentiation and regulation of immune system function.

Materials and Methods

Tissue culture and RNA extraction.

SCC25 cells were obtained from the American Type Cultures Collection (ATCC, Rockville, MD), and were cultured under recommended conditions. Cells cultured in 10 cm plates under conditions where controls cell could proliferate for at least 10 days prior to confluence (Akutsu et al., 2001a). Media was changed 24h prior to treatment with EB1089 or $1,25(OH)_2D_3$ (100nM) in DMSO for 0, 1, 3, 6, 12, 24, or 48h as previously described (Akutsu et al., 2001a). Total RNA was extracted with TRIZOL (GIBCO BRL, Burlington, Ontario), and 10μg of RNA isolated from EB1089-treated cells were used for microarray analysis. Cycloheximide (200nM; Sigma-Aldrich Canada, Ltd, Oakville, Ont.) was added 1 hr before addition of EB1089 as indicated. Ketoconazole (100nM; Sigma-Aldrich Canada, Ltd, Oakville, Ont.) was added along with EB1089 and $1\alpha,25(OH)_2D_3$ as indicated.

Microarray screening and data analysis.

Probe for microarray analysis was generated, and Affymetrix HuGene FL human gene oligonucleotide microarrays were screened as described in Novak et al (Novak et al., 2001). Screenings for EB1089-regulated genes were performed with three sets of probes generated from three independent tissue culture experiments. To test for statistically significant changes in signal intensity, compiled data was screened initially by non-parametric ANOVA (Sokal and Rohlf, 1995) using a P value of <0.05. Genes retained were then filtered for those whose expression was up- or downregulated a minimum of 2.5-fold at some point during the 48h time course, corresponding to a

minimum magnitude change of 200 fluorescence units. The data were filtered to eliminate genes with noisy expression profiles by calculation of cross correlations between individual profiles and hyperbolic tangents $[x(t)=tanh(nt/2)]$, where x is normalized fold induction, t is time, and n is a time constant controlling time of saturation. Profiles of upregulated genes with correlation coefficients of 0.8 or less, and downregulated genes with correlation coefficients of less than –0.8 were eliminated.

A method of clustering analysis was developed that classifies groups of genes based on time of regulation with respect to a threshold value, and does not take into account initial conditions. Maximal gene regulation was normalized to 1 for upregulated genes and –1 for downregulated genes. Given that experimental measurements were performed at 0, 1, 2, 6, 12, 24, and 48h, the number of intervals initially generates 6 clusters each for up- and downregulated genes. Clustering was evaluated for threshold values between .25 and .75, and -.25 and -.75 for induced and repressed genes, respectively. The number of clusters was then heuristically adjusted based on the following criteria: a) a cluster must contain at least two genes; b) the mean value of each cluster does not cross that of another cluster near the threshold. The optimum threshold was chosen as that generating the maximum cluster stability defined by the probability of a gene belonging to the same cluster in the average data set and the individual data sets. Based on these criteria, 0.50 and –0.50 were chosen as threshold values. The time the threshold is crossed was computed using a linear interpolation method. In order to avoid multiple threshold crossings, only the first crossing with a positive derivative for up-regulated genes, and negative derivative for down-regulated genes were considered. Analysis was carried out using Mathlab 6.12 (MathWorks Inc, Natick, MA).

Immunofluorescence.

SCC25 cells were plated on cover slips and treated with DMSO vehicle or 100nM EB1089 for 72h. Cells were processed for immunolabeling as described in (Petrecca et al., 2000). Briefly, cells were fixed in 2% paraformaldehyde and permeabilized, and blocked with Triton X-100/BSA. Cells were sequentially labeled with affinity purified rabbit anti-cystatin M (1:50; refs Sotiropoulou et al., 1997; Zeeuwen et al., 2001), mouse anti-protease M (1:150; ref Petraki et al., 2001) or rabbit anti-N-Cadherin (1;50; Sigma) primary antibodies for 1h at room temperature followed by Cy3-conjugated goat anti-rabbit or Cy2-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch) for 1h at room temperature. Immunofluorescence was visualized with a BioRad MicroRadiance confocal microscope at an optical thickness of ~10 μm using 25x or 63x objectives. For each pair of control and EB1089-treated samples, images were acquired and processed using identical parameters. Digital images were prepared using Adobe Photoshop.

Northern blotting and RT/PCR analysis of regulated gene expression.

Total RNA was extracted from SCC25 cells using Trizol (Gibco-BRL, Burlington, Ont.). Denatured RNA (3 μg) was reverse transcribed in a 20μl reaction at 42°C for 50 min with Superscript II (Gibco-BRL Burlington, Ont.) according to the supplier's instructions. Amplification conditions were optimized in preliminary experiments so that maximal amplification fell within the linear range. Products were diluted to 200μl, denatured at 95°C for 2 min, and then amplified as follows: Tenascin C,

(27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-CCACAGCTGGGAGATTTAGC-3' and reverse 5'-CTGGGAGCAAGTCCAGAGAG-3' primers; Nrf2, (21 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-ACCCTTGTCACCATCTCAGG-3' and reverse 5'-TTGCCATCTCTTGTTTGCTG-3' primers ; Dihydrodiol dehydrogenase, (21 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-GGTCACTTCATGCCTGTCCT-3' and reverse 5'-GGATGACATTCCACCTGGTT-3' primers ; Stromelysin, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-AACCTGTCCCTCCAGAACCT-3' and reverse 5'-TGGGTCAAACTCCAACTGTG -3'primers ; Collagenase 1, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-TGGACCTGGAGGAAATCTTG-3' and reverse 5'-GGGGTATCCGTGTAGCACAT -3' primers ; E1AF, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-CGCCTACGACTCAGATGTCA -3' and reverse 5'-GGAAGGCCAAAGAGAAGAGG -3'primers ; Protease M, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-GGGGTCCTTATCCATCCACT -3' and reverse 5'-GGGATGTTACCCCATGACAC -3' primers; G6PD, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-CAACCACATCTCCTCCCTGT -3' and reverse 5'-TCCCACCTCTCATTCTCCAC -3' primers; ST2, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-CAACTGGACAGCACCTCTTG -3'and reverse 5'-CAAATTCAGGGCCAGACAGT -3' primers;P-450, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-TTGCCCAGTATGGAGATGTG -3' and reverse 5'-GAACACTGCTCGTGGTTTCA -3' primers; 17β-hydroxysteroid dehydrogenase, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-

CACGAAGCCAGTGCAGATAA -3' and reverse 5'-GGAAATTCCGCTGTGCTAAG -3' primers; Cystatin M, (27 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-GGAGAACTCCGGGACCTGT-3' and reverse 5'-GGAACCACAAGGACCTCAAA -3' primers; Semaphorin V, (33 cycles; 94°C, 30sec; 60°C, 45sec; 72°C, 45sec) with forward 5'-AACCTGTGCCTTTGTGGAAG -3' and reverse 5'-AGCTGATCGAAGTGGGTGTC -3' primers; Collagenase 3, (26 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-ATGACTGAGAGGCTCCGAGA -3' and reverse 5'-ACCTAAGGAGTGGCCGAACT -3' primers; TRIP-14, (26 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-AAAGAGAGGCCATCATCCT-3' and reverse 5'-CAGGAACCTGGAAGGACAGA -3' primers; VRP, (33 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-TCTCTGTGGCGTGTTCTCTG -3' and reverse 5'-CACTGCAGCCCCTCACTATT -3' primers; SCCA, (26 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-TGATTTTGCAAATGCTCCAG -3' reverse and4 5'-TGGTTCTCAACGTGTCCTTG -3' primers; Interferon 56kD, (26 cycles; 94°C, 30sec; 57.5°C, 45sec; 72°C, 45sec) with forward 5'-GCTTCAGGATGAAGGACAGG-3' and reverse 5'-GAAATTCCTGAAACCGACCA -3' primers; GAPDH, (23 cycles; 94°C, 30sec; 55°C, 30sec; 72°C, 1min) with forward 5'-GGTGAAGGTCGGTGTCAACG -3' and reverse 5'-CAAAGTTGTCATGGATGACC -3' primers; Amphiregulin, (32 cycles; 94°C, 30sec; 55°C, 30sec; 72°C, 1min) with forward 5'-TTCGCACACCTGGGTGCCAG-3' and reverse 5'-AAGAGGATCCACTCATCATTTATGGCTATG-3' primers; Integrin α7B, (30 cycles; 94°C, 30sec; 53°C, 45sec; 72°C, 45sec) with forward 5'-GGTGAAGCTTCCTCGGGAAGAC-3' and reverse 5'-

GGAGCAAGCTTGAGTCAGTGACAC-3' primers; CRABP-II, (30 cycles; 94°C, 30sec; 53°C, 45sec; 72°C, 45sec) with forward 5'-GACAGGATCCAGTGCTCCAGCCTAGGAG' and reverse 5'-AGAGGGATCCTGCTCTGGGCTGGTTTGG-3' primers; 24-OH, (30 cycles; 94°C, 30sec; 55°C, 30sec; 72°C, 1min) with forward 5'-AAGGATCCTGTTCTGTCTTGCATCTTC-3' and reverse 5'-CCCTAAAGCTTTCACAGCAGAGAGAAAGC -3' primers; N-cadherin, (23 cycles; 94°C, 30sec; 50°C, 30sec; 72°C, 1min) with forward 5'-TTAGTCACCGTGGTCAAACCAATC-3' and reverse 5'-AGTGGATCCACTGCCTTCATAGTCAAACAC-3' primers. All of the above reactions were performed in 50µl of 2.5mM $MgCl_2$, 50mM KCl, and 10mM Tris-Cl (pH 9.0) using 2.5U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). Aliquots of 45µl of each amplified sample were subjected to electrophoresis on 2% agarose gels containing ethidium bromide and photographed. Fluorescent bands were quantified using Kodak digital science 1D Image Analysis software.

For Northern blotting, 20µg of total RNA or 1µg of poly A+ RNA were electrophoresed as described (Akutsu et al., 2001a). Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham, Baie d'Urfé, Québec). The blotted membrane was soaked in 3% SSC and 0.1% SDS at 50°C, and pre-hybridized at 42°C in 50 mM phosphate buffer pH 6.5, 50% formamide, 5% SSC, 10% Denhardt's solution containing 250 µg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled cDNA probes. Following hybridization, the membrane was washed 4 times in 2% SSC and 0.2% SDS for 5 min, 3 times in 0.1%

SSC and 0.2% SDS for 30 min at 50°C, dried, and autoradiographed. Band intensities were quantitated using the FluorChem digital imaging system and AlphaEaseFC software (Alpha Innotech Corp, San Leandro, CA).

Table 3.1

List of genes regulated by EB1089.

The Genbank accession number for each gene is provided in the first column. The second column lists the maximum fold regulation and time of maximal activation. The third column gives P value derived from non parametric ANOVA. Note that P values of less 0.00001 are listed as 0. The fourth column gives the cluster number derived in Fig. 3.2. Genes whose regulation has been confirmed by RT/PCR analysis and/or Northern blotting are in italics.

		Cluster	Adhesion/cytoskeleton
M69225	+3.2	U4	*bullous pemphigoid antigen (BPAG1)*
M76482	+2.4	U5	*desmoglein-3*
M95787	-7.2	D4	22kDa smooth muscle protein (SM22)
S42303	-6.6	D4	N-cadherin
U47634	+4.3	U2	beta-tubulin class III
X05608	-4.8	D2	NF-L
X07695	-7.7	D4	cytokeratin 4 C-terminal region
X74929	-5	D4	keratin 8 (KRT8)

		Cluster	Cell Cycle
M25753	-2.6	D4	cyclin B
U61836	+6.2	U3	putative cyclin G1 interacting protein
U66838	-2.9	D4	cyclin A1
U77949	-2.6	D3	Cdc6-related protein (HsCDC6)
X51688	-3	D4	cyclin A
X54942	-2.4	D3	c-shs2 Cks1 protein homolog
Z36714	-4.2	D4	cyclin F

		Cluster	Channels/transporters
L15296	+13.5	U2	rod cyclic nucleotide-gated cation channel
U73191	-5.3	D2	inward rectifier potassium channel Kir1.3
U81375	-2.9	D3	equilibrative nucleoside transporter 1 (hENT1)

		Cluster	ECM structure/remodeling
HG2197	+2.5	U4	Collage, Type VII, Alpha 1
M24486	+3.2	U3	prolyl 4-hydroxylase alpha subunit
M33653	+21.9	U3	type XIII collagen
M85289	+2.6	U4	heparan sulfate proteoglycan (HSPG2)
U20758	+6.5	U3	osteopontin
U50330	+3.5	U4	procollagen C-proteinase (pCP-2)
U62800	+20	U2	cystatin M
X05232	+9.4	U3	stromelysin
X16832	-4	D4	cathepsin H
X54925	+14	U4	collagenase 1
X74295	+4.4	U3	alpha 7B integrin
X75308	-20	D3	collagenase 3
X78565	-5	D3	tenascin-C, 7560bp
Z37976	+3.5	U4	latent TGFβ binding protein (LTBP-2)

		Cluster	Immune System
D12763	+26.7	U3	T1/ST2
D38037	+4.2	U4	FK506-binding protein 12kDa (hFKBP-12) homologue
J04164	-2.6	D4	interferon-inducible 9-27
M21005	-4.4	D3	migration inhibitory factor-related protein 8 (MRP8)
M21121	-3.0	D4	T cell-specific protein (RANTES)
M24594	-2	D3	interferon-inducible 56 Kd
M26311	-9.2	D1	migration inhibitory factor-related protein 14 (MRP14)
M28130	-5.1	D1	interleukin 8 (IL8) gene
M29696	-3.3	D3	IL-7 receptor
M33552	-4.3	D5	lymphocyte-specific protein 1 (LSP1)
M80254	-2.7	D2	cyclophilin (hCyP3)
M87507	+2.5	U3	interleukin-1 beta convertase (IL1BCE)
M93056	+11	U3	mononcyte/neutrophil elastase inh.
U04343	+4.8	U5	CD86 antigen
U67615	+2.8	U3	beige protein homolog chs
U90426	-2.7	D4	nuclear RNA helicase BAT1
X13334	+27	U3	CD14 myeloid cell-specific glycoprotein
X51441	-4.5	D4	serum amyloid A
X57351	-2.9	D5	interferon-inducible 1-8D gene
X59405	+2.8	U2	Membrane cofactor protein (CD46)
X68487	-2.9	D2	A2b adenosine receptor
Z49107	+3.5	U5	galectin

		Cluster	Intracellular Signaling
D67029	+2.8	U2	SEC14L
D89016	+2.7	U4	Rho GEF homolog
HG2167	+2.5	U3	A-kinase anchoring protein Ht31
L04510	+2.5	U2	ARD1
L13391	+2.8	U4	RGS2/G0S8
L15388	+3	U4	G protein-coupled receptor kinase (GRK5)
L24564	-21	D2	Rad
M14764	-2.6	D3	nerve growth factor receptor (nrgf)
M31724	+3.2	U2	PTP-1B
M58026	+3.5	U3	NB-1
M61906	+4	U2	P13-kinase p85
M97815	-3.7	D2	CRABP-II
U02081	+3	U2	guanine nucleotide regulatory protein NET1
U10550	+6	U1	Gem GTPase
U15932	+2.5	U3	dual-specificity protein phosphatase
U40271	+2.7	U4	receptor tyrosine kinase (PTK7)
U67733	+3.8	U4	cGMP-stim. 3,5 -cyclic nucleo. Phosphodiesterase PDE2A3(PDE2A)
U96922	+5.2	U3	inositol polyphosphate 4-phosphatase type II-α
X04828	+3.3	U4	G(I) protein alpha-subunit
X13461	+3.8	U4	calmodulin-like gene (CLP)
X13916	+2.5	U5	LDL-receptor related protein
X82676	+3.5	U2	PEZ/PTP36 tyrosine phosphatase
X89416	-8.9	D5	protein phosphatase 5

		Cluster	Redox
D00632	+2.8	U4	glutathione peroxidase
D87258	+6	U4	HtrA chaperon/protease
S73591	+8.2	U5	VDUP1
V00594	-3.1	D5	metallothionein from cadmium-treated cells
X55448	+5.8	U4	glucose-6-phosphate dehydrogenase (G6PDH)
X91247	+3	U1	thioredoxin reductase
Z11793	+4.1	U4	selenoprotein P

		Cluster	Signaling peptides/ growth factors
J05008	-5.6	D1	endothelin-1 (ENT-1)
K03183	-2.6	D3	chorionic gonadotropin (hcg) beta subunit
M22489	+3.7	U4	bone morphogenetic protein 2A (BMP-2A)
M30703	+2.7	U2	amphiregulin (AR)
M57293	-2.6	D3	PTHrP
M60315	+8.1	U4	transforming growth factor-beta (tgf-beta)
M77140	-4.2	D4	galanin
M94250	-3.4	D4	retinoic acid inducible factor (MK)
U03877	-3.1	D4	*extracellular protein S1-5*
U06863	+2.8	U4	follistatin-related protein precursor
U43142	-3.2	D1	*VEGF-related (VRP)*
U62015	-3.6	D2	Cyr61
X02530	-4	D3	gamma-interferon inducible IP-10
X54489	-2.7	D1	melanoma growth stimulatory activity (MGSA)
X57579	-2.6	D1	activin beta-A subunit
Y00787	-4.1	D1	monocyte-derived neutrophil chemotactic factor (MDNCF)

		Cluster	Steroid/lipid metabolism
D28235	+8.2	U2	Cox-2
J03600	+11	U5	5-lipoxygenase
L11708	+42	U4	type 2 17β-HSD
L13286	+196	U2	24-hydroxylase
M91432	+3.2	U2	medium-chain acyl-CoA dehydrogenase
U05861	+3.1	U3	hepatic dihydrodiol dehydrogenase
U07919	-2.5	D3	ALDH-6

		Cluster	Transcription factors
D31716	+2.5	U5	GC box bindig protein
D85131	-3.3	D2	Myc-associated zinc-finger protein of islet
HG1686	-3	D5	Transcription Factor E4tf1
HG2724	+3.6	U5	CHOP/GADD153
HG3510	+2.9	U4	COUP-TF1
HG4058	-2.6	D1	Oncogene Aml1-Evi-1
L40387	-2.5	D3	TRIP-14
L49054	+2.7	U5	fusion gene NPM-MLF1
M19720	+5.2	U5	L-myc gene
M99701	+3.1	U3	transc. elongation factor S-II homolog pp21
S74017	+2.6	U4	Nrf2 transcriptional activator
U18018	+2.6	U3	E1A enhancer binding protein (E1A-F)
U63824	-2.7	D2	transcription factor RTEF-1
U74612	-3.8	D4	HNF-3/fork head homolog 11
X52611	+4.6	U4	transcription factor AP-2
X56681	-2.8	D2	junD
X65644	+3.5	U2	MHC binding protein 2 (MBP-2)
X73358	-3	D2	hAES-1
X82209	+4.4	U3	MN1 mRNA
X84373	+4.4	U1	RIP140

		Cluster	Others
D00408	+36	U5	P-450 HFLa
D17793	+2.6	U3	KIAA0119
D31883	+3.5	U3	KIAA0059
D43636	+5.6	U1	KIAA0096
D78444	+2.8	U2	hkf-1
D78611	-2.6	D4	MEST
D86960	+2.9	U3	KIAA0205
HG4074	-2.6	D4	XPG
M32053	+4.3	U5	H19 RNA gene
M60047	-4.5	D2	*heparin binding protein (HBp17)*
M72885	+4	U2	G0S2
M90657	-6.2	D2	tumor antigen (L6)
M93036	-2.5	D4	carcinoma-associated antigen GA733-2
M97347	-2.7	D2	β-1,6-N-acetylglucosaminyltransferase (AGAT)
S66896	-7.2	D2	squamous cell carcinoma antigen (SCCA)
S81914	-3.5	D1	*radiation-inducible IEX-1*
S85655	-3.9	D3	prohibitin
U00115	+3.5	U5	bcl-6
U08021	-21	D2	nicotinamide N-methyltransferase (NNMT)
U15174	+3.7	U4	NIP3
U17077	+2.8	U3	BENE
U18467	+2.6	U4	pregnancy-specific beta 1-glycoprotein 7 (PSG7)
U21931	+2.4	U2	fructose-1,6-biphosphatase
U28369	+31	U3	semaphorin 3B (sema3B)
U38276	+2.9	U3	semaphorin 3F (Sema 3F)
U51010	-9.5	D2	nicotinamide N-methyltransferase
U52100	-3	D3	XMP
U52101	-3.6	D3	*YMP*
U62801	+32	U3	protease M
U84836	-3.2	D4	ERPROT 213-21
U95740	+5.7	U3	362G6.2 from BAC clone chromosome 16p13.1
X05299	-10.5	D3	mRNA (~95%) for major centromere autoantigen CENP-B
X06661	+2.8	U5	27-kDa calbindin
X12458	-6.7	D4	P3 protein (AA 1-1382)
X12517	-4.3	D4	U1 small nuclear RNP-specific C protein
X14850	-2.6	D4	histone H2A.X
X59303	-2.6	D4	G7a valyl-tRNA synthetase
Y00339	+5.1	U3	carbonic anhydrase II
Y00787	-2.9	D4	polypeptide 7B2
Z25535	-2.6	D3	*nuclear pore complex protein hnup153*
Z29331	+2.7	U5	(23k/3) ubiquitin-conjugating enzyme UbcH2

Results and Discussion

Time courses of EB1089-regulated gene expression in human SCC25 cells.

We previously found that proliferating human SCC25 HNSCC cells were arrested in G0/G1 by treatment with nanomolar concentrations of EB1089 (Akutsu et al., 2001a). To determine the molecular events underlying growth arrest, and to assess its potential as a chemopreventive agent, we analyzed the effects of EB1089 treatment on gene expression using Affymetrix HuGene FL oligonucleotide microarrays. SCC25 cells were treated for 0, 1, 2, 6, 12, 24 and 48h with EB1089 in 3 independent experiments. Prior to microarray screening, the response to EB1089 in each experiment was verified by Northern analysis of amphiregulin gene expression (data not shown), as our previous work demonstrated that the amphiregulin gene is a direct target of $1,25(OH)_2D_3$ (Akutsu et al., 2001a; Akutsu et al., 2001b).

Compiled raw data was initially analyzed by non-parametric ANOVA (Sokal and Rohlf. 1995) to eliminate genes whose change in expression was not statistically significant ($P<0.05$). Data were then filtered to eliminate genes included because of single chip artifacts, and those with erratic expression profiles that were not consistent between experiments (see below, and Materials and Methods for details). While previous microarray studies used variation filters as high as 3-fold regulation (Tamayo et al., 1999), we chose a filter of 2.5-fold, corresponding to a minimum magnitude change of 200 fluorescence units, so that genes whose induction was similar to that of amphiregulin (avg. +2.74-fold) would not be excluded. A list of 152 reproducibly regulated EB1089 targets composed of 89 upregulated and 63 downregulated genes is presented in Table

3.1. The results indicate that EB1089 signaling impinges upon every aspect of SCC cell function both in terms of intracellular metabolism, and communication with the extracellular milieu.

The list contains a number of previously identified $1,25(OH)_2D_3$ targets, including genes encoding integrin α7B, COX-2, and amphiregulin, which were identified in our earlier microarray analysis (Akutsu et al., 2001a). Sequences encoding another $1\alpha,25(OH)_2D_3$ target gene in SCC25 cells, $p21^{waf1/cip1}$, are not present on the HuGene FL chip. In addition to 24-hydroxylase, the list also contains other vitamin D responsive genes including those encoding osteopontin, carbonic anhydrase II, VDUP1, PTHrP, CD14 and TGFβ (Zou et al., 1997; Crawford et al., 1998; Quelo et al., 1994; Van Leewen et al., 1997; Kremer et al., 1996; Chen and DeLuca, 1994; Koli and Keskioja, 1995; Danilenko et al., 2001). One exception is the gene encoding GADD45α, which we showed is $1,25(OH)_2D_3$ responsive in mouse and human SCC lines (Prudencio et al., 2001; Akutsu et al., 2001a). Although it appeared upregulated, the gadd45α gene was not retained during the filtering process because of elevated levels of non-specific hybridization to control oligonucleotide sets (data not shown).

The range of fold regulation of target genes varied widely, with 24-hydroxylase exhibiting by far the highest upregulation (196-fold at 48h) of all genes identified. Expression of eight of these genes representing a range of fold regulations was further analyzed by Northern blotting (Fig. 3.1). The results of Northern and microarray analyses are in very good agreement. Most importantly, regulation of all genes identified on microarrays was confirmed on Northern blots, and the relative magnitudes of fold regulation observed were the same using the two techniques. There was also broad

agreement between the fold regulations observed using the two techniques. The exceptions were cystatin M and protease M, where fold inductions at 24h of 6.7- and 18-fold, and 8- and 32- fold were observed by Northern blotting and microarray analysis, respectively. However, other differences in fold regulation were less than 2-fold. Taken together, these experiments, coupled with RT/PCR analysis (Table 3.1, and see below), suggest that while the absolute magnitudes of fold regulation detected by microarray analysis may be somewhat higher in some cases than those detected by other techniques, the data compiled in Table 3.1 is highly reliable.

Initial clustering analysis of averaged data of reproducibly regulated genes processed by the K-mean algorithm with k=5 generated 4 clusters of upregulated genes distinguished based on rapidity of induction (data not shown). No such resolution was achieved for downregulated genes, arising from fact that the absolute value of the average fold activation of the upregulated genes at any given time point was substantially greater than that of the downregulated genes. In addition, the K-mean algorithm is strongly dependent upon the choice of initial points (K number of initial conditions). Therefore, different initial points will have different nearest neighbors, and refinement of calculating means with various neighbors can generate different clusters starting from different initial conditions. We have developed a method of clustering analysis that does not take into account initial conditions, and categorizes genes based on time of crossing of a threshold value (see Materials and Methods for details). The method generated symmetrical groups of clusters of up- and downregulated genes (Figs. 3.2A-L). The profiles of cluster genes were generally much less erratic than genes eliminated by filtering (Fig. 3.2M and N). For example, the compiled data for E2F4 (a transcription factor controlling cell cycle

Figure 3.1

Northern analysis of EB1089 target gene regulation.

Northern analyses were performed on RNA extracted from control SCC25 cells or cells treated for 24h with EB1089. Blots were hybridized with probes specific for integrin a-7B (a-7B), 24-hydroxylase (24-OH), protease M (prot. M), cystatin M (cyst. M), amphiregulin (AR), CRABP-II, N-cadherin (N-cad.), squamous cell carcinoma antigen (SCCA), and GAPDH control. Comparison of fold regulations after 24h detected by Northern blotting (North) and Affymetrix microarrays (Affy) are provided.

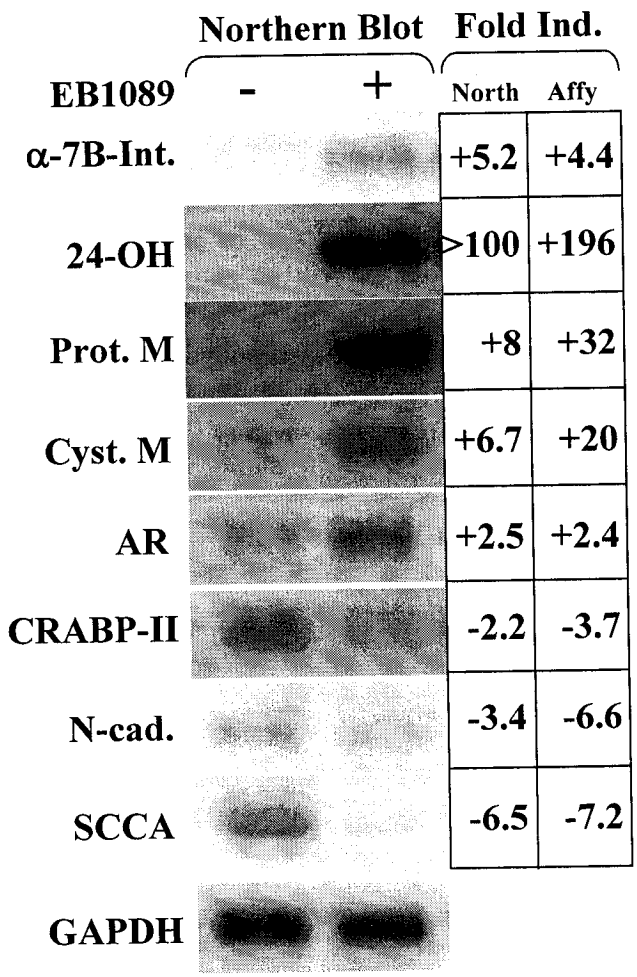

Northern Blot
Fold Ind.
EB1089
-
+
North
Affy
α-7B-Int.
+5.2
+4.4
24-OH
>100
+196
Prot. M
+8
+32
Cyst. M
+6.7
+20
AR
+2.5
+2.4
CRABP-II
-2.2
-3.7
N-cad.
-3.4
-6.6
SCCA
-6.5
-7.2
GAPDH

Figure 3.2

Profiles of genes listed in Table 1 were subjected to clustering analysis.

A-E. Normalized profiles of upregulated genes in clusters U1-U5 are presented, with the average trace shown in bold. F. Comparison of the average profiles for clusters U1-U5. G-K. Normalized profiles of downregulated genes in clusters D1–D5 are presented, with the average trace shown in bold. L. Comparison of the average profiles for clusters D1-D5. Numbers of genes in each cluster are indicated in brackets. M, N. Profiles of up- and downregulated genes eliminated by filtering prior to clustering analysis (see Materials and Methods for details). O. Analysis of transcription factor E2F4 regulation in EB1089-treated cells. The composite profile (in bold) and individual data sets are shown. The inset shows an analysis of E2F4 transcripts from EB1089-treated cells by RT/PCR using the same RNA preparations as in Fig. 3.5.

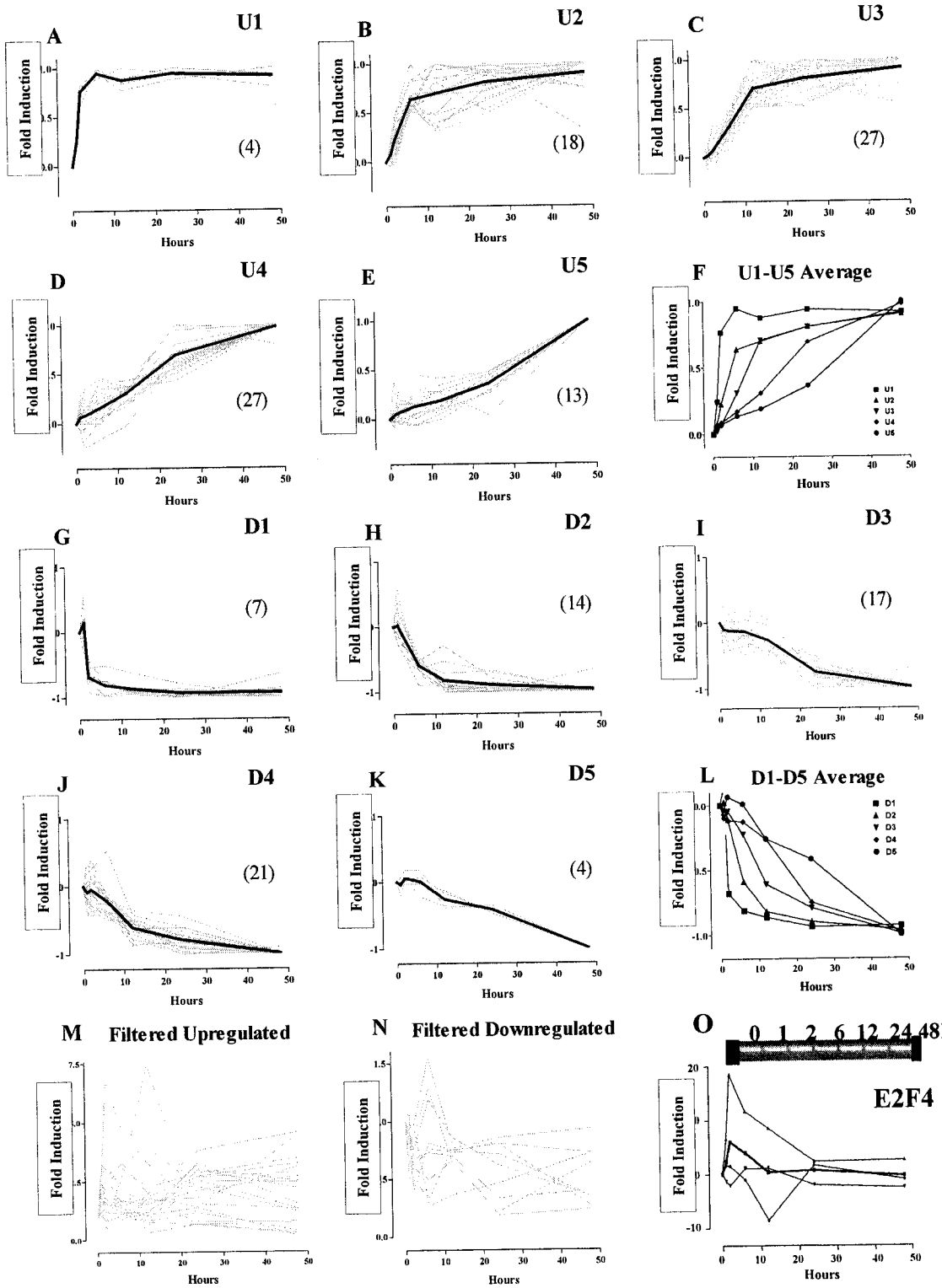

A
U1
Fold Induction
(4)
Hours
B
U2
(18)
C
U3
(27)
D
U4
(27)
E
U5
(13)
F
U1-U5 Average
U1
U2
U3
U4
U5
G
D1
(7)
H
D2
(14)
I
D3
(17)
J
D4
(21)
K
D5
(4)
L
D1-D5 Average
D1
D2
D3
D4
D5
M
Filtered Upregulated
N
Filtered Downregulated
O
0 1 2 6 12 24 48h
E2F4

Figure 3.3

Profiling of EB1089-regulated gene expression.

A. EB1089-dependent regulation of genes whose expression is generally disrupted or downregulated in cancer. B. Genes whose expression is upregulated in cancer. C. Genes controlling extracellular matrix structure and cell adhesion. Bottom. Cycloheximide does not block EB1089-dependent induction of collagenase 1 and stromelysin gene expression. SCC25 cells were treated with cycloheximide (C), and EB1089 (E) alone or in combination as indicated for 24h. Total RNA was analyzed by RT/PCR for expression of stromelysin, collagenase 1. GAPDH expression was not affected (not shown). D. Genes controlling non-GPCR-mediated intracellular signaling. E. Genes modulating GPCR function. F. Genes encoding signaling peptides. G. Genes controlling regulation of immune system function. H. Genes controlling intracellular redox balance. Note that these categories are not mutually exclusive, and some genes may appear under more than one category. In addition, not all genes listed in Table 3.1 are presented.

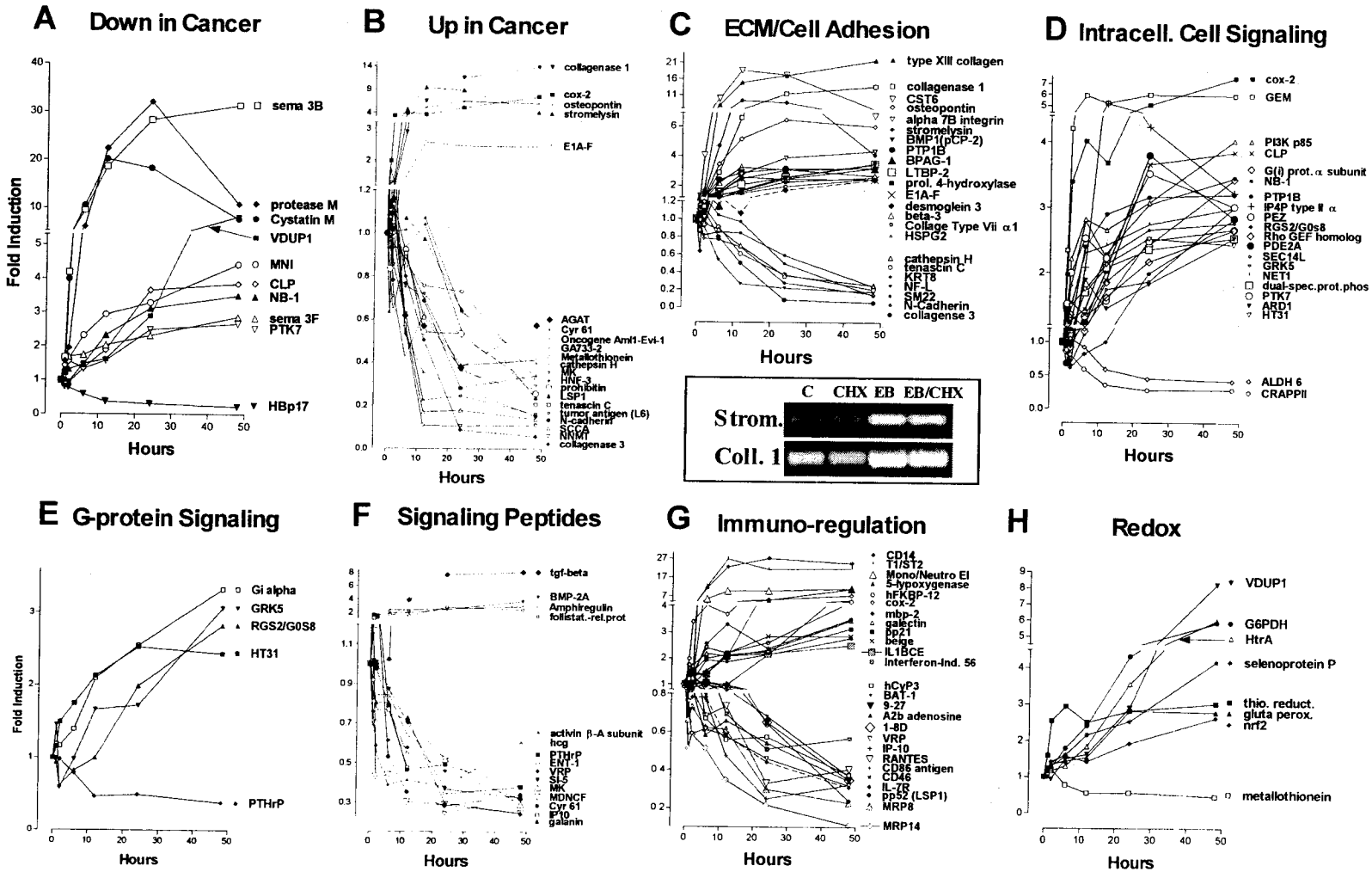
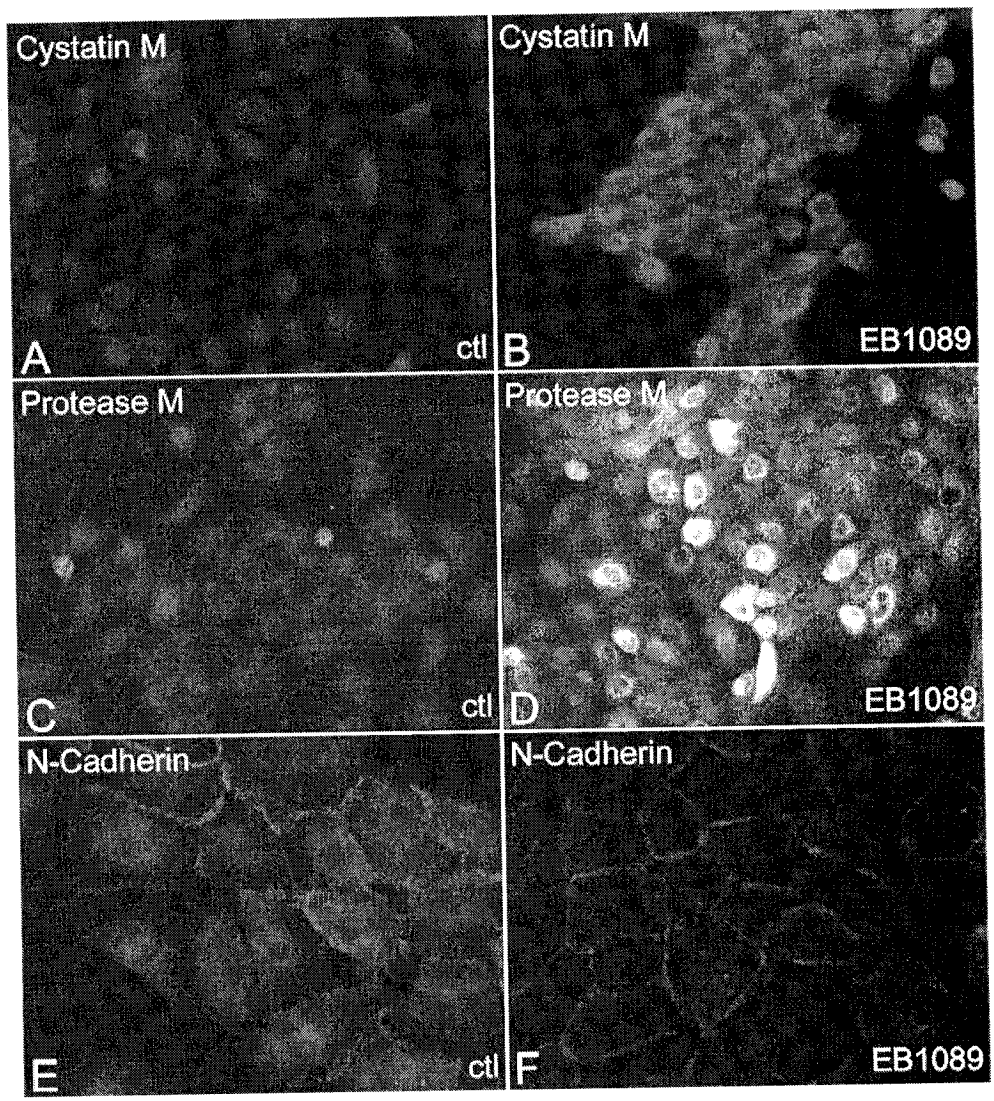

A Down in Cancer
Fold Induction
Hours
sema 3B
protease M
Cystatin M
VDUP1
MNI
CLP
NB-1
sema 3F
PTK7
HBp17
B Up in Cancer
collagenase 1
cox-2
osteopontin
stromelysin
E1A-F
AGAT
Cyr 61
Oncogene Aml1-Evi-1
GA733-2
Metallothionein
cathepsin H
MK
HNF-3
prohibitin
LSP1
tenascin C
tumor antigen (L6)
N-cadherin
SCCA
NKMT
collagense 3
C ECM/Cell Adhesion
type XIII collagen
collagenase 1
CST6
osteopontin
alpha 7B integrin
stromelysin
BMP1(pCP-2)
PTP1B
BPAG-1
LTBP-2
prol. 4-hydroxylase
E1A-F
desmoglein 3
beta-3
Collage Type Vii α1
HSPG2
cathepsin H
tenascin C
KRT8
NF-L
SM22
N-Cadherin
collagense 3
C CHX EB EB/CHX
Strom.
Coll. 1
D Intracell. Cell Signaling
cox-2
GEM
PI3K p85
CLP
G(i) prot. α subunit
NB-1
PTP1B
IP4P type II α
PEZ
RGS2/G0s8
Rho GEF homolog
PDE2A
SEC14L
GRK5
NET1
dual-spec.prot.phos
PTK7
ARD1
HT31
ALDH 6
CRAPPII
E G-protein Signaling
Gi alpha
GRK5
RGS2/G0S8
HT31
PTHrP
F Signaling Peptides
tgf-beta
BMP-2A
Amphiregulin
follistat.-rel.prot
activin β-A subunit
hcg
PTHrP
ENT-1
VRP
SLS
MK
MDNCF
Cyr 61
IP10
galanin
G Immuno-regulation
CD14
T1/ST2
Mono/Neutro El
5-lypoxygenase
hFKBP-12
cox-2
mbp-2
galectin
pp21
beige
IL1BCE
Interferon-Ind. 56
hCyP3
BAT-1
9-27
A2b adenosine
1-8D
VRP
IP-10
RANTES
CD86 antigen
CD46
IL-16
pp52 (LSP1)
MRP8
MRP14
H Redox
VDUP1
G6PDH
HtrA
selenoprotein P
thio. reduct.
gluta perox.
nrf2
metallothionein
Hours

Figure 3.4

Immunofluorescence analysis of cystatin M, protease M, and N-cadherin expression in EB1089-treated SCC25 cells.

Control (ctl; vehicle-treated) and EB1089-treated SCC25 cells were analyzed by immunofluorescence for expression of cystatin M (A and B), protease M (C and D), and N-cadherin (E and F). Primary antibodies were detected with Cy3-conjugated goat anti-rabbit (A, B, E and F) or Cy2-conjugated goat anti-mouse (C and D) secondary antibodies. No staining was seen in the absence of primary antibodies (data not shown). Images of each control and EB1089-treated sample pair were acquired by confocal microscopy and processed using identical parameters. See Materials and Methods for details. Magnifications: A-D, 25x; E and F 63x.


Cystatin M
A
ctl
Cystatin M
B
EB1089
Protease M
C
ctl
Protease M
D
EB1089
N-Cadherin
E
ctl
N-Cadherin
F
EB1089

progression), which suggests rapid upregulation, is composed of three distinctly different profiles (Fig. 3.2O). indeed, analysis of E2F4 transcripts from EB1089 treated cells by RT/PCR revealed no regulation (Fig. 3.2O, inset).

The 24-hydroxylase gene is among the most rapidly regulated genes in cluster U1, whereas regulation of the osteopontin gene is significantly slower (cluster U3; Fig. 3.2, Table 3.1). The promoters of both of these genes contain VDREs (Zou et al., 1997; Crawford et al., 1998). In addition, regulation of the carbonic anhydrase II gene, whose chicken homologue contains a VDRE (Quelo et al., 1994), fell into cluster U3. This indicates that the kinetics of gene induction by the EB1089/VDR bound to different VDREs is strongly promoter-specific. Several cell cycle regulatory genes whose products function after the G1/S boundary were among the more slowly regulated genes in clusters D3 and D4 (Table 3.1), likely reflecting the gradual diminution of cells in S phase or later. This is supported by observations that cyclin A1 and cyclin B levels in cells in G2 do not change during EB1089 treatment (not shown).

Regulation by EB1089 of markers associated with cancer cell progression.

EB1089 signaling regulates the expression of several markers associated with progression of cancer phenotypes. Of genes whose expression is reduced or eliminated in cancer cells, almost all are upregulated by EB1089 (Fig. 3.3A). Two of the more strongly induced genes, kallikrein protease protease M and the cysteine protease inhibitor cystatin M (Table 3.1, and Fig. 3.3A), are downregulated in breast cancers (Diamandis et al., 2000; Sotiropoulou et al., 1997), as is calmodulin-like protein (CLP; ref 41). CLP is a marker of epithelial cell differentiation (Rogers et al., 2001). Genes encoding semaphorin

3B and 3F lie in a region of chromosome 3 deleted in lung cancers (Xiang et al., 1996; Sekido et al., 1998; Lerman et al., and Minna, 2000). The exception to the above is HBp17, a putative regulator of FGF signaling that was expressed at lower levels in SCC than in primary cultures of keratinocytes (Velluci et al., 1995).

EB1089 also downregulates a large number of genes that are overexpressed in cancers (Fig. 3.3B), including tumor antigen L6, carcinoma associated antigen GA733-2, and squamous cell carcinoma antigen (SCCA). SCCA is a serum marker of uterine cervix, head and neck, lung and esophageal cancers, and ablation of its expression inhibits growth and induces natural killer cell infiltration of tumors (Suminami et al., 2001). Another downregulated gene, tenascin C, is an early marker of HNSCC progression (Ramos et al., 1998) Similarly, repression of overexpressed N-cadherin in head and neck squamous cell carcinoma is associated with restoration of an epithelial phenotype (Islam et al., 1996).

The above results suggest that EB1089 treatment reversed the malignant phenotype of SCC25 cells. This possibility was investigated further by immunofluorescence analysis of three markers that are differentially expressed in cancer cells, cystatin M, protease M, and N-cadherin. Both protease M and cystatin M transcripts are strongly induced by EB1089, and cystatin M is an ideal marker for these purposes because its expression is highly specific for differentiated epidermal keratinocytes (Zeeuwen et al., 2001). In addition, upregulation of N-cadherin in head and neck squamous, breast and prostate cancers ("cadherin switching") is associated with cancer progression, invasion and metastasis (Islam et al., 1996; Hazan et al., 2000; Tomita et al., 2000). Immunofluorescence studies in control and EB1089-treated cells

(Fig. 3.4) revealed a strong upregulation of cystatin M expression, giving rise to strong, relatively uniform cytoplasmic staining (Figs. 3.4A and B). Similar results were obtained with immunofluorescence analysis of protease M expression (Figs. 3.4C and D), with the exception that elevated levels of protease M expression varied somewhat in EB1089-treated cells. In contrast, EB1089 treatment downregulated N-cadherin expression (Figs. 3.4E and F). This downregulation included cell-cell contact sites, as well as the dotted pattern of non cell-cell contacts seen in other carcinoma cells (Tomita et al., 2000). The changes observed are in excellent agreement with the regulation of the genes encoding these markers (Table 3.1, Figs. 3.1 and 3.3). Moreover, in addition to providing evidence that EB1089 reverses the malignant phenotype of SCC25 cells, these studies provide sensitive new markers for following HNSCC progression and treatment.

Regulation of genes controlling ECM structure and remodeling, and cell adhesion consistent with induction of a basal keratinocyte phenotype.

EB1089 does induce expression of some genes that are often upregulated in cancers, many of which are implicated in extracellular matrix (ECM) structure and remodeling. Upregulated genes include those encoding transcription factor E1A-F, which controls matrix metalloproteinase (MMP) gene expression (Higashino et al., 1995), and two of its target genes, MMPs stromelysin and collagenase 1 (Figs. 3.2B and C). EB1089-dependent induction of stromelysin, collagenase 1 and E1A-F was confirmed by RT/PCR (Figs 3.2 and 3.3). Although E1A-F is a regulator of collagenase gene expression, cycloheximide did not block EB1089-induced expression of collagenase 1 or stromelysin (Fig. 3.3C, bottom). This indicates that induction of E1A-F expression by EB1089 is not

essential for observed regulation of collagenase 1 and stromelysin, and that EB1089 has both long- and short-term effects on matrix metalloproteinase expression. Expression of osteopontin, a non-collagen matrix protein implicated in ECM structure and remodeling was also upregulated. Several studies have indicated that osteopontin, collagenase 1, and stromelysin play key roles in ECM remodeling during wound healing (Singer and Clark, 1999; Pilcher et al., 1999; Agnihotri et al., 2001). Upregulation of their expression by EB1089 provides a molecular genetic basis for the proposed stimulatory role of $1\alpha,25(OH)_2D_3$ in wound healing (Tian et al., 1995).

The strong induction (22-fold) of expression of the type XIII collagen gene (COLXIII), a transmembrane collagen, provided further evidence that EB1089 induced keratinocytic differentiation of SCC25 cells. Interestingly, trimerization of type XIII collagen is activated by prolyl 4-hydroxylase (Snellman et al., 2000), whose gene is also upregulated (Fig. 3.3C). Type XIII collagen is expressed in normal human epidermis and is present at cell-cell contact sites and at the dermal-epidermal junction. It is highly colocalized with E-cadherin and may be a component of adherens-like junctions (Peltonen et al., 1999). In addition, expression of phosphotyrosine phosphatase PTP-1B, whose activity has been associated with enhanced cell adhesion (Cheng et al., 2001), is also increased.

EB1089 also upregulates BPAG-1 (Fig. 3.3C), a component of hemidesmosomes, structures essential for adhesion of epithelial cells to basement membranes (Borradori and Sonnenberg, 1999). Absence or disruption of hemidesmosomal components gives rise to devastating bullous pemphigoid blistering skin disorders. EB1089 also induces expression of desmoglein 3 (Fig. 3.3C), a cadherin component of desmosomes (Borradori

and Sonnenberg, 1999), and the autoantigen in pemphigus vulgaris. It is noteworthy that desmogleins are expressed in a gradient in the epidermis, with desmoglein 3 most abundant in the basal layer (Green and Gaudry, 2001). This observation, coupled with the upregulation of type XIII collagen and hemidesmosomal components, provides further evidence that EB1089 induces a more epithelial, less malignant phenotype in SCC25 cells, consistent with that of basal keratinocytes.

Pleiotropic effects of EB1089 on inter- and intracellular signaling.

Expression of several factors controlling intracellular signaling was altered in EB1089-treated cells (Figs. 3.2D-F), including a number of genes encoding proteins controlling G protein-coupled receptor signaling (Fig. 3.3E). Upregulated genes include those encoding the A kinase anchoring protein (AKAP) Ht31, and RGS2/G0S8, which is a selective inhibitor of Gq alpha signaling (Heximer et al., 1997). The induction of RGS2/G0S8 is intriguing, as its expression is also induced by parathyroid hormone in bone (Miles et al., 2000), which can signal through a G protein-coupled receptor linked to Gq alpha (Turner et al., 2000). $1\alpha,25(OH)_2D_3$ represses PTH receptor (PTH1R) signaling by inhibiting expression of the receptor and ligands PTH and PTHrP (Fig. 3.3F; refs Van Leewen et al., 1997; Kremer et al., 1996; Amizuka et al., 1999). In addition, EB1089 treatment induces expression of the G receptor kinase GRK5 (Fig. 3.3E), which can repress PTH1R function (Dicker et al., 1999). These results indicate that, in addition to inhibiting ligand and receptor expression, $1\alpha,25(OH)_2D_3$ signaling can also repress PTH1R function by inducing expression of factors that inhibit signaling via Gq alpha.

Expression of a number of signaling peptides was altered in treated cells (Fig. 3.3F), emphasizing the neuroendocrine nature of epidermal function (Slominski and Wortsman, 2000). Our previous studies have shown that induction of amphiregulin (Fig. 3.3G) can inhibit SCC25 proliferation (Akutsu et al., 2001). Downregulated genes include galanin, a neuropeptide implicated in nerve regeneration after injury (Wynick et al., 1998), and S1-5, a relatively uncharacterized factor with EGF-like domains (Lecka-Czernik et al., 1996). Consistent with its antiproliferative effects, EB1089 downregulated expression of several mitogenic factors. These include VEGF-related protein (VRP), which is mitogenic in Kaposi's Sarcoma and hematopoietic cells (Liu et al., 1997; Wang et al., 1997), Cyr61, which encodes a growth factor implicated in angiogenesis and tumorigenesis, whose expression is induced by estrogen in breast cancer cells (Sampath et al., 2001), and midkine (MK), mitogenic factor overexpressed in several carcinomas (Ikematsu et al., 2000).

Regulation of genes controlling immune system function.

Keratinocytes are considered to be an integral part of the immune system of the skin (Slominski and Wortsman, 2000). The intimate connection of epithelial cells to immune system function is reinforced by the large number of EB1089-related genes in SCC25 cells implicated in immunoregulation (Fig. 3.3G). The role of $1\alpha,25(OH)_2D_3$ in controlling the function of epithelial cells in innate immunity (Becker et al., 2000) is underlined by the strong induction by EB1089 of the gene encoding the pattern receptor CD14 (Fig. 3.3H), which is also a target gene in monocytic HL60 cells (Koli and Keskioja, 1995). Another strongly induced gene is that encoding T1/ST2, a member of

the interleukin-1 receptor family. Gene ablation studies in mice have revealed that T1/ST2 signaling is required for T helper 2, Th2, cell differentiation (Townsend et al., 2000).

EB1089 downregulated interferon γ-regulated genes encoding 9-27, 1-8D, interferon-inducible 56K protein, and the T cell chemokine IP-10, and the chemokine RANTES, which is also overexpressed in a number of cancers including more advanced breast cancer (Luboshits et al., 1999). Interferon γ signaling and overexpression of IP-10 underlie the inflammatory reactions in psoriasis (Giutizieri et al., 2001). Previous studies have suggested that $1,25(OH)_2D_3$ signaling can influence T helper cell differentiation (Jones et al., 1998). These data indicate that directs effects on epithelial cell signaling play a key role in the anti-inflammatory action of $1,25(OH)_2D_3$ analogues in skin. Our results are consistent with EB1089 stimulating Th2 responses, and inhibiting a number of genes associated with proinflammatory Th1 responses.

Control of genes regulating cellular redox balance.

EB1089 signaling regulates a number of genes encoding proteins that control cellular redox balance (Fig. 3.3H). Induction of these genes by EB1089 and $1,25(OH)_2D_3$ may represent a feedback response to epidermal vitamin D3 synthesis induced by sunlight, which is an effective inducer of reactive oxygen species in skin (Petersen et al., 2000; Peus et al., 2001). Upregulated genes include glucose-6-phosphate dehydrogenase (G6PDH), selenoprotein P, glutathione peroxidase, thioredoxin reductase, HtrA, and, importantly, the nrf2 transcription factor. Selenoprotein P is a plasma heparin binding protein with antioxidant properties (Hondal et al., 2001). HtrA is an extremely well

conserved protein whose prokaryotic homologue is essential for survival under conditions of oxidative stress (Hu et al., 1998). Ablation of nrf2 expression in mice rendered them more susceptible to carcinogenesis and resistant to the protective effects of chemoprevention agents (Ramos-Gomes et al., 2001). Nrf2 expression, which is induced by a number of chemopreventive agents, in turn induces expression of a number of phase II detoxifying enzymes. These events may provide a mechanism for protection by $1\alpha,25(OH)_2D_3$ against dimethyl-benzanthracene (DMBA) carcinogenesis in hampster cheek pouch carcinoma (Kawaura et al., 1998). DMBA is activated by a series of oxidation steps, and detoxified by phase II enzymes (Long et al., 2001).

Both G6PDH and thioredoxin reductase contribute to nucleotide biosynthesis in proliferating cells and are overexpressed in cancer cells (Powis and Montfort, 2001; Rahman et al., 1999). However, in quiescent cells they are source of reducing equivalents. G6PDH is at the head of the pentose-phosphate shunt, which is a source of NADPH, and thioredoxin reductase uses NADPH to reduce thioredoxins, proteins that in turn reduce oxidized cysteines. Elevated G6PDH and thioredoxin levels protect against apoptosis, which is sensitive to redox balance. Recent studies have shown that short-term $1,25(OH)_2D_3$ treatment of MCF-7 breast cancer cells has prooxidant effects (Koren et al., 2001). However, unlike the results of obtained in SCC25 cells (Fig. 3.3), G6PDH induction in MCF-7 cells was modest, and no changes in glutathione peroxidase levels were found. Significantly, however, $1,25(OH)_2D_3$ is an effective inducer of apoptosis in MCF-7 cells, whose onset can be controlled by redox balance, whereas no evidence for apoptosis was found in $1,25(OH)_2D_3$-treated SCC25 cells (Akutsu et al., 2001a). This suggests that the effects of $1,25(OH)_2D_3$ on redox balance may be cell-specific.

EB1089 and 1α,25(OH)$_2$D$_3$ regulate target gene expression with similar efficacy.

We have confirmed the regulation of a total of 30 genes by Northern blotting, RT/PCR (Figs. 3.1 and 3.5, Table 3.1). In addition to the 17 genes presented in Fig. 3.5, regulation of 9 other genes was confirmed at single time points (Table 3.1, and data not shown). We have also compared regulation by EB1089 and 1,25(OH)$_2$D$_3$ of several target genes. Structure/function studies have suggested the VDR forms structurally distinct complexes with EB1089 and 1,25(OH)$_2$D$_3$, possibly providing a molecular basis for gene-specific effects of the two compounds (Carlberg et al., 2001). In preliminary analyses by RT/PCR of the effects of 24 or 48h treatment with EB1089 or 1,25(OH)$_2$D$_3$, several target genes analyzed appeared to be differentially regulated by two compounds (data not shown). Therefore, we compared target gene regulation by EB1089 and 1,25(OH)$_2$D$_3$ over the entire 48h time course (Fig. 3.5). The results showed that 1,25(OH)$_2$D$_3$ regulated expression of several genes more transiently than EB1089, but did not provide any evidence for gene-specific differences in efficacy of the two compounds (Fig. 3.5).

To examine the potential role of 24-OHase in attenuation of 1,25(OH)$_2$D$_3$ signaling by 48h, we compared expression profiles in SCC25 cells treated with vehicle, EB1089 or 1α,25(OH)$_2$D$_3$ in the presence or absence of the cytochrome P450 inhibitor ketoconazole (Fig. 3.6). As expected, induction of T1/ST2 expression by EB1089 after 48h was strong, and was unaffected by ketoconazole. In contrast, while the effect of 1,25(OH)$_2$D$_3$ alone after 48h was weaker, T1/ST2 expression remained high in cells treated with 1,25(OH)$_2$D$_3$ and ketoconazole together, and was essentially identical to that observed in

Figure 3.5

Comparison of effects of EB1089- and 1,25(OH)$_2$D$_3$ on target gene expression.

SCC25 cells were cultured and treated with in parallel with EB1089 (dark grey bars) or 1,25(OH)$_2$D$_3$ (pale grey bars) as indicated and gene expression was analyzed by RT/PCR. Genes selected included both up- and downregulated targets and strongly (e.g. T1/ST2, protease M) and moderately (e.g. E1A-F, interferon-inducible 56kD protein) regulated genes.

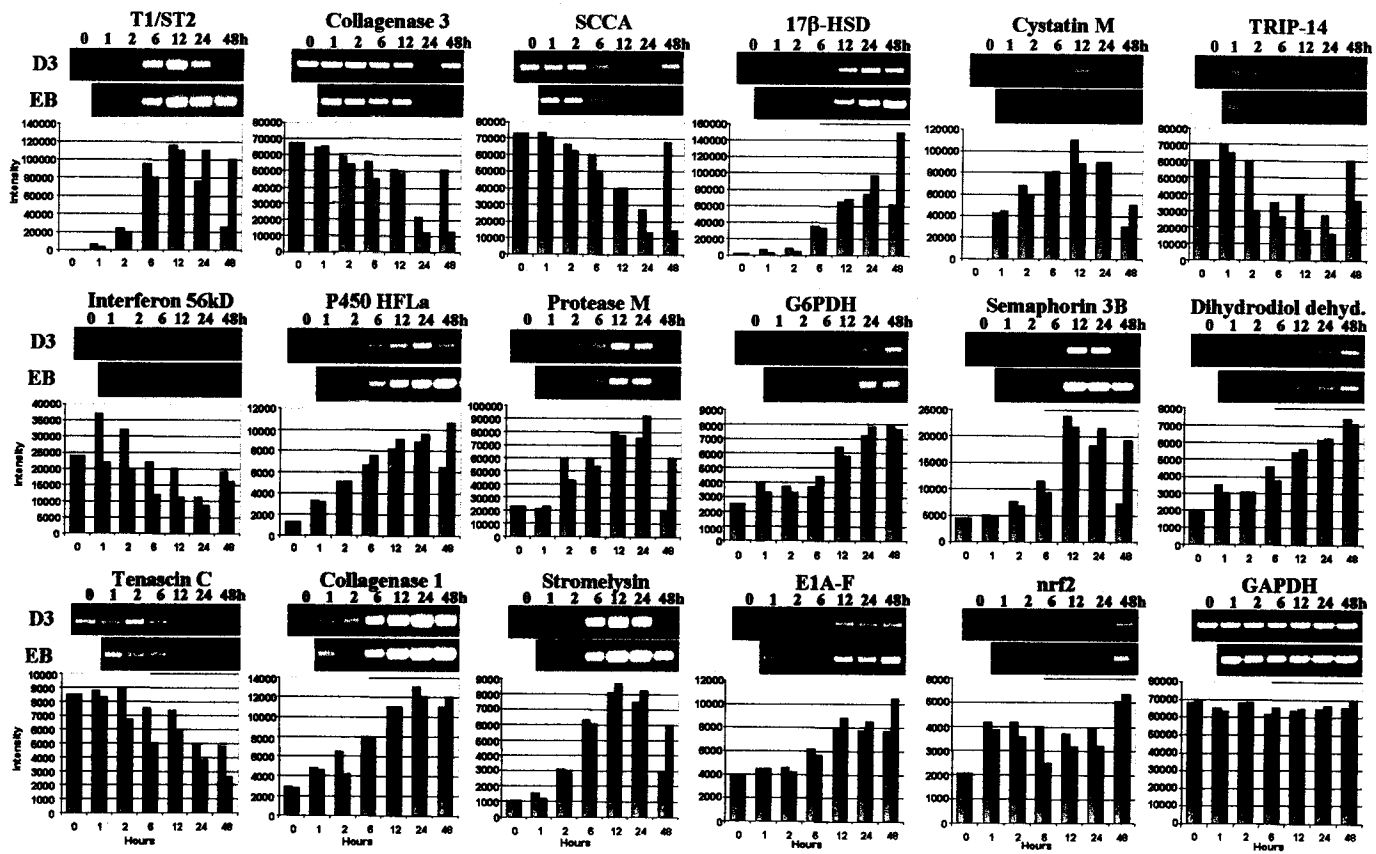
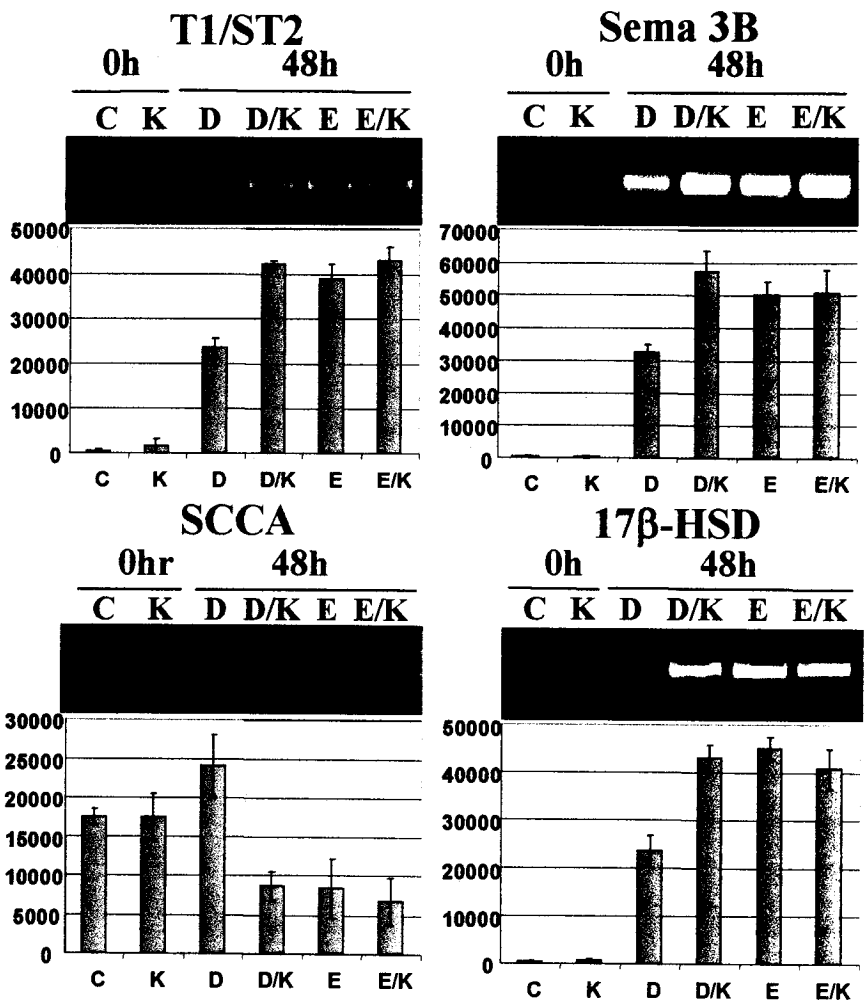

T1/ST2
Collagenase 3
SCCA
17β-HSD
Cystatin M
TRIP-14
Interferon 56kD
P450 HFLa
Protease M
G6PDH
Semaphorin 3B
Dihydrodiol dehyd.
Tenascin C
Collagenase 1
Stromelysin
E1A-F
nrf2
GAPDH
0 1 2 6 12 24 48h
D3
EB
Intensity
Hours

Figure 3.6

Analysis of the effects of cytochrome P450 inhibitor ketoconazole on EB1089- and 1,25(OH)$_2$D$_3$-regulated gene expression.

SCC25 cells were treated with vehicle alone (control; C), 100nM ketoconazole alone (K), 100nM 1,25(OH)$_2$D$_3$ alone (D), 100nM EB1089 alone (E), or in combination as indicated. Total RNA isolated from treated cells was analyzed by RT/PCR for expression of T1/ST2, Semaphorin 3B (Sema 3B), 17b-hydroxysteroid dehydrogenase (17b-HSD), and squamous cell carcinoma antigen (SCCA). Results of 3 independent experiments are presented.


T1/ST2
0h
48h
C K D D/K E E/K
50000
40000
30000
20000
10000
0
C K D D/K E E/K
Sema 3B
0h
48h
C K D D/K E E/K
70000
60000
50000
40000
30000
20000
10000
0
C K D D/K E E/K
SCCA
0hr
48h
C K D D/K E E/K
30000
25000
20000
15000
10000
5000
0
C K D D/K E E/K
17β-HSD
0h
48h
C K D D/K E E/K
50000
40000
30000
20000
10000
0
C K D D/K E E/K

the presence of EB1089 or EB1089 and ketoconazole. Similar effects of ketoconazole were observed on $1,25(OH)_2D_3$-dependent induction of semaphorin 3B, and type II 17β-hydroxysteroid dehydrogenase genes, and on repression of the SCCA gene (Fig. 3.6). No effects were observed of ketoconazole alone or with ligands on GAPDH expression (not shown). Thus, the more sustained regulation of several target genes by EB1089 is likely due to its insensitivity to induction of 24-OHase activity. The variability observed in the relative durations of the regulatory effects of EB1089 and $1,25(OH)_2D_3$ in Fig. 3.5 may reflect differences in stability of association of ligand-bound VDR with specific promoters, or with differing stabilities of target gene mRNAs. The data do not provide any evidence for gene-specific differences in efficacy of trans-activation or -repression by EB1089 and $1,25(OH)_2D_3$.

Conclusion

The studies above provide multiple insights into not only the potential of $1\alpha,25(OH)_2D_3$ analogues as agents of cancer chemoprevention, but also into the physiological actions of $1\alpha,25(OH)_2D_3$ in a number of tissues, including skin, bone, and the immune system. The data indicate that EB1089 performs key functions of a cancer chemoprevention agent; it is antiproliferative, it induces cellular differentiation, and it has potential genoprotective effects over and above our previous findings of the induction of GADD45α (Prudencio et al., 2001; Akutsu et al., 2001a). Differential effects on gene expression of EB1089 and $1\alpha,25(OH)_2D_3$ were attributable to the insensitivity of EB1089 to 24-OHase activity, suggesting that differences in action of the two compounds arise more from their sensitivity to metabolism and than from differential action of the VDR bound to each ligand.

Acknowledgment

We are grateful to Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark) for EB1089. We thank Dr. Jaroslav Novak (Montreal Genome Centre) for assistance with statistical analysis, and Dr. Leon Glass (Centre for Nonlinear Dynamics) for helpful comments on the manuscript. This work was supported by a grant from the Canadian Institutes of Health Research (CIHR; MT-15160) to J.H.W. R.L. was supported by a postgraduate scholarship from the CIHR. Y.N. was supported by a postdoctoral fellowship from the Heart and Stroke Foundation of Canada. R.S. is a postdoctoral fellow of the CIHR and T.J.H. is a clinician-scientist of the CIHR. J.H.W is a chercheur-boursier of the Fonds de Recherche en Santé du Québec (FRSQ).

CHAPTER 4

Amphiregulin is a Vitamin D3 target gene in Squamous Cell and Breast Carcinoma.

Preface

This chapter of my thesis is based on the following manuscript:

Akutsu, N., Bastien, Y. **Lin, R.,** Mader, S. and White, J.H. (2001) Amphiregulin is a vitamin D target gene in squamous cell and breast carcinoma. *Biochem. Biophys. Res. Comm.* 281, 1051-56.

We have screened gene arrays (Chapter 2 and Chapter 3) to identify targets of $1\alpha,25(OH)_2D_3$ and EB1089. The amphiregulin gene was identified as a putative target gene in screens with both ligands. Subsequent studies showed that amphiregulin expression is directly regulated by $1\alpha,25(OH)_2D_3$ in SCC25 cells.

Abstract

1α,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] inhibits growth of cells derived from a variety of tumors in vitro and in vivo. Proliferation in vitro of human SCC25 cells, derived from a primary squamous cell carcinoma (SCC) of the tongue, was blocked by 1,25$(OH)_2D_3$ and its analogue EB1089. A similar effect was observed with 13-cis retinoic acid (RA), which has been used in chemoprevention of SCC. We identified amphiregulin, a member of the epidermal growth factor family, as a 1,25$(OH)_2D_3$ target gene in SCC25 cells. Induction of amphiregulin mRNA by 1,25$(OH)_2D_3$ was rapid and sustained over 48h, and was unaffected by cycloheximide. 1,25$(OH)_2D_3$ also induced amphiregulin mRNA in estrogen receptor-positive and –negative human breast cancer cell lines, but not in LNCaP human prostate cancer cells. RAR- or RXR-specific retinoids did not affect amphiregulin mRNA levels in SCC25 cells, however, 13-cis RA partially blocked the response to 1,25$(OH)_2D_3$. Amphiregulin partially inhibited growth of SCC25 cells in culture. Our data show that amphiregulin is a 1,25$(OH)_2D_3$ target gene, and suggest that its induction may contribute to the growth inhibitory effects of 1,25$(OH)_2D_3$.

Introduction

The active form of vitamin D3, 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) is best known for its key role in calcium homeostasis (Jones et al., 1998). However, $1,25(OH)_2D_3$ and its analogues also inhibit proliferation and stimulate differentiation of cancer cells derived from a variety of tissues, including breast, prostate, colon, lung, endometrium, hematopoietic cells, and oral cavity (Jones et al., 1998; Hansen et al., 2000; Adam et al., 1999; Liu et al., 1996; Zhuang and Bernstein, 1998; Campbell and Koeffler, 1997; VanWeelden et al., 1998; Hershberger et al., 1999; Van den Bemd et al., 2000). The factor limiting the use of $1,25(OH)_2D_3$ itself has been hypercalcemia. However, over 800 analogues have been developed in an attempt to maintain the inhibitory effect on tumor cell proliferation while reducing the hypercalcemic effects (Hansen et al., 2000). For example, one such agent EB1089 caused apoptotic regression of xenografts of the breast carcinoma cell line MCF-7 in nude mice (VanWeelden et al., 1998). Dosages of EB1089 of 0.1 to 1.0 micrograms/kg/day showed no clinically significant hypercalcemia in animal models of prostate and breast carcinoma (Koshizuka et al., 1999; Lokeshwar et al., 1999).

$1α,25(OH)_2D_3$ and its analogues interact with the nuclear vitamin D receptor (VDR), which functions as a ligand-inducible transcription factor by forming heterodimers with members of the retinoid X receptor (RXR) family (Mangelsdorf et al., 1995). $1,25(OH)_2D_3$ thus exerts its antiproliferative effects by modulating target gene transcription. $1,25(OH)_2D_3$ analogues are attractive candidates for chemoprevention of squamous cell carcinomas (SCC) of the oral cavity (Vokes et al., 1993; Hong et al., 1994). Retinoids, such as 13-cis retinoic acid (13-cis RA; isotretinoin) have been used

clinically in chemoprevention of SCC since the 1980's (Hong et al., 1990). Signaling by 13-cis RA is very similar to that of $1,25(OH)_2D_3$, as retinoids bind to nuclear retinoic acid receptors (RARs), which function as RAR/RXR heterodimers. However, progression of SCC is associated with reduced expression of RARs, particularly RARs β and γ, loss of retinoid-regulated expression of terminal differentiation markers, and resistance to the antiproliferative effects of retinoids (Hu et al., 1991; Crowe, 1998; Lotan, 1994; Oridate et al., 1996; Lotan, 1997; McGregor et al., 1997).

We have found that $1,25(OH)_2D_3$ and its analogue EB1089 inhibit the proliferation in vitro of the human SCC line SCC25. A screen of a gene array was performed to identify D3 target genes in SCC25 cells. One target was found to be amphiregulin, a member of the epidermal growth factor family of peptides. Amphiregulin was originally isolated from serum-free medium of MCF-7 breast carcinoma cells treated with phorbol 12-myristate 13-acetate, which is growth inhibitory (Shoyab et al., 1998). It was so-named because of its capacity to inhibit proliferation of several human carcinoma cell lines and stimulate growth of other cell lines and primary cultures of normal fibroblasts (Plowman et al., 1990; Cook et al., 1991). Amphiregulin has moderate affinity for the epidermal growth factor receptor, and previous experiments have shown that it modulates cell proliferation at concentrations above 10nM (Solic and Davies, 1997). Here, we show that $1,25(OH)_2D_3$ rapidly and directly affects the regulation of amphiregulin gene expression in SCC25 cells and estrogen receptor-positive and -negative breast carcinoma cells, and that incubation of SCC25 cells with nM concentrations of recombinant amphiregulin partially reproduces the growth inhibitory effects of $1,25(OH)_2D_3$.

Materials and Methods

Nuclear receptor ligands and reagents.

$1,25(OH)_2D_3$ and EB1089 were kindly supplied by Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark). 13-cis RA was purchased from ICN, and LG1069 (LG) was a generous gift of Dr Tim Willson (Glaxo-Welcome Research and Development, Research Triangle Park, NC). All hormones were dissolved in dimethylsulfoxide (DMSO), and stock solutions were stored in the dark at –20°C. Recombinant human amphiregulin was purchased from R&D Systems (Minneapolis, MN) and cycloheximide was purchased from SIGMA (Oakville, Ontario).

Tissue Culture.

The squamous cell carcinoma cell line SCC25 and breast cancer cell lines MDA-MB361, MCF-7 and prostate cancer cell line, LNCaP were obtained from the American Type Cultures Collection (ATCC, Rockville, MD). Cell lines were cultured under recommended conditions. The effects of $1,25(OH)_2D_3$, EB1089, and retinoids on cell growth were analyzed by seeding cells in 6-well plates at 15,000 cells/well in 2ml of culture medium containing charcoal-stripped serum. After 24h the culture media were changed to charcoal-stripped medium containing vehicle or ligand at the indicated concentrations. Media were changed every two days and fresh ligand added as necessary. On the designated day, cells were washed with 2ml of phosphate buffered saline and removed from the plate by incubation with 0.7ml of 0.25% trypsin-EDTA. Cell numbers were determined using a hemacytometer. Four grid sections were counted for each well and the results were averaged. All treatment conditions were performed in triplicate

wells. For the effect of amphiregulin on SCC25 cell line cells were seeded in 6-well plates at 15,000 cells/well in 2ml of culture medium. After 24h the culture media were changed to charcoal-stripped medium containing vehicle or amphiregulin at the indicated concentrations. Media were changed every day. Cell counts were performed with same method as other cell growth experiments. All treatment conditions were tested in triplicate.

Total and polyA+ RNA isolation.

Cells were grown in 100-mm dishes. When appropriate, media were replaced with charcoal stripped medium containing ligand at the indicated concentrations. Cycloheximide was added to 200nM 1h prior to addition of ligand where indicated. After incubation, total RNA was extracted with TRIZOL (GIBCO BRL, Burlington, Ontario) according to the manufacturer's instruction. PolyA+ RNAs were isolated using an Oligotex mRNA Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

Differential screening.

Differential screening was performed with 100ng of polyA+ RNAs extracted from SCC25 cells treated for 24h with DMSO, $1,25(OH)_2D_3$ (100nM) or EB1089 (100nM) using an Atlas^{TM} cDNA Expression Array (CLONTECH, Palo Alto, CA). Preparation of probe and array screening were carried according to the manufacturer's instructions.

Complementary DNA probes and Northern blotting.

Complementary DNA probes were generated by reverse transcription-PCR amplification of polyA+ RNA from SCC-25 cells. RNAs were reverse transcripted by oligo dT priming and PCR amplification of amphiregulin sequences was performed using primer sets: 5'-TTCGCACACCTGGGTGCCAG-3' and 5'-AAGAGGATCCACTCATCATTTATGGCTATG-3'. Amplified fragments were subcloned into Bluescript SK+ (Stratagene, Aurora, Ontario) and verified by dideoxy sequencing.

For Northern blotting, 20μg of total RNA or 1μg of poly A+ RNA were separated on a 1.0% agarose gel containing 6.3% formaldehyde, 20 mM MOPS (pH 7.0), 15 mM sodium acetate, and 1 mM EDTA. Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham, Baie d'Urfé, Québec). The blotted membrane was soaked in 3% SSC and 0.1% SDS at 50°C, and pre-hybridized at 42°C in 50 mM phosphate buffer pH 6.5, 50% formamide, 5% SSC, 10% Denhardt's solution containing 250 μg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled cDNA probes. Following hybridization, the membrane was washed 4 times in 2% SSC and 0.2% SDS for 5 min, 3 times in 0.1% SSC and 0.2% SDS for 30 min at 50°C, dried, and autoradiographed. All Northern blots were performed at least three times with independent preparations of RNA from multiple plates.

Results

$1\alpha,25(OH)_2D_3$ and EB1089 block proliferation of SCC25 cells.

The growth inhibitory effects of $1,25(OH)_2D_3$ and its synthetic analogue EB1089 (Adam et al., 1999) were evaluated in SCC25 cells. SCC25 cells were chosen for these studies because they have maintained a relatively differentiated phenotype and are thus more representative of the targets of chemoprevention than less differentiated lines. For example, unlike other lines, SCC25 cells express near normal levels of RARs β and γ, and have retained retinoid-regulated K-19 expression (Vokes et al., 1993). Northern blotting studies of SCC25 cell polyA+ RNA detected the presence of mRNAs encoding the VDR and its heterodimeric partners RXRα and β (Fig. 4.1A). Growth of SCC25 cells was strongly inhibited in the presence of 10 nM and 100 nM $1,25(OH)_2D_3$ (Fig. 4.1B). Similar degrees of inhibition were observed in the presence of 10 nM and 100 nM 13-cis RA, used as a positive control in these studies. No significant differences were observed in the growth inhibitory effects of 10 nM $1,25(OH)_2D_3$ or 13-cis RA alone or in combination at any point over a 10-day period (Fig. 4.1B, and data not shown). Essentially complete growth inhibition was observed in the presence of 1 to 100 nM EB1089 (Fig. 4.1B), indicating that EB1089 is a more potent inhibitor of SCC25 cell growth than $1,25(OH)_2D_3$.

Identification of novel $1,25(OH)_2D_3$ target genes in SCC25 cells.

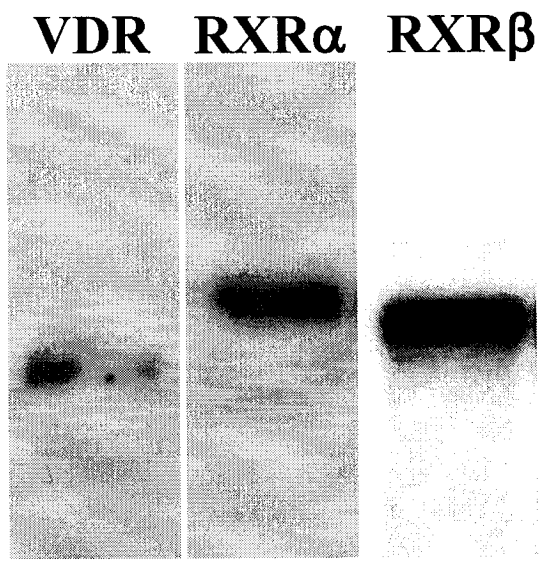
Given the sensitivity of SCC25 cells to $1,25(OH)_2D_3$ and EB1089, they represent ideal candidates for identification of $1,25(OH)_2D_3$ target genes. Regulated genes were

Figure 4.1

Inhibition of SCC25 cell proliferation by 1,25(OH)$_2$D$_3$ and EB1089.

A. Results of Northern blotting with 1mg of polyA+ RNA showing expression of mRNAs encoding the VDR, RXRa, and RXRb, as indicated. B. Relative growth of SCC25 cells incubated over a 10 day period in the presence of vehicle (-), 1, 10 or 100 nM 1,25(OH)$_2$D$_3$ (D3), EB1089 (EB) or 13-cis RA (RA), as indicated. Media was changed and fresh ligand added every two days over the period of the experiment. Each point represents the result obtained from triplicate wells. Cells were counted using a hemacytometer, and the numbers of cells in cultures treated with vehicle were given a value of 100%.

A


VDR
RXRα
RXRβ

B

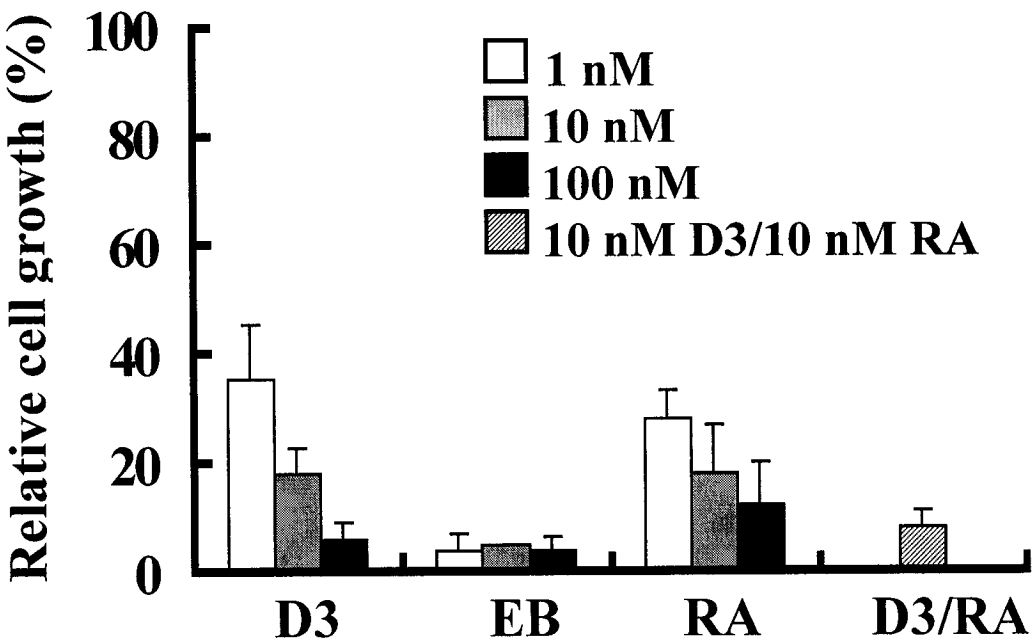
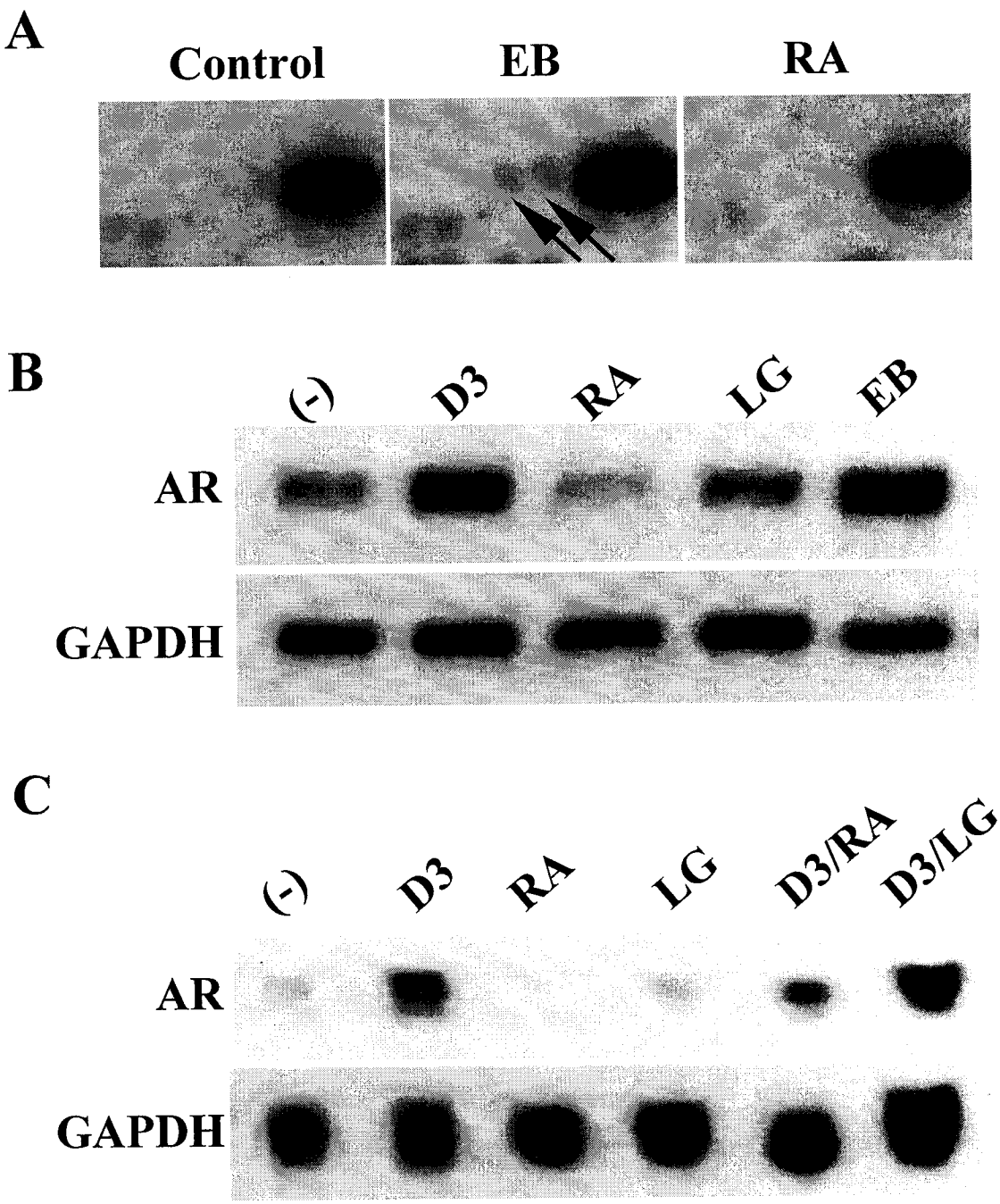

Relative cell growth (%)
100
80
60
40
20
0
1 nM
10 nM
100 nM
10 nM D3/10 nM RA
D3
EB
RA
D3/RA

Figure 4.2

A.Differential expression of amphiregulin in SCC25 cells treated with EB1089. Sections of autoradiograms of gene arrays hybridized with probe derived from RNA of SCC25 cells treated for 24h with vehicle (control), EB1089 (EB) or 13-cis RA (RA) are presented. The arrows indicate the position of amphiregulin cDNAs (spotted in duplicate) on the array.

B. Northern blots of total RNA probed for amphiregulin (AR) and GAPDH transcripts expressed in SCC25 cells treated with vehicle (-), or 100nM $1,25(OH)_2D_3$ (D3), 13-cis RA (RA), LG1069 (LG) or EB1089 (EB) for 24h.

C. Northern blots similar to B, except that cells were also treated with combinations of 1,25(OH)2D3 and LG1069 or 13-cis RA as indicated.


A
Control
EB
RA
B
(-)
D3
RA
LG
EB
AR
GAPDH
C
(-)
D3
RA
LG
D3/RA
D3/LG
AR
GAPDH

identified by duplicate screenings of gene arrays with probes derived from RNA purified from control cells or cells treated with $1,25(OH)_2D_3$ or EB1089 for 24h. Only genes that appeared to be regulated in both screenings were chosen for further study. A full list of target gene identified from multiple arrays will be reported elsewhere (manuscript submitted). Here, we will focus on the regulation of amphiregulin, one of the genes identified from the screens. Amphiregulin is a member of the epidermal growth factor family of peptide growth factors. Analysis of arrays showed that amphiregulin mRNA expression was elevated in $1,25(OH)_2D_3$-treated cells (not shown), and in EB1089-treated cells, but not in control or 13-cis RA-treated cells (Fig 4.2A). This regulation was confirmed by Northern analysis of RNA from cells treated with VDR-, RAR-, or RXR-specific ligands (Fig. 4.2B), which showed that amphiregulin expression was induced by $1,25(OH)_2D_3$ or EB1089, but not by 13-cis RA or the RXR-selective ligand LG1069.

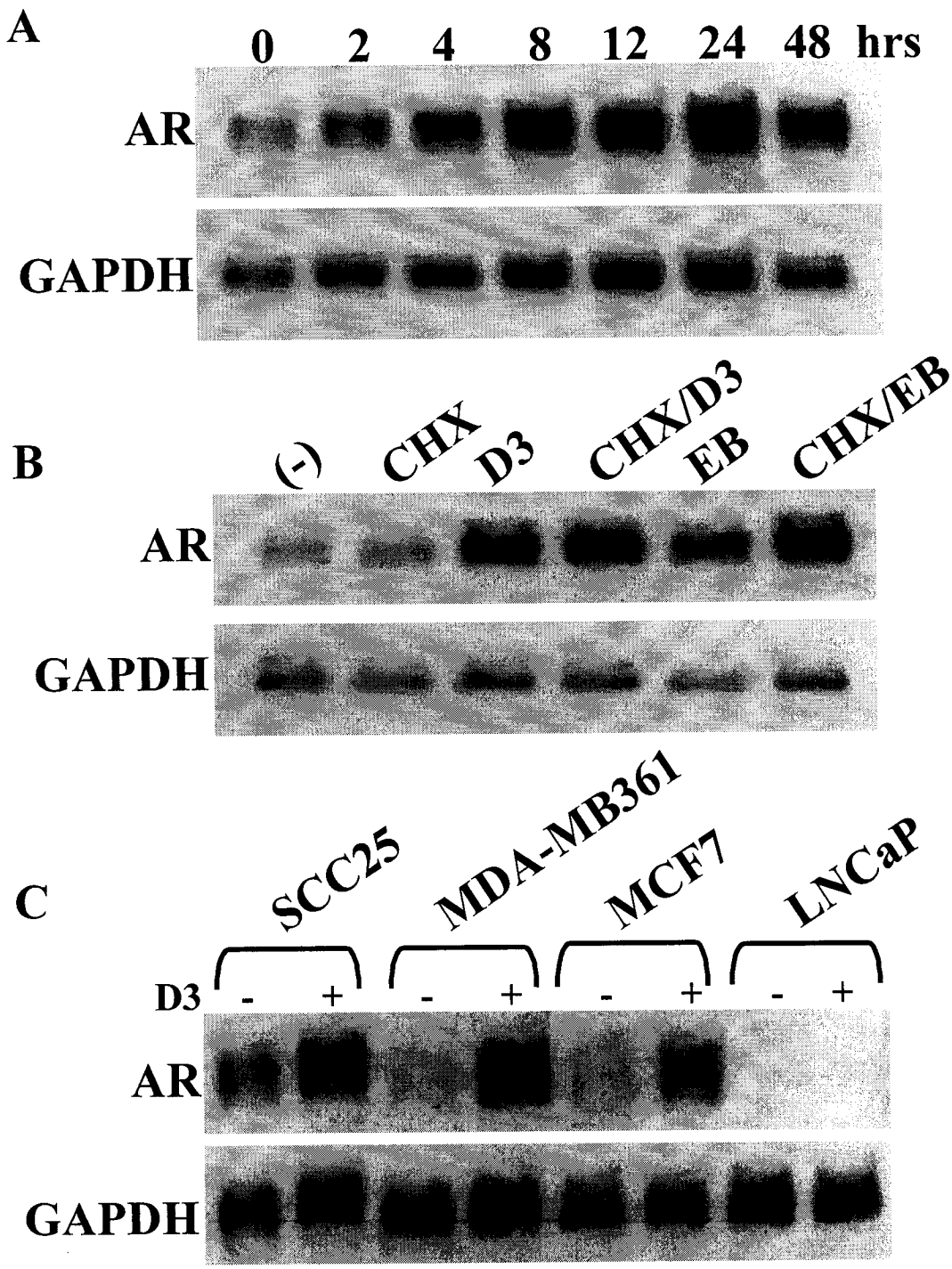
Remarkably, we found that 13-cis RA inhibited $1,25(OH)_2D_3$-dependent induction of amphiregulin expression, while the RXR-selective ligand LG1069 had no effect (Fig. 4.2C). This effect is not unique to the amphiregulin gene in SCC25 cells, as 13-cis RA also inhibited $1,25(OH)_2D_3$-dependent induced expression of the 24-hydroxylase gene (data not shown). In other studies, we have found that $1,25(OH)_2D_3$ inhibited expression of target genes of 13-cis RA (data not shown), indicating that, although $1,25(OH)_2D_3$ and 13-cis RA are growth inhibitory in combination (Fig. 4.1B), they can reciprocally inhibit expression of target genes.

Direct regulation of amphiregulin gene expression by $1,25(OH)_2D_3$ in SCC25 and breast carcinoma cells.

Figure 4.3

Further analysis of amphiregulin expression.

A. Induction of amphiregulin (AR) by $1,25(OH)_2D_3$ is rapid. SCC25 cells were treated with 100nM $1,25(OH)_2D_3$ for the times indicated prior to extraction of total RNA and analysis of amphiregulin expression by Northern blotting. B. $1,25(OH)_2D_3$ - or EB1089-stimulated expression of amphiregulin is not inhibited by cycloheximide (CHX) treatment. SCC25 cells were treated for 24 hrs with 100nM $1,25(OH)_2D_3$ (D3) or EB1089, or 200nM CHX alone or in combination as indicated. C. $1,25(OH)_2D_3$ regulates amphiregulin gene expression in breast but not prostate carcinoma cells. Northern analysis of amphiregulin expression in SCC25 cells, MDA-MB361 and MCF-7 breast cancer cells, which are ER-negative and -positive, respectively, and the prostate cancer cell line LNCaP. Cells were treated with vehicle (-) or (+) 100 nM $1,25(OH)_2D_3$ as indicated.


A
0 2 4 8 12 24 48 hrs
AR
GAPDH
B
(-) CHX D3 CHX/D3 EB CHX/EB
AR
GAPDH
C
SCC25 MDA-MB361 MCF7 LNCaP
D3 - + - + - + - +
AR
GAPDH

Further analysis showed that that effect of $1,25(OH)_2D_3$ on amphiregulin mRNA was rapid, with induction observable after 2-4h, and sustained over at least 48h (Fig. 4.3A). Treatment of cells with cycloheximide did not block induction of amphiregulin gene expression, indicating that *de novo* protein synthesis was not required for the effect of $1,25(OH)_2D_3$ or EB1089 (Fig. 4.3B). Amphiregulin expression was also induced in $1,25(OH)_2D_3$-sensitive human breast carcinoma cell lines MCF-7 and MDA-MB-361 (Fig. 4.3C), which are estrogen receptor α-positive and –negative, respectively, indicating that the stimulatory effect of $1,25(OH)_2D_3$ is not restricted to SCC of the oral cavity. However, no induction of amphiregulin gene expression was observed in the $1,25(OH)_2D_3$-sensitive human prostate cancer cell line LNCaP (Fig. 4.3C).

Amphiregulin inhibits SCC25 proliferation.

Amphiregulin has moderate affinity for the epidermal growth factor receptor, and has been shown to modulate cell proliferation at nanomolar concentrations. It was so-named for its capacity to inhibit proliferation of several human carcinoma cell lines and stimulate growth of other cell lines and primary cultures of normal fibroblasts (Shoyab et al., 1988; Plowman et al., 1990; Cook et al., 1991; Solic and Davies, 1997; Zvibel et al., 1999). SCC25 cells were treated over an 8-day period with amphiregulin concentrations ranging from 0.5 to 20 nM (Fig. 4.4). Statistically significant growth inhibition was observed after 8 days of treatment with 2 or 20nM amphiregulin, partially reproducing the inhibitory effects of $1,25(OH)_2D_3$ and EB1089 (see Fig. 4.1). Taken together, our results show that amphiregulin is a direct target gene of $1,25(OH)_2D_3$ in head and neck SCC and breast carcinoma cells. The antiproliferative effects of amphiregulin are

Figure 4.4

Amphiregulin inhibits growth of SCC25 cells in culture in a dose-dependent manner.

Cells in triplicate wells were treated with the concentrations of amphiregulin indicated. Media were changed and fresh amphiregulin was added daily. Statistical analysis was performed with a two-tailed student's T-test.

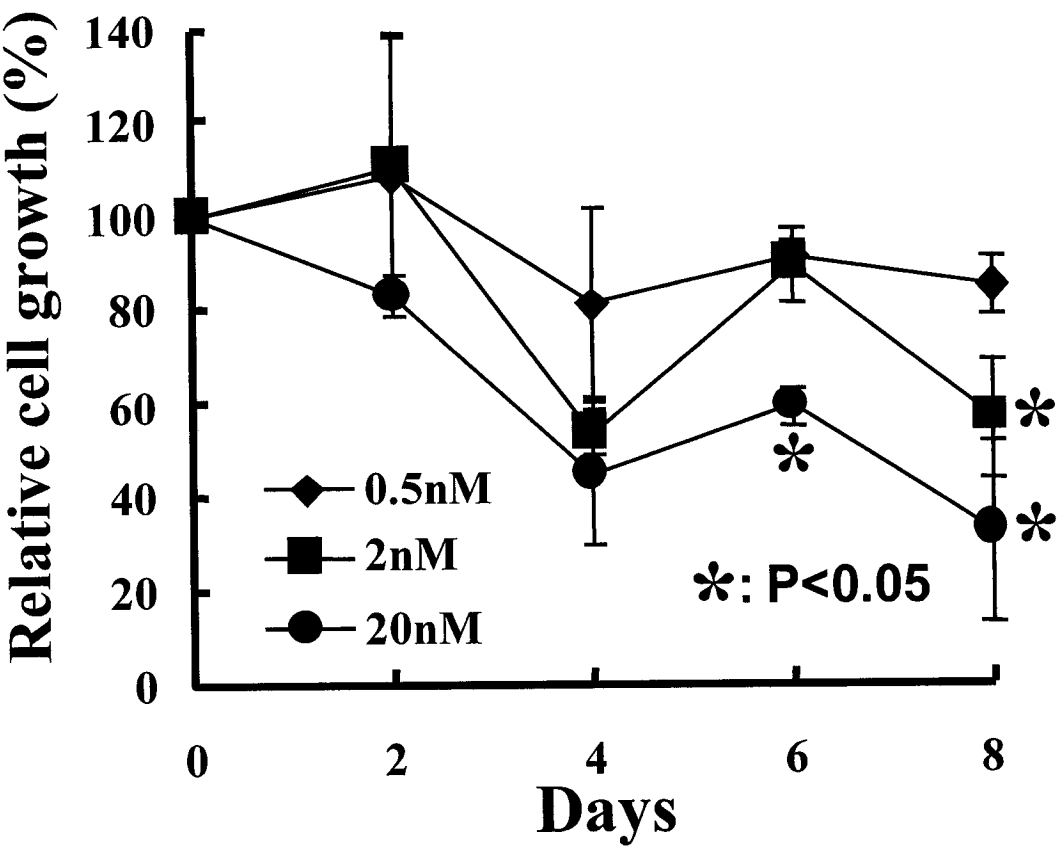
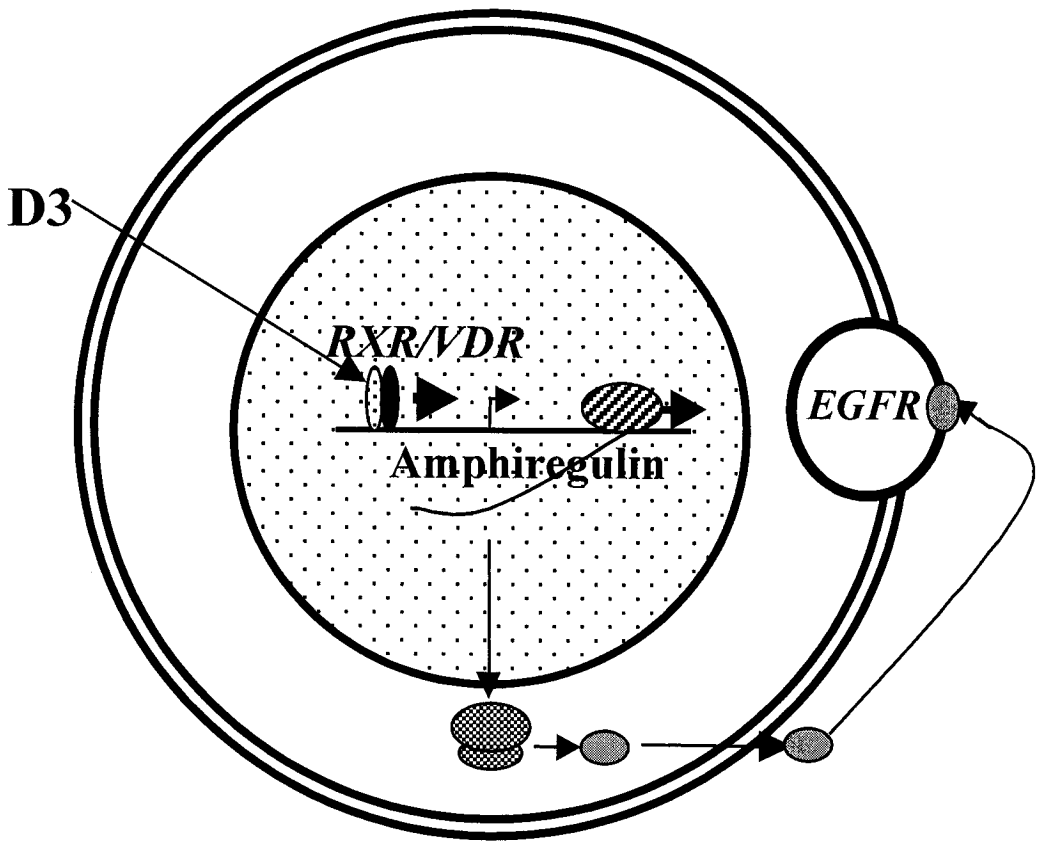

Relative cell growth (%)
140
120
100
80
60
40
20
0
0
2
4
6
8
Days
0.5nM
2nM
20nM
*: P<0.05
*
*
*

Figure 4.5

Schema presenting a potential model for 1,25(OH)$_2$D$_3$-dependent signaling through the nuclear vitamin D3 receptor (VDR) depicted as a heterodimer with its RXR partner.

Ligand dependent transactivation through the VDR leads to expression of amphiregulin and its action as an autocrine inhibitor of SCC25 cell proliferation.


D3
RXR/VDR
Amphiregulin
EGFR

consistent with it being a downstream effector of the growth inhibitory signal of $1,25(OH)_2D_3$ in SCC25 cells.

DISCUSSION

We have been interested in investigating the potential of $1,25(OH)_2D_3$ analogues as chemopreventive/ chemotherapeutic agents for treatment of head and neck SCC, and understanding their underlying mechanisms of action. SCC25 cells are a useful model for in vitro studies of head and neck SCC, because, although they are derived from a primary tumor (Hu et al., 1991), they retain a relatively differentiated phenotype that is more representative of the targets of chemoprevention. Both $1,25(OH)_2D_3$ and EB1089 completely inhibited SCC25 proliferation at nanomolar concentrations (Fig. 4.1). In other studies, we have observed similar growth inhibitory effects in a murine model of head and neck SCC (unpublished results), indicating that the potent antiproliferative activity of $1,25(OH)_2D_3$ and EB1089 in SCC is not restricted to SCC25 cells.

We have screened gene arrays to identify targets of $1,25(OH)_2D_3$ and EB1089. The amphiregulin gene was identified as a putative target gene in screens with both ligands. Subsequent studies showed that amphiregulin expression is directly regulated by $1,25(OH)_2D_3$ in SCC25 cells (Figs 4.2 and 4.3). The retinoid 13-cis RA, which also inhibits growth of SCC25 cells (Fig. 4.1), did not induce expression of amphiregulin (Fig. 4.2). Similarly, the RXR-specific retinoid LG1069 had no effect on amphiregulin expression, indicating that its induction is $1,25(OH)_2D_3$–specific. However, $1,25(OH)_2D_3$-dependent induction of amphiregulin expression was partially inhibited by 13-cis RA (Fig. 4.2C). This was part of a broader phenomenon of reciprocal inhibition of target gene expression observed in SCC25 cells. Thus, while $1,25(OH)_2D_3$ and 13-cis RA are growth inhibitory in SCC25 cells alone or in combination (Fig. 4.1), there is considerable cross-talk between the signals induced by the two ligands. Given that

$1,25(OH)_2D_3$ and 13-cis RA function by signaling through nuclear receptors, the reciprocal inhibition observed may be due to competition for limiting downstream factors.

Amphiregulin was identified in conditioned medium of MCF-7 breast cancer cells as a factor induced by phorbal 12-myristate 13-acetate, which is growth inhibitory (Shoyab et al., 1988). The growth regulatory characteristics of amphiregulin are complex and difficult to predict. Generally, amphiregulin stimulates proliferation of cultures of primary and immortalized cells. However, it can exhibit either mitogenic or growth inhibitory effects on transformed cells in culture (Plowman et al., 1990; Cook et al., 1991; Solic and Davies, 1997; Zvibel et al., 1999). Amphiregulin has moderate affinity for the epidermal growth factor receptor, and previous experiments have shown that it modulates cell proliferation at concentrations above 10nM (Solic and Davies, 1997). We observed a dose-dependent inhibition of SCC25 cell growth, and found that 2 and 20nM amphiregulin significantly inhibited proliferation (Fig. 4.4). Our experiments also showed that amphiregulin gene expression is induced by $1,25(OH)_2D_3$ in estrogen receptor-positive and negative breast cancer cells, but not in LNCaP prostate cancer cells (Fig. 4.3), indicating that the effect is cell-specific, but not limited to SCC. Taken together, our results show that induction of amphiregulin expression represents a primary response to $1,25(OH)_2D_3$ signaling, and that amphiregulin may act as a component of the antiproliferative response to $1,25(OH)_2D_3$ and its analogues by inhibiting SCC25 cell proliferation in an autocrine or paracrine manner (Fig. 4.5).

In addition to the mechanism depicted in Fig. 4.5, studies from other laboratories have raised the possibility that growth factors such as amphiregulin may affect signaling by

nuclear receptors including VDR/RXRs in other ways. Ligand-bound nuclear receptors modulate transcription of target genes by recruiting a series of factors known collectively as coregulators (Xu et al., 1999; Robyr et al., 2000). Of these, the p160 family, which includes SRC1, TIF2/GRIP1, and AIB1/ACTR/RAC3/ TRAM1/pCIP, is the best characterized. Recent experiments have shown that signaling through epidermal growth factor receptors can induce MAP kinase-dependent phosphorylation of SRC1 and AIB1 (Rowan et al., 2000; Font de Mora and Brown, 2000), which enhances their activity. It can be speculated, then, that amphiregulin signaling through the epidermal growth factor receptor may increase the activity of p160 family members expressed in target cells and lead to enhanced nuclear receptor function.

Conclusion

It should be noted that we have identified several $1,25(OH)_2D_3$ target genes in SCC25 cells and MCF-7 breast cancer cells (unpublished results), which suggest that $1,25(OH)_2D_3$ acts to modulate the function of several genes that control cell proliferation, as well as the function of adhesion proteins. Thus, while our results are consistent with induction of amphiregulin expression being a component of the antiproliferative response to $1,25(OH)_2D_3$ and its analogues, it is likely that these compounds exert their effects by modulating multiple facets of cell function.

Acknowledgement

We are grateful to Drs. Karin Hamberg and Lise Binderup (Leo Laboratories, Ballerup, Denmark) for $1\alpha,25(OH)_2D_3$ and EB1089, and Dr. Tim Willson (GlaxoWellcome Inc. Research Triangle Park, North Carolina) for LG1069. This work was supported by grant from the Cancer Research Society of Canada and a grant MT-13147 from the Canadian Institutes of Health Research. N.A. was supported by a postdoctoral fellowship from the Royal Victoria Research Institute, Montreal, Canada. J.H.W. and S.M. are holders of chercheur-boursier awards from the Fonds de Recherche en Santé du Québec (FRSQ).

CHAPTER 5

Inhibition of F-box protein $p45^{SKP2}$ expression and stabilization of cyclin-dependent kinase inhibitor $p27^{KIP1}$ in vitamin D analogue-treated cancer cells

Preface

This chapter of my thesis is based on the following manuscript:

Lin, R.*, Wang, T.T.*, and White, J.H. (2003) Inhibition of F-box protein $p45^{SKP2}$ expression and stabilization of cyclin-dependent kinase inhibitor $p27^{KIP1}$ in vitamin D analogue-treated cancer cells. *Endocrinology 144: 749-753.* ***Co-first authors**

$P27^{KIP1}$ is a key regulator of the G1/S checkpoint, and several studies have shown that its upregulation underlies the antiproliferative effects of $1,25(OH)_2D_3$ and its analogues. The effects of $1,25(OH)_2D_3$ on p27 RNA appear to vary with cell type as well. However, p27 mRNA did not seem to be regulated in our DNA microarrays. In the following chapter, we analyzed the effect of vitamin D3 on p27 in the mouse HNSCC model.

Abstract

Treatment of cancer cells with 1,25-dihydroxyvitamin D3 [$1,25(OH)_2D_3$] or its analogues induces growth arrest and expression of the cyclin-dependent kinase inhibitor $p27^{KIP1}$. While $1,25(OH)_2D_3$ transiently enhances $p27^{kip1}$ gene transcription in some cells, its effects on $p27^{KIP1}$ protein levels are generally more gradual and sustained. This suggests that $1,25(OH)_2D_3$ treatment may be stabilizing $p27^{KIP1}$ protein, which is sensitive to modification by the SCF^{SKP2} protein ubiquitin ligase and proteosomal degradation. Here, we show that treatment of AT-84 head and neck squamous carcinoma cells with the $1,25(OH)_2D_3$ analogue EB1089 increases $p27^{KIP1}$ protein levels without significantly affecting expression of its mRNA. EB1089 treatment repressed expression of mRNAs encoding the F-box protein $p45^{SKP2}$, a marker of poor head and neck cancer prognosis, and the cyclin kinase subunit CKS1, which is essential for targeting $p45^{SKP2}$ to $p27^{KIP1}$. This coincided with a reduction of total $p45^{SKP2}$ protein, and $p45^{SKP2}$ associated with $p27^{KIP1}$. Consistent with these findings, turnover of $p27^{KIP1}$ protein was strongly inhibited in the presence of EB1089. A similar reduction in $p45^{SKP2}$ expression and stabilization of $p27^{KIP1}$ protein was observed in $1,25(OH)_2D_3$-sensitive UF-1 promyelocytic leukemia cells, which also respond by transiently increasing $p27^{kip1}$ gene transcription. Our results reveal that $1,25(OH)_2D_3$ analogues increase levels of $p27^{KIP1}$ in different cell types by inhibiting expression of SCF^{SKP2} subunits, and reducing turnover of $p27^{KIP1}$ protein.

Introduction

Signal transduction by the active form of vitamin D3, 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] primarily controls calcium transport in the intestinal epithelia, and modulates bone resorption (1). However, 1,25$(OH)_2D_3$ and its analogues also stimulate cell differentiation and inhibit proliferation in a number of cellular and animal models, and its analogues are of interest because of their broad potential as anticancer agents (Jones et al., 1998). We are studying the antiproliferative and potential chemopreventive actions of 1,25$(OH)_2D_3$ analogues in head and neck squamous carcinoma (HNSCC; refs Prudencio et al., 2001; Akutsu et al., 2001; Lin et al., 2002), where development of second primary carcinomas after treatment of an initial malignancy is a major clinical problem (Benner et al., 1994).

1,25$(OH)_2D_3$ and its analogues regulate the expression cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$. 1,25$(OH)_2D_3$ affects $p21^{waf1/cip1}$ expression strongly cell-specifically (e.g. Prudencio et al., 2001; Liu et al., 1996; Munker et al., 1996;Hershberger et al., 1999), while its induction of $p27^{kip1}$ is generally more consistent. For example, $p27^{kip1}$ was induced in HNSCC lines under conditions where $p21^{waf1/cip1}$ was repressed (Prudencio et al., 2001; Hershberger et al., 1999). In addition, the rapid and transient induction of $p27^{kip1}$ transcripts in monocytic cells was accompanied by a delayed and more sustained increase in $p27^{KIP1}$ protein (Munker et al., 1996; Muto et al., 1999). $P27^{KIP1}$ protein is a substrate for of protein ubiquitin ligase (E3) SCF^{SKP2} (Spruck et al., 2001; Ganoth et al., 2001; Harper, 2001). The $p45^{SKP2}$ subunit of SCF^{SKP2} binds directly to the cyclin kinase subunit CKS1 (Spruck et al., 2001; Ganoth et al., 2001; Harper, 2001; Mongay et al., 2001), which directs $p45^{SKP2}$ to $p27^{KIP1}$. Significantly, $CKS^{-/-}$ mice express elevated levels of $p27^{KIP1}$ (Mongay et al., 2001).

We were interested in determining whether $p27^{KIP1}$ protein turnover was affected in cells treated with $1,25(OH)_2D_3$ analogues. Here, we show that $p27^{kip1}$ mRNA is unchanged in EB1089 treated AT-84 cells. Rather, treatment represses expression of $p45^{skp2}$ and cks1 mRNAs, leading to reduced association of $p45^{SKP2}$ protein with $p27^{KIP1}$, and strongly reduced $p27^{KIP1}$ protein turnover. A similar reduction in $p45^{SKP2}$ expression and stabilization of $p27^{KIP1}$ protein was observed in promyelocytic UF-1 cells. Taken together the results indicate that $1,25(OH)_2D_3$ analogues enhance $p27^{KIP1}$ expression in diverse cell types by reducing its turnover.

Materials and Methods

Tissue culture

Mouse AT-84 HNSCC cells and human promyelocytic UF-1 cells were cultured as described (Prudencio et al., 2001; Muto et al., 1999). Effects of EB1089 were analyzed by seeding cells in 100mm dishes at 60% confluence in 10ml of medium containing charcoal-stripped serum. Media were changed after 24h to charcoal-stripped medium containing 0.1 μM EB1089. Media were changed every 48h and fresh ligand was added.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed essential as described (Lin et al., 2002). For analysis of $p45^{skp2}$, $p27^{kip1}$ and cks1 mRNA expression, 1μl of RT reactions was amplified by PCR as follows: 30s denaturation at 94°C, 45s elongation at 72°C, and 30s annealing starting at 60°C, down 1°C per cycle to 55°C, and continuing 20 cycles amplification (94°C for 30s, 57.5°C for 30s, 72°C for 45s). Complementary DNAs for $p45^{skp2}$, $p27^{kip1}$ and cks1 were amplified using 5' primer 5'-CCTAAGCAGCTGTCCCAGAC-3', 3' primer 5'- GTGTCAGTCGGCATTTGATG-3' for $p45^{skp2}$, 5' primer 5'-GGATGGACGCCAGACAAG-3', 3' primer 5'-GGGGAACCGTCTGAAACATT-3' for $p27^{kip1}$, and 5' primer 5'-TTGGACAAATACGACGACGA-3', 3' primer 5'-CTTTGTTTTCTCGGGTAGTGG-3' for cks1. For amplification of GAPDH, 1μl of RT reaction was subjected to 18 cycles amplification (95°C for 30s, 56°C for 1min, 72°C for 25s) using 5' primer 5'-GGTGAAGGTCGGTGTCAACG-3', and 3' primer 5'-CAAAGTTGTCATGGATGACC-3'. All of the above reactions were performed in 25μl of 1.5mM $MgCl_2$, 50mM KCl, and 10mM Tris-

HCl (pH9.0) using 2.5U of Taq DNA polymerase (Pharmacia, Baie d'Urfe, Quebec). PCR reactions were loaded on 2% agarose gel, transferred for Southern blotting to a nylon membrane (Hybond-N+, Amersham, Baie d'Urfe, Quebec), and fixed by UV cross linker. The membrane was soaked in 3X SSC and 0.1% SDS at 50°C, and pre-hybridized at 42°C in 50mM-phosphate buffer pH6.5, 5X SSC, 10% Denhardt's solution containing 250μg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P end-labeled oligonucleotides 5'-GAGCTGAACCTCTCCTGGTG-3' for $p45^{skp2}$, 5'-CAAATGCCTGACTCGTCAGA-3' for $p27^{kip1}$, 5'-TCACATCTTGCTGTTCCGG-3' for cks1 and 5'-TCTTCACCACCATGGAGAAG-3' for GAPDH. Following hybridization, the membrane was washed four times in 2X SSC and 0.2% SDS for 5 min, three times in 0.1X SSC and 0.2% SDS for 30min at 50°C, dried, and autoradiographed. All experiments were repeated at least three times.

Immunoprecipitation and Western blot analysis

Cells were rinsed with PBS, harvested by scraping in 1 ml of PBS, and centrifuged (10,000 rpm, 5 min) at 4°C. Pellets were resuspended in 100 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP40) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and incubated on ice for 15 min. Lysates were centrifuged at 4°C (10,000 rpm, 10 min), and supernatants were recovered. Total protein was quantitated using the Bio-Rad (Richmond, CA) protein assay reagent. Lysates containing 100μg of total proteins normalized to a 1ml volume in lysis buffer were precleared by incubation with 30μl of 50% slurry protein A-agarose (Santa Cruz Biotechnology, CA) for 45 min on a rotator at 4°C. Protein complexes were immunoprecipitated from the precleared lysates by

addition of either 1μg of rabbit anti-$p27^{kip1}$ (Santa Cruz) or anti-$p45^{skp2}$ (Zymed Laboratories Inc., CA, US) for 1h at 4°C with rotation, followed by addition of 30μl of 50% slurry protein A-agarose for 1h on a rotator at 4°C. After 3 washes with lysis buffer, pelleted beads were boiled for 3min in 2X SDS-PAGE loading buffer. Immunoprecipitates and lysates (30μg) were resolved on 20% SDS-PAGE and analyzed by Western blotting with rabbit anti-$p45^{skp2}$ (0.5μg/ml) (Zymed Laboratories Inc., CA, US) and rabbit anti-p27 (0.4μg/ml), and secondary antibody goat anti-rabbit IgG HRP conjugate (0.04μg/ml) (Santa Cruz). The reaction was developed by the chemiluminescence method (NEN Life Science Product, Inc., MA, USA).

Metabolic labeling experiments

Cells treated with 10^{-7}M EB1089 or DMSO vehicle for 48h were rinsed with methionine-free RPMI-1640 medium, labeled with 5ml of 50μCi/ml [^{35}S]methionine for 3h, washed with medium containing charcoal-stripped serum and incubated in medium containing DMSO or EB1089. Cells were collected at 0, 1, 2, 3, 6 and 8h and lysed with lysis buffer. 100μg of total protein normalized to a 1ml volume in lysis buffer were precleared by incubation with 30μl of 50% slurry protein A-agarose (Santa Cruz Biotechnology, CA) for 45 min on a rotator at 4°C. Proteins were immunoprecipitated from the precleared lysates by addition of 1μg of rabbit anti-p27 (Santa Cruz) for 1h at 4°C with rotation, followed by 30μl of 50% slurry protein A-agarose for 1h on a rotator at 4°C. After 3 washes with lysis buffer, pelleted beads were boiled for 3 min in 2X SDS-PAGE loading buffer, resolved in 20% SDS-polyacrylamide gels and

autoradiographed. Signals were quantified using Kodak (Rochester, NY) digital science 1D Image Analysis software.

Results

Treatment of AT-84 cells with $1,25(OH)_2D_3$ or EB1089 induces G0/G1 arrest, and is accompanied by upregulation of $p27^{KIP1}$ protein (Prudencio et al., 2001). We probed further this regulation by RT/PCR analysis of $p27^{kip1}$ transcripts in EB1089-treated AT-84 cells. Strikingly, EB1089 treatment did not substantially alter $p27^{kip1}$ transcript levels over a 48h period (Fig. 5.1A), in contrast to results obtained in other cell lines where a strong but transient induction was observed (Liu et al., 1996; Munker et al., 1996; Muto et al., 1999). To investigate the possibility that mechanisms regulating the turnover of $p27^{KIP1}$ protein may be affected in EB1089-treated AT-84 cells, we analyzed the expression of transcripts encoding the F-box protein $p45^{SKP2}$ and the cyclin kinase subunit CKS1. RT/PCR analyses showed that expression of both transcripts was strongly and rapidly (<24h) repressed in treated cells (Figs. 5.1B and C). These results were consistent with those obtained by Northern blotting of RNA from EB1089-treated AT-84 cells (data not shown). In agreement with these and our previous studies (Prudencio et al., 2001), treatment with EB1089 for 72h led to a gradual accumulation of $p27^{KIP1}$ protein in AT-84 cells (Fig. 5.2A). Significant changes in $p27^{KIP1}$ protein were observed after 24h and levels continued to increase through 72h. No change in $p27^{KIP1}$ levels was observed in control experiments with vehicle-treated cells (data not shown). The accumulation of $p27^{KIP1}$ over 72h coincided with a progressive reduction in $p45^{SKP2}$ protein over the same period as assessed by direct Western blotting (Fig. 5.2B) or immunoprecipitation of $p45^{SKP2}$ following by Western blotting (Fig. 5.2C).

We were unable to detect by Western analysis expression of the SCF^{SKP} ubiquitin ligase subunit CKS1, which is essential for the interaction of $p45^{SKP2}$ with $p27^{KIP1}$ (Spruck et al., 2001; Ganoth et al., 2001; Harper, 2001; Mongay et al., 2001). Therefore, we analyzed by co-

Figure 5.1

Effects of EB1089 treatment on expression of p27kip1, p45skp2 and cks1 mRNA levels.

AT-84 cells in culture were treated with EB1089 for the times indicated, and expression of transcripts encoding p27KIP1 (*A*), p45SKP2 (*B*) and CKS1 (*C*) was analyzed by RT/PCR followed by Southern blotting with radiolabeled internal oligonucleotides. Southern blots of typical experiments are shown and the results of three experiments are presented in histograms. A control for GAPDH expression is shown at the bottom.

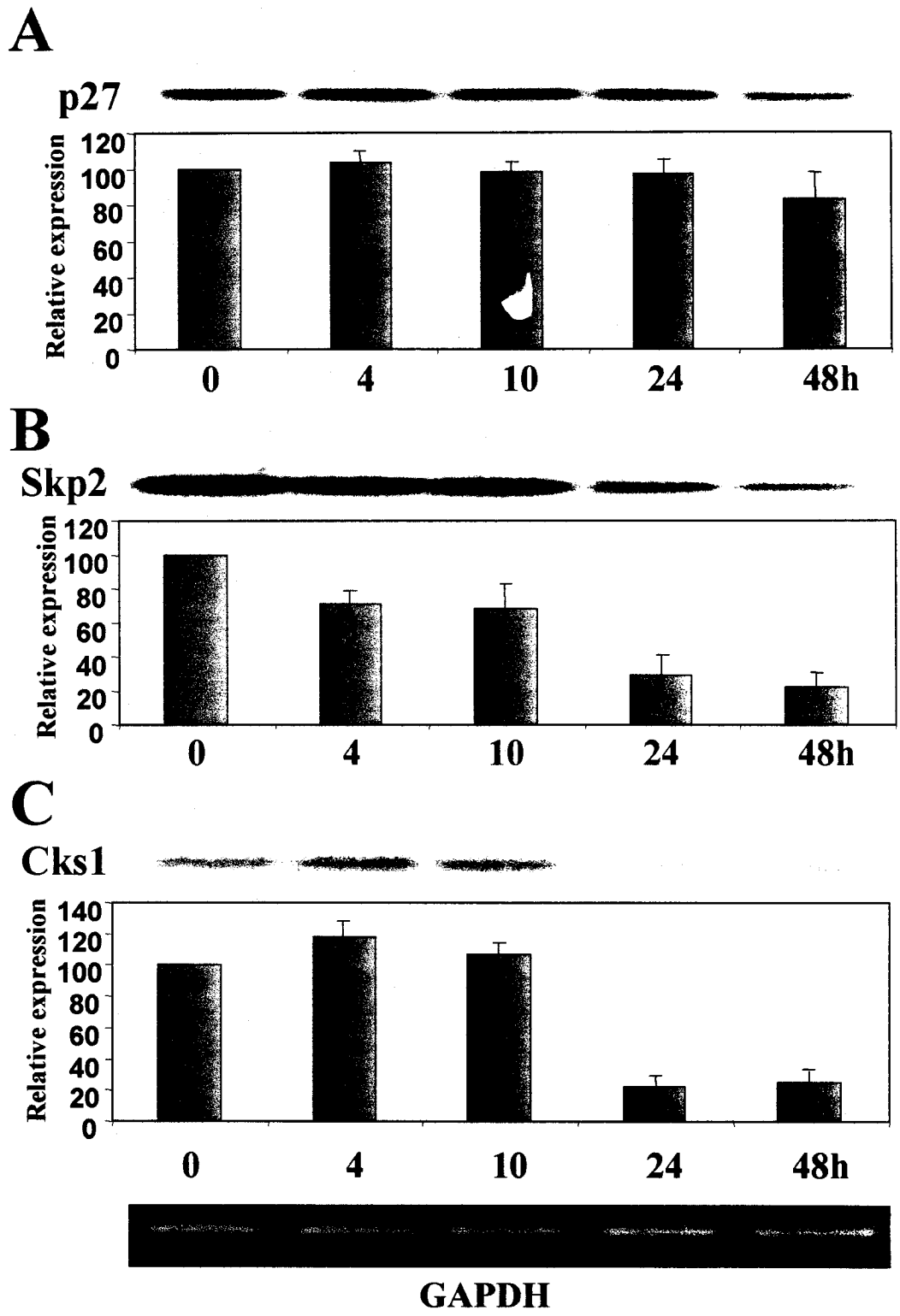
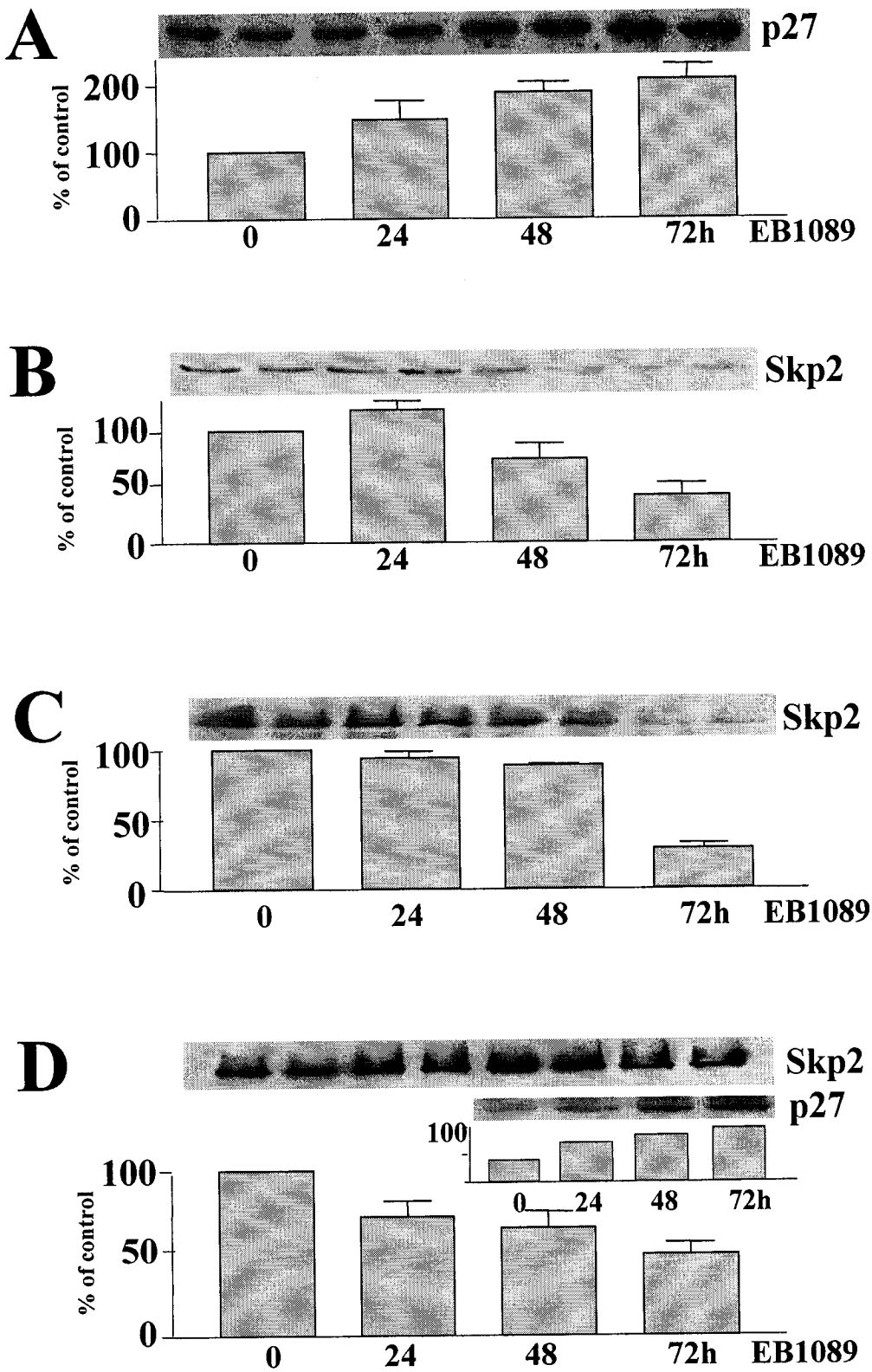

A
p27
Relative expression
120
100
80
60
40
20
0
0
4
10
24
48h
B
Skp2
Relative expression
120
100
80
60
40
20
0
0
4
10
24
48h
C
Cks1
Relative expression
140
120
100
80
60
40
20
0
0
4
10
24
48h
GAPDH

Figure 5.2

Effects of EB1089 treatment on p27KIP1 protein and its association with p45SKP2.

A and *B*. Extracts of EB1089-treated AT-84 cells were immunoblotted for expression of p27KIP1or p45SKP2. *C*. Cell extracts were immunoprecipitated with an anti-p45SKP2 antibody and immunoprecipitates were probed for p45SKP2. *D*. Cell extracts were immunoprecipitated with an anti-p27KIP1 antibody and immunoprecipitates were probed for p27KIP1 (inset) or p45SKP2. Note that in *A-D* Western blots show duplicate lanes corresponding to each time point. Histograms show the results of triplicate experiments.


A
p27
200
100
0
% of control
0
24
48
72h
EB1089
B
Skp2
100
50
0
% of control
0
24
48
72h
EB1089
C
Skp2
100
50
0
% of control
0
24
48
72h
EB1089
D
Skp2
p27
100
0
24
48
72h
100
50
0
% of control
0
24
48
72h
EB1089

immunoprecipitation the effects of EB1089 treatment on $p45^{SKP2}$ associated with $p27^{KIP1}$. Extracts of cells treated with EB1089 over 72h were immunoprecipitated with an anti-$p27^{KIP1}$ antibody, and immunoprecipitates were analyzed for expression of $p27^{KIP1}$ and $p45^{SKP2}$ (Fig. 5.2D). While immunoprecipitated $p27^{KIP1}$ increased in EB1089-treated cells (inset), 45^{SKP2} protein associated with $p27^{KIP1}$ fell 2-fold. Levels of co-immunoprecipitated $p45^{SKP2}$ were unaffected in a control experiment with DMSO-treated cells (data not shown). These observations indicate that a substantially reduced proportion of $p27^{KIP1}$ was complexed with $p45^{SKP2}$ in EB1089-treated cells. EB1089 treatment also led to a similar reduction in $p45^{SKP2}$ associated with $p27^{KIP1}$ in coimmunoprecipitation experiments performed with extracts of the $1,25(OH)_2D_3$–sensitive myelomonocytic cell line U937 (data not shown).

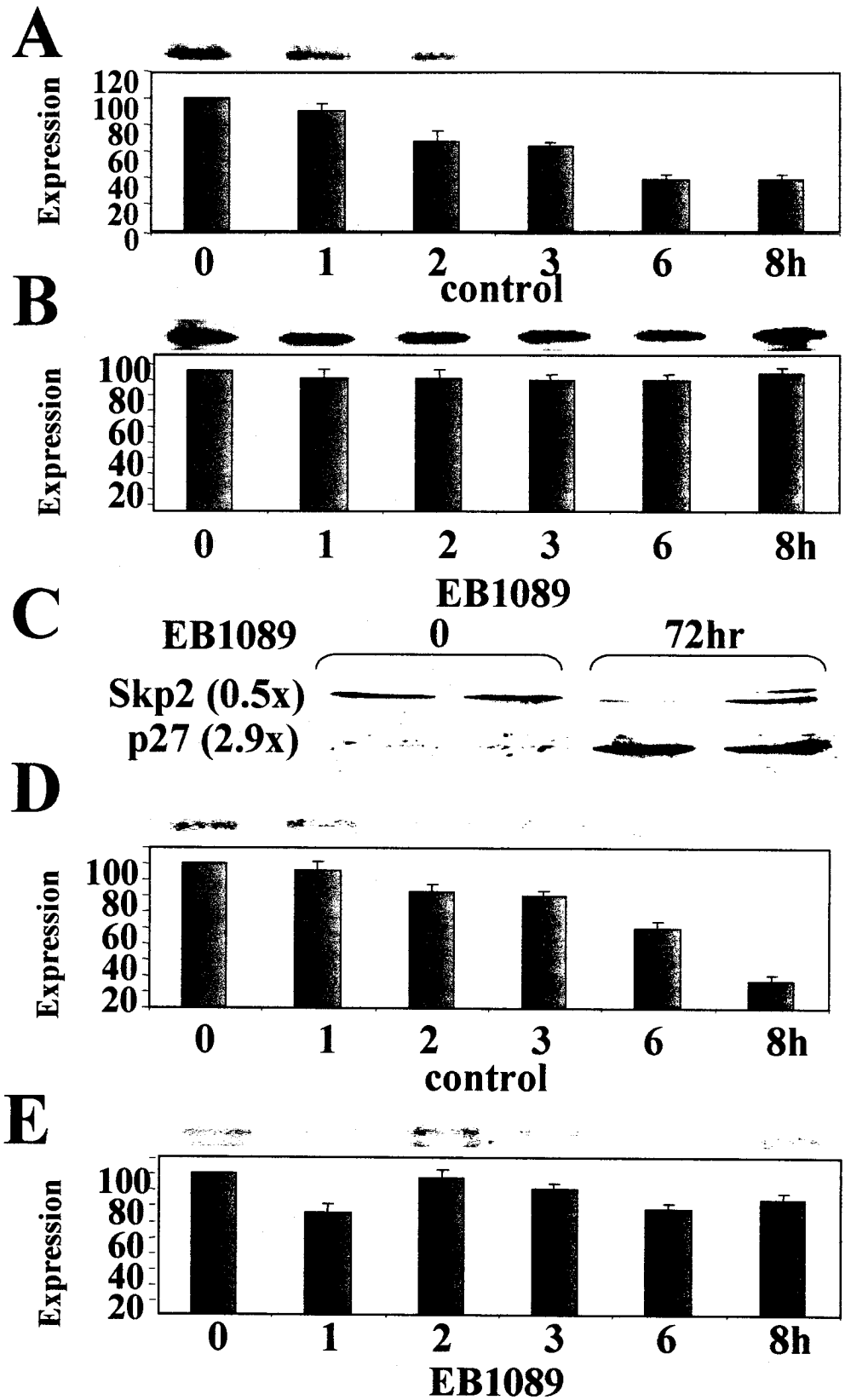
The reduced association of $p45^{SKP2}$ with $p27^{KIP1}$ suggested that $p27^{KIP1}$ was being turned over less rapidly in EB1089-treated cells. The effects of EB1089 on $p27^{KIP1}$ turnover in AT-84 cells were analyzed in metabolic labeling experiments (Fig. 5.3). Cells were treated with EB1089 or vehicle for 48h, labeled with ^{35}S-methionine, and chased with serum for the times indicated (Fig. 5.3). While radiolabeled $p27^{KIP1}$ immunoprecipitated from control cells diminished during the 8h chase, no $p27^{KIP1}$ turnover was observed in immunoprecipitates of EB1089-treated cells. Taken together our results indicate that EB1089 upregulates $p27^{KIP1}$ by inhibiting of expression of the $p45^{SKP2}$ subunit of the SCF^{SKP} ubiquitin ligase and reducing turnover of $p27^{KIP1}$ protein.

We were interested in determining whether EB1089 treatment stabilized $p27^{KIP1}$ protein in cells that also respond by transiently increasing $p27^{kip1}$ gene transcription. Previous studies have shown that UF-1 promyelocytic leukemia cells respond to $1,25(OH)_2D_3$ by transiently inducing $p27^{kip1}$ mRNA, followed by a more sustained increase in $p27^{KIP1}$ protein (Muto et al., 1999). Consistent with these findings, we observed a 2.9-fold increase in $p27^{KIP1}$ protein in UF-1 cells

Figure 5.3

EB1089 treatment stabilizes p27KIP1 protein.

Metabolic labeling experiments performed in vehicle- (A) and EB1089-treated AT-84 cells (B) are presented. Cells were labeled for 3h with 35S-methionine after 48h of treatment with either vehicle or EB1089, and chased as indicated. Extracts were immunoprecipitated with an anti-p27KIP1 antibody and immunoprecipitates were analyzed by gel electrophoresis. Histograms present the results of triplicate experiments. (C) Western analysis showing that EB1089 treatment enhances p27KIP1 protein expression and downregulates expression of p45SKP2 in human promyelocytic UF-1 cells. Metabolic labeling experiments performed as above in vehicle- (D) and EB1089-treated UF-1 cells (E) reveal that EB1089 treatment reduces p27KIP1 turnover in UF-1 cells (see Material and Methods for details).


A
Expression
120
100
80
60
40
20
0
0
1
2
3
6
8h
control
B
Expression
100
80
60
40
20
0
1
2
3
6
8h
EB1089
C
EB1089
0
72hr
Skp2 (0.5x)
p27 (2.9x)
D
Expression
100
80
60
40
20
0
1
2
3
6
8h
control
E
Expression
100
80
60
40
20
0
1
2
3
6
8h
EB1089

treated for 72h with EB1089 (Fig. 5.3C). Significantly, this increase was accompanied by a 50% decrease in $p45^{SKP2}$ levels over the same period (Fig. 5.3C). Consistent with these findings, $p27^{KIP1}$ protein turnover was decreased in EB1089-treated UF-1 cells (Figs. 5.3D and E). Taken together, the above results indicate that EB1089 treatment enhances $p27^{KIP1}$ expression in widely different cell types by reducing its turnover.

Discussion

$P27^{KIP1}$ is a key regulator of the G1/S checkpoint (14), and several studies have shown that its upregulation underlies the antiproliferative effects of $1,25(OH)_2D_3$ and its analogues (Prudencio et al., 2001; Liu et al., 1996; Munker et al., 1996; Hershberger et al., 1999; Muto et al., 1999). Our results indicate that $1,25(OH)_2D_3$ analogues can act by two cell type-dependent mechanisms to enhance expression of $p27^{KIP1}$; induction of expression of its gene, and inhibition of $p27^{KIP1}$ protein turnover. In contrast to results obtained in other cell lines (Liu et al., 1996; Munker et al., 1996; Muto et al., 1999), EB1089 had little effect on $p27^{kip1}$ mRNA levels in AT-84 cells over a 48h period. The varying effects of $1,25(OH)_2D_3$ analogues on $p27^{kip1}$ gene transcription may due in part to the fact that induction of expression is dependent on an Sp1 element and a CCAAT box in the $p27^{kip1}$ promoter and not a VDRE (Inoue et al., 1999), suggesting that these sites function cell-specifically.

In spite of the lack of response at the mRNA level, $p27^{KIP1}$ protein increased steadily over 72h in EB1089-treated AT-84 cells, consistent with studies in other cell lines (Liu et al., 1996; Munker et al., 1996; Muto et al., 1999). Metabolic labeling experiments indicated that the sustained increase was due to inhibition of $p27^{KIP1}$ degradation, which was consistent with the observed reduction in expression of $p45^{skp2}$ transcripts and protein. The inhibition of $p45^{skp2}$ gene expression by EB1089 was rapid, occurring within 24h, whereas the drop in $p45^{SKP2}$ protein levels continued over a 72h period. This reduced the total amount of $p45^{SKP2}$ associated with $p27^{KIP1}$ by coimmunoprecipitation even though $p27^{KIP1}$ protein levels were increasing. Taken together, these results indicate that the increase in levels of $p27^{KIP1}$ protein in EB1089-treated AT-84 calls arose from protein stabilization, and not from increased gene expression. A similar stabilization of $p27^{KIP1}$ protein was also observed in UF-1 promyelocytic leukemia cells.

The effect of EB1089 observed on $p27^{KIP1}$ protein expression in AT-84 cells (2-fold) is not dramatic. However, it is important to stress that progression through G1 into S phase is regulated by threshold levels of key regulators (Malumbres and Barbacid, 2001), and $p27^{KIP1}$ levels control the activity of the CDK2/cyclin E complex, whose function is critical for entry into S phase. Subtle changes in $p27^{KIP1}$ expression can have profound effects on cell function. Indeed, $p27^{KIP1}$ heterozygous mice are predisposed to tumors in a number of tissues after □ irradiation or exposure to carcinogens (Fero et al., 1998). Therefore, it is likely that the 2-fold increase observed in $p27^{KIP1}$ protein levels in treated AT-84 cells is critical for the antiproliferative effects of EB1089.

Just as reduced levels of $p27^{KIP1}$ are associated with cancer development, overexpression of $p45^{SKP2}$ is oncogenic. For example, elevated expression of $p45^{SKP2}$ was found in 49% of oral squamous cell carcinomas, and was correlated with a poor prognosis of affected patients (Kudo et al., 2001). Another study found an inverse correlation between $p45^{SKP2}$ and of $p27^{KIP1}$ levels in oral cancers (Gstaiger et al., 2001), and also showed that of $p45^{SKP2}$ could cooperate with H-Ras^{G12V} to transform primary fibroblasts. Similarly, $p45^{SKP2}$ cooperated with N-Ras in induction of T cell lymphomas in a mouse transgenic model, leading to significantly reduced survival times (Latres et al., 2001). Taken together, these studies strongly suggest that the downregulation of $p45^{SKP2}$ and consequent sustained upregulation of $p27^{KIP1}$ are key elements in the antiproliferative and anticancer actions of vitamin D analogues.

Acknowledgment

We are grateful to Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark) for the generous gift of EB1089, and to Dr. Masahiro Kizaki, Keio University School of Medicine, Tokyo, Japan for the generous gift of UF-1 cells.

CHAPTER 6

General Discussion

In the past few years, our understanding of the molecular mechanisms of nuclear receptor action has been improving rapidly. This is due to advancements in three fields, (i) 3D structure determination, (ii) analysis of the complexes formed between nuclear receptors and co-regulatory molecules, and (iii) the genetic analysis of nuclear receptors signal by gene "knock out" technology. The crystal structures of apo-, holo agonist-bound and antagonist- bound NR ligand-binding domain complexes have been important to our understanding of the structural principles, in particular of the ligand-induced allosteric alterations, that are the basis of nuclear actions. The identification and functional analysis of co-regulators (co-activators and co-repressors) previously predicted from squelching studies have provided us with the possibility to understand the propagation of the original signal from ligand binding through intramolecular allosteric effects to intermolecular interactions. However, much remains to be learned.

Many actions of $1,25(OH)_2D_3$ are believed to be mediated mainly by genomic mechanisms through the vitamin D_3 receptor, which controls gene expression by associating specifically with enhancer sequences in the promoter regions of $1,25(OH)_2D_3$ responsive genes. Thus, it could be speculated that the therapeutic effects of $1,25(OH)_2D_3$ and its analogues are obtained at least in part through an influence on the expression of genes with altered expression in cancer. Identification of differentially expressed genes following treatment of HNSCC with $1,25(OH)_2D_3$ and its analogue EB1089 would therefore be expected to increase our understanding of the activity of $1,25(OH)_2D_3$ and furthermore provide possible specific molecular targets for use in the development of new improved anticancer pharmaceuticals.

Recently, cDNA and oligonucleotide microarray technologies have provided powerful tools for analyzing simultaneously the expression of large numbers of genes. Arrays are ideally suited for analysis of the mechanisms underlying the antiproliferative effects of $1,25(OH)_2D_3$ and its analogue EB1089, given that they signal through a receptor that directly regulates target gene transcription.

Genomic studies of $1,25(OH)_2D_3$ analogue signaling in squamous cancer cells.

It is well known that the presence of functional VDRs (Chapter 2 and Chapter 4) is a prerequisite for most $1,25(OH)_2D_3$ -mediated effects to occur. Also, a direct relationship between the anti-proliferative effect of $1,25(OH)_2D_3$ and the level of receptors has been observed in some cell types in vitro (Reviewed in van den Bemd et al., 2000). Since the VDR is a direct regulator of gene transcription, $1,25(OH)_2D_3$ signaling is ideally suited to microarray analysis. In our studies, no evidence was found for loss of VDR expression or function in HNSCC cells. Moreover, no substantial differences were observed in induction of endogenous 24-hydroxylase gene expression, the transcription of which is controlled by VDRE-controlling promoter (Zou et al., 1997), or of a transiently transfected VDRE3-hsp68/lacZ reporter plasmid. This is consistent with other findings (Fig. 2.3) suggesting that VDR levels vary little among SCC lines (Ratnam et al., 1996; Xie and Bikle, 1998).

The growth inhibitory effects of $1,25(OH)_2D_3$ and its analogue EB1089 were evaluated in SCC25 cells in these studies (discussed in the next paragraph and see Fig. 2.1). SCC25 cells were chosen due to their relatively differentiated phenotype compared to SCC4, SCC9 and SCC15 cells, and are therefore more representative of the pre-

malignant targets of chemoprevention. Preclinical studies in HNSCC and other cancer models have shown that EB1089 has more potent antiproliferative effects than $1,25(OH)_2D_3$, but has less calcemic activity. We have analyzed the molecular mechanisms underlying the antiproliferative effects of EB1089 by screening over 10,000 genes on cDNA (Chapter 2) and oligonucleotide (Chapter 3) arrays. These studies have identified ~200 novel target genes (Table 2.1 and Table 3.1) of $1,25(OH)_2D_3$ /EB1089, many of which are key cell cycle regulators, are essential for normal DNA repair, or are markers of cellular differentiation. These experiments provide molecular genetic evidence that $1,25(OH)_2D_3$ and its analogues perform three key functions of chemopreventive agents: they are antiproliferative, they induce cellular differentiation, and have genoprotective effects. We further tested the expression of selected genes, which through northern blotting revealed that EB1089-stimulated expression in all genes tested were highly reproducible. The range of induction agreed well with that determined from our array analysis, and with that of a similar screening of thyroid hormone-regulated genes (Papathanasiou et al., 1991). In addition, we found an increase in the expression of 24-hydroxylase (CYP24) and of osteopontin in the EB1089-treated cells both of which are known to have a VDRE in the promoter region, which confirms the microarray techniques.

$1,25(OH)_2D_3$ and EB1089 regulate growth and target gene expression with similar efficacy

The inhibition of SCC cell proliferation by $1,25(OH)_2D_3$ is in agreement with previous results in many other cancer cells (Christakos et al., 1996; Gross et al., 1997),

and supports the hypothesis that $1,25(OH)_2D_3$ might be a factor protecting against HNSCC progress. $1,25(OH)_2D_3$ analogue EB1089 was a potent inhibitor of proliferation of SCC cells in culture at a 1nM-10nM (Fig. 2.1). Studies on other cancer cell types have suggested that EB1089 may be 50-200 times more potent than $1,25(OH)_2D_3$ in the regulation of cell growth (Hansen et al., 2000). This is again in accord with our results showing similar growth inhibition of 1nM EB1089 and 100nM $1,25(OH)_2D_3$ in SCC cells. The relatively high doses of $1,25(OH)_2D_3$ needed to obtain an inhibitory growth response many be due to the $1,25(OH)_2D_3$ -inactivating 24-hydroxylase enzyme, which was highly inducible by $1,25(OH)_2D_3$ and EB1089 in this study on HNSCC cells (Fig. 2.4). The ability of $1,25(OH)_2D_3$ to induce 24-hydroxylase through a vitamin D_3 receptor-dependent process is well known and is used as a marker of $1,25(OH)_2D_3$ action (Chen and DeLuca, 1995; Ohyama et al., 1996). Previously, induction of specific activity of 24-hydroxylase by $1,25(OH)_2D_3$ has been shown in cancer cells (Christopherson et al., 1986). Although, EB1089 up-regulated 24-hydroxylase mRNA expression here, the metabolism of EB1089 may not primarily involve 24-hydroxylase due to the altered side-chain of the compound (Shankar et al., 1997), and this may explain the higher potency of EB1089 compared to $1\alpha,25(OH)_2D_3$.

It is noteworthy in this regard that a comparison of several up-regulated and down-regulated target genes did not reveal any evidence for gene-specific differences in the efficacy of regulation by $1,25(OH)_2D_3$ and EB1089 (Fig. 3.5). However, in a number of instances, regulation by $1,25(OH)_2D_3$ was more transient than that of EB1089. The enhanced duration of EB1089-regulated gene expression was ascribed to its insensitivity to catabolism by 24-hydroxylase (Kissmeyer et al., 1997). Treatment of cells with

$1,25(OH)_2D_3$ and the cytochrome P450 inhibitor ketoconazole produced gene expression profiles that were essentially identical to those seen in cells treated by EB1089 alone (Fig. 3.6). The recently developed specific inhibitors of 24-hydroxylase might allow use of lower $1,25(OH)_2D_3$ concentrations to obtain growth inhibitory effects (Schuster et al., 2001a; Schuster et al., 2001b).

$1,25(OH)_2D_3$ and EB1089 are anti-proliferative and induce cellular differentiation.

$1,25(OH)_2D_3$ and its analogues likely exert their protective effects from their capacity to regulate cell proliferation and induce cellular differentiation (Sahpazidou et al., 2003). $1,25(OH)_2D_3$ analogues with low calcemic activity have been shown to have growth inhibitory effects on cells in tissue culture and in xenograft models of cancer (Gurlek et al., 2002; Colston et al., 1992; Campbell and Koeffler, 1997; Zhuang and Bernstein, 1998; Hershberger et al., 1999; Blutt et al., 2000; VanWeelden et al., 1998; Eisman et al., 1987; Honma et al., 1983). In vivo, in the presence of EB1089, we have shown a reduction in tumor size (80%) without inducing hypercalcemia in a mouse model of HNSCC (Prudencio et al., 2001). An identical dose of $1,25(OH)_2D_3$ induced hypercalcemia and reduced tumor growth to a lesser degree (Prudencio et al., 2001). The results are in accordance with the anti-tumor effects of EB1089 observed in xenograft models of breast and prostate cancer (VanWeelden et al., 1998; Blutt et al., 2000). Once again, EB1089 may be a potentially useful agent for cancer therapy.

The anti-proliferative effects of $1,25(OH)_2D_3$ are unlikely to be regulated by a single gene. Growth inhibition has been ascribed to several factors, including enhanced at transforming growth factor beta (TGFβ) signaling (Gurlek et al., 2002), epidermal growth

factor (EGF) and induction of cyclin-dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Gurlek et al., 2002; Zhuang and Bernstein, 1998; Prudencio et al., 2001; Liu et al., 1996; Munker et al., 1996). In Chapter 5, we have shown that the increased levels of $p27^{KIP1}$ in $1\alpha,25(OH)_2D_3$-treated HNSCC cells are due to reduced protein turnover rather than enhanced gene transcription. $1,25(OH)_2D_3$ down-regulates the expression of CKS1 and $p45^{SKP2}$, components of the SCF^{SKP2} ubiquitin ligase responsible for $p27^{KIP1}$ turnover (Chapter 5). The reduction in $p45^{SKP2}$ expression is significant because its overexpression is associated with a poor prognosis in HNSCC (Gstaiger et al., 2001; Hosomi et al., 1983).

It is well documented that cancer cells respond to a variety of paracrine/autocrine growth factors. EB1089 can reverse the growth stimulation effects of epidermal growth factor (EGF; Saez et al., 1994) and regulation of EGF receptor levels by $1,25(OH)_2D_3$ has been demonstrated (Koga et al., 1988; Desprez et al., 1991). In Chapter 4, we reported that the gene encoding amphiregulin (a gene identified on the Atlas microarray), a heparin-binding EGF-related growth factor, is transcriptionally regulated by $1,25(OH)_2D_3$ (Fig. 4.2 and Fig 4.3). Amphiregulin can exhibit either mitogenic or growth inhibitory effects on transformed cells in culture (Plowman et al., 1990; Cook et al., 1991; Solic and Davies, 1997; Zvibel et al., 1999). We observed dose-dependent inhibition of SCC25 cell growth and found that 2 and 20 nM amphiregulin significantly inhibited proliferation (Fig.ure 4.4).

In addition, microarray studies revealed that EB1089 down-regulated expression of several mitogenic factors in HNSCC cells. These included VEGF-related protein, which is mitogenic in Kaposi's Sarcoma and hematopoeitic cells (Liu et al., 1997), Cyr61,

which encodes a growth factor implicated in angiogenesis and tumorigenesis, whose expression is induced by estrogen in breast cancer cells (Sampath et al., 2001), and midkine (MK), a mitogenic factor overexpressed in several carcinomas (Ikematsu et al., 2000).

$1,25(OH)_2D_3$ and its analogues can induce differentiation of both primary cultures of non-malignant cells and cancer cells alike (Chida et al., 1985;Liu et al., 1996; Hosomi et al., 1983; Islam et al., 1996). In our microarray studies, EB1089 appear to drive cancer cells to a more differentiated state (less malignant state). By observing the gene expression profiles of treated and non-treated SCC25 cells, we found that EB1089 tended to repress the expression of several markers associated with cancer progression (e.g. N-cadherin, squamous cell carcinoma antigen (SCCA), tenascin C, tumour antigen L6, carcinoma associated antigen GA733-2) (Fig. 3.3 and Table 3.1) and induced the expression of several genes associated with differentiated epithelial cells (cystatin M, protease M, type XIII collagen, desmoglein 3) (Fig. 3.3 and Table 3.1). For example, SCCA is a serum marker of uterine cervix, head and neck, lung and esophageal cancers. Ablation of its expression inhibits growth and induces natural killer cell infiltration of tumors (Suminami et al., 2001). Tenascin C is an extracellular matrix protein with growth-, invasion-, and angiogenesis-promoting activities, and is an early marker of HNSCC progression (Ramos et al., 1998). The enhanced expression of N-cadherin in HNSCC cells is consistent with the phenomenon of "cadherin switching" observed in cancer cells. Other work has shown that repression of overexpressed N-cadherin in HNSCC is associated with restoration of an epithelial phenotype (Islam et al., 1996; Tomita et al., 200).

Molecular evidence for the chemopreventive action of $1\alpha,25(OH)_2D_3$.

DNA is generally considered to be the most critical cellular target when considering the carcinogenic and mutagenic effects of drugs, radiation and environmental chemicals. These agents may damage DNA by altering bases or disrupting the sugar phosphate. Our results have provided insights into the molecular basis of the chemopreventive effects of $1,25(OH)_2D_3$. Gene expression profiling in HNSCC cells revealed that the growth arrest and DNA damage gene (GADD45α) is induced in cells treated with EB1089 (Chapter 2; Prudencio et al., 2001), apparently through a p53-independent mechanism. A similar $1\alpha,25(OH)_2D_3$-dependent induction of GADD45α expression was seen *in vitro* and tumour xenografts of a mouse model of HNSCC (Prudencio et al., 2001). This is consistent with the induction of GADD45α expression recently observed in studies of the antineoplastic effects of $1,25(OH)_2D_3$ in insulinoma cells (Galbiati et al., 2002). The identification of GADD45α as a $1,25(OH)_2D_3$ target gene is important as it is directly implicated in DNA repair and is required for maintenance of global genomic stability (Hollander et al., 1999). This suggests that treatment with $1,25(OH)_2D_3$ or its analogues provides genomic protection against accumulation of mutations which are essential for cellular transformation and cancer progression.

Metallothionein genes are particularly noteworthy for their preferential induction by $1,25(OH)_2D_3$ in normal but not cancer-derived primary epithelial cells. In fact, several metallothionein genes are significantly downregulated by $1,25(OH)_2D_3$ in cancer cells.

Certain metallothioneins are reportedly overexpressed in cancer (Zhang et al., 1996). Thus, the downregulation of one of the metallothionein in HNSCC by EB1089, as we noted in Chapter 3, might be beneficial for therapy. $1,25(OH)_2D_3$ signaling is believed to participate in controlling the antioxidant machinery (Garcion et al., 1999). This is supported by our observations that EB1089 induces expression of several genes controlling redox balance in HNSCC (Table 2.1 and Table 3.1), including glucose-6-phosphate dehydrogenase (G6PD), which lies at the beginning of the pentose phosphate shunt, whose activity warrants a ready availability of reducing equivalent in times of need, glutathione peroxidase and thioredoxin reductase. Induction of Glutathione peroxidase protects cell proteins and membranes against oxidation by inhibiting the initiation of peroxidative attacks on membrane lipids. It has been suggested to be the first of line of defense against oxidative damage (Sardar, 1996). Induction of thioredoxin reductase activity has been observed in $1\alpha,25(OH)_2D_3$ -treated prostate and breast carcinoma cells (Swami et al., 2000; Krishnan et al., 2003). These results are consistent with the observation that treatment of leukemic cells reduces intracellular levels of reactive oxygen species (ROS) (Danilenko et al., 2003). The protective effects of $1,25(OH)_2D_3$ may be due to photochemical synthesis of vitamin D in skin by ultraviolet light, which is a DNA damaging agent and an inducer of (ROS) (Petersen et al., 2000).

$1,25(OH)_2D_3$ also stimulated expression of the gene encoding the NRF2 transcription factor (Ramos-Gomez et al., 2001). NRF2 expression is induced by a number of chemopreventive agents, and in turn stimulates expression of several phase II detoxifying enzymes. nrf2 -/- mice are more susceptible to carcinogenesis and resistant to protective effects of chemopreventive agents (Ramos-Gomez et al., 2001). The genes

mentioned above are unlikely to be directly involved in growth inhibition but are likely be key elements in mediating the chemopreventive activity of $1,25(OH)_2D_3$ and its analogue EB1089 by preventing DNA damage caused by ROS.

Insights from the microarray studies into immuno-modulatory effects of $1,25(OH)_2D_3$.

The VDR is expressed in most cells of the immune system, including T lymphocytes, and antigen presenting cells such as macrophages and dendritic cells (Provvedini et al., 1983; Brennan et al., 1987; Bhalla et al., 1984; Adorini, 2002). Several important data on the role of $1,25(OH)_2D_3$ signaling in controlling immune system function have come out of the experiments with VDR knock-out mice. For example, abnormal development of pro-inflammatory T helper 1 (Th1) cell development was observed in VDR knock-out mice (O'Kelly et al., 2002). Furthermore, mice rendered $1,25(OH)_2D_3$ deficient by knockout of the gene encoding 25-hydroxyvitamin D_3 1α-hydroxylase were deficient in peripheral T lymphocytes (Panda et al., 2001).

Chapter 3 gave more insights into the effects of $1,25(OH)_2D_3$ in squamous carcinoma cells derived from epithelial keratinocytes. Keratinocytes are considered to be an important part of the immune system of the skin (Slominski and Wortsman, 2000). In our array studies, EB1089 downregulated interferon γ-regulated genes encoding 9-27, 1-8D, interferon-inducible 56K protein, the T cell chemokine IP-10, and the chemokine RANTES (Table 3.1), consistent with an inhibition of IFNγ signaling. This is noteworthy because enhanced IFNγ signaling and overexpression of IP-10 underlie the inflammatory reactions in psoriasis (Giustizieri et al., 2001). In addition, $1,25(OH)_2D_3$ strongly induced

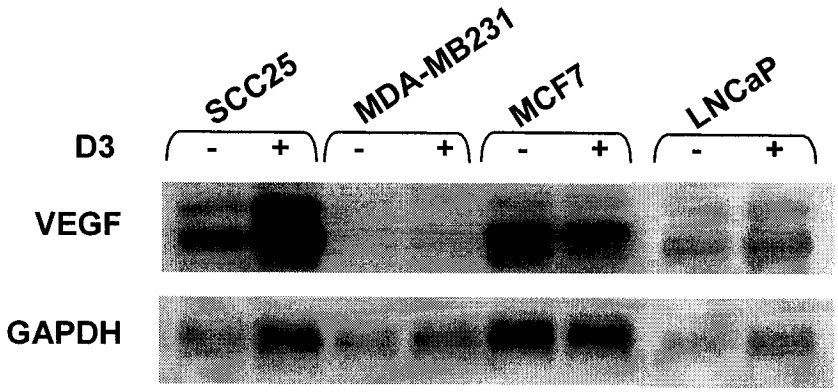
expression of T1/ST2, a member of the interleukin-1 receptor family (Fig. 3.5). In T1/ST2 knock-out mice experiments have revealed that T1/ST2 signaling is essential for normal T helper 2, Th2, cell differentiation (Townsend et al., 2000). These results are consistent with EB1089 stimulating Th2 responses, and inhibiting a number of genes associated with pro-inflammatory Th1 responses. This is in accordance with the protective effects of $1\alpha,25(OH)_2D_3$ analogues against a number of T-cell-driven autoimmune diseases in model studies and in the clinic (Adorini, 2002).

Future Direction

More than 50 genes are described in the literature as being responsive to vitamin D including at least 26 genes that contain promoters in which VDRE are identified (Hannah and Norman, 1994; Carlberg and Polly, 1998; Toell et al., 2000). With the sequence of the human genome available to us now, there is an additional technique for identification of target genes of $1,25(OH)_2D_3$ and its analogues.

Recently, we screened the human genome for sequences that correspond to high affinity DR3 or ER6 elements. These are elements that are either perfect consensus sequences, or which deviate by a single base pair from the consensus in one of the two half-sites. The advantage to this technique is that it will identify genes independent of their tissue of expression as opposed to the microarray technique. For example, we found in our array studies that the gene encoding vascular endothelial growth factor was regulated by $1,25(OH)_2D_3$ in SCC25 cells. Further experiments showed that the gene is not regulated in $1,25(OH)_2D_3$ -sensitive cells derived from breast, prostate or colon cancer (Fig. 6.1). The VDRE-scanning approach yielded over 2000 genes; however, it

only identified promoter proximal VDREs in some of the genes identified in our microarray screen. These results emphasize that although binding of the VDR to DNA is important for $1,25(OH)_2D_3$ action, it is possible that additional mechanisms may



significantly contribute to the regulation of many $1,25(OH)_2D_3$ -target genes.

Figure 6.1: VEGF expression in different cell lines in the presence of $1,25(OH)_2D_3$ (D3)

In conclusion, the microarray studies described above reveal many biologically relevant molecular targets of $1,25(OH)_2D_3$ in HNSCC cells and provide a starting point for additional investigations to more fully elucidate the mechanism of $1,25(OH)_2D_3$ action in cancer. The identification of genes primarily responsible for the anti-cancer effects of $1,25(OH)_2D_3$ could lead to a tissue or serum marker. Up and down regulation of various genes associated with neoplastic process by $1,25(OH)_2D_3$ is expected to give insight into the significance of this sterol in tumor biology. The evidence presented in our studies reveal that $1,25(OH)_2D_3$ is acting as a master steroid hormone by its critical involvement in a multitude of biological processes essential for maintaining normal life.

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Original Contribution to Knowledge

- We showed the use of DNA and Oligo Microarrays can generate reproducible results.
- We showed that $1,25(OH)_2D_3$ analogues block proliferation of head and neck squamous carcinoma cells (HNSCC) in vitro
- We identified ~200 novel $1,25(OH)_2D_3$ -regulated genes in HNSCC.
- We identified GADD45 alpha as a $1,25(OH)_2D_3$ -regulated gene which is essential for normal DNA repair, and is up-regulated in vitamin D3 analogue-treated tumors in vivo, indicating that vitamin D analogues have genoprotective effects.
- We showed that amphiregulin is a $1,25(OH)_2D_3$ target gene, and suggested that its induction may contribute to the growth inhibitory effects of $1,25(OH)_2D_3$.
- We provided numerous insights into the mechanisms by which $1,25(OH)_2D_3$ signaling controls cell proliferation, differentiation and immune system function.
- We showed that vitamin D analogues inhibit expression of p45 skp2, a component of the ubiquitin ligase that targets cell cycle regulator p27 kip1, and a marker of poor HNSCC prognosis, thus providing insights into how $1,25(OH)_2D_3$ analogues control cell proliferation.

Appendix

Regulation of Gene Expression by 1α,25-Dihydroxyvitamin D_3 and Its Analog EB1089 under Growth-Inhibitory Conditions in Squamous Carcinoma Cells

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Analogs of 1α,25-dihydroxyvitamin D_3 (1α, 25$(OH)_2D_3$) inhibit growth *in vitro* and *in vivo* of cells derived from a variety of tumors. Here, we examined the effects of 1α,25$(OH)_2D_3$ and its analog EB1089 on proliferation and target gene regulation of human head and neck squamous cell carcinoma (SCC) lines SCC4, SCC9, SCC15, and SCC25. A range of sensitivities to 1α,25$(OH)_2D_3$ and EB1089 was observed, from complete G_0/G_1 arrest of SCC25 cells to only 50% inhibition of SCC9 cell growth. All lines expressed similar levels of vitamin D_3 receptor (VDR) mRNA and protein, and no significant variation was observed in 1α,25$(OH)_2D_3$-dependent induction of the endogenous 24-hydroxylase gene, or of a transiently transfected 1α,25$(OH)_2D_3$-sensitive reporter gene. The antiproliferative effects of 1α,25$(OH)_2D_3$ and EB1089 in SCC25 cells were analyzed by screening more than 4,500 genes on two cDNA microarrays, yielding 38 up-regulated targets, including adhesion molecules, growth factors, kinases, and transcription factors. Genes encoding factors implicated in cell cycle regulation were induced, including the growth arrest and DNA damage gene, gadd45α, and the serum- and glucocorticoid-inducible kinase gene, sgk. Induction of GADD45α protein in EB1089-treated cells was confirmed by Western blotting. Moreover, while expression of proliferating cell nuclear antigen (PCNA) was reduced in EB1089-treated cells, coimmunoprecipitation studies revealed increased association between GADD45α and PCNA in treated cells, consistent with the capacity of GADD45α to stimulate DNA repair. While 1α,25$(OH)_2D_3$ and EB1089 modestly induced transcripts encoding the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, no changes in protein levels were observed, indicating that $p21^{waf1/cip1}$ induction does not contribute to the antiproliferative effects of 1α,25$(OH)_2D_3$ and EB1089 in SCC cells. Finally, in partially resistant SCC9 cells, there was extensive loss of target gene regulation (10 of 10 genes tested), indicating that resistance arises from widespread loss of 1α,25$(OH)_2D_3$-dependent gene regulation in the presence of normal levels of functional VDRs. (Molecular Endocrinology 15: 1127–1139, 2001)

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INTRODUCTION

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] modulates gene expression by binding to the vitamin D_3 receptor (VDR), which is a member of the nuclear receptor family of transcriptional regulators. 1α,25$(OH)_2D_3$-bound VDR heterodimerizes with retinoid X receptors (RXRs) and binds to specific DNA sequences in target genes known as vitamin D_3 response elements (VDREs) (1, 2). Apart from its well characterized role in calcium homeostasis (3), 1α,25$(OH)_2D_3$ also inhibits growth and stimulates differentiation of cancer cells derived from a variety of tissues, including breast, prostate,

colon, lung, endometrium, hematopoietic cells, and oral cavity (4–10). A side chain analog of $1\alpha,25(OH)_2D_3$, EB1089, caused apoptotic regression of MCF-7 breast carcinoma xenografts in nude mice (9), and animal studies and early clinical testing have shown that therapeutic doses of EB1089 can be tolerated without inducing hypercalcemia (10).

Analogs of $1\alpha,25(OH)_2D_3$ are potential candidates for chemoprevention of squamous cell carcinomas (SCCs) of the oral cavity, where formation of second primary carcinomas after surgical removal of tumors is a major concern (11, 12). Retinoids, such as 13-*cis* retinoic acid (13-*cis*-RA; isotretinoin) have been used clinically in SCC chemoprevention (13). 13-*cis*-RA functions by binding to retinoic acid receptors (RARs), which, like the VDR, are nuclear receptors and function as heterodimers with RXRs (1). However, SCC progression is associated with reduced expression of RARs, particularly RARs β and γ, loss of retinoid-regulated differentiation markers, and resistance to the antiproliferative effects of retinoids (14–19).

Here, we have examined the effect of $1\alpha,25(OH)_2D_3$ and EB1089 on proliferation and target gene regulation of four human SCC lines, SCC4, SCC9, SCC15, and SCC25, which were derived from the floor of mouth/base of tongue lesions (14). SCC25 cells express near normal levels of RARs β and γ and retain retinoid regulation of keratin-19 (K-19) gene expression, whereas SCC4, SCC9, and SCC15 cells express reduced levels of RARγ, no RARβ, and have lost regulated K-19 expression (14). The SCC lines display differing sensitivities to $1\alpha,25(OH)_2D_3$ and EB1089. SCC25 cell growth was completely blocked by $1\alpha,25(OH)_2D_3$ and EB1089, while the other lines were partially resistant. We have identified 38 $1\alpha,25(OH)_2D_3$ target genes in SCC25 cells, which encode several components of signal transduction pathways. Our results indicate that the antiproliferative effects of $1\alpha,25(OH)_2D_3$ and its analogs are mediated by multiple downstream components. Moreover, resistance to $1\alpha,25(OH)_2D_3$ in SCC9 cells was accompanied by widespread loss of target gene regulation in spite of normal levels of functional VDRs.

RESULTS

Effect of $1\alpha,25(OH)_2D_3$ and EB1089 on Growth of SCC Lines

The growth-inhibitory effects of $1\alpha,25(OH)_2D_3$, EB1089, and 13-*cis*-RA were evaluated in human lines SCC4, SCC9, SCC15, and SCC25, derived from SCCs of the oral cavity. The four lines displayed different sensitivities to $1\alpha,25(OH)_2D_3$ or EB1089 (Fig. 1). Over 10 days, SCC25 cell growth was completely inhibited by 100 nM $1\alpha,25(OH)_2D_3$, and 1–100 nM EB1089 (Fig. 1, A and B), while SCC4, SCC9, and SCC15 cells displayed partial resistance to both compounds (Fig. 1, D, E, G, H, J, and K). Similarly, SCC25 cell growth was strongly inhibited by 13-*cis*-RA (100 nM; Fig. 1C), whereas growth of SCC4, SCC9, and SCC15 cells was partially resistant (Fig. 1, F, I, and L). Flow cytometric analysis showed that treatment of SCC25 cells with 100 nM EB1089 for 72 h reduced the number of cells in S phase by 2.5-fold and significantly increased the percentage in G_0/G_1 (Fig. 2A). No evidence for DNA fragmentation was observed by terminal deoxynucleotidyltransferase dUTP-biotin nick end-labeling (TUNEL) assays under these conditions or over extended periods (Fig. 2B).

Resistance to $1\alpha,25(OH)_2D_3$ in SCC4, SCC9, and SCC15 cells Is Not Accompanied by Loss of Expression of Functional VDR

Given that resistance to 13-*cis*-RA correlated with lost or reduced expression of RARs β and γ, respectively (14), it was of interest to examine the levels of functional VDR in SCC cells. Northern and Western blots showed that VDR transcript and protein levels were essentially identical in all four lines (Fig. 3, A and B). Similarly, no evidence was found for loss of expression of the two major RXRs expressed in SCC, RXRα and RXRβ (data not shown). VDR function was tested by transient transfection of a $1\alpha,25(OH)_2D_3$-sensitive reporter-promoter plasmid containing a bacterial lacZ gene under control of a synthetic promoter containing three VDREs (20). High levels of $1\alpha,25(OH)_2D_3$-inducible β-galactosidase activity were detected in all cell extracts (Fig. 4A), suggesting that the lines expressed similar levels of functional VDRs. Both $1\alpha,25(OH)_2D_3$ and EB1089 induced similar levels of expression of the endogenous 24-hydroxylase (24-OHase) gene (Fig. 4B), whose promoter contains VDREs (21). Moreover, EB1089 induced 24-OHase expression with essentially identical potencies in $1\alpha,25(OH)_2D_3$-sensitive SCC25 cells and the partially resistant lines SCC4 and SCC9 (Fig. 4C). Taken together, the results of Figs. 3 and 4 suggest that resistance to $1\alpha,25(OH)_2D_3$ does not arise through loss of expression of functional VDRs.

Effects of $1\alpha,25(OH)_2D_3$ and EB1089 on Cell Cycle Regulators in SCC25 Cells

We were interested in analyzing the mechanisms underlying the antiproliferative effects of $1\alpha,25(OH)_2D_3$ and EB1089 in SCC25 cells. Previous work has shown that $1\alpha,25(OH)_2D_3$ rapidly (4 h) and strongly stimulated expression of the cyclin-dependent kinase inhibitor genes $p21^{waf1/cip1}$ and $p27^{kip1}$ in myeloid leukemia cells under conditions where it induced differentiation and inhibited cell growth (4, 22). However, the magnitude of the effect of $1\alpha,25(OH)_2D_3$ on $p21^{waf1/cip1}$ expression varies widely in different cell lines (4, 6, 22–24). We found that $1\alpha,25(OH)_2D_3$- or EB1089-dependent induction of $p21^{waf1/cip1}$ transcripts in SCC25 cells was gradual and modest (Fig. 5A and data not shown), whereas no effect was observed on expression of $p27^{kip1}$ or p53 mRNA levels (Fig. 5B). The modest effect of $1\alpha,25(OH)_2D_3$ and EB1089 on $p21^{waf1/cip1}$ mRNA levels did not give rise to significant changes in $p21^{waf1/cip1}$ protein, however. In addition, no effect of $1\alpha,25(OH)_2D_3$ or EB1089 was observed on $p27^{kip1}$ protein levels (Fig. 5C).

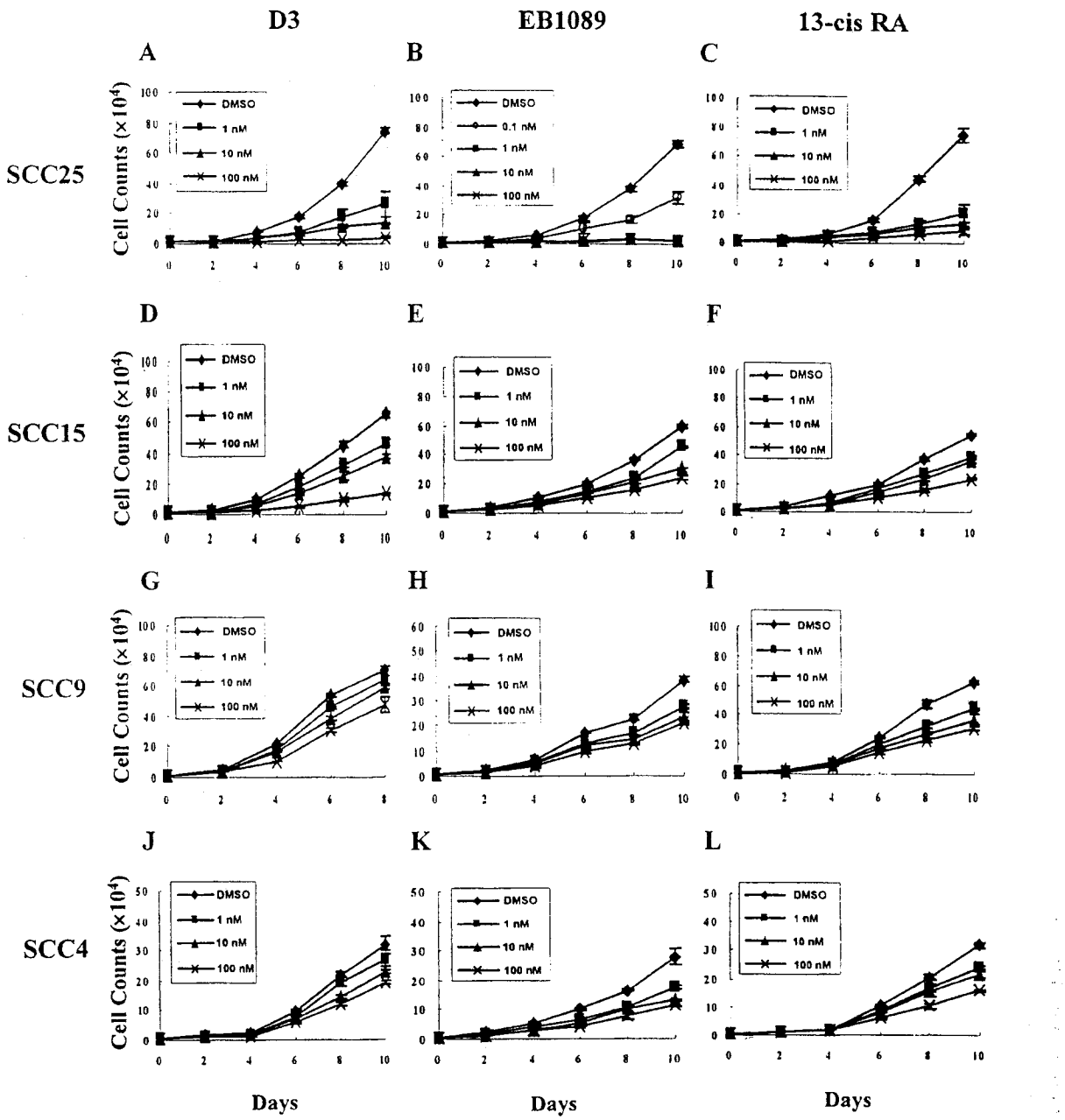

Fig. 1. Dose-Dependent Effects of $1\alpha,25(OH)_2D_3$, EB1089, and 13-*cis*-RA on Proliferation of SCC Lines in Culture

SCC lines were treated with 1, 10, or 100 nM $1\alpha,25(OH)_2D_3$ (A, D, G, and J), 0.1, 1, 10, or 100 nM EB1089 (B, E, H, and K), and 1, 10, or 100 nM 13-*cis*-RA (C, F, I, and L). Media were changed and fresh ligand added every 2 days over the 10-day period of the experiment. Each *point* represents the result obtained from triplicate wells (see *Materials and Methods* for details).

Identification of Target Genes of $1\alpha,25(OH)_2D_3$ and EB1089 by Screening of cDNA Microarrays

We screened cDNA microarrays for novel target genes of $1\alpha,25(OH)_2D_3$ and EB1089 in SCC25 cells to identify factors mediating their antiproliferative effects. More than 4,500 genes on two different gene arrays [Atlas array, 588 genes; (CLONTECH Laboratories, Inc., Palo Alto, CA); Named Genes filter, 4,000+ genes (Research Genetics, Inc., Huntsville, AL)] were screened with probes derived from vehicle-treated cells or cells treated with EB1089 for 24 h. Previous work has shown that there is considerable variation in gene expression levels associated with screening gene arrays (25–28). Arrays were therefore screened multiple times, and only reproducibly regulated genes were retained. Two rounds of screening of Atlas arrays yielded 10 candidate genes, of which 6 were revealed

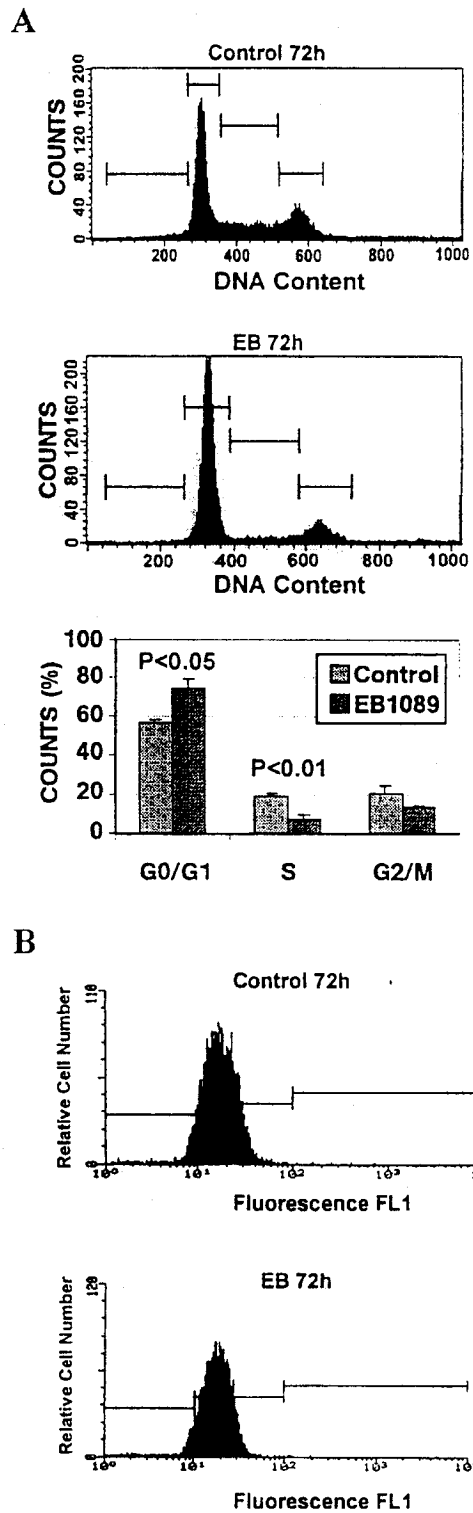

Fig. 2. Flow Cytometric Analysis and TUNEL Assay of Control or EB1089-Treated SCC25 Cells

A, SCC25 cells were treated with vehicle (SCC25 cont) or EB1089 (SCC25 EB) for 72 h. A histogram of fractions of cells in G_0/G_1, S, or G_2 from three independent experiments is presented. Statistical significance was determined using Student's *t* test. B, Representative histograms of three experiments assessing 3′-OH end labeling characteristic of apoptotic cells (TUNEL assay). Control cells and cells treated for 72 h with 100 nM EB1089 display minimal DNA fragmentation.

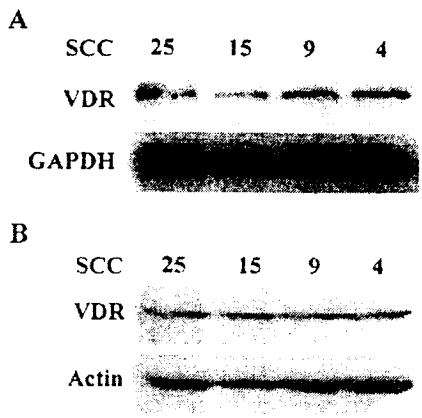

Fig. 3. Expression of VDR Transcripts and Protein in SCC Lines

A, Northern analyses are presented of transcripts encoding the VDR in SCC lines, along with GAPDH controls. B, Western blots are presented of VDR and β-actin protein levels in SCC lines.

by Northern blotting to be regulated by $1\alpha,25(OH)_2D_3$ and EB1089 (Fig. 6A, and data not shown; Table 1). In addition to $p21^{waf1/cip1}$ (not shown), these included novel target genes amphiregulin, a member of the epidermal growth factor family, the transcription factor fos-related antigen-1 (fra-1), the growth arrest and DNA damage (gadd45α) gene, and integrin α7B. We also found that the vascular endothelial growth factor (VEGF), which has been shown to be a $1\alpha,25(OH)_2D_3$ target gene in osteoblast-like cells (29, 30), was regulated by EB1089 in SCC25 cells.

Initial analysis of Research Genetics, Inc. gene filters screened with duplicate preparations of probe from vehicle-treated cells revealed a substantial number of differentially expressed genes (data not shown), which likely corresponded to random fluctuations in gene expression observed in expression profiling (25–28). Therefore, filters were screened three times each with probe from independent preparations of vehicle- and EB1089-treated cells, generating nine sets of cross-comparisons. Genes that were reproducibly regulated at least 1.5-fold in all comparisons were conserved. This yielded 32 additional up-regulated genes representing several different classes of proteins (Table 1). Screening under these conditions did not reveal any reproducibly down-regulated genes. Up-regulated genes included calmodulin, which has previously been shown to be a $1\alpha,25(OH)_2D_3$ target gene (31). Northern blotting, used to further test expression of 10 of these genes, revealed EB1089-stimulated expression in all

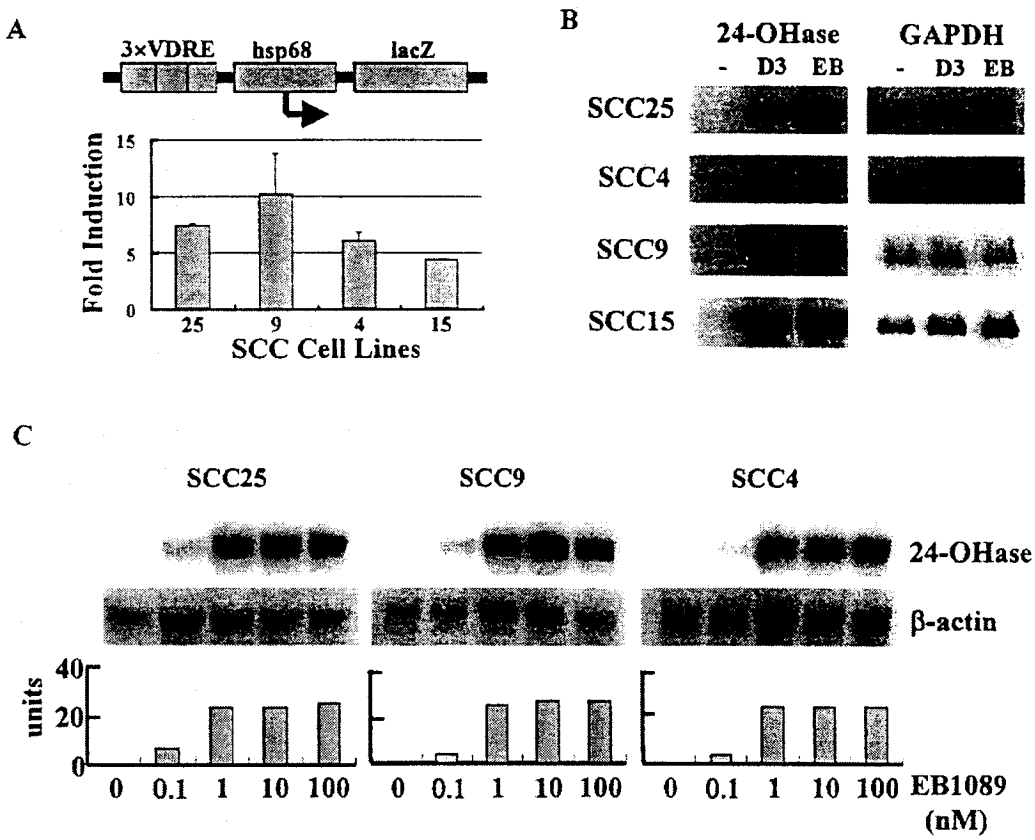

Fig. 4. Assessment of VDR Function in SCC Lines

A, Cells were transfected with the $1\alpha,25(OH)_2D_3$-sensitive reporter plasmid VDRE3-hsp68-lacZ and treated with vehicle or 10 nM $1\alpha,25(OH)_2D_3$ for 24 h (see *Materials and Methods* for details). Data are presented as fold induction of lacZ expression observed in the presence of $1\alpha,25(OH)_2D_3$. B, Induction of endogenous 24-OHase gene expression by $1\alpha,25(OH)_2D_3$ or EB1089 in SCC lines. Northern blots of total RNA extracted from cells treated for 24 h with vehicle (−), $1\alpha,25(OH)_2D_3$-, or EB1089-treated cells are presented. C, Dose-dependence of 24-hydroxylase induction in $1\alpha,25(OH)_2D_3$-sensitive SCC25 cells and partially resistant SCC4 and SCC9 cells. Northern blots of 24-OHase and β-actin controls are presented above, along with the normalized results of densitometric scanning of the 24-OHase blots below.

cases (Fig. 6B), indicating that the data in Table 1 are highly reliable. Most of the genes retained from phosphorimager analysis of the Research Genetics, Inc. arrays were up-regulated 2- to 4-fold (Table 1). This range of induction agrees well with that of up-regulated targets identified in a similar screen of thyroid hormone-regulated genes (33).

Broad but Selective Loss of Target Gene Expression in $1\alpha,25(OH)_2D_3$-Resistant SCC Lines

Given the resistance of SCC9 cells to the inhibitory effects of $1\alpha,25(OH)_2D_3$ and EB1089, we analyzed the regulation of target genes in these cells. Remarkably, in spite of apparently normal induction of 24-OHase expression (Fig. 4), regulation of all of the target genes tested in SCC9 cells was either lost, or in the case of calmodulin and GAP SH3 binding protein, attenuated (Fig. 6C). These results provide a strong correlation between increased resistance to the antiproliferative effects of $1\alpha,25(OH)_2D_3$ and a broad but selective loss of $1\alpha,25(OH)_2D_3$ target gene regulation in the presence of apparently normal levels of functional VDR.

EB1089 Treatment Induces Expression of GADD45α Protein and Enhances Formation of GADD45α-Proliferating Cell Nuclear Antigen (PCNA) Complexes

One of the more intriguing genes identified from the array screening presented above was gadd45α (Fig. 6 and Table 1). Gadd45α is a p53 target gene induced by a variety of agents that damage DNA and arrest cell growth (33–36), and overexpression of GADD45α inhibits cell proliferation (34). Ablation of the gadd45α gene provided evidence that GADD45α functions to maintain global genomic stability (35). Peak expression of GADD45α occurs in G_1. DNA repair is enhanced at the G_1/S checkpoint, and several studies have suggested that GADD45α enhances DNA repair,

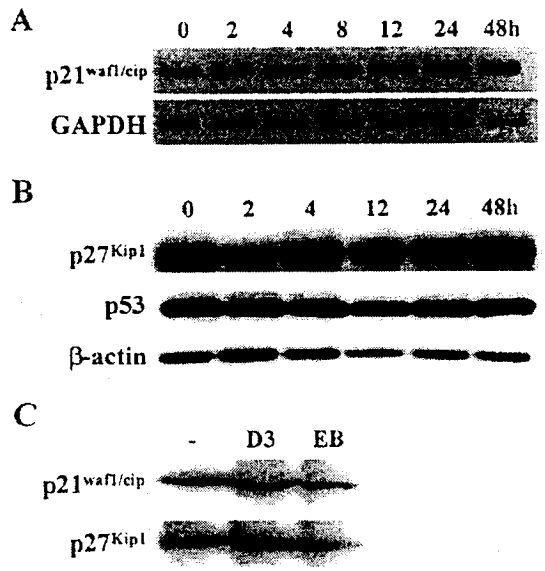

Fig. 5. EB1089-Inducible Expression of $p21^{waf1/cip1}$, but Not $p27^{kip1}$ or p53, in SCC25 Cells

A, The effect of EB1089 on expression in SCC25 cells of $p21^{waf1/cip1}$ and a GAPDH control were analyzed by Northern blotting of 20 μg of total RNA from cells treated with 10 nM $1\alpha,25(OH)_2D_3$ for the times indicated. B, The effect of EB1089 on expression in SCC25 cells of $p27^{kip1}$ and p53 along with a β-actin control was analyzed by RT-PCR. Amplified products were probed with ^{32}P-labeled internal oligonucleotides as detailed in *Materials and Methods*. C, Western blotting of immunoprecipitates of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ from SCC25 cells treated for 48 h with vehicle (−), or 100 nM 1,25-$(OH)_2D_3$ (D3) or EB1089 (EB).

at least in part, through its interaction with PCNA (36–38).

Induction of gadd45α mRNA by EB1089 was only partially blocked by protein synthesis inhibitor cycloheximide (Fig. 7A, and data not shown), indicating that the effect of EB1089 is at least partially direct. In related studies, we found no effect of cycloheximide on induction of gadd45α transcripts by EB1089 in the mouse SCC line AT-84 (38a). Immunoprecipitations from control and treated SCC25 cells revealed that EB1089 induced expression of GADD45α protein (Fig. 7B), consistent with its effects on gadd45α mRNA levels. Previous studies have demonstrated that γ and UV irradiation induce GADD45α and enhance its interaction with PCNA (36, 37). It was therefore of interest to determine whether a similar interaction was induced by EB1089, which is not a DNA damaging agent. While EB1089 treatment of SCC25 cells consistently reduced expression of PCNA protein (Fig. 7B and data not shown), reciprocal coimmunoprecipitations revealed an increased association between PCNA and GADD45α in EB1089-treated cells (Fig. 7B). Thus, $1\alpha,25(OH)_2D_3$ analog EB1089 induces expression of GADD45α, leading to increased formation of GADD45α-PCNA complexes. Taken together, our results suggest that induction of GADD45α contributes to the growth-inhibitory effects of $1\alpha,25(OH)_2D_3$ and EB1089 in SCC25 cells.

DISCUSSION

The results presented above show that $1\alpha,25(OH)_2D_3$ and EB1089 were as or more potent, respectively, than 13-*cis*-RA in inhibiting growth of SCC25 cells in culture. SCC4, SCC9, and SCC15 cells were partially resistant to 13-*cis*-RA and to $1\alpha,25(OH)_2D_3$ and EB1089 (Fig. 1), raising the possibility of a common underlying mechanism of resistance. Expression of RARs β and γ is lost or reduced, respectively, in SCC4, SCC9, and SCC15 cells (14). However, no evidence was found for loss of VDR expression or function in these lines. No substantial differences were observed in induction of endogenous 24-hydroxylase gene expression, the transcription of which is controlled by a VDRE-containing promoter (21), or of a transiently transfected VDRE3-hsp68/lacZ reporter plasmid. This is consistent with other findings suggesting that VDR levels vary little among SCC lines, including SCC4 (39, 40). Our results showed that VDRs expressed in all four lines studied retained the capacity to activate transcription from VDRE-containing promoters. We have also characterized a mouse SCC line, AT-84, which is highly sensitive to $1\alpha,25(OH)_2D_3$ and EB1089 but resistant to the growth- inhibitory effects of retinoids (38a), showing that resistance to $1\alpha,25(OH)_2D_3$ and retinoids is not necessarily coupled.

Several results suggest that many factors contribute to the growth-inhibitory effects of $1\alpha,25(OH)_2D_3$ in a cell-specific manner. Transcripts encoding the cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ were strongly and rapidly up-regulated by $1\alpha,25(OH)_2D_3$ in myeloid leukemia cells, and forced expression of $p21^{waf1/cip1}$ induced myeloid cell differentiation (4, 22). Moreover, a VDRE that functioned in U937 cells was identified in the p21 promoter (4). However, the effect of $1\alpha,25(OH)_2D_3$ on $p21^{waf1/cip1}$ and $p27^{kip1}$ expression is highly cell specific. The induction of $p21^{waf1/cip1}$ mRNA by EB1089 in SCC25 cells was gradual and modest, but no effect on protein levels was observed (Fig. 5). $1\alpha,25(OH)_2D_3$ treatment modestly increased $p21^{waf1/cip1}$ protein in LNCaP prostate cancer cells (23). However, no significant effect on transcript levels and no $1\alpha,25(OH)_2D_3$-dependent induction of the $p21^{waf1/cip1}$ promoter was observed in gene transfer experiments in LNCaP cells (23). Hershberger *et al.* (6) found that $1\alpha,25(OH)_2D_3$ repressed $p21^{waf1/cip1}$ expression in the mouse SCCVII/SF line, and we have observed a similar repression of $p21^{waf1/cip1}$ transcripts and protein in the mouse SCC line AT-84 (38a).

The lack of induction of cyclin-dependent kinase inhibitors in $1\alpha,25(OH)_2D_3$- or EB1089-treated SCC25 cells led us to screen gene arrays to identify other regulated genes in SCC25 cells. A total of 38 target genes, including $p21^{waf1/cip1}$, were identified in two

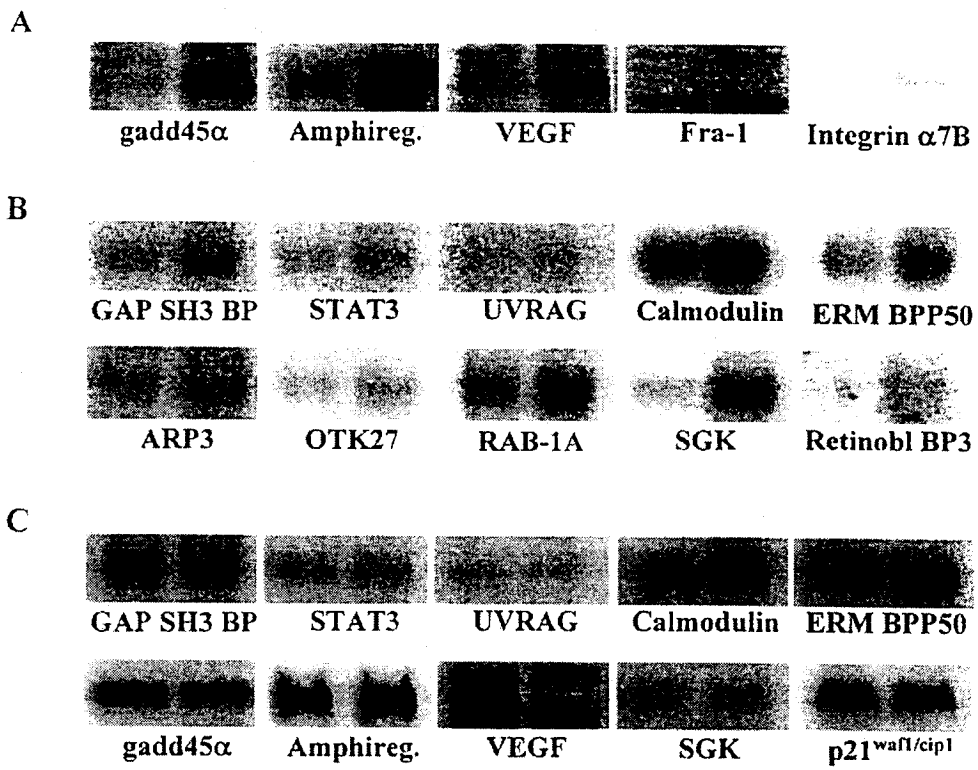

Fig. 6. Northern Analysis of EB1089-Regulated Target Gene Expression

A, Northern analyses of target genes identified using a CLONTECH Laboratories, Inc. Atlas array. Transcripts expressed in SCC25 cells encoding amphiregulin (amphireg.), GADD45α, FRA-1, integrin α7B, and VEGF are shown. Cells were treated with vehicle (*left lane*) or 10 nM EB1089 (*right lane*) for 24 h, and 1 μg of poly A+ RNA was loaded on each lane. B, Northern analyses, performed as in panel A, of target genes identified using a Research Genetics, Inc. gene filter, as follows: GAP SH3 BP, GAP SH3 binding protein; STAT3; UVRAG, UV resistance-associated gene; calmodulin; ERM BPP50, ezrin-radixin-meoisin binding phosphorprotein-50; ARP3, actin-related protein 3; OTK27; RAB-1A, ras-related protein 1A; SGK, serum- and glucocorticoid-inducible kinase; Retinobl BP3, retinoblastoma binding protein 3. C, Northern analysis of target gene regulation in SCC9 cells. Cells were treated and blots were performed as in panel A. Note that GAPDH controls were performed for blots in A–C and showed no significant variations (not shown).

screens of more than 4,500 genes (Table 1). The 32 targets identified on the Research Genetics, Inc. filter were retained after 9 sets of cross- comparisons of data derived from screening with probe derived from vehicle- and EB1089-treated SCC25 cells, using a minimum induction of 1.5-fold as a cut-off. We confirmed that 10 of 10 candidates analyzed by Northern blotting showed $1\alpha,25(OH)_2D_3$-regulated expression (Fig. 6), indicating that the data obtained from the array screening are highly reliable. Most genes were up-regulated 2- to 4-fold, a range in good agreement with that of up-regulated targets identified in a similar screen of thyroid hormone- regulated genes (33), and generally more modest than the levels of gene regulation observed by forced overexpression of the tumor suppressor genes BRCA1 (41) and WT1 (42).

The genes identified in this study encode several different classes of proteins, many of which are components of different signal transduction pathways. They include cell adhesion proteins (*e.g.* galectin-2, integrin α7B), growth factors (*e.g.* amphiregulin, VEGF), cytoskeletal proteins (*e.g.* actin-related protein 3), protein kinases (*e.g.* serum- and glucocorticoid-regulated kinase, sgk), other intracellular signaling molecules, and transcription factors (AP-4, STAT-3, FRA-1). Some of the genes identified here have been implicated in regulation of the cell cycle and growth arrest. One example is serum- and glucocorticoid-inducible kinase, SGK, which is shuttled between the nucleus and the cytoplasm during the cell cycle. Its forced retention in either compartment suppressed serum-induced growth and DNA synthesis in mammary tumor cells (43).

We also found that $1\alpha,25(OH)_2D_3$ and EB1089 induced expression of gadd45α, which like $p21^{WAF1/CIP1}$ is a p53 target gene. However, neither compound affected p53 expression in SCC25 cells. A similar induction of GADD45α expression by $1\alpha,25(OH)_2D_3$ and EB1089 was observed *in vitro* and *in vivo* in the murine SCC line AT-84 under conditions in which expression of p53 was unaffected and $p21^{WAF1/CIP1}$ was repressed. In contrast, DNA damaging agents induced p53, $p21^{WAF1/CIP1}$, and

Table 1. Summary of Target Genes Identified Using cDNA Microarrays

Function	Gene	Array	Fold Induction (± SEM)	RG cDNA id	UniGene Accession No.
Cell adhesion/Extracellular matrix	*Integrin α7B*	C	NA	NA	AF032108
	GALECTIN-2	RG	2.75 (0.77)	1472743	AA872397
	Ninjurin1	RG	2.45 (0.49)	744917	AA625806
Growth factors/receptors	*Amphiregulin*	C	NA	NA	M30704
	VEGFα	C	NA	NA	AF022375
	Macrophage stimulating 1 (hepatocyte growth factor-like)	RG	2.62 (0.72)	72395	T51539
	Platelet-derived growth factor receptor, β-polypeptide	RG	2.35 (0.58)	40643	R56211
Cytoskeletal proteins	*Ezrin-radixin-moesin binding phosphoprotein-50*	RG	3.93 (1.70)	773286	AA425299
	Actin-related protein (ARP3)	RG	3.05 (0.90)	593251	AA164562
	Sarcospan-2 (SPN2)	RG	2.78 (0.60)	1049330	AA620859
	Keratin 4	RG	1.95 (0.17)	1035889	AA629189
Signal transduction	*GADD45α*	C	NA	NA	HUMGADD45
	Calmodulin	RG	5.16 (2.50)	321389	W44860
	Ras-related protein (RAB-1A)	RG	3.95 (1.76)	293715	N69689
	GAP SH3 binding protein	RG	3.21 (1.21)	564756	AA129537
	SGK	RG	3.05 (0.90)	840776	AA486082
	Protein kinase (MLK-3)	RG	2.72 (0.91)	146868	R80779
	Retinoblastoma binding protein	RG	2.48 (0.51)	768260	AA424950
	Aplysia ras-related homolog 12	RG	2.50 (0.74)	897158	AA676955
	LDL-receptor related protein	RG	1.98 (0.28)	810551	AA64566
	Melanoma differentiation-associated gene (MDA-7)	RG	1.60 (0.25)	712049	AA281635
Transcription factors/ nuclear proteins	*Fra-1*	C	NA	NA	HSFRA1M
	OTK27	RG	3.44 (1.20)	950709	AA608583
	Activating protein 4 (AP-4)	RG	3.28 (1.04)	713839	AA284693
	STAT 3 (acute response factor)	RG	2.95 (1.02)	725746	AA399410
	IL-4 Stat	RG	2.65 (0.74)	85541	T72202
	Cytokine-inducible nuclear protein	RG	2.44 (0.55)	840683	AA488072
	GCN5-like 1	RG	1.81 (0.20)	230218	H94857
Other	SCP-1	RG	3.48 (1.76)	1031799	AA609655
	HsPex13p	RG	3.28 (1.24)	128783	R16849
	T245 protein (T245)	RG	2.92 (0.81)	252382	H87106
	IAI.3B	RG	2.86 (0.59)	882511	AA676470
	Enigma gene	RG	2.67 (0.57)	502682	AA127096
	Homo sapiens PAP	RG	2.54 (0.52)	511066	AA100296
	Prostaglandin-endoperoxide synthase 1 (PGG/H synthase and cyclooxygenase)	RG	2.52 (0.50)	811927	AA454668
	PM5 protein	RG	2.50 (0.56)	884673	AA629923
	UV Radiation resistance associated gene	RG	2.48 (0.53)	823901	AA490501

EB1089-dependent regulation of genes listed in *italics* has been verified by Northern blotting. RG, Research Genetics Gene Filter; C, Clontech Atlas Array; LDL, low-density lipoprotein; PAP, poly(A) polymerase.

GADD45α in AT-84 cells (38a). Taken together, these results suggest that $1\alpha,25(OH)_2D_3$- and EB1089-dependent induction of gadd45α occurs by a p53-independent mechanism.

Consistent with its effects on gadd45α mRNA, EB1089 treatment of SCC25 cells enhanced expression of GADD45α protein and stimulated formation of GADD45α-PCNA complexes. Previous studies have shown that DNA damaging agents, such as γ or UV irradiation, induce formation of GADD45α-PCNA complexes (36, 37). Induction of GADD45α-PCNA complexes by EB1089, which is a growth inhibitor, but not a DNA damaging agent, indicates that increased DNA damage is not necessary to induce complex formation.

PCNA function is required for DNA replication in S phase, and for DNA repair through its association with

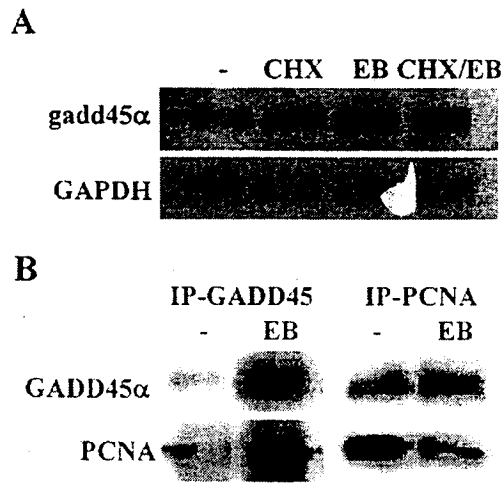

Fig. 7. Induction of gadd45α Expression and Enhanced Formation of GADD45α-PCNA Complexes in EB1089-Treated SCC25 Cells

A, The effect of 200 nM cycloheximide (CHX) on induction of gadd45α expression by 100 nM EB1089 (EB) was analyzed by Northern blotting. SCC25 cells were treated for 48 h with vehicle (−), cycloheximide, or EB1089 as indicated. B, Induction GADD45α protein and association of GADD45α with PCNA was assessed by reciprocal coimmunoprecipitation of extracts of SCC25 cells treated for 48 h with 100 nM EB1089 (EB) using either anti-GADD45α antibody (*left panel*) or anti-PCNA antibody (*right panel*) followed by Western blotting (see *Materials and Methods* for details).

polymerases δ and ϵ (44). Association of GADD45α with PCNA is considered to divert PCNA from sites of DNA replication to sites of DNA repair. GADD45α modifies DNA accessibility on damaged chromatin and can stimulate DNA repair *in vitro* (36, 45, 46). In addition, DNA damaging agents induce changes in the nuclear distribution of PCNA (47). It should be noted, however, that PCNA also interacts with a number of other regulatory proteins, including $p21^{WAF1/CIP1}$ (48), at sites that overlap those recognized by GADD45α (49). The relative roles and importance of interactions of $p21^{WAF1/CIP1}$ and GADD45α with PCNA remain to be fully elucidated. Nonetheless, the induction of GADD45α expression and its central role in enhancing DNA repair suggest that treatment of SCC cells with $1\alpha,25(OH)_2D_3$ or EB1089 would provide a genoprotective effect. This would be an important characteristic of a potential chemopreventive agent.

The observation that $1\alpha,25(OH)_2D_3$ induced expression of VEGF in SCC cells was surprising given that increased VEGF levels are associated with tumor vascularization and tumor progression (50). Elevated VEGF levels have been correlated with a higher rate of disease recurrence and a shorter disease-free interval in SCC of the oral cavity (51). These results highlight the complexity of cellular responses to growth regulators such as $1\alpha,25(OH)_2D_3$ and its analogs, where a combination of regulatory signals is induced under conditions in which the overall effect of $1\alpha,25(OH)_2D_3$ is growth inhibitory. It should also be noted that $1\alpha,25(OH)_2D_3$-regulated expression of VEGF is highly cell specific. We did not observe any induction of VEGF expression in MCF-7 and MBA-MD231 breast cancer or LNCaP prostate cancer cells (data not shown), whereas others have shown that VEGF expression is regulated by $1\alpha,25(OH)_2D_3$ in osteoblast-like cells (29, 30).

The partial resistance of SCC9 cells to the growth-inhibitory effects of $1\alpha,25(OH)_2D_3$ correlated with broad deregulation of target gene expression (10 of 10 genes tested). It is unlikely that loss of regulation arises through repressed expression due to target gene methylation, since transcripts of all genes refractory to $1\alpha,25(OH)_2D_3$ were detected in vehicle-treated SCC9 cells (Fig. 6). It is possible that $1\alpha,25(OH)_2D_3$-dependent induction of these genes requires synergism of the VDR with other transcription factor(s) or downstream regulators, the function of which is defective in SCC9 cells. Such factors would not be required for regulated expression of the endogenous 24-hydroxylase gene or the synthetic VDRE3-hsp68 promoter. One possible candidate is AP1, whose function is enhanced by $1\alpha,25(OH)_2D_3$ signaling (52–54). However, this enhancement apparently requires, at least in part, up-regulation of expression of AP1 components, particularly c-jun. We have also found here that $1\alpha,25(OH)_2D_3$ modestly up-regulates fra-1 mRNA levels. This suggests that if loss of induced AP1 activity contributes to deregulation of $1\alpha,25(OH)_2D_3$ target gene expression in resistant SCC lines, it may not be a primary defect. It should also be noted that we have tested the effect of cycloheximide on the six target genes identified on the Atlas array, $p21^{waf1/cip1}$, amphiregulin, VEGF, fra-1, gadd45α, and integrin α7B, and found in each case there was no effect on $1\alpha,25(OH)_2D_3$-stimulated expression (Fig. 7, and data not shown). Therefore, in these instances, the stimulatory effect of $1\alpha,25(OH)_2D_3$ did not require protein synthesis. Moreover, with the exceptions of integrin α7B and fra-1, which were not tested, regulation of all of these genes was lost in SCC9 cells (Fig. 6).

In summary, our studies have shown that $1\alpha,25(OH)_2D_3$ analogs can be potent inhibitors of SCC proliferation and control the expression of several regulators of cell proliferation. However, partial resistance to $1\alpha,25(OH)_2D_3$ can arise even in the presence of apparently normal levels of functional VDR. Resistance arises from a broad, but selective, loss in $1\alpha,25(OH)_2D_3$-regulated gene expression in the presence of normal levels of functional VDRs.

MATERIALS AND METHODS

Plasmids and Reagents

The VDRE3-hsp68-lacZ reporter contains three VDREs (20), inserted upstream of minimal hsp68 promoter in the plasmid p610AZ (55). The plasmid tk-LUC contains a truncated Her-

pes Simplex Virus thymidine kinase promoter inserted upstream of a promoterless luciferase reporter gene in pXP1 (56). 1α,25(OH)$_2$D3 and EB1089 were kindly supplied by Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark). 13-*cis*-RA was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). All hormones were dissolved in dimethylsulfoxide (DMSO), and stock solutions were stored in the dark at −20 C.

Tissue Culture

The SCC lines, SCC4, SCC9, SCC15, and SCC25, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were cultured under recommended conditions. Effects of 1α,25(OH)$_2$D$_3$, EB1089, and 13-*cis*-RA on cell growth were analyzed by seeding cells in 6-well plates at 15,000 cells per well in 2 ml of culture medium containing charcoal-stripped serum. Media were changed after 24 h to charcoal-stripped medium containing vehicle or ligand as indicated. Media were changed every 48 h and fresh ligand added. Cells were harvested by washing with 2 ml of PBS and incubation with 0.7 ml of 0.25% trypsin-EDTA. Cell numbers were determined using a hemacytometer. Four grid sections were counted for each well and the results were averaged. All treatments were performed in triplicate.

Transient Transfections

SCC cells were grown to 60% confluency in six-well plates in charcoal-stripped medium, washed with 2 ml of Opti-MEM I-reduced serum media (Life Technologies, Inc., Burlington, Ontario, Canada), and cultured in 1 ml of Opti-MEM I. Cells were transfected with 500 ng of VDRE3-LacZ reporter plasmid and 500 ng of tk-LUC internal control using Lipofectin (Life Technologies, Inc.) according to the manufacturer's protocol. After 18 h media were replaced with charcoal- stripped medium containing ligands as indicated. Cells were lysed 24 h later using lysis buffer (Promega Corp., Madison, WI), and β-galactosidase assays were performed as described (57). Transfections were performed in triplicate and standardized using the Luciferase Assay System with reporter lysis buffer (Promega Corp.).

RNA Isolation and Northern Blotting

Cells were grown in 100-mm dishes. Media were replaced with charcoal-stripped medium containing ligand as indicated. Total RNA was extracted with TRIZOL (Life Technologies, Inc.). PolyA+ RNAs were isolated using an Oligotex mRNA Kit (QIAGEN, Valencia, CA). One microgram of polyA+ RNA was separated on a 1.0% agarose gel containing 6.3% formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), 15 mM sodium acetate, and 1 mM EDTA. Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Baie d'Urfe, Quebec), which then was soaked in 3×saline-sodium citrate (SSC) and 0.1% SDS at 50 C, and prehybridized at 42 C in 50 mM phosphate buffer, pH 6.5, 50% formamide, 5× SSC, 10% Denhardt's solution containing 250 μg/ml sheared, denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled cDNA probes. Membranes were washed four times in 2×SSC and 0.2% SDS for 5 min, three times in 0.1×SSC and 0.2% SDS for 30 min at 50 C, dried, and autoradiographed. All blots were performed at least three times with independent preparations of RNA.

RT-PCR

Ten micrograms of total RNA were subjected to oligo dT priming first-strand cDNA synthesis by SuperScript II (Life Technologies, Inc., Burlington, Ontario, Canada). Twenty microliter aliquots were diluted 5-fold with water. For RT-PCR analysis of p53 and p27 ^{kip1} mRNA, expression of 1 μl of RT reactions was analyzed by PCR amplification as follows: 30 sec denaturation at 94 C, 45 sec elongation at 72 C, and 30 sec annealing starting at 60 C, down 1 C per cycle to 55 C, and continuing 20 cycles of amplification (94 C for 30 sec, 57.5 C for 30 sec, 72 C for 45 sec). Complementary DNAs for p53 and p27 ^{kip1} were amplified using 5′-primer 5′-CAAGTCTGTGACTTGCACGTA-3′ and 3′-primer 5′-TTCTT-GCGGAGATTCTCTTCC-3′ for p53, and 5′-primer 5′-CCG-GAATTCATGTCAAACGTGCGAGTGTCT-3′ and 3′-primer 5′-CCGGAATTCTTACGTTTGACGTCTTCTGAGGC-3′ for p27^{kip1}. For β-actin, 1 μl of RT reaction was subjected to 18 cycles of amplification (95 C for 30 sec, 56 C for 1 min, 72 C for 25 sec) using 5′-primer 5′-GCTGTGCTATCCCTGTACGC-3′ and 3′-primer 5′-CCAATGGTGATGACCTGGC-3′. All of the above reactions were performed in 25 μl of 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-Cl (pH 9.0) using 2.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). PCR reactions were loaded on a 2% agarose gel, transferred for Southern blotting to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech), and fixed by UV cross-linker. The membrane was soaked in 3× SSC and 0.1% SDS at 50 C, and prehybridized at 42 C in 50 mM phosphate buffer, pH 6.5, 5× SSC, 10% Denhardt's solution containing 250 μg/ml sheared and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P end-labeled oligonucleotides 5′-CTACAAGCAGTCACAGCACAT-3′ for p53, 5′-CTAACTCTGAGGACACGCATT-3′ for p27^{kip1}, and 5′-CGAGAAGCTGTGCTACGTCG-3′ for β-actin. After hybridization, the membrane was washed four times in 2× SSC and 0.2% SDS for 5 min, three times in 0.1× SSC and 0.2% SDS for 30 min at 50 C, dried, and autoradiographed. All experiments were repeated at least three times.

Immunoprecipitation and Western Blotting

After incubation with ligands, cells were washed twice with PBS and harvested by scraping in 1 ml of PBS and centrifuged at 4 C. The pellet was resuspended in 500 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% NP40) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany), incubated on ice for 10 min. Lysates were centrifuged at 4 C (14,000 rpm, 10 min), and supernatants were recovered. For p21$^{WAF1/CIP1}$ and p27^{KIP1} immunoprecipitations, protein extracts (200 μg) were immunoprecipitated at 4 C overnight with 3 μg of F-5 and F-8 anti-p21$^{WAF1/CIP1}$ and −p27^{KIP1} monoclonal antibodies, respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using 30 μl of 50% slurry protein S-Sepharose (Amersham Pharmacia Biotech). Beads were centrifuged, and pellets were washed four times each with lysis buffer and boiled for 3 min in 2× SDS-polyacrylamide gel loading buffer. Immunoprecipitates were resolved on 20% SDS-polyacrylamide gels and analyzed by Western blotting with the same antibodies. Immunoprecipitations of GADD45α and PCNA were performed with anti-GADD45α antibody 4T-27 or with anti-PCNA antibody PC-10 (Santa Cruz Biotechnology, Inc.). Immunoprecipitates were harvested, processed for Western blotting as above and probed with anti-GADD45 antibody (H-165) (Santa Cruz Biotechnology, Inc.) or with anti-PCNA (PC-10) (Santa Cruz Biotechnology, Inc.).

Western analysis of VDR expression was performed with 30 μg of total cell protein resolved on a 15% SDS-polyacrylamide gel. VDRs were probed with 800 ng of a rabbit polyclonal anti-VDR antibody (Santa Cruz Biotechnology, Inc.). Proteins were detected by enhanced chemiluminescence (ECL: NEN Life Science Products, Boston, MA).

Flow Cytometry and TUNEL Assays

SCC25 cells treated with 100 nM EB1089 or DMSO for 72 h were harvested with 0.25% trypsin-EDTA, fixed with 70% ethanol for 1 h at 4 C, treated with 200 μg/ml RNase A for 30 min, stained with 5 μg/ml propidium iodide for DNA, and analyzed for cell cycle status by flow cytometry (Becton Dickinson and Co., Franklin Lakes, NJ). Experiments were repeated three times. TUNEL assays were performed using an Apoptag kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, after incubation with vehicle or ligand, cells were fixed for 15 min in 1% paraformaldehyde, washed twice with PBS, and stored in 70% ethanol at −20 C. Cells (100 μl) were then incubated for 30 min at 37 C with terminal deoxynucleotidyl transferase and digoxigenin-dUTP. After two washes with 0.1% Triton X-100 in PBS, cells were incubated with fluorescein-conjugated antidigoxigenin antibody for 30 min at room temperature. After two washes with 0.1% Triton X-100 in PBS, cells were treated with RNase A and processed for flow cytometry as above.

Array Screening

SCC25 cells were treated for 24 h with DMSO or EB1089 (100 nM). Atlas cDNA Expression Arrays containing 588 genes (CLONTECH Laboratories, Inc. Palo Alto, CA) were screened with 100 ng of polyA+ RNA. GF211 Named Human Genes arrays containing more than 4,000 genes (Research Genetics, Inc.) were probed with 1 μg of total RNA. Probe preparation and array screening were carried out according to manufacturers' instructions. Duplicate Atlas arrays were screened twice each with probe derived from control or treated cells and arrays were visualized by autoradiography. Genes that appeared reproducibly regulated were studied by Northern analysis. GF211 filters were probed three times each with probe derived from control cells and EB1089-treated cells, and visualized by phosphorimaging. Relative expression levels were compared using Pathways software (Research Genetics, Inc.). Genes that were up-regulated at least 1.5-fold in nine sets of cross-comparisons were retained. Of these, 10 were further analyzed by Northern analysis using cDNA probes from Research Genetics, Inc.

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Expression Profiling in Squamous Carcinoma Cells Reveals Pleiotropic Effects of Vitamin D_3 Analog EB1089 Signaling on Cell Proliferation, Differentiation, and Immune System Regulation

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The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is key mediator of calcium homeostasis and is a component of the complex homeostatic system of the skin. 1,25-$(OH)_2D_3$ regulates cellular differentiation and proliferation and has broad potential as an anticancer agent. Oligonucleotide microarrays were used to assess profiles of target gene regulation at several points over a 48 h period by the low calcemic 1,25-$(OH)_2D_3$ analog EB1089 in human SCC25 head and neck squamous carcinoma cells. One hundred fifty-two targets were identified, composed of 89 up- and 63 down-regulated genes distributed in multiple profiles of regulation. Results are consistent with EB1089 driving SCC25 cells toward a less malignant phenotype, similar to that of basal keratinocytes. Targets identified control inter- and intracellular signaling, G protein-coupled receptor function, intracellular redox balance, cell adhesion, and extracellular matrix composition, cell cycle progression, steroid metabolism, and more than 20 genes modulating immune system function. The data indicate that EB1089 performs three key functions of a cancer chemoprevention agent; it is antiproliferative, it induces cellular differentiation, and has potential genoprotective effects. While no evidence was found for gene-specific differences in efficacy of 1,25-$(OH)_2D_3$ and EB1089, gene regulation by 1,25-$(OH)_2D_3$ was generally more transient. Treatment of cells with 1,25-$(OH)_2D_3$ and the cytochrome P450 inhibitor ketoconazole produced profiles of regulation essentially identical to those observed with EB1089 alone, indicating that the more sustained regulation by EB1089 was due to its resistance to inactivation by induced 24-hydroxylase activity. This suggests that differences in action of the two compounds arise more from their relative sensitivities to metabolism than from differing effects on VDR function. (*Molecular Endocrinology* 16: 1243–1256, 2002)

NATURALLY OCCURRING VITAMIN D_3 is found in a limited number of dietary sources (*e.g.* cod liver oil, oily fish), and is produced through the action of ultraviolet light on 7-dehydrocholesterol in the skin (1). Vitamin D_3 is one of several factors produced by the complex homeostatic system in the skin, which, as a protective barrier and environmental sensor, is intimately connected to the body's immune and neuroendocrine functions (2). Vitamin D_3 is 25-hydroxylated in the liver and converted into its active 1α,25-dihydroxy form [1,25-$(OH)_2D_3$] in the kidney and several peripheral organs, including skin (2, 3). 1,25-$(OH)_2D_3$ signals through its cognate nuclear vitamin D receptor, which is a direct regulator of gene transcription. Signal transduction by 1,25-$(OH)_2D_3$ has a broad range of physiological effects (2, 3). Primarily, 1,25-$(OH)_2D_3$ controls calcium transport in the intestinal epithelia, and modulates bone resorption. However, it has widespread effects on cellular proliferation and differentiation. 1,25-$(OH)_2D_3$ stimulated differentiation of the OB 17 preadipocyte cell line (4) and induced immature basal layer skin cells to differentiate into keratinocytes (5). Hematopoietic cell lines can be induced to differentiate along the macrophage/monocyte pathway (6–8). 1,25-$(OH)_2D_3$ inhibits proliferation of cells in several models of cancer, including myeloid leukemia, melanoma, and carcinomas of the breast, prostate, colon, and head and neck (3).

Abbreviations: G6PDH, Glucose-6-phosphate dehydrogenase; HNSCC, head and neck squamous cell carcinoma; MMP, metalloproteinase; PTH1R, PTH receptor; SCC, squamous cell carcinoma; SCCA, squamous cell carcinoma antigen; SPC, second primary carcinomas; VDRE, vitamin D response element.

It is unlikely that regulation of a single gene provides the key to the growth inhibitory properties of 1,25-$(OH)_2D_3$ and its analogs. Expression of genes encoding the cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ was strongly but transiently induced by 1,25-$(OH)_2D_3$ in myeloid leukemia cells, and forced expression of $p21^{waf1/cip1}$ induced myeloid cell differentiation (9, 10). However, the effect of 1,25-$(OH)_2D_3$ on $p21^{waf1/cip1}$ expression varies widely in different cell types. Whereas 1,25-$(OH)_2D_3$ treatment modestly increased $p21^{waf1/cip1}$ protein levels in LNCaP prostate cancer cells, no effect was observed on $p21^{waf1/cip1}$ mRNA or the $p21^{waf1/cip1}$ promoter in these cells (11). Moreover, Hershberger *et al.* (12) and ourselves (13) found that 1,25-$(OH)_2D_3$ repressed $p21^{waf1/cip1}$ expression in mouse head and neck squamous cell carcinoma (HNSCC) lines. The effect of 1,25-$(OH)_2D_3$ on $p27^{kip1}$ expression is generally more consistent. Rapid and transient induction of $p27^{kip1}$ transcripts is accompanied by substantially delayed and more sustained increase in $p27^{kip1}$ protein (10, 14), suggesting that additional mechanisms may control its expression.

The limiting factor for use of 1,25-$(OH)_2D_3$ in cancer therapy has been hypercalcemia. However, many potent analogs have been developed with reduced calcemic effects (15, 16). One such analog, EB1089, contains a side chain modified to render it less susceptible to catabolic degradation (17, 18). *In vivo* studies of prostate and breast carcinomas using EB1089 dosages up to 1.0 μg/kg/d showed no clinically significant hypercalcemia (19, 20). Our previous experiments with a mouse model of HNSCC showed that an EB1089 dose of 0.25 μg/kg/d reduced tumor growth by up to 80% in the absence of hypercalcemia (13).

We are interested in investigating the potential chemopreventive effects of 1,25-$(OH)_2D_3$ analogs using HNSCC as a model. Early stage HNSCC can be successfully treated with surgery and/or radiation therapy. However, primary tumors are often associated with areas of dysplastic epithelia, which lead to the development of second primary carcinomas (SPC) at an annual rate of 3–7%. Thus, it is important to identify chemopreventive agents in HNSCC. Accumulating epidemiological evidence suggests that 1,25-$(OH)_2D_3$ analogs may have widespread chemopreventive effects (16). Preclinical studies with models of colon (16, 22), cheek pouch (23), gastrointestinal (24), and skin carcinogenesis (25, 26) have also provided evidence for chemoprevention. We found that 1,25-$(OH)_2D_3$ and EB1089 induced the expression of the growth arrest and DNA damage (gadd45α) gene in human and mouse HNSCC lines *in vitro* and in tumors by an apparently p53-independent mechanism (13, 21). GADD45α is required for normal DNA repair and maintenance of global genomic stability (27). This strongly suggests that 1,25-$(OH)_2D_3$ and its analogs can act as a genoprotective agents. Induction of DNA repair mechanisms may represent a feedback response to the stimulation of cutaneous vitamin D synthesis by ultraviolet light.

Here, we have used oligonucleotide microarrays to perform large-scale profiling of the effects of EB1089 and 1,25-$(OH)_2D_3$ on gene expression in human HNSCC cells at several times over a 48-h period. Nuclear receptor signaling is ideally suited for microarray analysis, as ligand-bound receptors bind to promoter regions and directly regulate the expression of most of their target genes. These studies provide numerous insights into the effects of 1,25-$(OH)_2D_3$ and its analogs on cell proliferation, differentiation and regulation of immune system function.

RESULTS AND DISCUSSION

Time Courses of EB1089-Regulated Gene Expression in Human SCC25 Cells

We previously found that proliferating human SCC25 HNSCC cells were arrested in G0/G1 by treatment with nanomolar concentrations of EB1089 (21). To determine the molecular events underlying growth arrest, and to assess its potential as a chemopreventive agent, we analyzed the effects of EB1089 treatment on gene expression using Affymetrix HuGene FL oligonucleotide microarrays. SCC25 cells were treated for 0, 1, 2, 6, 12, 24, and 48 h with EB1089 in three independent experiments. Before microarray screening, the response to EB1089 in each experiment was verified by Northern analysis of amphiregulin gene expression (data not shown), as our previous work demonstrated that the amphiregulin gene is a direct target of 1,25-$(OH)_2D_3$ (21, 28).

Compiled raw data was initially analyzed by nonparametric ANOVA (29) to eliminate genes whose change in expression was not statistically significant ($P < 0.05$). Data were then filtered to eliminate genes included because of single chip artifacts, and those with erratic expression profiles that were not consistent between experiments (see below and *Materials and Methods* for details). While previous microarray studies used variation filters as high as 3-fold regulation (30), we chose a filter of 2.5-fold, corresponding to a minimum magnitude change of 200 fluorescence units, so that genes whose induction was similar to that of amphiregulin (average +2.74-fold) would not be excluded. A list of 152 reproducibly regulated EB1089 targets composed of 89 up-regulated and 63 down-regulated genes is presented in Table 1. The results indicate that EB1089 signaling impinges upon every aspect of HNSCC cell function both in terms of intracellular metabolism, and communication with the extracellular milieu.

The list contains a number of previously identified 1,25-$(OH)_2D_3$ targets, including genes encoding integrin α7B, COX-2, and amphiregulin, which were identified in our earlier microarray analysis (21). Sequences encoding another 1,25-$(OH)_2D_3$ target gene in SCC25 cells, $p21^{waf1/cip1}$, are not present on the HuGene FL chip. In addition to 24-hydroxylase, the list also con-

Table 1. List of Genes Regulated by EB1089

		P<0.05	Cluster	Adhesion/cytoskeleton
M68225	+3.2/48	0.00098	U4	*bullous pemphigoid antigen (BPAG1)*
M76482	+2.4/48	0.00009	U6	*desmoglein-3*
M95787	-7.2/48	0.00776	D4	22kDa smooth muscle protein (SM22)
S42303	-6.6/48	0.00132	D4	*N-cadherin*
M47634	+4.3/12	0.00332	U2	beta-tubulin class IV
X06808	-4.8/48	0.0002	D2	NF-L
X74929	-5/48	0.00007	D4	keratin 8 (KRT8)
				Cell Cycle
M25753	-2.6/48	0.01613	D4	cyclin B
U66838	-2.9/48	0.0249	D4	cyclin A1
U77949	-2.6/48	0.00056	D5	Cdc6-related protein (HsCDC6)
X51688	-3/48	0.01417	D4	cyclin A
X54942	-2.4/48	0.00035	D3	cksHs2 Cks1 protein homolog
Z36714	-4.2/48	0.0013	D4	*cyclin F*
				Channels/transporters
L18296	+13.5/12	0.00002	U3	rod cyclic nucleotide-gated cation channel
U73191	-5.3/48	0.00257	D2	inward rectifier potassium channel Kir1.3
U81375	-2.9/24	0.00035	D3	equilibrative nucleoside transporter 1 (hENT1)
				ECM structure/remodeling
HG2197	+2.5/48	0.00749	U4	Collage, Type VII, Alpha 1
M24486	+3.2/48	0.04366	U3	prolyl 4-hydroxylase alpha subunit
M33653	+21.9/48	0.0003	U3	type XIII collagen
M85289	+2.6/48	0.00015	U4	heparan sulfate proteoglycan (HSPG2)
U20758	+6.5/24	0.0002	U3	osteopontin
U50330	+3.5/48	0.00007	U4	procollagen C-proteinase (pCP-2)
U62800	+20/12	0.00045	U2	*cystatin M*
X05232	+9.4/12	0.00749	U3	*stromelysin*
X16832	-4/48	0.00098	D4	cathepsin H
X54925	+14/48	0.00219	U4	*collagenase 1*
X74295	+4.4/48	0.00219	U3	*alpha 7B integrin*
X75308	-20/48	0.00612	D3	*collagenase 3*
X78565	-5/48	0.00787	D3	*fanaecin-C, 7580bp*
Z37976	+3.5/48	0.00007	U4	latent TGF binding protein (LTBP-2)
				Immune System
D12763	+26.7/12	0.00533	U3	*T1/ST2*
D38037	+4.2/48	0.00132	U4	FK506-binding protein 12kDa (hFKBP-12) homologue
J04164	-2.8/24	0.01145	D4	interferon-inducible 9-27
M21005	-4.4/48	0.00009	D3	migration inhibitory factor-related protein 8 (MRP8)
M21121	-3.0/24	0	D4	T cell-specific protein (RANTES)
M24594	-1.96/24	0.003	D3	*interferon-inducible 56 Kd*
M26311	-9.2/48	0.00031	D1	migration inhibitory factor-related protein 14 (MRP14)
M29696	-3.3/48	0.00045	D3	IL-7 receptor
M33682	-4.1/48	0.02265	D5	lymphocyte-specific protein 1 (LSP1)
M60254	-2.7/48	0.00219	D2	cyclophilin (hCyP3)
M87507	+2.5/48	0.00009	U3	interleukin-1 beta convertase (IL1BCE)
M93056	+11/48	0.00289	U3	monocyte/neutrophil elastase inh.
U04343	+4.8/48	0.01613	U6	CD98 antigen
U67615	+2.8/48	0.00582	U3	beige protein homolog chs
U80428	-2.7/48	0.00749	D4	nuclear RNA helicase BAT1
X13334	+27/24	0.0006	U3	CD14 myeloid cell-specific glycoprotein
X57351	-2.9/48	0.02539	D6	interferon-inducible 1-8D gene
X59405	+2.8/48	0.00009	U2	Membrane colector protein (CD46)
X68487	-2.5/48	0.00009	D2	A2b adenosine receptor
Z49107	+3.5/48	0.00009	U5	galectin
				Intracellular Signaling
D87029	+2.8/48	0.0002	U2	SEC14L
D89016	+2.7/48	0.00219	U4	Rho GEF homolog
HG2167	+2.6/24	0.00058	U3	A-kinase anchoring protein Ht31
L04510	+2.5/48	0.00219	U2	ARD1
L13391	+2.8/48	0.0158	U4	RGS2/G0S8
L15388	+3/48	0.00219	U4	G protein-coupled receptor kinase (GRK5)
M31724	+3.2/48	0.00289	U2	PTP-1B
M58026	+3.5/48	0.00132	U3	NB-1
M61906	+4/48	0	U2	P13-kinase p85
M97815	-4.2/48	0.00045	D2	*CRABP-II*
U02081	+3/48	0.00035	U2	guanine nucleotide regulatory protein NET1
U16550	+6/24	0.00219	U1	Gem GTPase
U15932	+2.5/48	0.00045	U3	dual-specificity protein phosphatase
U40371	+2.7/48	0.01324	U4	receptor tyrosine kinase (PTK7)
U67733	+3.8/24	0.03061	U4	cGMP-stim. 3,5 -cyclic nucleo. Phosphodiesterase PDE2A3(PDE2A)
U36922	+5.2/12	0.00056	U3	inositol polyphosphate 4-phosphatase type II-
X04828	+3.3/48	0.00007	U4	G(i) protein alpha-subunit
X13461	+3.8/48	0.00007	U4	calmodulin-like protein (CLP)
X82676	+3.5/24	0.00439	U2	PEZ/PTP36 tyrosine phosphatase
D00632	+2.8/24	0.00035	U4	glutathione peroxidase
D87258	+8/48	0.00098	U4	HtrA chaperon/protease
S73591	+8.2/48	0.00015	U6	VDUP1
V00594	-3.1/48	0.00533	D5	metallothionein from cadmium-treated cells
X65448	+5.8/48	0.00749	U4	glucose-6-phosphate dehydrogenase (G6PDH)
X91247	+3/48	0	U1	thioredoxin reductase
Z11793	+4.1/48	0.00009	U4	selenoprotein P
				Signaling peptides/ growth factors
J05008	-5.6/24	0.00318	D1	endothelin-1 (ENT-1)
K03183	-2.6/48	0.01613	D3	chorionic gonadotropin (hcg) beta subunit
M22488	+3.7/48	0.00016	U4	bone morphogenetic protein 2A (BMP-2A)
M30703	+2.7/48	0.00045	U2	*amphiregulin (AR)*
M57283	-2.6/48	0.00749	D3	PTHrP
M60315	+6.1/48	0.00043	U4	transforming growth factor-beta (tgf-beta)
M77140	-4.2/48	0	D4	galanin
M84250	-3.4/48	0.00132	D4	retinoic acid inducible factor (MK)
U03877	-3.1/48	0.01146	D4	*astrocalixtr protein 31-5*
U06863	+2.8/24	0.00046	U4	follistatin-related protein precursor
U43142	-3.2/48	0	D1	*VEGF-related (VRP)*
U62015	-3.6/24	0.00436	D2	Cyr61
X02530	-4/24	0.00098	D3	gamma-interferon inducible IP-10
X67879	-2.6/24	0.01417	D1	activin beta-A subunit
Y00787	-4.1/48	0.00332	D1	monocyte-derived neutrophil chemotactic factor (MDNCF)
				Steroid/lipid metabolism
D28235	+8.2/48	0.00015	U2	Cox-2
J03606	+10.9/48	0.00002	U5	5-lipoxygenase
L11708	+42/48	0.0002	U4	*type 2 17 beta-HSD*
L13386	+196/48	0.0013	U2	*24-hydroxylase*
M91432	+3.2/48	0.00035	U2	medium-chain acyl-CoA dehydrogenase
U05861	+3.1/48	0.00612	U3	*hepatic dihydrodiol dehydrogenase*
U07919	-2.5/48	0.0177	D3	ALDH-6
				Transcription factors
D31716	+2.5/48	0.01016	U5	GC box bindig protein
HG1684	-3/48	0.00098	D5	Transcription Factor E4tf1
HG3510	+2.9/48	0.02285	U4	COUP-TF1
HG4068	-2.6/24	0.01472	D1	Oncogene Aml1-Evi-1
L40387	-2.5/48	0.00332	D3	*TRIP-14*
L49054	+2.7/48	0.00812	U6	fusion gene NPM-MLF1
M19720	+6.2/48	0.00015	U6	L-myc gene
M99701	+3.1/48	0.00007	U3	transc. elongation factor S-II homolog pp21
S74017	+2.6/48	0.00009	U4	*Nrf2 transcriptional activator*
U18018	+2.6/12	0.01324	U3	*E1A enhancer binding protein (E1A-F)*
U63824	-2.7/48	0.00098	D2	transcription factor RTEF-1
U74612	-3.8/48	0.00289	D4	HNF-3/fork head homolog 11
X52611	+4.6/48	0.04366	U4	transcription factor AP-2
X66581	-2.8/24	0.00219	D2	junD
X66644	+3.5/48	0.00805	U2	MHC binding protein 2 (MBP-2)
X82209	+4.4/48	0.00009	U3	MN1 mRNA
X84373	+4.4/24	0.00056	U1	RIP140
				Others
D00408	+36/48	0.00069	U5	*P-450 HFL4*
D17793	+2.6/48	0.01417	U1	KIAA0119
D31883	+3.56/48	0.0002	U3	KIAA0059
D43636	+6.8/12	0.00797	U1	KIAA0096
D76444	+2.8/48	0.00045	U2	hkl-1
D79611	-2.6/48	0.00812	D4	MEST
D86960	+2.9/12	0.00797	U3	KIAA0205
HG4074	-2.6/48	0.00797	D4	XPG
M32053	+4.3/48	0.02539	U5	H19 RNA gene
M60847	-4.5/48	0	D2	*heparin binding protein (HBp17)*
M72885	+4/12	0.0002	U2	G8S2
M90657	-6.2/48	0.00078	D2	tumor antigen (L6)
M93036	-2.5/48	0.01417	D4	carcinoma-associated antigen GA733-2
M97347	-2.7/24	0.00039	D2	Beta-1,6-N-acetylglucosaminyltransferase (AGAT)
S66896	-7.2/48	0.00223	D3	squamous cell carcinoma antigen (SCCA)
S81914	-3.5/48	0.00106	D1	*radiation-inducible IEX-1*
S85655	-3.9/48	0.00007	D3	prohibitin
U00115	+3.5/48	0.01324	U5	bcl-6
U08021	-21/24	0.00196	D2	nicotinamide N-methyltransferase (NNMT)
U15174	+3.7/24	0.00078	U4	MP3
U17077	+2.8/48	0.00039	U3	BENE
U18467	+2.6/48	0.00289	U4	pregnancy-specific beta 1-glycoprotein 7 (PSG7)
U21931	+2.4/24	0.00035	U2	fructose-1,6-bisphosphatase
U28368	+31/48	0.00035	U3	*semaphorin 3B (sema3B)*
U38276	+2.9/48	0.00132	U5	semaphorin 3F (Sema 3F)
U51010	-8.5/24	0.01106	D2	nicotinamide N-methyltransferase
U62100	-3/24	0.00132	D3	XMP
U63191	-3.6/48	0.00007	D3	YMP
U63801	+32/24	0.00287	U3	*protease M*
U89740	+6.7/48	0.00612	U3	362G6.2 from BAC clone chromosome 16p13.1
X06981	+2.9/48	0.00612	U5	27-kDa calbindin
X12517	-4.3/48	0.00581	D4	U1 small nuclear RNP-specific C protein
X14850	-2.6/48	0.00098	D4	histone H2A.X
Y00339	+6.1/48	0.00287	U3	carbonic anhydrase II
Y00787	-2.5/24	0.01981	D4	polypeptide TB2
Z25635	-2.6/48	0.00287	D3	*nuclear pore complex protein hnup153*
Z28331	+2.7/48	0.00228	U5	(23/3) ubiquitin-conjugating enzyme Ubch2

The GenBank accession no. for each gene is provided in the *first column*. The *second column* lists the maximum fold regulation and time of maximal activation. The *third column* gives *P* value derived from nonparametric ANOVA. Note that *P* values of less than 0.00001 are listed as 0. The *fourth column* gives the cluster number derived in Fig. 2. Genes whose regulation has been confirmed by RT-PCR analysis and/or Northern blotting are in *italics*. The profiles of individual experiments used to compile the above data are shown in Table 2, which is published as supplemental data on The Endocrine Society's Journals Online web site, http://mend.endojournals.org/.

tains other vitamin D-responsive genes including those encoding osteopontin, carbonic anhydrase II, VDUP1 (vitamin D up-regulated 1), PTHrP, CD14, and TGFβ (31–38). One exception is the gene encoding GADD45α, which we showed is 1,25-$(OH)_2D_3$ responsive in mouse and human HNSCC lines (13, 21). Although it appeared up-regulated, the gadd45α gene was not retained during the filtering process because

of elevated levels of nonspecific hybridization to control oligonucleotide sets (data not shown).

The range of fold regulation of target genes varied widely, with 24-hydroxylase exhibiting by far the highest up-regulation (196-fold at 48 h) of all genes identified. Expression of eight of these genes representing a range of fold regulations was further analyzed by Northern blotting (Fig. 1). The results of Northern and microarray analyses are in very good agreement. Most importantly, regulation of all genes identified on microarrays was confirmed on Northern blots, and the relative magnitudes of fold regulation observed were the same using the two techniques. There was also broad agreement between the fold regulations observed using the two techniques. The exceptions were cystatin M and protease M, where fold inductions at 24 h of 6.7- and 18-fold, and 8- and 32-fold were observed by Northern blotting and microarray analysis, respectively. However, other differences in fold regulation were less than 2-fold. Taken together, these experiments, coupled with RT-PCR analysis (Table 1, and see below), suggest that while the absolute magnitudes of fold regulation detected by microarray analysis may be somewhat higher in some cases than those detected by other techniques, the data compiled in Table 1 is highly reliable.

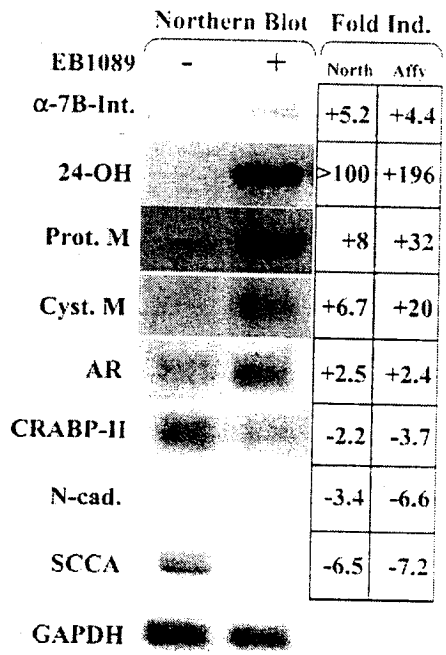

Fig. 1. Northern Analysis of EB1089 Target Gene Regulation

Northern analyses were performed on RNA extracted from control SCC25 cells or cells treated for 24 h with EB1089. Blots were hybridized with probes specific for integrin α-7B (α-7B), 24-hydroxylase (24-OH), protease M (prot. M), cystatin M (cyst. M), amphiregulin (AR), CRABP-II, N-cadherin (N-cad.), squamous cell carcinoma antigen (SCCA), and GAPDH control. Comparison of fold regulations after 24 h detected by Northern blotting (North) and Affymetrix microarrays (Affy) are provided.

Initial clustering analysis of averaged data of reproducibly regulated genes processed by the K-mean algorithm with k=5 generated 4 clusters of up-regulated genes distinguished based on rapidity of induction (data not shown). No such resolution was achieved for down-regulated genes, arising from fact that the absolute value of the average fold activation of the up-regulated genes at any given time point was substantially greater than that of the down-regulated genes. In addition, the K-mean algorithm is strongly dependent upon the choice of initial points (K number of initial conditions). Therefore, different initial points will have different nearest neighbors, and refinement of calculating means with various neighbors can generate different clusters starting from different initial conditions.

We have developed a method of clustering analysis that does not take into account initial conditions, and categorizes genes based on time of crossing of a threshold value (see *Materials and Methods* for details). The method generated symmetrical groups of clusters of up- and down-regulated genes (Fig. 2, A–L). The profiles of cluster genes were generally much less erratic than genes eliminated by filtering (Fig. 2, M and N). For example, the compiled data for E2F4 (a transcription factor controlling cell cycle progression), which suggests rapid up-regulation, is composed of three distinctly different profiles (Fig. 2O). Indeed, analysis of E2F4 transcripts from EB1089 treated cells by RT-PCR revealed no regulation (Fig. 2O, *inset*).

The 24-hydroxylase gene is among the most rapidly regulated genes in cluster U1, whereas regulation of the osteopontin gene is significantly slower (cluster U3; Fig. 2, Table 1). The promoters of both of these genes contain vitamin D response elements (VDREs) (31, 32). In addition, regulation of the carbonic anhydrase II gene, whose chicken homolog contains a VDRE (33), fell into cluster U3. This indicates that the kinetics of gene induction by the EB1089/VDR bound to different VDREs is strongly promoter specific. Several cell cycle regulatory genes whose products function after the G1/S boundary were among the more slowly regulated genes in clusters D3 and D4 (Table 1), likely reflecting the gradual diminution of cells in S phase or later. This is supported by observations that cyclin A1 and cyclin B levels in cells in G2 do not change during EB1089 treatment (not shown).

Regulation by EB1089 of Markers Associated with Cancer Cell Progression

EB1089 signaling regulates the expression of several markers associated with progression of cancer phenotypes. Of genes whose expression is reduced or eliminated in cancer cells, almost all are up-regulated by EB1089 (Fig. 3A). Two of the more strongly induced genes, kallikrein protease protease M and the cysteine protease inhibitor cystatin M (Table 1 and Fig. 3A), are down-regulated in breast cancers (39, 40), as is calm-

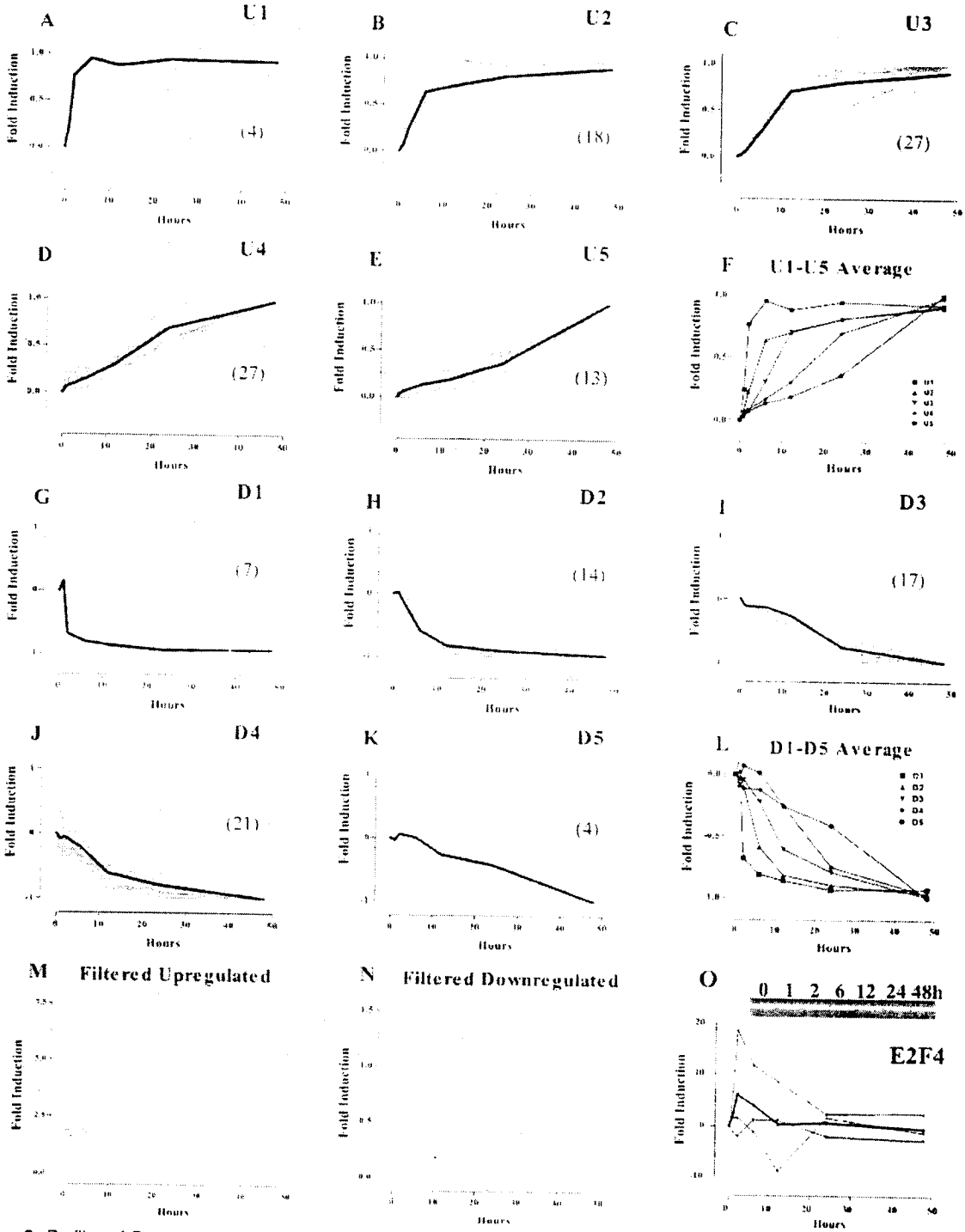

Fig. 2. Profiles of Genes Listed in Table 1 were Subjected to Clustering Analysis

A–E, Normalized profiles of up-regulated genes in clusters U1–U5 are presented, with the average trace shown in *bold*. F, Comparison of the average profiles for clusters U1–U5. G–K, Normalized profiles of down-regulated genes in clusters D1–D5 are presented, with the average trace shown in *bold*. L, Comparison of the average profiles for clusters D1–D5. Numbers of genes in each cluster are indicated in *brackets*. M, N, Profiles of up- and down-regulated genes eliminated by filtering before clustering analysis (see *Materials and Methods* for details). O, Analysis of transcription factor E2F4 regulation in EB1089-treated cells. The composite profile (in *bold*) and individual data sets are shown. The *inset* shows an analysis of E2F4 transcripts from EB1089-treated cells by RT-PCR using the same RNA preparations as in Fig. 5.

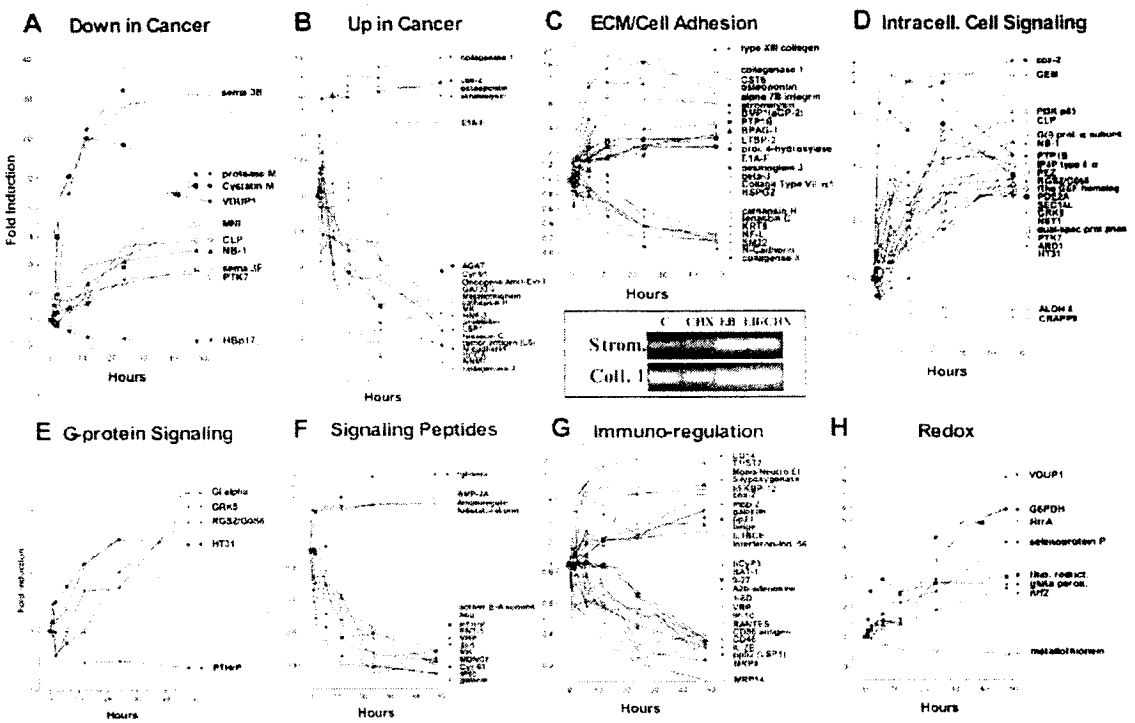

Fig. 3. Profiling of EB1089-Regulated Gene Expression

A, EB1089-dependent regulation of genes whose expression is generally disrupted or down-regulated in cancer. B, Genes whose expression is up-regulated in cancer. C, Genes controlling extracellular matrix structure and cell adhesion. *Bottom*, Cycloheximide does not block EB1089-dependent induction of collagenase 1 and stromelysin gene expression. SCC25 cells were treated with cycloheximide (C), and EB1089 (E) alone or in combination as indicated for 24 h. Total RNA was analyzed by RT-PCR for expression of stromelysin, collagenase 1. GAPDH expression was not affected (not shown). D, Genes controlling non-GPCR-mediated intracellular signaling. E, Genes modulating GPCR function. F, Genes encoding signaling peptides. G, Genes controlling regulation of immune system function. H, Genes controlling intracellular redox balance. Note that these categories are not mutually exclusive, and some genes may appear under more than one category. In addition, not all genes listed in Table 1 are presented.

odulin-like protein (41). Calmodulin-like protein is a marker of epithelial cell differentiation (41). Genes encoding semaphorin 3B and 3F lie in a region of chromosome 3 deleted in lung cancers (42–44). The exception to the above is HBp17, a putative regulator of FGF signaling that was expressed at lower levels in SCC than in primary cultures of keratinocytes (45).

EB1089 also down-regulates a large number of genes that are overexpressed in cancers (Fig. 3B), including tumor antigen L6, carcinoma associated antigen GA733-2, and squamous cell carcinoma antigen (SCCA). SCCA is a serum marker of uterine cervix, head and neck, lung, and esophageal cancers, and ablation of its expression inhibits growth and induces natural killer cell infiltration of tumors (46). Another down-regulated gene, tenascin C, is an early marker of HNSCC progression (47) Similarly, repression of overexpressed N-cadherin in head and neck squamous cell carcinoma is associated with restoration of an epithelial phenotype (48).

The above results suggest that EB1089 treatment reversed the malignant phenotype of SCC25 cells. This possibility was investigated further by immunofluorescence analysis of three markers that are differentially expressed in cancer cells, cystatin M, protease M, and N-cadherin. Both protease M and cystatin M transcripts are strongly induced by EB1089, and cystatin M is an ideal marker for these purposes because its expression is highly specific for differentiated epidermal keratinocytes (49). In addition, up-regulation of N-cadherin in head and neck squamous, breast and prostate cancers ("cadherin switching") is associated with cancer progression, invasion and metastasis (48, 50, 51). Immunofluorescence studies in control and EB1089-treated cells (Fig. 4) revealed a strong up-regulation of cystatin M expression, giving rise to strong, relatively uniform cytoplasmic staining (Fig. 4, A and B). Similar results were obtained with immunofluorescence analysis of protease M expression (Fig. 4, C and D), with the exception that elevated levels of protease M expression varied somewhat in EB1089-treated cells. In contrast, EB1089 treatment down-regulated N-cadherin expression (Fig. 4, E and F). This down-regulation included cell-cell contact sites, as well as the dotted pattern of non-cell-to-cell contacts seen in other carcinoma cells (51). The changes observed are in excellent agreement with the regulation of the genes encoding these markers (Table 1, Figs. 1 and 3). Moreover, in addition to providing evidence

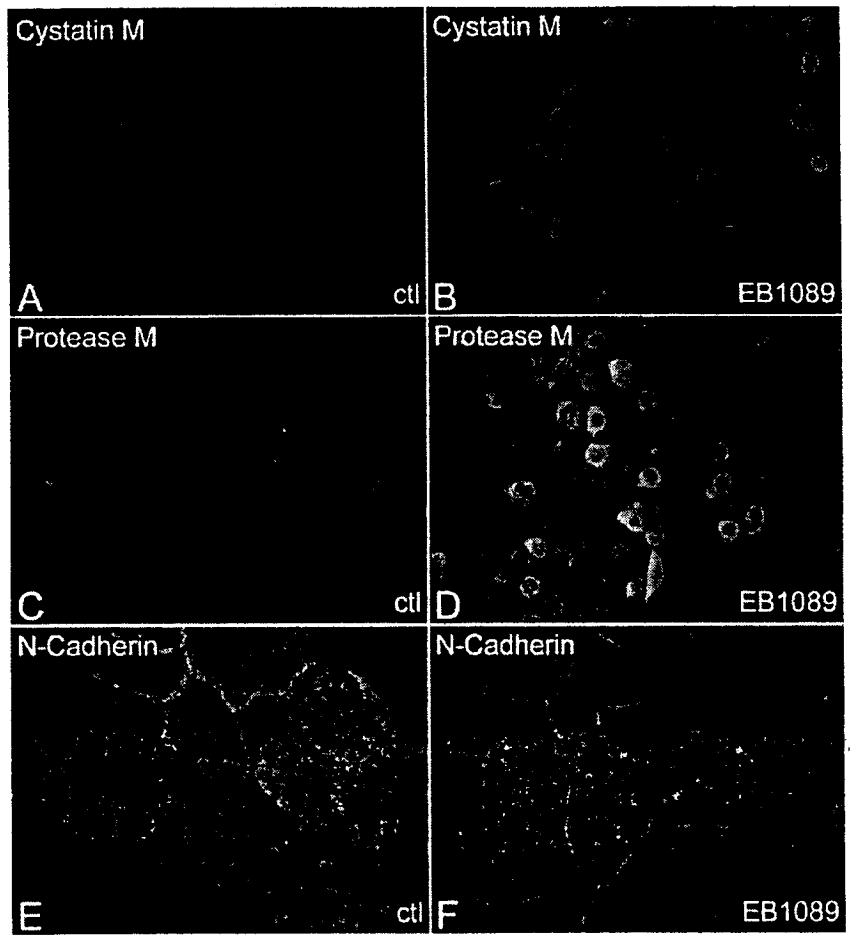

Fig. 4. Immunofluorescence Analysis of Cystatin M, Protease M, and N-Cadherin Expression in EB1089-Treated SCC25 Cells

Control (ctl; vehicle-treated) and EB1089-treated SCC25 cells were analyzed by immunofluorescence for expression of cystatin M (A and B), protease M (C and D), and N-cadherin (E and F). Primary antibodies were detected with Cy3-conjugated goat antirabbit (A, B, E, and F) or Cy2-conjugated goat antimouse (C and D) secondary antibodies. No staining was seen in the absence of primary antibodies (data not shown). Images of each control and EB1089-treated sample pair were acquired by confocal microscopy and processed using identical parameters. See *Materials and Methods* for details. Magnifications: A–D, ×25; E and F, ×63.

that EB1089 reverses the malignant phenotype of SCC25 cells, these studies provide sensitive new markers for HNSCC progression and treatment.

Regulation of Genes Controlling ECM Structure and Remodeling, and Cell Adhesion Consistent with Induction of a Basal Keratinocyte Phenotype

EB1089 does induce expression of some genes that are often up-regulated in cancers, many of which are implicated in extracellular matrix (ECM) structure and remodeling. Up-regulated genes include those encoding transcription factor E1A-F, which controls matrix metalloproteinase (MMP) gene expression (52), and two of its target genes, MMPs stromelysin and collagenase 1 (Fig. 2, B and C). EB1089-dependent induction of stromelysin, collagenase 1 and E1A-F was confirmed by RT-PCR (Figs. 2 and 3). Although E1A-F is a regulator of collagenase gene expression, cycloheximide did not block EB1089-induced expression of collagenase 1 or stromelysin (Fig. 3C, *bottom*). This indicates that induction of E1A-F expression by EB1089 is not essential for observed regulation of collagenase 1 and stromelysin, and that EB1089 has both long- and short-term effects on matrix metalloproteinase expression. Expression of osteopontin, a noncollagen matrix protein implicated in ECM structure and remodeling was also up-regulated. Several studies have indicated that osteopontin, collagenase 1, and stromelysin play key roles in ECM remodeling during wound healing (53–55). Up-regulation of their expression by EB1089 provides a molecular genetic basis for the proposed stimulatory role of 1,25-$(OH)_2D_3$ in wound healing (56).

The strong induction (22-fold) of expression of the type XIII collagen gene, a transmembrane collagen, provided further evidence that EB1089 induced kera-

tinocytic differentiation of SCC25 cells. Interestingly, trimerization of type XIII collagen is activated by prolyl 4-hydroxylase (57), whose gene is also up-regulated (Fig. 3C). Type XIII collagen is expressed in normal human epidermis and is present at cell-to-cell contact sites and at the dermal-epidermal junction. It is highly colocalized with E-cadherin and may be a component of adherens-like junctions (58). In addition, expression of phosphotyrosine phosphatase PTP-1B, whose activity has been associated with enhanced cell adhesion (59), is also increased.

EB1089 also up-regulates BPAG-1 (bullous pemphigoid antigen-1; Fig. 3C), a component of hemidesmosomes, structures essential for adhesion of epithelial cells to basement membranes (60). Absence or disruption of hemidesmosomal components gives rise to devastating bullous pemphigoid blistering skin disorders. EB1089 also induces expression of desmoglein 3 (Fig. 3C), a cadherin component of desmosomes (60), and the autoantigen in pemphigus vulgaris. It is noteworthy that desmogleins are expressed in a gradient in the epidermis, with desmoglein 3 most abundant in the basal layer (61). This observation, coupled with the up-regulation of type XIII collagen and hemidesmosomal components, provides further evidence that EB1089 induces a more epithelial, less malignant phenotype in SCC25 cells, consistent with that of basal keratinocytes.

Pleiotropic Effects of EB1089 on Inter- and Intracellular Signaling

Expression of several factors controlling intracellular signaling was altered in EB1089-treated cells (Figs. 2, D–F), including a number of genes encoding proteins controlling G protein-coupled receptor signaling (Fig. 3E). Up-regulated genes include those encoding the A kinase anchoring protein Ht31, and RGS2/G0S8, which is a selective inhibitor of Gq α signaling (62). The induction of RGS2/G0S8 is intriguing, as its expression is also induced by PTH in bone (63), which can signal through a G protein-coupled receptor linked to Gq α (64). 1,25-$(OH)_2D_3$ represses PTH receptor (PTH1R) signaling by inhibiting expression of the receptor and ligands PTH and PTHrP (Fig. 3F; Refs. 34, 35, 65). In addition, EB1089 treatment induces expression of the G receptor kinase GRK5 (Fig. 3E), which can repress PTH1R function (66). These results indicate that, in addition to inhibiting ligand and receptor expression, 1,25-$(OH)_2D_3$ signaling can also repress PTH1R function by inducing expression of factors that inhibit signaling via Gq α.

Expression of a number of signaling peptides was altered in treated cells (Fig. 3F), emphasizing the neuroendocrine nature of epidermal function (2). Our previous studies have shown that induction of amphiregulin (Fig. 3G) can inhibit SCC25 proliferation (28). Down-regulated genes include galanin, a neuropeptide implicated in nerve regeneration after injury (67), and S1–5, a relatively uncharacterized factor with EGF-like domains (68). Consistent with its antiproliferative effects, EB1089 down-regulated expression of several mitogenic factors. These include VEGF-related protein, which is mitogenic in Kaposi's Sarcoma and hematopoietic cells (69, 70), Cyr61, which encodes a growth factor implicated in angiogenesis and tumorigenesis, whose expression is induced by estrogen in breast cancer cells (71), and midkine, mitogenic factor overexpressed in several carcinomas (72).

Regulation of Genes Controlling Immune System Function

Keratinocytes are considered to be an integral part of the immune system of the skin (2). The intimate connection of epithelial cells to immune system function is reinforced by the large number of EB1089-related genes in SCC25 cells implicated in immunoregulation (Fig. 3G). The role of 1,25-$(OH)_2D_3$ in controlling the function of epithelial cells in innate immunity (73) is underlined by the strong induction by EB1089 of the gene encoding the pattern receptor CD14 (Fig. 3H), which is also a target gene in monocytic HL60 cells (37). Significantly, another strongly induced gene is that encoding T1/ST2, a member of the IL-1 receptor family. Gene ablation studies in mice have revealed that T1/ST2 signaling is required for T helper 2, Th2, cell differentiation (74).

EB1089 down-regulated interferon γ-regulated genes encoding 9–27, 1–8D, interferon-inducible 56K protein, and the T cell chemokine IP-10, and the chemokine RANTES, which is also overexpressed in a number of cancers including more advanced breast cancer (75). Interferon γ signaling and overexpression of IP-10 underlie the inflammatory reactions in psoriasis (76). Previous studies have suggested that 1,25-$(OH)_2D_3$ signaling can influence T helper cell differentiation (3). These data indicate that directs effects on epithelial cell signaling play a key role in the antiinflammatory action of 1,25-$(OH)_2D_3$ analogs in skin. Our results are consistent with EB1089 stimulating Th2 responses, and inhibiting a number of genes associated with proinflammatory Th1 responses.

Control of Genes Regulating Cellular Redox Balance

EB1089 signaling regulates a number of genes encoding proteins that control cellular redox balance (Fig. 3H). Induction of these genes by EB1089 and 1,25-$(OH)_2D_3$ may represent a feedback response to epidermal vitamin D_3 synthesis induced by sunlight, which is an effective inducer of reactive oxygen species in skin (77, 78). Up-regulated genes include glucose-6-phosphate dehydrogenase (G6PDH), selenoprotein P, glutathione peroxidase, thioredoxin reductase, HtrA, and, importantly, the nrf2 transcription factor. Selenoprotein P is a plasma heparin binding protein with antioxidant properties (79). HtrA is an extremely well conserved protein whose prokaryotic homolog is essential for survival under conditions of

oxidative stress (80). Ablation of nrf2 expression in mice rendered them more susceptible to carcinogenesis and resistant to the protective effects of chemoprevention agents (81). Nrf2 expression, which is induced by a number of chemopreventive agents, in turn induces expression of a number of phase II detoxifying enzymes. These events may provide a mechanism for protection by 1,25-$(OH)_2D_3$ against dimethyl-benzanthracene carcinogenesis in hampster cheek pouch carcinoma (24). Dimethyl-benzanthracene is activated by a series of oxidation steps, and detoxified by phase II enzymes (82).

Both G6PDH and thioredoxin reductase contribute to nucleotide biosynthesis in proliferating cells and are overexpressed in cancer cells (83, 84). However, in quiescent cells they are source of reducing equivalents. G6PDH is at the head of the pentose-phosphate shunt, which is a source of NADPH, and thioredoxin reductase uses NADPH to reduce thioredoxins, proteins that in turn reduce oxidized cysteines. Elevated G6PDH and thioredoxin levels protect against apoptosis, which is sensitive to redox balance. Recent studies have shown that short-term 1,25-$(OH)_2D_3$ treatment of MCF-7 breast cancer cells has prooxidant effects (85). However, unlike the results of obtained in SCC25 cells (Fig. 3), G6PDH induction in MCF-7 cells was modest, and no changes in glutathione peroxidase levels were found. Significantly, however, 1,25-$(OH)_2D_3$ is an effective inducer of apoptosis in MCF-7 cells, whose onset can be controlled by redox balance, whereas no evidence for apoptosis was found in 1,25-$(OH)_2D_3$-treated SCC25 cells (21). This suggests that the effects of 1,25-$(OH)_2D_3$ on redox balance may be cell specific.

EB1089 and 1α,25(OH)$_2$D$_3$ Regulate Target Gene Expression with Similar Efficacy

We have confirmed the regulation of a total of 30 genes by Northern blotting and/or RT-PCR (Figs. 1 and 5, Table 1). In addition to the 17 genes presented in Fig. 5, regulation of 9 other genes was confirmed at single time points (Table 1, and data not shown). We have also compared regulation by EB1089 and 1,25-$(OH)_2D_3$ of several target genes. Structure/function studies have suggested the VDR forms structurally distinct complexes with EB1089 and 1,25-$(OH)_2D_3$, possibly providing a molecular basis for gene-specific effects of the two compounds (86). In preliminary analyses by RT-PCR of the effects of 24 or 48 h treatment with EB1089 or 1,25-$(OH)_2D_3$, several target genes analyzed appeared to be differentially regulated by two compounds (data not shown). Therefore, we compared target gene regulation by EB1089 and 1,25-$(OH)_2D_3$ over the entire 48 h time course (Fig. 5). The results showed that 1,25-$(OH)_2D_3$ regulated expression of several genes more transiently than EB1089 but did not provide any evidence for gene-specific differences in efficacy of the two compounds (Fig. 5).

To examine the potential role of 24-OHase in attenuation of 1,25-$(OH)_2D_3$ signaling by 48 h, we compared expression profiles in SCC25 cells treated with vehicle, EB1089 or 1,25-$(OH)_2D_3$ in the presence or absence of the cytochrome P450 inhibitor ketoconazole (Fig. 6). As expected, induction of T1/ST2 expression by EB1089 after 48 h was strong, and was unaffected by ketoconazole. In contrast, while the effect of 1,25-$(OH)_2D_3$ alone after 48 h was weaker, T1/ST2 expression remained high in cells treated with 1,25-$(OH)_2D_3$ and ketoconazole together and was essentially identical to that observed in the presence of EB1089 or EB1089 and ketoconazole. Similar effects of ketoconazole were observed on 1,25-$(OH)_2D_3$-dependent induction of semaphorin 3B, and type II 17β-hydroxysteroid dehydrogenase genes, and on repression of the SCCA gene (Fig. 6). No effects were observed of ketoconazole alone or with ligands on GAPDH expression (not shown). Thus, the more sustained regulation of several target genes by EB1089 is likely due to its insensitivity to induction of 24-OHase activity. The variability observed in the relative durations of the regulatory effects of EB1089 and 1,25-$(OH)_2D_3$ in Fig. 5 may reflect differences in stability of association of ligand-bound VDR with specific promoters, or with differing stabilities of target gene mRNAs. The data do not provide any evidence for gene-specific differences in efficacy of *trans*-activation or -repression by EB1089 and 1,25-$(OH)_2D_3$.

CONCLUSIONS

The studies above provide multiple insights into not only the potential of 1,25-$(OH)_2D_3$ analogs as agents of cancer chemoprevention, but also into the physiological actions of 1,25-$(OH)_2D_3$ in a number of tissues, including skin, bone, and the immune system. The data indicate that EB1089 performs key functions of a cancer chemoprevention agent; it is antiproliferative, it induces cellular differentiation, and it has potential genoprotective effects over and above our previous findings of the induction of GADD45α (13, 21). Differential effects on gene expression of EB1089 and 1,25-$(OH)_2D_3$ were attributable to the insensitivity of EB1089 to 24-OHase activity, suggesting that differences in action of the two compounds arise more from their sensitivity to metabolism and than from differential action of the VDR bound to each ligand.

MATERIALS AND METHODS

Tissue Culture and RNA Extraction

SCC25 cells were obtained from the American Type Culture Collection (Manassas, VA), and were cultured under recommended conditions. Cells cultured in 10-cm plates under conditions where controls cell could proliferate for at least 10 d before confluence (21). Media were changed 24 h before treatment with EB1089 or 1,25-$(OH)_2D_3$ (100 nM) in dimethylsulfoxide for 0, 1, 3, 6, 12, 24, or 48 h as previously de-

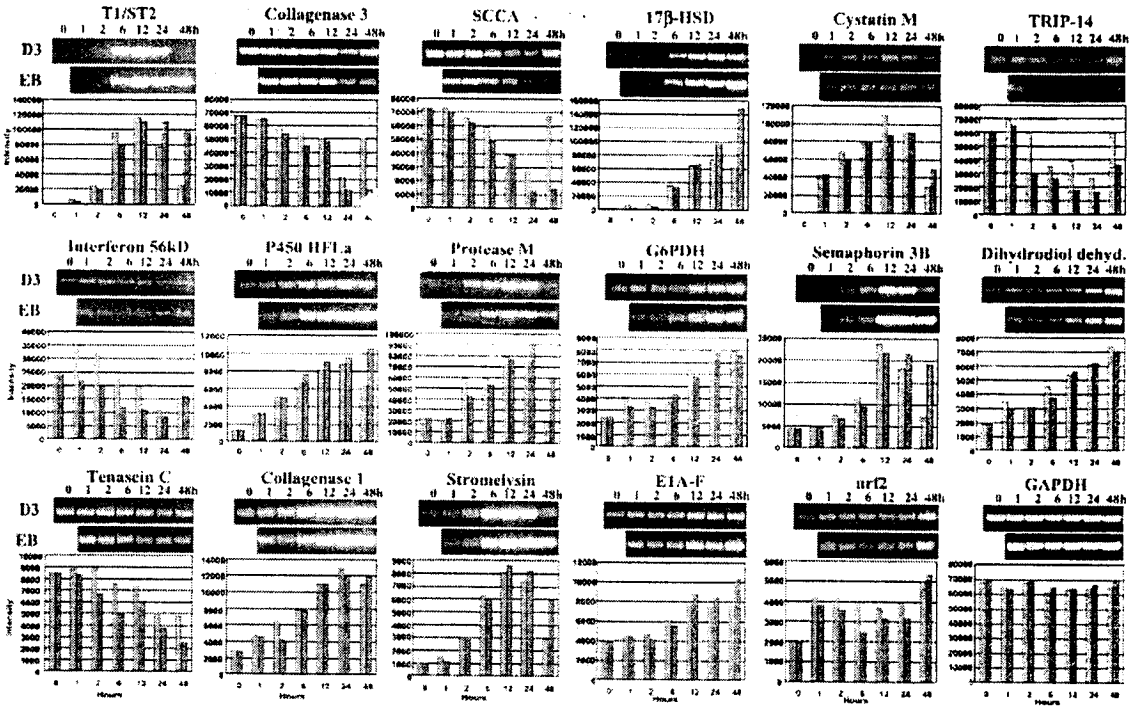

Fig. 5. Comparison of Effects of EB1089 and 1,25-$(OH)_2D_3$ on Target Gene Expression

SCC25 cells were cultured and treated with in parallel with EB1089 (*dark gray bars*) or 1,25-$(OH)_2D_3$ (*pale gray bars*) as indicated and gene expression was analyzed by RT-PCR. Genes selected included both up- and down-regulated targets and strongly (*e.g.* T1/ST2, protease M) and moderately (*e.g.* E1A-F, interferon-inducible 56-kDa protein) regulated genes.

scribed (21). Total RNA was extracted with TRIZOL (Life Technologies, Inc., Burlington, Ontario, Canada), and 10 μg of RNA isolated from EB1089-treated cells were used for microarray analysis. Cycloheximide (200 nM; Sigma-Aldrich Canada, Oakville, Ontario, Canada) was added 1 h before addition of EB1089 as indicated. Ketoconazole (100 nM; Sigma-Aldrich Canada) was added along with EB1089 and 1,25-$(OH)_2D_3$ as indicated.

Microarray Screening and Data Analysis

Probe for microarray analysis was generated, and Affymetrix HuGene FL human gene oligonucleotide microarrays were screened as described in Novak *et al.* (87). Screenings for EB1089-regulated genes were performed with three sets of probes generated from three independent tissue culture experiments. To test for statistically significant changes in signal intensity, compiled data was screened initially by nonparametric ANOVA (29) using a P value of < 0.05. Genes retained were then filtered for those whose expression was up- or down-regulated a minimum of 2.5-fold at some point during the 48-h time course, corresponding to a minimum magnitude change of 200 fluorescence units. The data were filtered to eliminate genes with noisy expression profiles by calculation of cross correlations between individual profiles and hyperbolic tangents [$x(t)=tanh(nt/2)$], where x is normalized fold induction, t is time, and n is a time constant controlling time of saturation. Profiles of up-regulated genes with correlation coefficients of 0.8 or less, and down-regulated genes with correlation coefficients of less than -0.8 were eliminated.

A method of clustering analysis was developed that classifies groups of genes based on time of regulation with respect to a threshold value, and does not take into account initial conditions. Maximal gene regulation was normalized to 1 for up-regulated genes and -1 for down-regulated genes. Given that experimental measurements were performed at 0, 1, 2, 6, 12, 24, and 48 h, the number of intervals initially generates 6 clusters each for up- and down-regulated genes. Clustering was evaluated for threshold values between 0.25 and 0.75, and -0.25 and -0.75 for induced and repressed genes, respectively. The number of clusters was then heuristically adjusted based on the following criteria: 1) a cluster must contain at least two genes; 2) the mean value of each cluster does not cross that of another cluster near the threshold. The optimum threshold was chosen as that generating the maximum cluster stability defined by the probability of a gene belonging to the same cluster in the average data set and the individual data sets. Based on these criteria, 0.50 and -0.50 were chosen as threshold values. The time the threshold is crossed was computed using a linear interpolation method. To avoid multiple threshold crossings, only the first crossing with a positive derivative for up-regulated genes, and negative derivative for down-regulated genes were considered. Analysis was carried out using Mathlab 6.12 (MathWorks Inc., Natick, MA).

Immunofluorescence

SCC25 cells were plated on cover slips and treated with dimethylsulfoxide vehicle or 100 nM EB1089 for 72 h. Cells were processed for immunolabeling as described in (88). Briefly, cells were fixed in 2% paraformaldehyde and permeabilized, and blocked with Triton X-100/BSA. Cells were sequentially labeled with affinity purified rabbit anticystatin M (1:50; Refs. 40, 49), mouse antiprotease M (1:150; Ref. 89) or rabbit anti-N-Cadherin (1:50; Sigma) primary antibodies for 1 h at room temperature followed by Cy3-conjugated goat antirabbit or Cy2-conjugated goat antimouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West

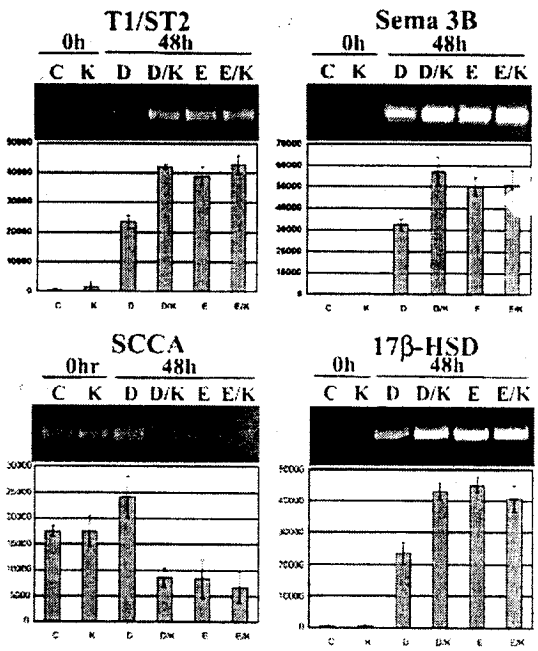

Fig. 6. Analysis of the Effects of Cytochrome P450 Inhibitor Ketoconazole on EB1089- and 1,25-$(OH)_2D_3$-Regulated Gene Expression

SCC25 cells were treated with vehicle alone (control; C), 100 nM ketoconazole alone (K), 100 nM 1,25-$(OH)_2D_3$ alone (D), 100 nM EB1089 alone (E), or in combination as indicated. Total RNA isolated from treated cells was analyzed by RT-PCR for expression of T1/ST2, Semaphorin 3B (Sema 3B), 17β-hydroxysteroid dehydrogenase (17β-HSD), and SCCA. Results of three independent experiments are presented.

Grove, PA) for 1 h at room temperature. Immunofluorescence was visualized with a Bio-Rad Laboratories, Inc. (Hercules, CA) MicroRadiance confocal microscope at an optical thickness of approximately 10 μm using 25− or 63− objectives. For each pair of control and EB1089-treated samples, images were acquired and processed using identical parameters. Digital images were prepared using Adobe Photoshop.

Northern Blotting and RT-PCR Analysis of Regulated Gene Expression

Total RNA was extracted from SCC25 cells using Trizol (Life Technologies, Inc.). Denatured RNA (3 μg) was reverse transcribed in a 20 μl reaction at 42 C for 50 min with SuperScript II (Life Technologies, Inc.) according to the supplier's instructions. Amplification conditions were optimized in preliminary experiments so that maximal amplification fell within the linear range. Products were diluted to 200 μl, denatured at 95 C for 2 min, and then amplified as follows: Tenascin C, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-CCACAGCTGGGAGATTTAGC-3′ and reverse 5′-CTGGGAGCAAGTCCAGAGAG-3′ primers; Nrf2, (21 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-ACCCTTGTCACCATCTCAGG-3′ and reverse 5′-TTGCCATCTCTTGTTTGCTG-3′ primers; dihydrodiol dehydrogenase, (21 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-GGTCACTTCATGCCTGTCCT-3′ and reverse 5′-GGATGACATTCCACCTGGTT-3′ primers; stromelysin (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-AACCTGTCCCTCCAGAACCT-3′ and reverse 5′-TGGGTCAAACTCCAACTGTG-3′primers; Collagenase 1, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-TGGACCTGGAGGAAATCTTG-3′ and reverse 5′-GGGGTATCCGTGTAGCACAT-3′ primers; E1AF, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-CGCCTACGACTCAGATGTCA-3′ and reverse 5′-GGAAGGCCAAAGAGAAGAGG-3′primers; Protease M, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-GGGGTCCTTATCCATCCACT-3′ and reverse 5′-GGGATGTTACCCCATGACAC-3′ primers; G6PD, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-CAACCACATCTCCTCCCTGT-3′ and reverse 5′-TCCCACCTCTCATTCTCCAC-3′ primers; ST2, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-CAACTGGACAGCACCTCTTG-3′and reverse 5′-CAAATTCAGGGCCAGACAGT-3′ primers; P-450 (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-TTGCCCAGTATGGAGATGTG-3′ and reverse 5′-GAACACTGCTCGTGGTTTCA-3′ primers; 17β-hydroxysteroid dehydrogenase, (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-CACGAAGCCAGTGCAGATAA-3′ and reverse 5′-GGAAATTCCGCTGTGCTAAG-3′ primers; Cystatin M (27 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-GGAGAACTCCGGGACCTGT-3′ and reverse 5′-GGAACCACAAGGACCTCAAA-3′ primers; Semaphorin V, (33 cycles; 94 C, 30 sec; 60 C, 45 sec; 72 C, 45 sec) with forward 5′-AACCTGTGCCTTTGTGGAAG-3′ and reverse 5′-AGCTGATCGAAGTGGGTGTC-3′ primers; Collagenase 3 (26 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-ATGACTGAGAGGCTCCGAGA-3′ and reverse 5′-ACCTAAGGAGTGGCCGAACT-3′ primers; TRIP-14, (26 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-AAAGAGAGGCCATCATCCT-3′ and reverse 5′-CAGGAACCTGGAAGGACAGA-3′ primers; VEGF-related protein (33 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-TCTCTGTGGCGTGTTCTCTG-3′ and reverse 5′-CACTGCAGCCCCTCACTATT-3′ primers; SCCA, (26 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-TGATTTTGCAAATGCTCCAG-3′ reverse and 4 5′-TGGTTCTCAACGTGTCCTTG-3′ primers; Interferon 56 kDa, (26 cycles; 94 C, 30 sec; 57.5 C, 45 sec; 72 C, 45 sec) with forward 5′-GCTTCAGGATGAAGGACAGG-3′ and reverse 5′-GAAATTCCTGAAACCGACCA-3′ primers; GAPDH (23 cycles; 94 C, 30 sec; 55 C, 30 sec; 72 C, 1 min) with forward 5′-GGTGAAGGTCGGTGTCAACG-3′ and reverse 5′-CAAAGTTGTCATGGATGACC-3′ primers; Amphiregulin, (32 cycles; 94 C, 30 sec; 55 C, 30 sec; 72 C, 1 min) with forward 5′-TTCGCACACCTGGGTGCCAG-3′ and reverse 5′-AAGAGGATCCACTCATCATTTATGGCTATG-3′ primers; Integrin α7B, (30 cycles; 94 C, 30 sec; 53 C, 45 sec; 72 C, 45 sec) with forward 5′-GGTGAAGCTTCCTCGGGAAGAC-3′ and reverse 5′-GGAGCAAGCTTGAGTCAGTGACAC-3′ primers; CRABP-II, (30 cycles; 94 C, 30 sec; 53 C, 45 sec; 72 C, 45 sec) with forward 5′-GACAGGATCCAGTGCTCCAGCCTAGGAG′ and reverse 5′-AGAGGGATCCTGCTCTGGGCTGGTTTGG-3′ primers; 24-OH (30 cycles; 94 C, 30 sec; 55 C, 30 sec; 72 C, 1 min) with forward 5′-AAGGATCCTGTTCTGTCTTGCATCTTC-3′ and reverse 5′-CCCTAAAGCTTTCACAGCAGAGAGAAAGC-3′ primers; N-cadherin, (23 cycles; 94 C, 30 sec; 50 C, 30 sec; 72 C, 1 min) with forward 5′-TTAGTCACCGTGGTCAAACCAATC-3′ and reverse 5′-AGTGGATCCACTGCCTTCATAGTCAAACAC-3′ primers. All of the above reactions were performed in 50 μl of 2.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-Cl (pH 9.0) using 2.5U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). Aliquots of 45 μl of each amplified sample were subjected to electrophoresis on 2% agarose gels containing ethidium bromide and photographed. Fluorescent bands were quantified using Kodak (Rochester, NY) digital science 1D Image Analysis software.

For Northern blotting, 20 μg of total RNA or 1 μg of poly A+ RNA were electrophoresed as described (21). Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). The blotted membrane was

soaked in 3% SSC and 0.1% SDS at 50 C, and prehybridized at 42 C in 50 mM phosphate buffer pH 6.5, 50% formamide, 5% SSC, 10% Denhardt's solution containing 250 μg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled cDNA probes. After hybridization, the membrane was washed 4 times in 2% SSC and 0.2% SDS for 5 min, 3 times in 0.1% SSC and 0.2% SDS for 30 min at 50 C, dried, and autoradiographed. Band intensities were quantitated using the FluorChem digital imaging system and AlphaEaseFC software (Alpha Innotech Corp., San Leandro, CA).

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Amphiregulin Is a Vitamin D_3 Target Gene in Squamous Cell and Breast Carcinoma

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1α,25-Dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] inhibits growth of cells derived from a variety of tumors *in vitro* and *in vivo*. Proliferation *in vitro* of human SCC25 cells, derived from a primary squamous cell carcinoma (SCC) of the tongue, was blocked by 1,25(OH)$_2$D$_3$ and its analog EB1089. A similar effect was observed with 13-*cis* retinoic acid (RA), which has been used in chemoprevention of SCC. We identified amphiregulin, a member of the epidermal growth factor family, as a 1,25(OH)$_2$D$_3$ target gene in SCC25 cells. Induction of amphiregulin mRNA by 1,25(OH)$_2$D$_3$ was rapid and sustained over 48 h, and was unaffected by cycloheximide. 1,25(OH)$_2$D$_3$ also induced amphiregulin mRNA in estrogen receptor-positive and -negative human breast cancer cell lines, but not in LNCaP human prostate cancer cells. RAR- or RXR-specific retinoids did not affect amphiregulin mRNA levels in SCC25 cells; however, 13-*cis* RA partially blocked the response to 1,25(OH)$_2$D$_3$. Amphiregulin partially inhibited growth of SCC25 cells in culture. Our data show that amphiregulin is a 1,25(OH)$_2$D$_3$ target gene, and suggest that its induction may contribute to the growth inhibitory effects of 1,25(OH)$_2$D$_3$. © 2001 Academic Press

Key Words: **1α,25-dihydroxyvitamin D_3; retinoic acid; head and neck squamous cell carcinoma; chemoprevention; growth inhibition; gene expression.**

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) is best known for its key role in calcium homeostasis (1). However, 1,25(OH)$_2$D$_3$ and its analogs also inhibit proliferation and stimulate differentiation of cancer cells derived from a variety of tissues, including breast, prostate, colon, lung, endometrium, hematopoietic cells, and oral cavity (1–9). The factor limiting the use of 1,25(OH)$_2$D$_3$ itself has been hypercalcemia. However, over 800 analogs have been developed in an attempt to maintain the inhibitory effect on tumor cell proliferation while reducing the hypercalcemic effects (10). For example, one such agent EB1089 caused apoptotic regression of xenografts of the breast carcinoma cell line MCF-7 in nude mice (7). Dosages of EB1089 of 0.1 to 1.0 μg/kg/day showed no clinically significant hypercalcemia in animal models of prostate and breast carcinoma (11, 12).

1,25(OH)$_2$D$_3$ and its analogs interact with the nuclear vitamin D receptor (VDR), which functions as a ligand-inducible transcription factor by forming heterodimers with members of the retinoid X receptor (RXR) family (13). 1,25(OH)$_2$D$_3$ thus exerts its antiproliferative effects by modulating target gene transcription. 1,25(OH)$_2$D$_3$ analogs are attractive candidates for chemoprevention of squamous cell carcinomas (SCC) of the oral cavity (14, 15). Retinoids, such as 13-*cis* retinoic acid (13-*cis* RA; isotretinoin) have been used clinically in chemoprevention of SCC since the 1980s (16). Signaling by 13-*cis* RA is very similar to that of 1,25(OH)$_2$D$_3$, as retinoids bind to nuclear retinoic acid receptors (RARs), which function as RAR/RXR heterodimers. However, progression of SCC is associated with reduced expression of RARs, particularly RARs β and γ, loss of retinoid-regulated expression of terminal differentiation markers, and resistance to the antiproliferative effects of retinoids (17–22).

We have found that 1,25(OH)$_2$D$_3$ and its analog EB1089 inhibit the proliferation *in vitro* of the human SCC line SCC25. A screen of a gene array was performed to identify D3 target genes in SCC25 cells. One target was found to be amphiregulin, a member of the epidermal growth factor family of peptides. Amphiregulin was originally isolated from serum-free medium of MCF-7 breast carcinoma cells treated with phorbol 12-myristate 13-acetate, which is growth inhibitory (23). It was so-named because of its capacity to

Abbreviations used: CHX, cycloheximide; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SCC, squamous cell carcinoma; VDR, vitamin D$_3$ receptor.

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inhibit proliferation of several human carcinoma cell lines and stimulate growth of other cell lines and primary cultures of normal fibroblasts (24, 25). Amphiregulin has moderate affinity for the epidermal growth factor receptor, and previous experiments have shown that it modulates cell proliferation at concentrations above 10 nM (26). Here, we show that $1,25(OH)_2D_3$ rapidly and directly affects the regulation of amphiregulin gene expression in SCC25 cells and estrogen receptor-positive and -negative breast carcinoma cells, and that incubation of SCC25 cells with nM concentrations of recombinant amphiregulin partially reproduces the growth inhibitory effects of $1,25(OH)_2D_3$.

MATERIALS AND METHODS

Nuclear receptor ligands and reagents. 1α,25-Dihydroxyvitamin D_3 and EB1089 were kindly supplied by Dr. Lise Binderup (Leo Laboratories, Ballerup, Denmark). 13-*cis* RA was purchased from ICN, and LG1069 (LG) was a generous gift of Dr. Tim Willson (Glaxo-Welcome Research and Development, Research Triangle Park, NC). All hormones were dissolved in dimethyl sulfoxide (DMSO), and stock solutions were stored in the dark at −20°C. Recombinant human amphiregulin was purchased from R&D Systems (Minneapolis, MN) and cycloheximide was purchased from Sigma (Oakville, Ontario).

Tissue culture. The squamous cell carcinoma cell line SCC25 and breast cancer cell lines MDA-MB361, MCF-7 and prostate cancer cell line, LNCaP were obtained from the American Type Cultures Collection (ATCC, Rockville, MD). Cell lines were cultured under recommended conditions. The effects of $1,25(OH)_2D_3$, EB1089, and retinoids on cell growth were analyzed by seeding cells in 6-well plates at 15,000 cells/well in 2 ml of culture medium containing charcoal-stripped serum. After 24 h the culture media were changed to charcoal-stripped medium containing vehicle or ligand at the indicated concentrations. Media were changed every two days and fresh ligand added as necessary. On the designated day, cells were washed with 2 ml of phosphate buffered saline and removed from the plate by incubation with 0.7 ml of 0.25% trypsin-EDTA. Cell numbers were determined using a hemacytometer. Four grid sections were counted for each well and the results were averaged. All treatment conditions were performed in triplicate wells. For the effect of amphiregulin on SCC25 cell line cells were seeded in 6-well plates at 15,000 cells/well in 2 ml of culture medium. After 24 h the culture media were changed to charcoal-stripped medium containing vehicle or amphiregulin at the indicated concentrations. Media were changed every day. Cell counts were performed with same method as other cell growth experiments. All treatment conditions were tested in triplicate.

Total and poly(A)$^+$ RNA isolation. Cells were grown in 100-mm dishes. When appropriate, media were replaced with charcoal stripped medium containing ligand at the indicated concentrations. Cycloheximide was added to 200 nM 1 h prior to addition of ligand where indicated. After incubation, total RNA was extracted with TRIZOL (GIBCO BRL, Burlington, Ontario) according to the manufacturer's instruction. Poly(A)$^+$ RNAs were isolated using an Oligotex mRNA Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Differential screening. Differential screening was performed with 100 ng of poly(A)$^+$ RNAs extracted from SCC25 cells treated for 24 h with DMSO, $1,25(OH)_2D_3$ (100 nM) or EB1089 (100 nM) using an Atlas cDNA Expression Array (Clontech, Palo Alto, CA). Preparation of probe and array screening were carried according to the manufacturer's instructions.

Complementary DNA probes and Northern blotting. Complementary DNA probes were generated by reverse transcription-PCR amplification of poly(A)$^+$ RNA from SCC-25 cells. RNAs were reverse transcripted by oligo dT priming and PCR amplification of amphiregulin sequences was performed using primer sets: 5′-TTCGCACACCTGGGTGCCAG-3′ and 5′-AAGAGGATCCACTCATCATTTATGGCTATG-3′. Amplified fragments were subcloned into Bluescript SK+ (Stratagene, Aurora, Ontario) and verified by dideoxy sequencing. For Northern blotting, 20μg of total RNA or 1 μg of poly(A)$^+$ RNA were separated on a 1.0% agarose gel containing 6.3% formaldehyde, 20 mM Mops (pH 7.0), 15 mM sodium acetate, and 1 mM EDTA. Separated RNAs were transferred to a Nylon membrane (Hybond-N+, Amersham, Baie d'Urfé, Québec). The blotted membrane was soaked in 3% SSC and 0.1% SDS at 50°C, and pre-hybridized at 42°C in 50 mM phosphate buffer pH 6.5, 50% formamide, 5% SSC, 10% Denhardt's solution containing 250 μg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-labeled cDNA probes. Following hybridization, the membrane was washed 4 times in 2% SSC and 0.2% SDS for 5 min, 3 times in 0.1% SSC and 0.2% SDS for 30 min at 50°C, dried, and autoradiographed. All Northern blots were performed at least three times with independent preparations of RNA from multiple plates.

RESULTS

$1,25(OH)_2D_3$ and EB1089 block proliferation of SCC25 cells. The growth inhibitory effects of $1,25(OH)_2D_3$ and its synthetic analog EB1089 (2) were evaluated in SCC25 cells. SCC25 cells were chosen for these studies because they have maintained a relatively differentiated phenotype and are thus more representative of the targets of chemoprevention than less differentiated lines. For example, unlike other lines, SCC25 cells express near normal levels of RARs β and γ, and have retained retinoid-regulated K-19 expression (14). Northern blotting studies of SCC25 cell poly(A)$^+$ RNA detected the presence of mRNAs encoding the VDR and its heterodimeric partners RXRα and β (Fig. 1A). Growth of SCC25 cells was strongly inhibited in the presence of 10 and 100 nM $1,25(OH)_2D_3$ (Fig. 1B). Similar degrees of inhibition were observed in the presence of 10 and 100 nM 13-*cis* RA, used as a positive control in these studies. No significant differences were observed in the growth inhibitory effects of 10 nM $1,25(OH)_2D_3$ or 13-*cis* RA alone or in combination at any point over a 10-day period (Fig. 1B, and data not shown). Essentially complete growth inhibition was observed in the presence of 1 to 100 nM EB1089 (Fig. 1B), indicating that EB1089 is a more potent inhibitor of SCC25 cell growth than $1,25(OH)_2D_3$.

Identification of novel $1,25(OH)_2D_3$ target genes in SCC25 cells. Given the sensitivity of SCC25 cells to $1,25(OH)_2D_3$ and EB1089, they represent ideal candidates for identification of $1,25(OH)_2D_3$ target genes. Regulated genes were identified by duplicate screenings of gene arrays with probes derived from RNA purified from control cells or cells treated with $1,25(OH)_2D_3$ or EB1089 for 24 h. Only genes that appeared to be regulated in both screenings were chosen

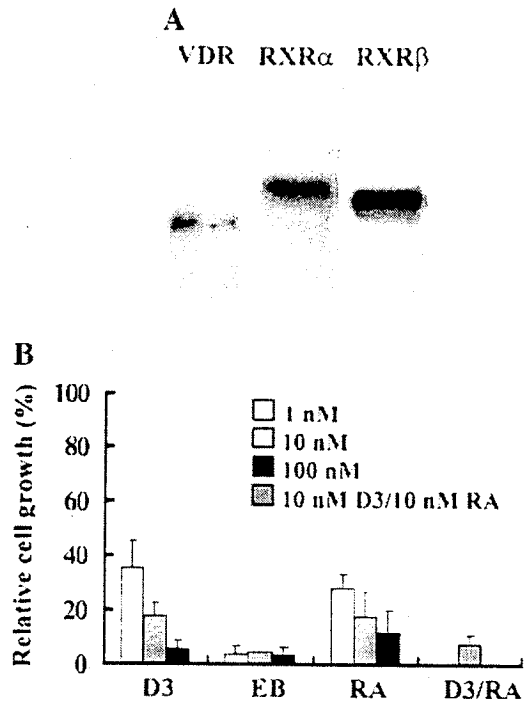

FIG. 1. Inhibition of SCC25 cell proliferation by $1,25(OH)_2D_3$ and EB1089. (A) Results of Northern blotting with 1 μg of poly(A)$^+$ RNA showing expression of mRNAs encoding the VDR, RXRα, and RXRβ, as indicated. (B) Relative growth of SCC25 cells incubated over a 10-day period in the presence of vehicle (−), 1, 10, or 100 nM $1,25(OH)_2D_3$ (D3), EB1089 (EB), or 13-*cis* RA (RA), as indicated. Medium was changed and fresh ligand added every 2 days over the period of the experiment. Each point represents the result obtained from triplicate wells. Cells were counted using a hemacytometer, and the numbers of cells in cultures treated with vehicle were given a value of 100%.

for further study. A full list of target gene identified from multiple arrays will be reported elsewhere (manuscript submitted). Here, we will focus on the regulation of amphiregulin, one of the genes identified from the screens. Amphiregulin is a member of the epidermal growth factor family of peptide growth factors. Analysis of arrays showed that amphiregulin mRNA expression was elevated in $1,25(OH)_2D_3$-treated cells (not shown), and in EB1089-treated cells, but not in control or 13-*cis* RA-treated cells (Fig 2A). This regulation was confirmed by Northern analysis of RNA from cells treated with VDR-, RAR-, or RXR-specific ligands (Fig. 2B), which showed that amphiregulin expression was induced by $1,25(OH)_2D_3$ or EB1089, but not by 13-*cis* RA or the RXR-selective ligand LG1069.

Remarkably, we found that 13-*cis* RA inhibited $1,25(OH)_2D_3$-dependent induction of amphiregulin expression, while the RXR-selective ligand LG1069 had no effect (Fig. 2C). This effect is not unique to the amphiregulin gene in SCC25 cells, as 13-*cis* RA also inhibited $1,25(OH)_2D_3$-dependent induced expression of the 24-hydroxylase gene (data not shown). In other studies, we have found that $1,25(OH)_2D_3$ inhibited expression of target genes of 13-*cis* RA (data not shown), indicating that, although $1,25(OH)_2D_3$ and 13-*cis* RA are growth inhibitory in combination (Fig. 1B), they can reciprocally inhibit expression of target genes.

Direct regulation of amphiregulin gene expression by $1,25(OH)_2D_3$ in SCC25 and breast carcinoma cells. Further analysis showed that that effect of $1,25(OH)_2D_3$ on amphiregulin mRNA was rapid, with induction observable after 2–4 h, and sustained over at least 48 h (Fig. 3A). Treatment of cells with cycloheximide did not block induction of amphiregulin gene expression, indicating that *de novo* protein synthesis was not required for the effect of $1,25(OH)_2D_3$ or EB1089 (Fig. 3B). Amphiregulin expression was also induced in $1,25(OH)_2D_3$-sensitive human breast carcinoma cell lines MCF-7 and MDA-MB-361 (Fig. 3C), which are estrogen receptor α-positive and -negative, respectively, indicating that the stimulatory effect of $1,25(OH)_2D_3$ is not restricted to SCC of the oral cavity. However, no induction of amphiregulin gene expression was observed in the $1,25(OH)_2D_3$-sensitive human prostate cancer cell line LNCaP (Fig. 3C).

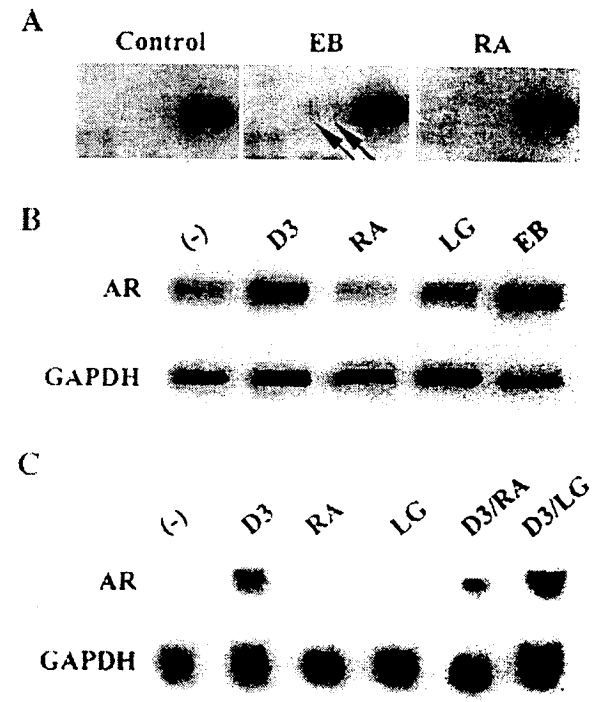

FIG. 2. (A) Differential expression of amphiregulin in SCC25 cells treated with EB1089. Sections of autoradiograms of gene arrays hybridized with probe derived from RNA of SCC25 cells treated for 24 h with vehicle (control), EB1089 (EB), or 13-*cis* RA (RA) are presented. The arrows indicate the position of amphiregulin cDNAs (spotted in duplicate) on the array. (B) Northern blots of total RNA probed for amphiregulin (AR) and GAPDH transcripts expressed in SCC25 cells treated with vehicle (−), or 100 nM $1,25(OH)_2D_3$ (D3), 13-*cis* RA (RA), LG1069 (LG), or EB1089 (EB) for 24 h. (C) Northern blots similar to B, except that cells were also treated with combinations of $1,25(OH)_2D_3$ and LG1069 or 13-*cis* RA as indicated.

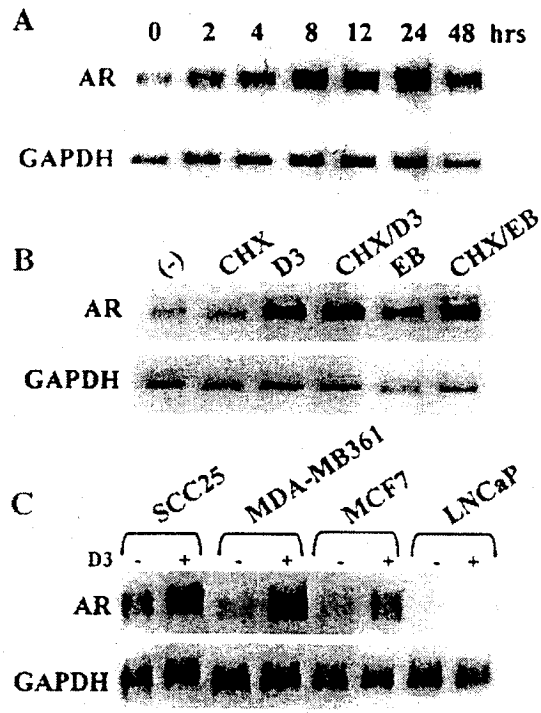

FIG. 3. Further analysis of amphiregulin expression. (A) Induction of amphiregulin (AR) by $1,25(OH)_2D_3$ is rapid. SCC25 cells were treated with 100 nM $1,25(OH)_2D_3$ for the times indicated prior to extraction of total RNA and analysis of amphiregulin expression by Northern blotting. (B) $1,25(OH)_2D_3$- or EB1089-stimulated expression of amphiregulin is not inhibited by cycloheximide (CHX) treatment. SCC25 cells were treated for 24 h with 100 nM $1,25(OH)_2D_3$ (D3) or EB1089, or 200 nM CHX alone or in combination as indicated. (C) $1,25(OH)_2D_3$ regulates amphiregulin gene expression in breast but not prostate carcinoma cells. Northern analysis of amphiregulin expression in SCC25 cells, MDA-MB361 and MCF-7 breast cancer cells, which are ER-negative and -positive, respectively, and the prostate cancer cell line LNCaP. Cells were treated with vehicle (−) or (+) 100 nM $1,25(OH)_2D_3$ as indicated.

Amphiregulin inhibits SCC25 proliferation. Amphiregulin has moderate affinity for the epidermal growth factor receptor, and has been shown to modulate cell proliferation at nanomolar concentrations. It was so-named for its capacity to inhibit proliferation of several human carcinoma cell lines and stimulate growth of other cell lines and primary cultures of normal fibroblasts (23–27). SCC25 cells were treated over an 8-day period with amphiregulin concentrations ranging from 0.5 to 20 nM (Fig. 4). Statistically significant growth inhibition was observed after 8 days of treatment with 2 or 20 nM amphiregulin, partially reproducing the inhibitory effects of $1,25(OH)_2D_3$ and EB1089 (see Fig. 1). Taken together, our results show that amphiregulin is a direct target gene of $1,25(OH)_2D_3$ in head and neck SCC and breast carcinoma cells. The antiproliferative effects of amphiregulin are consistent with it being a downstream effector of the growth inhibitory signal of $1,25(OH)_2D_3$ in SCC25 cells.

DISCUSSION

We have been interested in investigating the potential of $1,25(OH)_2D_3$ analogs as chemopreventive/chemotherapeutic agents for treatment of head and neck SCC, and understanding their underlying mechanisms of action. SCC25 cells are a useful model for *in vitro* studies of head and neck SCC, because, although they are derived from a primary tumor (17), they retain a relatively differentiated phenotype that is more representative of the targets of chemoprevention. Both $1,25(OH)_2D_3$ and EB1089 completely inhibited SCC25 proliferation at nanomolar concentrations (Fig. 1). In other studies, we have observed similar growth inhibitory effects in a murine model of head and neck SCC (unpublished results), indicating that the potent antiproliferative activity of $1,25(OH)_2D_3$ and EB1089 in SCC is not restricted to SCC25 cells.

We have screened gene arrays to identify targets of $1,25(OH)_2D_3$ and EB1089. The amphiregulin gene was identified as a putative target gene in screens with both ligands. Subsequent studies showed that amphiregulin expression is directly regulated by $1,25(OH)_2D_3$ in SCC25 cells (Figs. 2 and 3). The retinoid 13-*cis* RA, which also inhibits growth of SCC25 cells (Fig. 1), did not induce expression of amphiregulin (Fig. 2). Similarly, the RXR-specific retinoid LG1069 had no effect on amphiregulin expression, indicating that its induction is $1,25(OH)_2D_3$-specific. However, $1,25(OH)_2D_3$-dependent induction of amphiregulin expression was partially inhibited by 13-*cis* RA (Fig. 2C). This was part of a broader phenomenon of reciprocal inhibition of target gene expression observed in SCC25 cells. Thus, while $1,25(OH)_2D_3$ and 13-*cis* RA are growth inhibitory in SCC25 cells alone or in combination (Fig. 1), there is considerable cross-talk between the signals induced by the two ligands. Given that $1,25(OH)_2D_3$ and 13-*cis* RA function by signaling through nuclear receptors, the reciprocal inhibition observed may be due to competition for limiting downstream factors.

Amphiregulin was identified in conditioned medium of MCF-7 breast cancer cells as a factor induced by

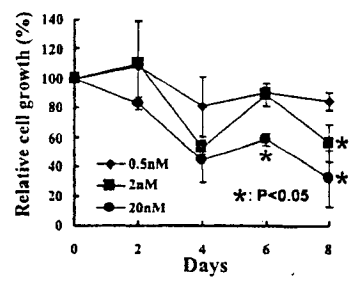

FIG. 4. Amphiregulin inhibits growth of SCC25 cells in culture in a dose-dependent manner. Cells in triplicate wells were treated with the concentrations of amphiregulin indicated. Media were changed and fresh amphiregulin was added daily. Statistical analysis was performed with a two-tailed Student's *t* test.

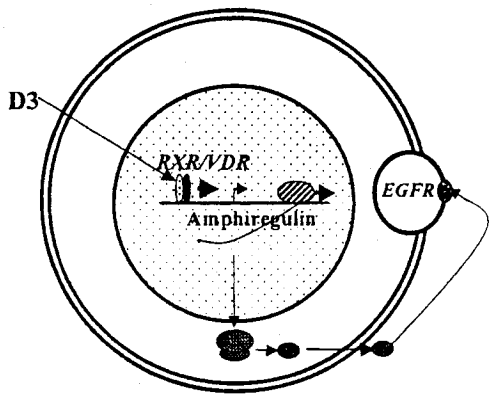

FIG. 5. Schema presenting a potential model for $1,25(OH)_2D_3$-dependent signaling through the nuclear vitamin D_3 receptor (VDR) depicted as a heterodimer with its RXR partner. Ligand-dependent transactivation through the VDR leads to expression of amphiregulin and its action as an autocrine inhibitor of SCC25 cell proliferation.

phorbol 12-myristate 13-acetate, which is growth inhibitory (23). The growth regulatory characteristics of amphiregulin are complex and difficult to predict. Generally, amphiregulin stimulates proliferation of cultures of primary and immortalized cells. However, it can exhibit either mitogenic or growth inhibitory effects on transformed cells in culture (24–27). Amphiregulin has moderate affinity for the epidermal growth factor receptor, and previous experiments have shown that it modulates cell proliferation at concentrations above 10 nM (26). We observed a dose-dependent inhibition of SCC25 cell growth, and found that 2 and 20 nM amphiregulin significantly inhibited proliferation (Fig. 4). Our experiments also showed that amphiregulin gene expression is induced by $1,25(OH)_2D_3$ in estrogen receptor-positive and negative breast cancer cells, but not in LNCaP prostate cancer cells (Fig. 3), indicating that the effect is cell-specific, but not limited to SCC. Taken together, our results show that induction of amphiregulin expression represents a primary response to $1,25(OH)_2D_3$ signaling, and that amphiregulin may act as a component of the antiproliferative response to $1,25(OH)_2D_3$ and its analogs by inhibiting SCC25 cell proliferation in an autocrine or paracrine manner (Fig. 5).

In addition to the mechanism depicted in Fig. 5, studies from other laboratories have raised the possibility that growth factors such as amphiregulin may affect signaling by nuclear receptors including VDR/RXRs in other ways. Ligand-bound nuclear receptors modulate transcription of target genes by recruiting a series of factors known collectively as coregulators (28, 29). Of these, the p160 family, which includes SRC1, TIF2/GRIP1, and AIB1/ACTR/RAC3/TRAM1/pCIP, is the best characterized. Recent experiments have shown that signaling through epidermal growth factor receptors can induce MAP kinase-dependent phosphorylation of SRC1 and AIB1 (30, 31), which enhances their activity. It can be speculated, then, that amphiregulin signaling through the epidermal growth factor receptor may increase the activity of p160 family members expressed in target cells and lead to enhanced nuclear receptor function.

In closing, it should be noted that we have identified several $1,25(OH)_2D_3$ target genes in SCC25 cells and MCF-7 breast cancer cells (unpublished results), which suggest that $1,25(OH)_2D_3$ acts to modulate the function of several genes that control cell proliferation, as well as the function of adhesion proteins. Thus, while our results are consistent with induction of amphiregulin expression being a component of the antiproliferative response to $1,25(OH)_2D_3$ and its analogs, it is likely that these compounds exert their effects by modulating multiple facets of cell function.

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Inhibition of F-Box Protein $p45^{SKP2}$ Expression and Stabilization of Cyclin-Dependent Kinase Inhibitor $p27^{KIP1}$ in Vitamin D Analog-Treated Cancer Cells

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Treatment of cancer cells with 1,25-dihydroxyvitamin D3 [$1,25(OH)_2D_3$] or its analogs induces growth arrest and expression of the cyclin-dependent kinase inhibitor $p27^{KIP1}$. Although $1,25(OH)_2D_3$ transiently enhances $p27^{kip1}$ gene transcription in some cells, its effects on $p27^{KIP1}$ protein levels are generally more gradual and sustained. This suggests that $1,25(OH)_2D_3$ treatment may be stabilizing $p27^{KIP1}$ protein, which is sensitive to modification by the SCF^{SKP2} protein ubiquitin ligase and proteosomal degradation. Here, we show that treatment of AT-84 head and neck squamous carcinoma cells with the $1,25(OH)_2D_3$ analog EB1089 increases $p27^{KIP1}$ protein levels without significantly affecting expression of its mRNA. EB1089 treatment repressed expression of mRNAs encoding the F-box protein $p45^{SKP2}$, a marker of poor head and neck cancer prognosis, and the cyclin kinase subunit CKS1, which is essential for targeting $p45^{SKP2}$ to $p27^{KIP1}$. This coincided with a reduction of total $p45^{SKP2}$ protein, and $p45^{SKP2}$ associated with $p27^{KIP1}$. Consistent with these findings, turnover of $p27^{KIP1}$ protein was strongly inhibited in the presence of EB1089. A similar reduction in $p45^{SKP2}$ expression and stabilization of $p27^{KIP1}$ protein was observed in $1,25(OH)_2D_3$-sensitive UF-1 promyelocytic leukemia cells, which also respond by transiently increasing $p27^{kip1}$ gene transcription. Our results reveal that $1,25(OH)_2D_3$ analogs increase levels of $p27^{KIP1}$ in different cell types by inhibiting expression of SCF^{SKP2} subunits and reducing turnover of $p27^{KIP1}$ protein. (*Endocrinology* 144: 749–753, 2003)

SIGNAL TRANSDUCTION BY the active form of vitamin D_3, $1\alpha,25$-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], primarily controls calcium transport in the intestinal epithelia and modulates bone resorption (1). However, $1,25(OH)_2D_3$ and its analogs also stimulate cell differentiation and inhibit proliferation in a number of cellular and animal models, and its analogs are of interest because of their broad potential as anticancer agents (1). We are studying the antiproliferative and potential chemopreventive actions of $1,25(OH)_2D_3$ analogs in head and neck squamous carcinoma (HNSCC; Refs. 2–4), where development of second primary carcinomas after treatment of an initial malignancy is a major clinical problem (5).

$1,25(OH)_2D_3$ and its analogs regulate the expression cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$. $1,25(OH)_2D_3$ affects $p21^{waf1/cip1}$ expression strongly in a cell specific matter (*e.g.* Refs. 2, 6–8), whereas its induction of $p27^{kip1}$ is generally more consistent. For example, $p27^{kip1}$ was induced in HNSCC lines under conditions where $p21^{waf1/cip1}$ was repressed (2, 8). In addition, the rapid and transient induction of $p27^{kip1}$ transcripts in monocytic cells was accompanied by a delayed and more sustained increase in $p27^{KIP1}$ protein (7, 9). $p27^{KIP1}$ protein is a substrate for protein ubiquitin ligase (E3) SCF^{SKP2} (10–12). The $p45^{SKP2}$ subunit of SCF^{SKP2} binds directly to the cyclin kinase subunit CKS1 (10–13), which directs $p45^{SKP2}$ to $p27^{KIP1}$. Significantly, $CKS^{-/-}$ mice express elevated levels of $p27^{KIP1}$ (13).

We were interested in determining whether $p27^{KIP1}$ protein turnover was affected in cells treated with $1,25(OH)_2D_3$ analogs. Here, we show that $p27^{kip1}$ mRNA is unchanged in EB1089-treated AT-84 cells. Rather, treatment represses expression of $p45^{skp2}$ and cks1 mRNAs, leading to reduced association of $p45^{SKP2}$ protein with $p27^{KIP1}$ and strongly reduced $p27^{KIP1}$ protein turnover. A similar reduction in $p45^{SKP2}$ expression and stabilization of $p27^{KIP1}$ protein was observed in promyelocytic UF-1 cells. Taken together, the results indicate that $1,25(OH)_2D_3$ analogs enhance $p27^{KIP1}$ expression in diverse cell types by reducing its turnover.

Materials and Methods

Tissue culture

Mouse AT-84 HNSCC cells and human promyelocytic UF-1 cells were cultured as described (2, 9). Effects of EB1089 were analyzed by seeding cells in 100-mm dishes at 60% confluence in 10 ml of medium containing charcoal-stripped serum. Media were changed after 24 h to charcoal-stripped medium containing 0.1 μM EB1089. Media were changed every 48 h, and fresh ligand was added.

RT-PCR

RT-PCR analysis was performed essential as described (4). For analysis of $p45^{skp2}$, $p27^{kip1}$, and cks1 mRNA expression, 1 μl of reverse transcription reactions was amplified by PCR as follows: 30-sec denaturation at 94 C, 45-sec elongation at 72 C, and 30-sec annealing starting at 60 C, down 1 C per cycle to 55 C, and continuing 20 cycles amplification (94 C for 30 sec, 57.5 C for 30 sec, 72 C for 45 sec). cDNAs for $p45^{skp2}$, $p27^{kip1}$, and cks1 were amplified using 5′ primer 5′-CCTAAGCAGCTGTCCCAGAC-3′, 3′ primer 5′-GTGTCAGTCGGCATTTGATG-3′

Abbreviations: CKS, Cyclin kinase subunit; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNSCC, head and neck squamous carcinoma; $1,25(OH)_2D_3$, $1\alpha,25$-dihydroxyvitamin D_3; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

for $p45^{skp2}$, 5′ primer 5′-GGATGGACGCCAGACAAG-3′, 3′ primer 5′-GGGGAACCGTCTGAAACATT-3′ for $p27^{kip1}$, and 5′ primer 5′-TTGGACAAATACGACGACGA-3′, 3′ primer 5′-CTTTGTTTTCTC-GGGTAGTGG-3′ for cks1. For amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1 μl of reverse transcription reaction was subjected to 18 cycles amplification (95 C for 30 sec, 56 C for 1 min, 72 C for 25 sec) using 5′ primer 5′-GGTGAAGGTCGGTGTCAACG-3′, and 3′ primer 5′-CAAAGTTGTCATGGATGACC-3′. All of the above reactions were performed in 25 μl of 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 9.0) using 2.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, [illegible]e d'Urfé, Québec, Canada). PCRs were loaded on 2% agarose gel, transferred for Southern blotting to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech), and fixed by UV cross-linker. The membrane was soaked in 3× standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 50 C, and prehybridized at 42 C in 50 mM-phosphate buffer (pH 6.5), 5× SSC, 10% Denhardt's solution containing 250 μg/ml sheared, and denatured salmon sperm DNA. Hybridization was carried out in the same solution by the addition of ^{32}P-end-labeled oligonucleotides 5′-GAGCTGAACCTCTCCTGGTG-3′ for $p45^{skp2}$, 5′-CAAATGCCTGACTCGTCAGA-3′ for $p27^{kip1}$, 5′-TCA-CATCTTGCTGTTCCGG-3′ for cks1 and 5′-TCTTCACCACCATG-GAGAAG-3′ for GAPDH. Following hybridization, the membrane was washed four times in 2× SSC and 0.2% SDS for 5 min, three times in 0.1× SSC. and 0.2% SDS for 30 min at 50 C, dried, and autoradiographed. All experiments were repeated at least three times.

Immunoprecipitation and Western blot analysis

Cells were rinsed with PBS, harvested by scraping in 1 ml of PBS, and centrifuged (10,000 rpm, 5 min) at 4 C. Pellets were resuspended in 100 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0; 60 mM KCl; 1 mM EDTA; 1 mM dithiothreitol; 0.5% Nonidet P-40) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and incubated on ice for 15 min. Lysates were centrifuged at 4 C (10,000 rpm, 10 min), and supernatants were recovered. Total protein was quantitated using the Bio-Rad Laboratories, Inc. (Richmond, CA) protein assay reagent. Lysates containing 100 μg of total proteins normalized to a 1-ml volume in lysis buffer were precleared by incubation with 30 μl of 50% slurry protein A-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 45 min on a rotator at 4 C. Protein complexes were immunoprecipitated from the precleared lysates by addition of either 1 μg of rabbit anti-$p27^{kip1}$ (Santa Cruz Biotechnology, Inc.) or anti-$p45^{skp2}$ (Zymed Laboratories, Inc., South San Francisco, CA) for 1 h at 4 C with rotation, followed by addition of 30 μl of 50% slurry protein A-agarose for 1 h on a rotator at 4 C. After three washes with lysis buffer, pelleted beads were boiled for 3 min in 2× SDS-PAGE loading buffer. Immunoprecipitates and lysates (30 μg) were resolved on 20% SDS-PAGE and analyzed by Western blotting with rabbit anti-$p45^{skp2}$ (0.5 μg/ml) (Zymed Laboratories, Inc.) and rabbit anti-p27 (0.4μg/ml), and secondary antibody goat antirabbit IgG horseradish peroxidase conjugate (0.04 μg/ml) (Santa Cruz Biotechnology, Inc.). The reaction was developed by the chemiluminescence method (NEN Life Science Products, Beverly, MA).

Metabolic labeling experiments

Cells treated with 10^{-7} M EB1089 or dimethylsulfoxide (DMSO) vehicle for 48 h were rinsed with methionine-free RPMI 1640, labeled with 5 ml of 50 μCi/ml [^{35}S]methionine for 3 h, washed with medium containing charcoal-stripped serum and incubated in medium containing DMSO or EB1089. Cells were collected at 0, 1, 2, 3, 6, and 8 h and lysed with lysis buffer. One hundred micrograms of total protein normalized to a 1-ml volume in lysis buffer were precleared by incubation with 30 μl of 50% slurry protein A-agarose (Santa Cruz Biotechnology, Inc.) for 45 min on a rotator at 4 C. Proteins were immunoprecipitated from the precleared lysates by addition of 1 μg of rabbit anti-p27 (Santa Cruz Biotechnology, Inc.) for 1 h at 4 C with rotation, followed by 30 μl of 50% slurry protein A-agarose for 1 h on a rotator at 4 C. After three washes with lysis buffer, pelleted beads were boiled for 3 min in 2× SDS-PAGE loading buffer, resolved in 20% SDS-polyacrylamide gels and autoradiographed. Signals were quantified using Kodak (Rochester, NY) digital science 1D Image Analysis software.

Results

Treatment of AT-84 cells with $1,25(OH)_2D_3$ or EB1089 induces G0/G1 arrest and is accompanied by up-regulation of $p27^{KIP1}$ protein (2). We probed further this regulation by RT-PCR analysis of $p27^{kip1}$ transcripts in EB1089-treated AT-84 cells. Strikingly, EB1089 treatment did not substantially alter $p27^{kip1}$ transcript levels over a 48-h period (Fig. 1A), in contrast to results obtained in other cell lines where a strong but transient induction was observed (6, 7, 9). To investigate the possibility that mechanisms regulating the turnover of $p27^{KIP1}$ protein may be affected in EB1089-treated AT-84 cells, we analyzed the expression of transcripts encoding the F-box protein $p45^{SKP2}$ and the cyclin kinase subunit CKS1. RT-PCR analyses showed that expression of both transcripts was strongly and rapidly (<24 h) repressed in treated cells (Fig. 1, B and C). These results were consistent with those obtained by Northern blotting of RNA from EB1089-treated AT-84 cells (data not shown). In agreement with these and our previous studies (2), treatment with EB1089 for 72 h led to a gradual accumulation of $p27^{KIP1}$ protein in AT-84 cells (Fig. 2A). Significant changes in $p27^{KIP1}$

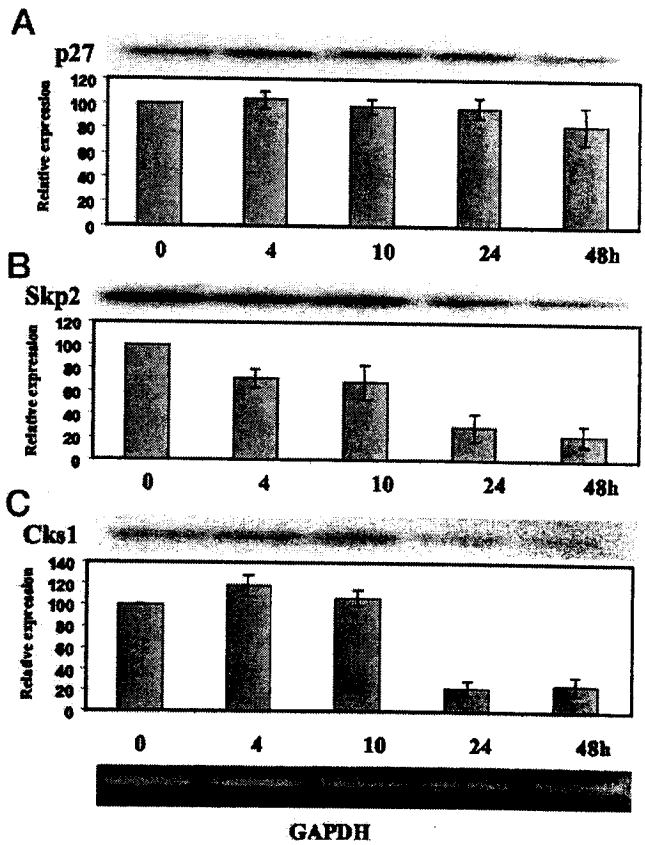

FIG. 1. Effects of EB1089 treatment on expression of $p27^{kip1}$, $p45^{skp2}$, and cks1 mRNA levels. AT-84 cells in culture were treated with EB1089 for the times indicated, and expression of transcripts encoding $p27^{KIP1}$ (A), $p45^{SKP2}$ (B), and CKS1 (C) was analyzed by RT-PCR followed by Southern blotting with radiolabeled internal oligonucleotides. Southern blots of typical experiments are shown and the results of three experiments are presented in histograms. A control for GAPDH expression is shown at the *bottom*.

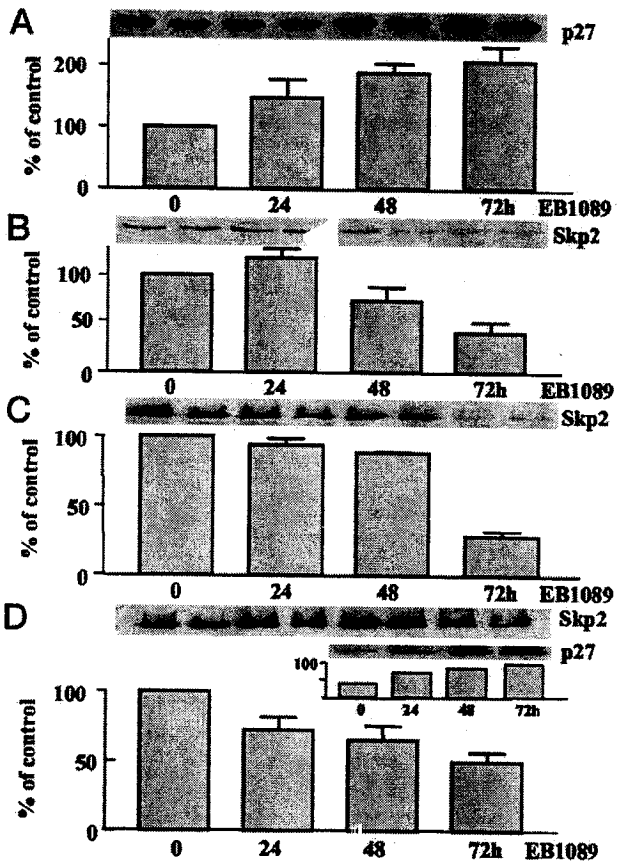

FIG. 2. Effects of EB1089 treatment on $p27^{KIP1}$ protein and its association with $p45^{SKP2}$. A and B, Extracts of EB1089-treated AT-84 cells were immunoblotted for expression of $p27^{KIP1}$or $p45^{SKP2}$. C, Cell extracts were immunoprecipitated with an anti-$p45^{SKP2}$ antibody and immunoprecipitates were probed for $p45^{SKP2}$. D, Cell extracts were immunoprecipitated with an anti-$p27^{KIP1}$ antibody and immunoprecipitates were probed for $p27^{KIP1}$ (*inset*) or $p45^{SKP2}$. Note that in A–D, Western blots show duplicate lanes corresponding to each time point. Histograms show the results of triplicate experiments.

protein were observed after 24 h and levels continued to increase through 72 h. No change in $p27^{KIP1}$ levels was observed in control experiments with vehicle-treated cells (data not shown). The accumulation of $p27^{KIP1}$ over 72 h coincided with a progressive reduction in $p45^{SKP2}$ protein over the same period as assessed by direct Western blotting (Fig. 2B) or immunoprecipitation of $p45^{SKP2}$ following by Western blotting (Fig. 2C).

We were unable to detect by Western analysis expression of the SCF^{SKP} ubiquitin ligase subunit CKS1, which is essential for the interaction of $p45^{SKP2}$ with $p27^{KIP1}$ (10–13). Therefore, we analyzed by coimmunoprecipitation the effects of EB1089 treatment on $p45^{SKP2}$ associated with $p27^{KIP1}$. Extracts of cells treated with EB1089 over 72 h were immunoprecipitated with an anti-$p27^{KIP1}$ antibody, and immunoprecipitates were analyzed for expression of $p27^{KIP1}$ and $p45^{SKP2}$ (Fig. 2D). Although immunoprecipitated $p27^{KIP1}$ increased in EB1089-treated cells (*inset*), 45^{SKP2} protein associated with $p27^{KIP1}$ fell 2-fold. Levels of coimmunoprecipitated $p45^{SKP2}$ were unaffected in a control experiment with DMSO-treated cells (data not shown). These observations indicate that a substantially reduced proportion of $p27^{KIP1}$ was complexed with $p45^{SKP2}$ in EB1089-treated cells. EB1089 treatment also led to a similar reduction in $p45^{SKP2}$ associated with $p27^{KIP1}$ in coimmunoprecipitation experiments performed with extracts of the $1,25(OH)_2D_3$-sensitive myelomonocytic cell line U937 (data not shown).

The reduced association of $p45^{SKP2}$ with $p27^{KIP1}$ suggested that $p27^{KIP1}$ was being turned over less rapidly in EB1089-treated cells. The effects of EB1089 on $p27^{KIP1}$ turnover in AT-84 cells were analyzed in metabolic labeling experiments (Fig. 3). Cells were treated with EB1089 or vehicle for 48 h, labeled with ^{35}S-methionine, and chased with serum for the times indicated (Fig. 3). Although radiolabeled $p27^{KIP1}$ immunoprecipitated from control cells diminished during the 8-h chase, no $p27^{KIP1}$ turnover was observed in immunoprecipitates of EB1089-treated cells. Taken together, our results indicate that EB1089 up-regulates $p27^{KIP1}$ by inhibiting of expression of the $p45^{SKP2}$ subunit of the SCF^{SKP} ubiquitin ligase and reducing turnover of $p27^{KIP1}$ protein.

We were interested in determining whether EB1089 treatment stabilized $p27^{KIP1}$ protein in cells that also respond by transiently increasing $p27^{kip1}$ gene transcription. Previous studies have shown that UF-1 promyelocytic leukemia cells respond to $1,25(OH)_2D_3$ by transiently inducing $p27^{kip1}$ mRNA, followed by a more sustained increase in $p27^{KIP1}$ protein (9). Consistent with these findings, we observed a 2.9-fold increase in $p27^{KIP1}$ protein in UF-1 cells treated for 72 h with EB1089 (Fig. 3C). Significantly, this increase was accompanied by a 50% decrease in $p45^{SKP2}$ levels over the same period (Fig. 3C). Moreover, $p27^{KIP1}$ protein turnover was decreased in EB1089-treated UF-1 cells (Fig. 3, D and E). Taken together, the above results indicate that EB1089 treatment enhances $p27^{KIP1}$ expression in widely different cell types by reducing its turnover.

Discussion

$P27^{KIP1}$ is a key regulator of the G1/S checkpoint (14), and several studies have shown that its up-regulation underlies the antiproliferative effects of $1,25(OH)_2D_3$ and its analogs (2, 6–9). Our results indicate that $1,25(OH)_2D_3$ analogs can act by two cell type-dependent mechanisms to enhance expression of $p27^{KIP1}$; induction of expression of its gene, and inhibition of $p27^{KIP1}$ protein turnover. In contrast to results obtained in other cell lines (6, 7, 9), EB1089 had little effect on $p27^{kip1}$ mRNA levels in AT-84 cells over a 48-h period. The varying effects of $1,25(OH)_2D_3$ analogs on $p27^{kip1}$ gene transcription may due in part to the fact that induction of expression is dependent on an Sp1 element and a CCAAT box in the $p27^{kip1}$ promoter and not a vitamin D response element (15), suggesting that these sites function cell specifically.

In spite of the lack of response at the mRNA level, $p27^{KIP1}$ protein increased steadily over 72 h in EB1089-treated AT-84 cells, consistent with studies in other cell lines (6, 7, 9). Metabolic labeling experiments indicated that the sustained increase was due to inhibition of $p27^{KIP1}$ degradation, which was consistent with the observed reduction in expression of $p45^{skp2}$ transcripts and protein. The inhibition of $p45^{skp2}$ gene expression by EB1089 was rapid, occurring within 24 h,

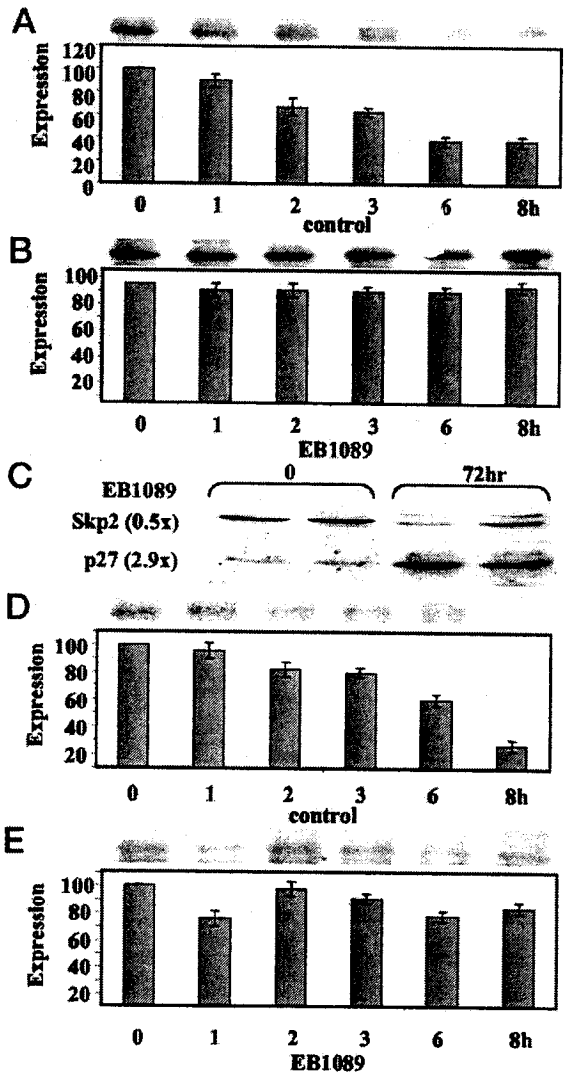

FIG. 3. EB1089 treatment stabilizes $p27^{KIP1}$ protein. Metabolic labeling experiments performed in vehicle- (A) and EB1089-treated AT-84 cells (B) are presented. Cells were labeled for 3 h with ^{35}S-methionine after 48 h of treatment with either vehicle or EB1089, and chased as indicated. Extracts were immunoprecipitated with an anti-$p27^{KIP1}$ antibody and immunoprecipitates were analyzed by gel electrophoresis. Histograms present the results of triplicate experiments. C, Western analysis showing that EB1089 treatment enhances $p27^{KIP1}$ protein expression and down-regulates expression of $p45^{SKP2}$ in human promyelocytic UF-1 cells. Metabolic labeling experiments performed as above in vehicle- (D) and EB1089-treated UF-1 cells (E) reveal that EB1089 treatment reduces $p27^{KIP1}$ turnover in UF-1 cells (see *Materials and Methods* for details).

whereas the drop in $p45^{SKP2}$ protein levels continued over a 72-h period. This reduced the total amount of $p45^{SKP2}$ associated with $p27^{KIP1}$ by coimmunoprecipitation even though $p27^{KIP1}$ protein levels were increasing. Taken together, these results indicate that the increase in levels of $p27^{KIP1}$ protein in EB1089-treated AT-84 calls arose from protein stabilization, and not from increased gene expression. A similar stabilization of $p27^{KIP1}$ protein was also observed in UF-1 promyelocytic leukemia cells.

The effect of EB1089 observed on $p27^{KIP1}$ protein expression in AT-84 cells (2-fold) is not dramatic. However, it is important to stress that progression through G1 into S phase is regulated by threshold levels of key regulators (14), and $p27^{KIP1}$ levels control the activity of the CDK2/cyclin E complex, whose function is critical for entry into S phase. Subtle changes in $p27^{KIP1}$ expression can have profound effects on cell function. Indeed, $p27^{KIP1}$ heterozygous mice are predisposed to tumors in a number of tissues after γ irradiation or exposure to carcinogens (16). Therefore, it is likely that the 2-fold increase observed in $p27^{KIP1}$ protein levels in treated AT-84 cells is critical for the antiproliferative effects of EB1089.

Just as reduced levels of $p27^{KIP1}$ are associated with cancer development, overexpression of $p45^{SKP2}$ is oncogenic. For example, elevated expression of $p45^{SKP2}$ was found in 49% of oral squamous cell carcinomas and was correlated with a poor prognosis of affected patients (17). Another study found an inverse correlation between $p45^{SKP2}$ and of $p27^{KIP1}$ levels in oral cancers (18) and also showed that of $p45^{SKP2}$ could cooperate with $H\text{-}Ras^{G12V}$ to transform primary fibroblasts. Similarly, $p45^{SKP2}$ cooperated with N-Ras in induction of T cell lymphomas in a mouse transgenic model, leading to significantly reduced survival times (19). Taken together, these studies strongly suggest that the down-regulation of $p45^{SKP2}$ and consequent sustained up-regulation of $p27^{KIP1}$ are key elements in the antiproliferative and anticancer actions of vitamin D analogs.

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