CHAPTER

94

Overview of Vitamin D Actions in Cancer

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OUTLINE

INTRODUCTION

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] is the most potent metabolite of vitamin D_3 and is an important regulator of calcium homeostasis and bone metabolism via actions in the intestine, bone, kidney, and parathyroid glands. $1,25(OH)_2D_3$ exerts its effects via an intracellular receptor that is a member of the steroid hormone receptor family (see chapters in Section II on Mechanism of Action). Throughout the last few decades it has become evident that the vitamin D receptor (VDR) is not limited to cells and tissues involved in regulation of calcium and bone metabolism but is also present in a wide variety of other cells and tissues including cancer cells of various origins. This has led to a vast series of studies on the role of vitamin D in tumor cell growth regulation, treatment of cancer and development of potent synthetic vitamin D analogs. Various specialized chapters will discuss in detail the effect of vitamin D on specific cancers (see chapters in Section X) and the development of analogs (see chapters in Section IX). In this chapter our goal is to set the stage by providing an overview of the history and current state of knowledge of the field. We will address several areas: recent developments in studies of vitamin D and cancer, regulation of tumor cells, possible mechanisms, and clinical applications. Since the field has become so vast of course we could not cite all of the relevant papers, and the reader is referred to the specialized chapters on the various cancers that follow this chapter for more detail.

VITAMIN D AND CANCER

Vitamin D Receptor

As exemplified in Table 94.1, the VDR has been demonstrated in a broad range of tumors and malignant cell types. VDR level is increased in ovarian carcinoma compared to

TABLE 94.1 Vitamin D Receptor in Tumors and Malignant Cell Types

Basal Cell Carcinoma	Myeloid Leukemia
Breast Carcinoma	Multiple Myeloma
Bladder Cancer	Osteogenic Sarcoma
Cervical Carcinoma	Ovarian Carcinoma
Colonic Adenocarcinoma	Neuroblastoma
Colorectal Carcinoma	Non-Hodgkin's Lymphoma
Gall Bladder Carcinoma	Pancreatic Carcinoma
Glioblastoma	Parathyroid Adenoma
Kaposi Sarcoma	Pituitary Adenoma
Lung Carcinoma	Prostate Carcinoma
Lymphocytic Leukemia	Renal cell Carcinoma
Malignant B-Cell Progenitors	Squamous cell Carcinoma
Malignant Melanoma	Transitional cell Bladder Carcinoma
Medullary Thyroid Carcinoma	Uterine Carcinosarcoma

normal ovarian tissue [1]. For colon and breast cancer cells an inverse relationship between VDR level and degree of differentiation has been described by some investigators [2,3]. For colorectal cancer it was shown that VDR expression is associated with the degree of tumor differentiation [4] and with a more favorable prognosis [5]. Accordingly, VDR expression in colon tumor stromal fibroblasts predicted a favorable clinical outcome [6]. This is an important aspect of the anticancer actions of vitamin D: interacting with surrounding stromal cells and not only with the cancer cells. In pancreatic cancer, the VDR regulates transcription of pancreatic stellate cells, which results in stromal remodeling that results in reduced tumor volume and increased chemotherapeutic response [7]. In hepatocellular carcinoma, p62/SQSTM1 protein was found to acts as a negative regulator of liver inflammation and fibrosis through VDR signaling in hepatic stellate cells [8].

A VDR immunoreactivity score showed an increase in VDR in breast carcinoma specimens compared to normal breast tissue but no clear relation with proliferative status could be assessed [9]. A later study by the same group showed that VDR expression is not a prognostic factor for breast cancer but the strong VDR immunoreactivity in the breast cancer specimens supports the evidence that it may be a target for intervention [10]. Also in other studies no associations between VDR concentration and clinical and biochemical parameters of breast cancer were found [11–13]. These outcomes could be the result of the fact that in clinical human breast tumor samples, variable expression of the VDR was found in different cohorts [14].

Albeit that the association studies on VDR expression and predictive and/or prognostic characteristics for cancer are so far not conclusive, depending also on other features like VDR functionality or 25(OH)D levels, the widespread distribution of the VDR in malignant cells indicates that regulation of cancer cell function might be a new target in the action of $1,25(OH)_2D_3$ and provides a biological basis for the epidemiological observations discussed below.

An interesting observation has put the VDR in relation to cancer in another perspective. It was shown that VDR can function as a receptor for the secondary bile acid lithocholic acid (Mangelsdorf third edition Vitamin D). This compound is hepatotoxic and a potential enteric carcinogen. Interestingly, both binding of lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [15,16] (see also Chapter 84). It is postulated that vitamin D and lithocholic acid, by binding to the VDR, activate a feed-forward catabolic pathway that increases CYP3A expression leading to detoxification of carcinogenic bile acids.

A relationship between the presence of VDR and carcinogenesis was also shown for the skin. Absence of VDR increased the sensitivity for chemically induced tumorigenesis [17]. Moreover, in mice the vitamin D analogs EB1089 prevented β-catenin-induced trichofolliculomas, while low levels of VDR associated with the induction by β-catenin of infiltrative basal cell carcinomas [18].

The β-catenin as well as the Hedgehog signaling and the recently found long noncoding RNA pathways underlie the protective role of the VDR as a tumor suppressor in the skin [19,20]. In addition, regulation of c-MYC by the VDR may lie at the basis for cancer preventive actions [21]. In stroma from pancreatic tumors, the VDR is a master transcriptional regulator of the conversion to quiescent cells after calcipotriol treatment leading to reduced tumor volume and increase in survival compared to chemotherapy [7].

Although cellular effects of $1,25(OH)_2D_3$ traditionally have been attributed to activation of the nuclear VDR, over the years research has been performed to identify a membrane $1,25(OH)_2D_3$ receptor (see also Chapter 16 (vol. 1 of this book)). As discussed in Chapter 16 (vol. 1 of this book), the best evidence suggests that this rapid acting membrane receptor is related to the VDR.

Epidemiology

The first to document an association of cancer mortality with sun exposure and latitude was Hoffman in 1915 [22]. Later studies in 1980 by Garland et al., provided additional data showing that death rates from colon cancer tended to increase with increasing latitude and decreasing sunlight [23]. The sunlight/ecological concept is discussed in Chapters 61 and 95. Later more direct evidence about a correlation between vitamin D concentration and colon cancer came from the inverse relationship between levels of serum 25-hydroxyvitamin 25(OH)D and the incidence of colon cancer [24,25]. In a metaanalysis Gorham et al. estimated that an increase of 84 nmol/L (33 ng/mL) in serum 25(OH)D level would lead to a 50% reduction in the incidence of colon cancer [26]. A study of National Health and Nutrition Examination Survey III (NHANES III) data also found an association between 25(OH)D concentration and colorectal cancer mortality. Individuals with a 25(OH)D level over 80 nmol/L (32 ng/mL) had a 75% lower risk of death from colorectal cancer than those with lower levels of 25(OH)D. A concentration over 95 nmol/L correlated with a 55% reduction in colon cancer risk compared to those with a level below 40 nmol/L [27]. Several studies confirmed that a higher concentration of vitamin D was associated with lower colon cancer incidence and patients have a better overall survival [28].

From the NHANES III study it was reported that women with a serum concentration of 25(OH)D more than 62 nmol/L had a 75% decrease in mortality due to breast cancer [27]. From two other studies the authors concluded that there was a 58% lower risk of breast cancer in women with 25(OH)D concentration more than 95 nmol/L compared to women with levels lower than 37.5 nmol/L [29,30]. In a metaanalyses 1750 women were stratified into 5 groups of 25(OH)D concentrations ranging from high to low and this showed a clear dose-response association [31]. The highest breast cancer rates were found in the group with the lowest 25(OH)D concentration (<32 nmol/L), while the cancer rates were lower at higher levels (>130 nmol/L). Later studies confirmed the relationship between higher 25(OH)D levels and a lower risk for breast cancer progression and mortality [32]. A large Finnish epidemiological study showed an association of low serum 25(OH)D with prostate cancer [33,34]. The incidence of prostate cancer was twice as high in men with a 25(OH)D concentration below 70 nmol/L and $1,25(OH)_2D_3$ levels below 77 pmol/L.

A full discussion of the epidemiologic data linking vitamin D and cancer can be found in Chapter 95. It is strongly suggestive that avoiding vitamin D deficiency may be a way to reduce cancer risk and progression, while results of ongoing clinical trials are still awaited [35].

Studies showed that the association between UVB irradiance and prostate cancer incidence depends on the season of irradiance [36]. The relationship between sunlight exposure and cancer, especially with respect to vitamin D, had been carefully reviewed earlier by Studzinski and Moore [37]. The dual relationship between sunlight and cancer is of interest and remains the subject of many studies [38–40]. A relation between skin type and prostate cancer has been described [41–43] and an article discussing the skin, sunlight, vitamin D and cancer from an evolutionary perspective has been published [44]. Grant et al. estimated that between 50,000 and 63,000 Americans and between 19,000 and 25,000 adults from the United Kingdom die every year from cancer due to vitamin D deficiency [45]. An analysis of the economic burden due to vitamin D insufficiency from inadequate exposure to solar UVB, diet and supplements was $40–56 billion in 2004 versus an economic burden for excess UV irradiation of $6–7 billion [46]. In Multiple myeloma, lower 25(OH)D levels were associated with higher plasma cell number in the bone marrow and a high incidence of vitamin D deficiency was found in myeloma patients [47].

In addition, the relationship between cancer, diet, and calcium intake and vitamin D has been addressed in several studies [48–50]. A study on intake of micronutrients suggested that vitamin D and calcium might interact with antioxidants like vitamin C and E in reducing colorectal cancer risk [51]. It is clear that sunlight exposure, vitamin D intake, and other dietary components such as calcium and fat should be considered as possibly interacting with one another when the relationship between vitamin D and cancer risk is assessed. The data on VDR as bile acid sensor and its postulated role in detoxification provide a direct biological basis for the relation between increased colon cancer and high-fat diets [52] and that colon cancer occurs in areas with higher prevalence of rickets [53]. In addition, mice lacking VDR have been reported to have a higher proliferation rate in the colon [54,55]. A survey of possible mutations in the VDR in osteosarcomas, several other sarcomas, nonsmall cell lung cancers, and a large number of cell lines representing many tumor types did not show that mutations or rearrangements in the VDR gene play a role in these cancers [56]. Aspects of sunlight and the epidemiology of vitamin D and calcium will be discussed in greater detail in Chapters 61 and 95.

However, data on the associations between vitamin D and cancer are not consistent. This has been observed in prostate cancer [32]. In a large prospective study by Ahn et al. the hypothesis that vitamin D is associated with decreased risk of prostate cancer was not supported; in contrast higher circulating $25(OH)D_3$ concentrations may be associated with increased risk of aggressive disease [57]. Also in other types of cancer the same association showing benefit by vitamin D was not always found. In breast cancer similar vitamin D intakes were found in breast cancer patients and control subjects [58]. Moreover, in a mouse model no relationship was found between dietary intake of a wide range of doses of calcium or vitamin D on carcinogen-induced skin tumors [59]. Also for ovarian cancer a similar discrepancy was observed. For example, Grant et al. reported a strong association between vitamin D levels, geographical latitude and ovarian cancer mortality [38,60], while more recently Toriola et al. in a case-control study with the Finnish Maternity Cohort did not find a significant association between ovarian cancer and serum $25(OH)D_3$ levels [61].

A concluding comment is that a high number, but by no means not all, observational, epidemiological, and preclinical studies suggest a protective anticancer action of vitamin D. The Cochrane review [62] warns for study bias in randomized trials due to low numbers of participants and selective groups of participants. More trials are necessary on vitamin D supplementation, involving younger participants, men/women and taking into account vitamin D status, longer treatment/higher doses and longer follow-up of all participants.

Vitamin D Receptor Gene Polymorphisms

Several polymorphisms have been identified in the VDR gene and studied in relation to various endpoints including osteoporosis and other diseases (discussed in Chapter 65). Over the last 15–20 years an increasing number of studies have examined the association of polymorphisms in the VDR and cancer. An early study showed an association between polymorphisms at the 3′ end of the VDR gene and prostate cancer [63]. This was shortly followed by a study showing an association of prostate cancer with variations in the 3′ poly-A stretch in the VDR gene [64]. Subsequently several other studies also showed associations of polymorphisms in the 3′ region of the VDR gene and prostate cancer risk [65–68] albeit other studies did not confirm this association [69–71]. For the Cdx-2 VDR promoter polymorphism an increased risk for prostate cancer was reported to be dependent on UV radiation exposure [72]. For breast cancer both the presence [73,74] and absence [75] of an association with polymorphisms in the VDR gene have been reported. Also

for colon cancer both presence [76,77] and absence [78] of an association with VDR polymorphisms have been reported. In a recent study that compared cases to unaffected sibling controls, no association between any of the VDR single nucleotide polymorphisms and risk for colorectal cancer was observed [79]. No association of VDR polymorphisms with basal cell carcinoma was reported [80]. An association with the aggressive renal cell carcinoma was found for the TaqI VDR polymorphism [81], while the FokI but not with TaqI polymorphism was associated with altered risk for malignant melanoma [82], Another study on rectal cancer reported a correlation between VDR gene polymorphisms and erbB-2/HER-2 expression [83]. It can be concluded that so far the studies searching for a link between VDR gene polymorphisms and cancer risk are far from conclusive with some studies finding a relationship to cancer risk and others failing to find one. A major reason might be the limited size of most of the studies so that they do not have the power to identify with statistical significance a small increase in risk. In the absence of a large definitive study, more association studies of VDR gene polymorphisms and specific cancers are needed, which should be followed by a metaanalysis to more definitively assess whether there is an association and if so, what the size of the effect is. In an updated metaanalysis including newer studies, an overall significant association of FokI polymorphism was found with any type of cancer [84].

In studies of VDR gene polymorphisms it also is important to take into account the potential impact of environmental factors interacting with the genetic variance. Diet, vitamin D intake and sun exposure may modify the association with cancer risk. Interaction between vitamin D and calcium intake and cancer was found in some of the VDR gene polymorphism studies [76,85–87]. They reported decreased risk of prostate cancer [85] and colorectal adenomas [86] in those with lower vitamin D levels and a particular VDR gene polymorphism. However, results of these studies are unusual in light of the fact that higher calcium and vitamin D intake are generally associated with a modestly reduced risk of colorectal neoplasia. In the study by Poynter et al. calcium and vitamin D intake derived from the food frequency questionnaire did not change their observation about the absence of an association between VDR gene variations and colorectal cancer [79]. Finally, and most importantly, it should be realized that except for the FokI translational start site polymorphism, all other polymorphisms analyzed so far are anonymous with no change in the coded protein. Thus functionality of the polymorphism or linkage with other polymorphisms that may be functional still needs to be proven. The 3′ polymorphisms have been shown to be in linkage with 3′-UTR polymorphisms but no relation with VDR mRNA stability could be demonstrated [88]. In the VDR promoter region 1a two functional polymorphism have been identified. The Cdx-2 promoter polymorphism has been reported to lead to different VDR gene expression [89,90] and the G-1521-C polymorphism to binding of different complexes in gel shift analyses [91,92]. Further detailed discussion of possible functional consequences of VDR gene polymorphisms and impact of vitamin D levels is beyond the scope of this chapter but will be addressed in Chapter 65.

Growth and Development

In addition to the epidemiological studies and demonstration of VDR in cancer cells, since the early 1980s there is also an increasing amount of cell biological data supporting a role for vitamin D as an inhibitor of cancer growth [35,93–95]. Multiple studies have shown that at elevated concentrations (10^{-9}–10^{-7} M), $1,25(OH)_2D_3$ inhibits the growth of tumor cells in vitro. It was demonstrated as early as 1981 that $1,25(OH)_2D_3$ inhibits the growth of malignant melanoma cells and stimulates the differentiation of immature mouse myeloid leukemia cells in culture [96–98]. $1,25(OH)_2D_3$ also induces differentiation of normal bone marrow cells. Immature bone marrow cells of the monocyte-macrophage lineage are believed to be the precursors of osteoclasts, and $1,25(OH)_2D_3$ induces differentiation of immature myeloid cells toward monocytes-macrophages and also stimulates the activation and fusion of some macrophages. From these results it has been postulated that $1,25(OH)_2D_3$ stimulates differentiation and fusion of osteoclast progenitors into osteoclasts [99–101]. In addition, in the intestine, $1,25(OH)_2D_3$ has important effects on cellular proliferation and differentiation [102]. Thus the differentiation inducing capacity of bone and interstitial cells, $1,25(OH)_2D_3$ may play an important role in the regulation of calcium and bone metabolism. These in vitro findings were followed by the in vivo observation that $1,25(OH)_2D_3$ prolongs the survival time of mice inoculated with myeloid leukemia cells [103]. As shown in Table 94.2, over the years $1,25(OH)_2D_3$ has been shown to have beneficial effects in several other in vivo animal models of various types of cancers [104–126]. For more detailed reviews of breast, prostate, colon and other cancers see other chapters in this section of the book.

An important aspect and limitation of the treatment of cancer with $1,25(OH)_2D_3$ was revealed by this limited set of clinical trials (See section Clinical Studies); to achieve growth inhibition, relatively higher doses of $1,25(OH)_2D_3$ are needed (confirming the in vitro data), which can cause the side effect of hypercalcemia. This has prompted the development of analogs of $1,25(OH)_2D_3$ to dissociate the antiproliferative effect from the calcemic and bone metabolism effects (see Section IX in this book). Although the precise mechanism for this dissociation of activities is not completely understood, at the moment several $1,25(OH)_2D_3$ analogs are available that seem to fulfill these criteria. In Table 94.3 the in vivo animal studies using $1,25(OH)_2D_3$ analogs on various cancer types are summarized [114,120,121,123–145] and more fully discussed in Section IX of this volume.

Clinical Studies

Only a limited number of clinical trials of vitamin D in cancer have been performed up to now, which may be attributed in part to the calcemic activity of $1,25(OH)_2D_3$. Alfacalcidol (1α-hydroxyvitamin D_3;1α-$(OH)D_3$), which is converted to $1,25(OH)_2D_3$ in vivo, caused a beneficial response in low-grade non-Hodgkin's lymphoma patients [146,147]. In addition, in a study treating patients with myelodysplasia with alfacalcidol, transient improvement in peripheral blood

TABLE 94.2 In Vivo Effects of $1,25(OH)_2D_3$ and 1α-$(OH)D_3$ in Animal Models of Cancer[a] (Partial Listing)

Tumor	Model	Effect	References
Adenocarcinoma	CAC-8 cells injected in nude mice	Reduction in tumor volume	[124]
Breast	NMU- and DMBA-induced breast cancer in rats	Tumor suppression	[110,113]
Colon	Human colon cell line implanted into nude mice; DMH-induced colon cancer in rats; APCmin mice	Tumor suppression; Reduction of the incidence of colon adenocarcinomas; decrease in polyp number and tumor load	[107,109,112,493]
Kaposi sarcoma	KS Y-1 cells implanted in nude mice	Tumor growth retardation	[122]
Leydig tumor	Leydig cell tumor implanted into rats	Tumor suppression	[114]
Liver tumor	Injection of liver carcinogen diethylnitrosamine in mice and *low* vitamin D diet	Increase in tumor growth	[370]
Lung	Implantation of Lewis lung carcinoma into mice	Reduction of the number of metastases (without suppression of primary tumor); Tumor suppression; increased antitumor immunity	[104,118,397,494]
Melanoma	Human melanoma cells implanted into nude mice	Tumor suppression	[107]
Osteosarcoma	Human osteosarcoma cells implanted into nude mice	Tumor suppression	[115]
Prostate	Dunning MAT LyLu rat prostate model; LNCaP xenografts in nude mice; PAIII tumors in Lobund-Wistar rats.	Reduction in lung metastasis; Tumor suppression	[120,121,123,125,126]
Retinoblastoma	Retinoblastoma cell line implanted into nude mice; Transgenic mice with retinoblastoma	Tumor suppression	[108,111]
Walker carcinoma	Walker carcinoma cells injected in rats	Tumor suppression	[117]
Skin	DMBA/TPA-induced skin tumors in mice Human squamous cell carcinoma cells (A431) injected in nude mice	Inhibition of tumor formation Tumor cell death	[105,106] [414]

[a]*The dosage, duration of treatment, diet, and effects on serum/urinary calcium vary among the studies.*
DMBA, 7,12-dimethylbenz[a]anthracene; *DMH*, 1,2-dimethylhydrazine dihydrochloride; *NMU*, nitrosomethylurea; *TPA*, 12-O-tetradecanoylphorbol-13-acetate.

counts were seen, however, half of the patients developed hypercalcemia [148]. Another study reported a sustained hematological response in six myelodysplasia patients treated with high doses of alfacalcidol [149]. These patients were restricted in their dietary calcium intake; nevertheless, four patients developed hypercalcemia due to increased bone resorption. With respect to treatment of cutaneous T-cell lymphoma with a combination of $1,25(OH)_2D_3$ and retinoids, contrasting results have been obtained. It has been suggested that the variability was due to differences in phenotype of the various lymphomas [150–152].

A study on early recurrent prostate cancer showed that daily treatment with $1,25(OH)_2D_3$ slowed the rise in prostate-specific antigen (PSA) [153]. Using a regime of once weekly treatment with very high-dose calcitriol in patients with rising PSA after prostatectomy was found to be safe but did not result in a significant reduction in PSA [154]. Two studies were specifically designed to examine the route and schedule of administration and calcemic response in patients with advanced malignancies [155,156]. The complicated set of trials using very high dose $1,25(OH)_2D_3$ plus taxotere in advanced prostate cancer has recently been reviewed [157]. Further discussion on clinical trials can be found in the chapters on the specific malignancies that follow.

Clinical trials using vitamin D analogs have been initiated over the last years. However, these were mostly limited clinical trials focusing on small groups of patients for whom regular treatment had failed. Only a relatively few studies have been published. The analogs calcipotriol (Daivonex/Dovonex/MC903) has been used for topical treatment of advanced breast cancer; however, several of the patients still developed hypercalcemia [158]. Studies have been carried out in advanced breast cancer [159] and pancreatic cancer [160], but the clinical results were limited. In a single case of Kaposi sarcoma and topical application of calcipotriol good success in tumor regression was reported [122]. Also the impact of inhibition of CYP24 to enhance the anticancer activity of vitamin D has been studied and a potentiation of the vitamin D effect was found as had been shown in cells work previously [161]. Data on clinical studies with vitamin D and vitamin D analogs are reviewed by Vijayakumar et al. [162,163], Feldman et al. [35], Giammanco et al. [164] and Scaranti et al. [165]. Still more randomized controlled trials are necessary to overcome some unsolved issues in previous studies.

TABLE 94.3 In Vivo Effects $1,25(OH)_2D_3$ Analogs in Animal Models for Cancer (Partial Listing)

Analogs	Model	Antitumor Effect	References
$1,25(OH)D_2$	Retinoblastoma	Tumor suppression	[143]
$1,25(OH)D_5$	Breast	Tumor suppression	[144]
CB966	Breast	Tumor suppression	[129]
CB1093	Prostate	Tumor suppression No effect on angiogenesis	[125]
DD-003	Colon	Tumor suppression	[135]
EB1089	Adenocarcinoma	Tumor suppression	[124]
EB1089	Breast	Tumor suppression	[129,132,140,413]
EB1089	Colon	Tumor suppression	[139]
EB1089	Hepatocellular carcinoma	Inhibition of tumor incidence	[495]
EB1089	Leydig cell tumor	Tumor suppression	[114]
EB1089	Prostate	Tumor suppression Reduction lung metastases No effect on angiogenesis	[121,123,125,126,141,142]
KH1060	Prostate	Tumor suppression	[126]
LG190119	Prostate	Tumor suppression	[123]
OCT	Breast	Tumor suppression	[128,133]
OCT	Breast	Tumor suppression	[130]
OCT	Breast	Tumor suppression	[133]
OCT	Colon	Decreased tumor incidence	[136]
MC903	Breast	Tumor suppression	[131]
Ro 23-7553	Prostate	Tumor suppression	[137]
Ro 23-7553	Leukemia	Increased survival	[127]
Ro 24-5531	Breast	Decreased tumor incidence	[134]
Ro 24-5531	Colon	Decreased tumor incidence	[138]
Ro-25-6760	Prostate	Tumor suppression	[120]
Ro-26-9114	Colon	decrease in polyp number and tumor load	[493]
Ro-26-9114	Prostate	Tumor suppression	[126]

CB966, 24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D_3; *CB1093*, 20-epi-22(S)-ethoxy-23yne-24a, 26a,27a-trihomo-1α,25-dihydroxyvitamin D3; *DD-003*, 22(S)-24-homo-26,26,26,27,27,27-hexafluoro-1α,22,25-trihydroxy-vitamin D_3; *EB1089*, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D3; *MC903*, 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D3; *OCT*, 22-Oxacalcitriol; *Ro 23–7553*, 1,25dihydroxy-16-ene-23-yne-vitamin D_3; *Ro 24–5531*, 1,25dihydroxy-16-ene-23-yne-26,27-hexafluorovitamin D_3; *Ro 26–9114*, 1α,25-$(OH)_2$-16-ene-19-nor-24-oxo-D_3.

Angiogenesis and Metastasis

For the tumor suppressive activity of vitamin D_3 compounds in vivo, besides growth inhibition and differentiation, two additional aspects contribute to potential benefits including: (1) effects to inhibit angiogenesis and (2) actions that inhibit invasion and metastasis. First we will discuss vitamin D and angiogenesis. Angiogenesis is an essential requirement for the growth of solid tumors. Compounds that inhibit angiogenesis might therefore contribute to antitumor therapy. Antiangiogenic drugs may lead to inhibition of tumor progression, stabilization of tumor growth, tumor regression, and prevention of metastasis. Antiangiogenic effects may play a role in the tumor suppressive activity of vitamin D_3 compounds [166]. The effect of $1,25(OH)_2D_3$ on angiogenesis may be due to inhibition of tumor cell proliferation, resulting in fewer angiogenic cells. However, inhibition of angiogenesis could also be observed when the tumor cells were treated in vitro with $1,25(OH)_2D_3$ and, after cell washing, were injected into mice [167]. Under these conditions both control and $1,25(OH)_2D_3$-treated mice were injected with similar numbers of cells. Therefore, these data indicate that $1,25(OH)_2D_3$

inhibits the release of angiogenic factors (vascular endothelium growth factor, transforming growth factor-α, basic fibroblast growth factor, epidermal growth factor, etc.) or stimulates anti-angiogenic factors. $1,25(OH)_2D_3$ treatment caused a reduction in the angiogenic signaling molecule, angiopoietin-2 in squamous cell carcinoma and radiation-induced fibrosarcoma-1 cells [168]. In retinoblastomas in mice, $1,25(OH)_2D_3$ has also been shown to reduce angiogenesis [169]. A study by Oades et al., however, showed that the $1,25(OH)_2D_3$ analogs EB1089 and CB1093 inhibited tumor growth in two prostate animal models but did not inhibit angiogenesis in a rat aorta assay [125]. Whether this implicates that vitamin D affects angiogenesis in a tumor situation and not in a nonmalignant condition is not clear. This may resemble the effects of endostatin, which inhibits pathological but not normal vascularization [170,171]. In support of this possibility is the finding that $1,25(OH)_2D_3$ and its analogs EB1089, Ro-25-6760, and ILX23-7553 potently inhibit growth of endothelial cells derived from tumors but less potent against normal aortic or yolk sac endothelial cells [168]. In SW480-ADH colon cancer cells $1,25(OH)_2D_3$ has a complex regulatory effect on the angiogenic phenotype: it increases the expression of VEGF and TSP-1, but not that of PDGF-B, through the activation of their respective promoters [172]. Finally, an interesting observation is deglycosylated vitamin D-binding protein (DBP-maf) has also been reported to inhibit angiogenesis [173,174] and to inhibit growth of pancreatic tumor in nude mice [174]. Whether $1,25(OH)_2D_3$ may interfere with DBP-maf in tumor growth inhibition and antiangiogenesis remains to be established. Interaction with another factor, interleukin-12, in the inhibition of angiogenesis has been reported [175].

The second mechanism of antitumor activity to be discussed, and one that is related to angiogenesis, is invasion and metastasis. Metastasis is the primary cause of the fatal outcome of cancer diseases. A study by Mork Hansen et al. indicated that $1,25(OH)_2D_3$ may be effective in reducing the invasiveness of breast cancer cells [176]. They showed that $1,25(OH)_2D_3$ inhibited the invasion and migration of a metastatic human breast cancer cell line (MDA-MB-231) using the Boyden chamber invasion assay. In support of this, it was shown that $1,25(OH)_2D_3$, and the analogs KH1060, EB1089, and CB1093, all inhibited secretion of tissue-type and urokinase plasminogen activator and increase plasminogen activator inhibitor 1 in the MDA-MB-231 metastatic breast cancer cell line [177].

In line with decreasing the capability of breast cancer cells to metastasize $1,25(OH)_2D_3$ also inhibited the epithelial-mesenchymal transition, an important step in metastatic behavior [178]. Current understanding of the role of vitamin D in the epithelial-mesenchymal transition is reviewed by Larriba et al. [179].

The vitamin D analogs EB1089 also prevented skeletal metastasis in vivo and prolonged survival time in nude mice transplanted with human breast cancer cells [180]. Interestingly, it was shown that vitamin D deficiency promotes the growth of human breast cancer cells in the bones of nude mice [181]. A recent study found that ablation of VDR expression in BCa cells accelerated primary tumor growth and enabled the development of metastases, demonstrating a tumor autonomous effect of vitamin D signaling to suppress BCa metastases [182]. The authors went on to show that vitamin D signaling inhibited the expression of the tumor progression gene Id1, and this pathway was abrogated in vitamin D deficiency in vivo in 2 murine models of BCa. The findings are relevant to humans, because they discovered that the mechanism of VDR regulation of inhibitor of differentiation 1 (ID1) was conserved in BCa derived from human breast cancer cells, and there was a negative correlation between serum 25(OH)D levels and the level of ID1 in primary tumors from patients with BCa. Interestingly, the "prohormone" 25(OH)D could delay neoplasia, tumor growth and metastasis in a nonimmunodeficient MMTV-PyMT mouse model of metastatic breast cancer [183].

Vitamin D also inhibited the invasive ability of human prostate cancer cell lines, LNCaP, PC-3, and DU145. $1,25(OH)_2D_3$ decreased MMP-9 and cathepsins, while it increased the activity of tissue inhibitors of metalloproteinase-1 and cathepsin inhibitors [184]. $1,25(OH)_2D_3$ decreased androgen-stimulated progression of prostate cancer, but prolonged treatment with $1,25(OH)_2D_3$ increased metastatic behavior in a model of transgenic adenocarcinoma of mouse prostate. This shows the need for further mechanistic studies to elucidate both antineoplastic as well as possible prometastatic effects of vitamin D in prostate cancer [185].

In an in vivo study it was shown that $1,25(OH)_2D_3$ reduces the metastasis to the lung of subcutaneously implanted Lewis lung carcinoma cells [118]. In two animal models of prostate cancer $1,25(OH)_2D_3$ and the analogs EB1089 and RO25-6760 inhibited lung metastases [120,121]. In these models the tumors were implanted subcutaneously and therefore, in contrast to the model of direct tumor cell injection in the left ventricle [186], no bone metastases occurred. In pancreatic cancer, the vitamin D analogs MART-10 as well as $1,25(OH)_2D_3$ repressed migration and invasion of tumor cells via blocking the epithelial-mesenchymal transition [187]. MART-10 was also reported to repress metastases of head and neck squamous carcinoma cells [188].

A fact to be considered in relation to metastasis is that bone is the most frequent site of metastasis of advanced breast and prostate cancer. There are some indications from clinical studies that bone metastases develop preferentially in areas with high bone turnover [189,190]. In contrast, agents that inhibit bone resorption like bisphosphonates and Denosumab have been reported to reduce the incidence of skeletal metastasis and improve survival [191–194]. Promising are also studies that focus on bone anabolic therapies [195]. Akech et al. showed that Runx2 is a key regulator of events associated with prostate and breast cancer metastatic bone disease [196]. Runx2 is intimately involved in vitamin D actions in osteoblast development [197]. As $1,25(OH)_2D_3$ may stimulate bone turnover, treatment of cancer with $1,25(OH)_2D_3$ might theoretically increase the risk of skeletal metastases. This aspect of $1,25(OH)_2D_3$ therapy certainly needs further study. Considering the use of vitamin D_3 analogs with reduced

calcemic activity or treatment with parental vitamin D_3 in combination with other compounds to reduce bone turnover may be helpful (see section Combination Therapy below). The versatile aspects of endocrine interplay (including vitamin D) in the cross talk between bone cells and metastatic cancer cells were reviewed by Hofbauer et al. [198].

The data obtained so far on angiogenesis and metastasis show that these two processes contribute to the multiple mechanisms by which vitamin D_3 exerts anticancer activity.

Parathyroid Hormone-Related Peptide

$1,25(OH)_2D_3$ and parathyroid hormone (PTH) mutually regulate synthesis and secretion of one another (see Chapter 27 (vol. 1 of this book)). Production and secretion of PTH are inhibited by $1,25(OH)_2D_3$ via a transcriptional effect, and a vitamin D responsive element (VDRE) in the promoter of the PTH gene has been identified [199,200]. Parathyroid hormone-related peptide (PTHrP) was initially isolated from several carcinomas and is responsible for the syndrome of humoral hypercalcemia of malignancy [201,202] (see Chapter 46 (vol. 1 of this book)). Although originally identified in carcinomas, PTHrP has also been identified in normal cells. As will be discussed now, vitamin D effects to inhibit PTH and PTHrP may have a role in its anticancer actions and in reducing metastases to bone [203,204].

In normal human mammary epithelial cells, $1,25(OH)_2D_3$ did not affect basal but inhibited growth factor-stimulated PTHrP expression via an effect on transcription [205]. In normal keratinocytes $1,25(OH)_2D_3$ had no effect on PTHrP secretion in basal culture conditions [206] but did inhibit growth factor-stimulated PTHrP production as well [207]. Likewise, $1,25(OH)_2D_3$ as well as the analogs 22-oxacalcitriol and MC903 inhibited PTHrP secretion in immortalized human keratinocytes (HPK1A), but this inhibition was less in the more malignant ras-transfected clone HPK1A-ras [208,209]. $1,25(OH)_2D_3$ and the analogs EB1089 and 22-oxacalcitriol inhibit the PTHrP gene transcription in and release from the squamous cancer cell line NCI H520 [210]. In addition, in the human T-cell lymphotropic virus type I transfected T-cell line MT-2, $1,25(OH)_2D_3$, and 22-oxacalcitriol inhibited PTHrP gene expression and PTHrP secretion [211] and in rat H-500 Leydig tumor cells [212], and $1,25(OH)_2D_3$ inhibited PTHrP secretion by PC-3 prostate cancer cells. However, another study demonstrated a prostate cancer-specific or cell-specific effect. Vitamin D and the analogs EB1089 inhibit the PTHrP expression via a negative VDRE in LNCaP but not PC3 prostate cancer cells [213,214]. It was suggested that this might play a role in the growth inhibition by vitamin D as PTHrP stimulates prostate cancer growth, tumor invasion and metastasis [215–217]. In vivo observations comparable to these in vitro observations have also been made. When H-500 Leydig tumor cells were implanted in Fisher rats, treatment with $1,25(OH)_2D_3$ and the analogs EB1089 resulted in reduced levels of tumor PTHrP mRNA and PTHrP serum levels [114]. EB1089 also reduced serum levels of PTHrP in nude mice implanted with squamous cancer cells [218]. In Fisher rats implanted with the Walker carcinoma, $1,25(OH)_2D_3$ caused a decrease in serum PTHrP but the ratio of PTHrP levels and tumor weight was similar in rats receiving vehicle or $1,25(OH)_2D_3$. The data point to an indirect effect on PTHrP via growth inhibition. However, the PTHrP mRNA levels appeared to be decreased by $1,25(OH)_2D_3$ [117]. In nude mice bearing the FA-6 cell line of a pancreas carcinoma lymph node metastasis, 22-oxacalcitriol inhibits PTHrP gene expression, which is related to inhibition of tumor-induced hypercalcemia [219]. Together, the overall picture that emerges from these studies is that an important additional anticancer effect of vitamin D_3 and analogs could be the inhibition of the syndrome of humoral hypercalcemia of malignancy due to PTHrP.

In contrast to these inhibitory effects in human tumor cells and tumor models, a stimulatory effect of $1,25(OH)_2D_3$ and EB1089 on PTHrP gene transcription and PTHrP production by a canine oral squamous carcinoma cell line (Sec 2/88) has been observed [220,221]. Also in vivo with the canine adenocarcinoma CAC-8 in nude mice, stimulation of PTHrP by $1,25(OH)_2D_3$ and EB1089 was observed [221]. These findings indicate that the effect of vitamin D and analogs on canine tumors differ from the action on human tumors.

VITAMIN D EFFECTS ON TUMOR CELLS

Cell Cycle

It has now been well established that vitamin D inhibits growth of cells by interfering with the cell cycle (see Chapter 96). In a randomized clinical trial an inverse relation of vitamin D metabolite levels and Ki67 intensity (proliferative activity) in prostate cancer tissue was found after vitamin D treatment [222]. Both in breast cancer [223] as well as in colon cancer inhibition of cell proliferation via vitamin D is associated with JNK1. JNK1 interacts with the VDR and regulates its expression, influencing $1,25(OH)_2D_3$ mediated inhibition of proliferation of cancer cells [224]. Proliferating cells progress through the cell cycle, which comprises the G_0/G_1 phase (most differentiated, nondividing cells are in the G_1 phase), the S phase in which new DNA is synthesized, and the G_2 phase, which is followed by mitosis (M phase) whereon the cells reenter the G_0/G_1 phase. In most of the cells studied so far treatment with $1,25(OH)_2D_3$ and its analogs results in a blockade at a specific check-point, i.e., the restriction point (R), in the G_1 phase limiting the transition of G_1 to S and reducing the number of cells in S phase. Some studies also have examined the effect on the G_2 phase, but these results are somewhat more diverse. In general it can be concluded that blocking the transition from the G_0/G_1 phase to the S phase plays an important role in the growth inhibitory effect of $1,25(OH)_2D_3$. Numerous genes and proteins have been described that participate in the regulation of the cell cycle. It is beyond the scope of this chapter to discuss in detail the regulation of all of the genes/proteins by vitamin D. In Fig. 94.1, an overview is given of the interacting genes/proteins that are involved in intracellular signaling and regulating the cell cycle. These genes and proteins are part

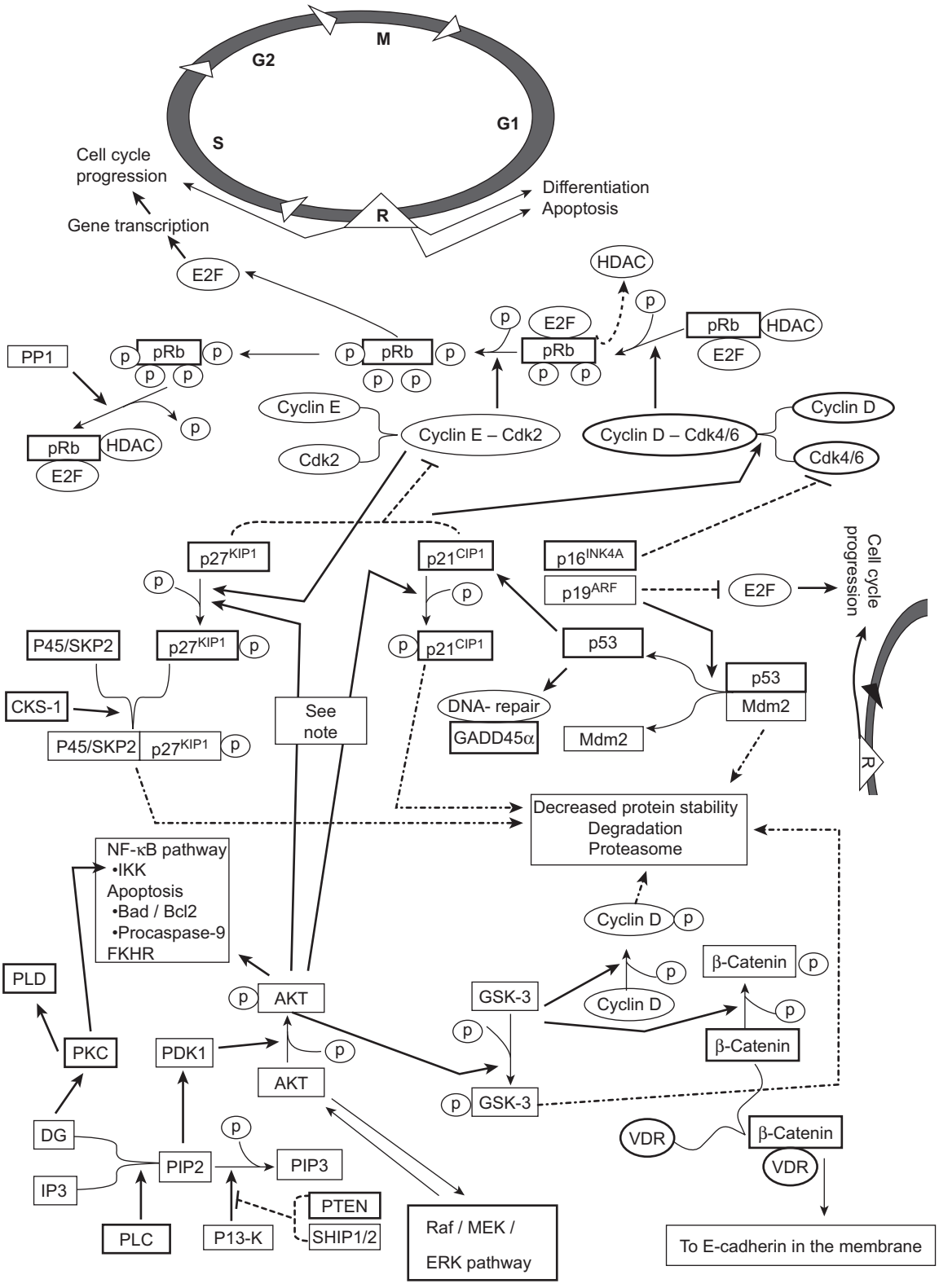

FIGURE 94.1 Schematic representation summarizing the intracellular pathways and signaling pathways involved regulation of the cell cycle shown to be regulated by $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ analogs in regulating cell proliferation. Targets shown to be affected by $1,25(OH)_2D_3$ and/or its analogs are indicated in the *bold boxes* and *ovals*. *Bold arrows* and *fine dotted lines* indicate stimulation and inhibition, respectively. Coarse *dotted lines* indicate processing to the proteasome. p indicates phosphorylation. The effects on these cellular targets are not demonstrated in all types of cancer cells but this diagram is aimed to give an overview of demonstrated targets and potential targets. Note: Dependent on the site of phospharylation proteins can either be destabilized or degraded or be stabilized and activated. For example: phosphorylation of p21 at T145 by AKT leads to degradation, while phosphorylation of S146 by AKT leads to increased stability. *AKT (PKB)*, Protein kinase B; *Bad*, BCL2-antagonist of cell death; *Bcl2*, B-cell leukemia/lymphoma 2; *Cdk*, Cyclin-dependent kinase; *CKS-1*, Cyclin kinase subunit 1; *DG*, Diacylglycerol; *E2F*, Transcription factor; *ERK*, Extracellular-signal regulated kinase; *FHKR (AFX/FOX)*, Forkhead family of transcription factors; *GSK-3*, Glycogen synthase kinase-3; *HDAC*, Histone deacetylase; *IKK*, I-κB kinase; *IP3*, Inositol 1,4,5-trisphosphate; *Mdm2*, Mouse double minute 2; *MEK*, Raf-1-MAPK/ERK kinase; *PDK1*, Phosphatidylinositol-dependent kinase 1; *PI3–K*, Phosphatidylinositol 3 kinase; *PIP2*, Phosphatidylinositol (4,5)-phosphate; *PIP3*, Phosphatidylinositol (3,4,5) phosphate; *PKC*, Protein kinase C; *PLC*, Phospholipase C; *PLD*, Phospholipase D; *PP!*, Protein phosphatase 1-like protein; *pRB*, Retinoblastoma protein; *PTEN*, Phosphatase and tensin homologue; *SHIP 1 and 2*, Src homology 2 (SH2) containing phosphatases 1 and 2; *SKP2*, Ubiquitin ligase; *VDR*, Vitamin D receptor.

of the cascade of events on which vitamin D exerts its effects. The components shown to be regulated by vitamin D are indicated. Fig. 94.1 is a compilation of data presented thus far and it is important to realize that probably not all of the genes/proteins are affected by vitamin D in all tumor cells. However, in this way, one can get an overview and appreciate the broad-range of effects mediated by vitamin D on intracellular signaling pathways involved in regulation of (tumor) cell growth. More details on the regulation will be discussed in more detail in various other chapters in this section of the book especially Chapter 96.

Besides effects on cell cycle regulation vitamin D has recently been implicated to be involved in control of genomic stability [225]. $1,25(OH)_2D_3$ has been reported to inhibit hepatic chromosomal aberrations and DNA strand breaks [226]. This is supported by the finding that $1,25(OH)_2D_3$ and EB1089 stimulated the expression of GADD45, which stimulates DNA repair [227] and might be coupled to release of p53 from Mdm2 (see Fig. 94.1). Notably, a recent study has shown that supplemental vitamin D_3 and calcium, separately but not together, decreased the level of the DNA damage marker 8-hydroxy-2′-deoxyguanosine in normal colorectal mucosa in a randomized clinical trial [228].

(Proto)-oncogenes and Tumor Suppressor Genes

Oncogenes and tumor suppressor genes generally are involved in control of the cell cycle and apoptosis. One of the most widely studied oncogenes in relation to vitamin D is *c-myc*. *c-Myc* suppresses expression of cell cycle/growth arrest genes gas1, p15, p21, p27, and gadd34, -45, and -153 [229] and has been postulated to play an early role in the following cascade of events in G_1: cyclins activate cyclin-dependent kinases (CDKs), which in turn can phosphorylate the retinoblastoma tumor suppressor gene product ($p110^{RB}$), resulting in transition from G_1 to S phase (see Fig. 94.1). In several cancer cell types $1,25(OH)_2D_3$ has been reported to decrease *c-myc* oncogene expression [230]. Analysis of HL-60 sublines showed a relation between reduction of c-*myc* expression and inhibition of proliferation [231]. Similar observations were made for neuroblastoma cells treated with $1,25(OH)_2D_3$, EB1089 and KH1060 [232]. The mechanism of c-myc inhibition appears to be both direct, by inducing the binding of proteins to an intron element and the involvement of HOXB4 [233,234], and at least in colon cancer cells also indirect via the inhibition of the transcriptional activity of β-catenin and T cell factor (TCF) complexes [235]. In earlier studies, we did not observe a $1,25(OH)_2D_3$-induced change in *c-myc* expression in MCF-7 and ZR-75.1 breast cancer cells, while they were both growth inhibited [236], and a similar observation has been made for the colon-adenocarcinoma CaCo-2 cell line [237]. Nontransformed embryonic fibroblasts are growth inhibited by $1,25(OH)_2D_3$, whereas *c-myc* expression is not changed or is even increased [238,239]. In the MG-63 osteosarcoma cell line, $1,25(OH)_2D_3$ has been shown to enhance *c-myc* expression [240], whereas we observed growth inhibition by $1,25(OH)_2D_3$ [241]. Likewise, 1,25(OH)2D3 inhibits proliferation and increases c-MYC expression in fibroblasts from psoriatic patients [242].

In a recent study inhibition of c-myc was implicated as playing a major role in the ability of $1,25(OH)_2D_3$ to inhibit prostate cancer proliferation [243]. As an underlying mechanism, $1,25(OH)_2D_3$ and the VDR regulate the functional balance of c-MYC and its repressor MAD1/MXD1, to suppress c-MYC function [21]. Collectively, these data show that regulation of c-myc expression may be part of growth inhibition by vitamin D but that this is not generally applicable to all cells. $1,25(OH)_2D_3$ has also been reported to regulate expression of other oncogenes [244–246]; however, these data are rather limited.

Nevertheless, it is clear that $1,25(OH)_2D_3$ has effects on the expression of various proto-oncogenes. The data so far are not conclusive with respect to that genes are crucial in the growth inhibitory action of $1,25(OH)_2D_3$. This can be attributed to the fact that these (proto)oncogenes encode for transcription factors, growth factor receptors or components or intracellular signaling cascades. The effects of these genes may differ between cells dependent on the presence or absence of additional cell type–specific conditions. Therefore, their postulated role is often complex. For example, increased *c-myc* expression can be related not only to induction of apoptosis but also to stimulation of cell cycle progression. Interestingly, in oncogene-induced senescence, functional relationships were revealed between Ras, the vitamin D/VDR axis and DNA repair factors [247].

In contrast to the oncogenes, the effect of $1,25(OH)_2D_3$ on tumor suppressor genes like the retinoblastoma gene is much clearer. This may be related to the fact that, in contrast to oncogenes, retinoblastoma and p53 take well-defined positions in the control of cell cycle and DNA repair (see Fig. 94.1). The $p110^{RB}$ retinoblastoma gene product can either be phosphorylated or dephosphorylated. In the phosphorylated form it can activate several transcription factors and cause transition to S phase and DNA synthesis [248]. In human chronic myelogenous leukemia cells [249], breast cancer cells [250], and HL-60 cells [251,252], $1,25(OH)_2D_3$ caused a dephosphorylation of $p110^{RB}$, which is related to growth inhibition and cell cycle arrest in G_0/G_1 and in one study also in G_2 [252]. In the leukemic cells $1,25(OH)_2D_3$ also caused a reduction in the cellular level of $p110^{RB}$ [249,251]. In nontransformed keratinocytes $1,25(OH)_2D_3$ induced dephosphorylation of $p110^{RB}$ as well [253]. The other major tumor suppressor gene is *p53* (in humans). For leukemic U937 cells it was reported that presence of p53 is important for $1,25(OH)_2D_3$-induced differentiation [254]. In rat glioma cells $1,25(OH)_2D_3$ induces expression of p53 [255]. However, $1,25(OH)_2D_3$ can inhibit cell growth and induce differentiation in cancer cells with defective p53 [256] and also p53-independent induction of apoptosis by EB1089 has been demonstrated [257]. These latter observations might be explained by the fact that vitamin D also interferes at levels in the cascade of cell cycle control downstream of p53 (see Fig. 94.1). Recently, novel interesting data were added to the story of p53 and $1,25(OH)_2D_3$ [258]. It was shown that a mutant p53, often present in tumors, physically and functionally interacts with VDR. Mutant p53 is recruited to vitamin D target genes and can stimulate gene expression and relieve suppression of

other genes. Mutant p53 increases nuclear accumulation of VDR and transforms vitamin D into an antiapoptotic agent [258]. An interesting unique relationship between tumor suppressor genes and vitamin D has been shown for the Wilms' tumor suppressor gene WT1. This zinc-finger containing transcription factor induces transcription of the VDR gene [259].

Several interesting additional genes, interactions and vitamin D targets in cancer treatment should be mentioned. It has been demonstrated that $1,25(OH)_2D_3$ can trigger NF-κB activity through PI3K/Akt pathways [260,261] and also, treatment of NB4 leukemic cells with vitamin D causes a rapid phosphorylation of IκBα [262]. Contrary to these observations, vitamin D has been shown to inhibit NF-κB activity by increasing IκBα expression in different cell lines [263–265]. Sun et al. [266], using mouse embryonic fibroblasts derived from $Vdr^{-/-}$ mice, demonstrated that VDR plays an inhibitory role in NF-κB activation by regulating IκBα levels and VDR-p65 interaction. This role for VDR was supported by a recent study that also demonstrated that $1,25(OH)_2D_3$ inhibits transcriptional activity of NF-κB in breast cancer cells via histone deacetylase (HDAC3 and SMRT) mediated p65 transrepression [267]. Kovalenko et al. showed direct transcriptional regulation by $1,25(OH)_2D_3$ of NF-κB in RWPE1 immortalized but nontumerogenic prostate cells [268]. Fekrmandi et al. found that $1,25(OH)_2D_3$ suppressed NF-κB function by enhancing the turnover of the FBW7-dependent subunit [269]. $1,25(OH)_2D_3$ also indirectly inhibits NF-κB by directly stimulating expression of IGFBP-3, an inhibitor of NF-κB [270].

Interestingly, in relation to NF-κB regulation, as early as 1994, Chen and DeLuca isolated and characterized a vitamin D-induced gene in HL-60 cells [271]. The encoded protein, named vitamin D-upregulated protein-1 (VDUP1), is a thioredoxin-binding protein-2 [272]. Thioredoxin has several roles in processes such as proliferation or apoptosis. It also promotes DNA binding of transcription factors such as NF-κB, AP-1, p53, and PEBP2. In addition, overexpression of thioredoxin suppresses the degradation of IκB and the transactivation of NF-κB, whereas overexpression of nuclear-targeted thioredoxin exhibits enhancement of NF-κB-dependent transactivation [273]. However, it is in only more recent studies that a coupling between VDUP1 and cancer has been made. The expression of VDUP1 was found to correlate with malignant status of colorectal and gastric cancers [274]. 5-fluorouracil, which is widely used for treatment of colon cancer, induces VDUP1 expression in the SW620 colon cancer cell line [275]. In smooth muscle cells and cardiomyocytes VDUP1 inhibits proliferation and is involved in induction of apoptosis [276,277]. A relation with vitamin D effects on cancer is made by two recent studies showing induction of VDUP1 by $1,25(OH)_2D_3$ in tumor cells and that VDUP1 induces cell cycle arrest [278,279]. Moreover, interaction with histone deacetylase (HDAC; see Fig. 94.1), and promyelocytic leukemia zinc-finger (PLZF) was demonstrated. Interestingly and further complicating the story, PLZF inhibits $1,25(OH)_2D_3$ induced differentiation of U937 leukemic cells by binding to the VDR and inhibiting gene transcription [280,281]. Interestingly, a new related gene, DRH1, was cloned and its expression was found to be strongly reduced in hepatocellular carcinoma tissue compared to normal liver [282]. DRH1 is 41% homologous with VDUP1. Whether this points to a new family of cancer genes remains to be established but it certainly opens new venues for intervening in cancer cell growth. PIM-1 kinase was identified as a new VDR interacting protein, regulating $1,25(OH)_2D_3$ target gene (osteopontin) transcription and DR3 reporter response [283].

Important in the regulation of gene expression is the involvement of microRNAs (miRNAs). These small endogenous RNAs target mRNAs and cause translational repression or degradation [284]. In gastric cancer cells it was found that miR-145 is induced by $1,25(OH)_2D_3$ and mediates antiproliferative and effects on gene regulation by vitamin D, with as a direct target transcription factor E2F3 [285]. Also in other cancers vitamin D regulates miRNA expression that opens new routes for therapeutic targeting [286,287]. The VDR itself is also regulated by miRNAs: miR-125b repressed endogenous levels of VDR in MCF-7 cells. Because miR-125b is downregulated in cancer, this may result in upregulation of the VDR and positively influence the antitumor effects of vitamin D [288]. The regulation of miRNAs by vitamin D in cancer model systems and impact on $1,25(OH)_2D_3$ signaling is reviewed by Ma et al. [289].

Several alternate therapeutic targets for vitamin D anticancer activity can be mentioned here that are discussed in more detail in the following various chapters on specific cancers. One is vitamin D regulation of enzymes involved in estrogen and androgen synthesis and metabolism because these pathways drive the growth of breast and prostate cancer, respectively [290–294]. Vitamin D downregulates the expression of estrogen receptor (ER) alpha. Two negative VDREs in the ER promotor act together in inhibiting ER expression by $1,25(OH)_2D_3$ [295].

Next, telomerase activity provides a mechanism for unlimited cell division. In HL-60 cells $1,25(OH)_2D_3$ inhibits telomerase activity [296]. Additionally, whether the homeobox genes will prove to be a major target for vitamin D action in cancer remains to be elucidated but in a differential expression screen in the human U937 leukemic cells the HoxA10 gene was shown to be regulated by $1,25(OH)_2D_3$ [297]. A final area is the antiinflammatory activity of vitamin D especially its ability to inhibit of COX-2 and the prostaglandin pathway [298]. Inflammation and carcinogenesis are intimately related and vitamin D inhibits many proinflammatory pathways perhaps contributing to its chemoprevention as well as its therapeutic activity [270]. Stromal-epithelial cross talk is important in the effects of $1,25(OH)_2D_3$ on the inflammatory process, as was shown in prostate cancer [299]. Vice versa, proinflammatory cytokines such as TNFα and IL-6 can decrease the expression of CYP27B1 in colon cancer, impairing activation of vitamin D, so limiting its antiinflammatory action again [300].

It was further suggested that the inhibitory effects on prostate cancer cell growth by vitamin D were related to the ability of $1,25(OH)_2D_3$ to modulate assembly of C x 32 proteins into gap junctions, a way of cell–cell communication that is important in cell growth and differentiation [301].

It is to be expected that as a result of the increasing application of large-scale microarray gene expression analyses a vast number of new cell cycle and vitamin D regulated genes will be identified and these additional findings will add to the unraveling and further understanding of the mechanism of vitamin D control of cancer cell proliferation [302–304]. Recently, RNA sequence data revealed 523 genes that were differentially expressed in breast cancer tissue after vitamin D treatment (compared to 127 genes in normal breast tissue). These genes were mainly involved in processes as cellular adhesion, metabolic pathways and tumor suppressor-like pathways [305].

Apoptosis

The blockade in the cell cycle that prevents transition into S phase may cause cells to either go into apoptosis (programmed cell death) or enter a specific differentiation pathway. What exactly determines the decision between apoptosis or differentiation remains to be elucidated. It is suggested that early G_1 phase may be the point at which switching between cell cycle progression and induction of apoptosis occurs [306,307]. Induction of apoptosis by $1,25(OH)_2D_3$ is an orderly and characteristic sequence of biochemical, molecular, and structural changes resulting in the death of the cell [308]. Apoptosis is a mechanism by which $1,25(OH)_2D_3$ inhibits tumor cell growth and may be the explanation for the tumor suppression and reduction in tumor volume found in various in vivo animal studies (see section Growth and Development).

$1,25(OH)_2D_3$ has been shown to regulate expression of apoptosis genes and to induce apoptosis of cancer cells of various origins. For example, $1,25(OH)_2D_3$ and the analogs Ro 25-6760 induce a cell cycle blockade in HT-29 human colon cancer cells causing growth inhibition and induction of apoptosis [309]. The *bcl-2* oncogene decreases the rate of programmed cell death [310]. However, protection of HL-60 cells against apoptosis occurred despite downregulation of *bcl-2* gene expression [311]. In several breast cancer cell lines (MCF-7, BT-474, MDA-MB-231) $1,25(OH)_2D_3$ and the analogs KH1060 and EB1089, decreased *bcl-2* expression [256,312] and also CB1093 reduced *bcl-2* expression in MCF-7 cells related to induction of apoptosis [313]. However, only in MCF-7 cells this change in *bcl-2* expression was accompanied by apoptosis. The apoptosis induced by $1,25(OH)_2D_3$ and the analogs EB1089 and CB1093 in MCF-7 and T47D breast cancer cells does not involve caspases or p53 activation [314]. $1\alpha,25(OH)_2D_3$ induced apoptosis in MCF-7 cells via disruption of mitochondrial function, which is associated with Bax translocation to mitochondria, cytochrome c release, and production of reactive oxygen species [315]. It was shown that for MCF-7 cells calpain, a calcium-dependent cysteine protease, may take over the role of the major execution protease in apoptosis-like death induced by vitamin D and EB1089 [316].

In B-cell chronic lymphocytic leukemia cells in vitro, the vitamin D_3 analogs EB1089 also induces apoptosis via a p53-independent mechanism involving p38 MAP kinase activation and suppression of ERK activity [257]. In prostate cancer, the effects of vitamin D on apoptosis of tumor cells is caspase dependent and the human VDR is a target of caspase-3, suggesting that activation of caspase-3 may limit VDR activity [317].

Effects on other apoptosis genes/proteins such as BAX and BAK have been reported [318] and microarray gene expression analyses and differential screening will also definitively reveal additional vitamin D targets involved in regulating apoptosis [304,319]. Remarkably, treatment of patients with vitamin D_3 and calcium increased BAK immunostaining in the interior of colonic polyps [320] without affecting BCL2 expression in the same polyps [320] or in normal colon mucosa [321]. In a squamous cell carcinoma model system, the $1,25(OH)_2D_3$ analogs Inecalcitol showed antitumor activity via apoptosis through the activation of the caspase 8/10- caspase 3 pathway [322].

A central role for apoptosis in the action of $1,25(OH)_2D_3$ is unclear because growth inhibition of several other breast cancer cells besides MCF-7 cells appeared to be independent of apoptosis [256]. In addition, MCF-7 cells that showed growth inhibition by $1,25(OH)_2D_3$ could, after removal of the hormone, again be stimulated to grow, implying transient growth inhibition and not cell death [236]. Stable transfection of leukemic U937 cells with the wild-type p53 tumor suppressor gene resulted in a reduced growth rate and produced cells that can undergo either apoptosis or maturation. In these cells $1,25(OH)_2D_3$ protects against p53-induced apoptosis and enhances p53-induced maturation [254], In two independent studies with HL-60 cells, $1,25(OH)_2D_3$ was found either to protect against or to have no effects on apoptosis [311,323]. Vitamin D protection against apoptosis was also detected in human U937 leukemic cells treated with tumor necrosis factor α [324]. Absence of a vitamin D effect on apoptosis might be explained by the expression of the antiapoptotic protein BAG-1 p50 isoform. This protein has been shown to bind to the VDR and block vitamin D induced transcription [325]. Presence of additional interacting factors might also be important for the eventual effect on apoptosis as in the study with HL-60 cells that, in the presence but not the absence of 9-cis-retinoic acid, $1,25(OH)_2D_3$ did induce apoptosis [323]. Role of vitamin D interaction with other factors will be discussed in more detail in Combination Therapy section.

In summary, the data obtained so far show that $1,25(OH)_2D_3$-induced growth inhibition can be related to apoptosis in some cases but that growth inhibition also can be observed independent of apoptosis. Possibly in these latter cases induction of differentiation is more prominent. The factor(s) that decide whether cells undergo apoptosis or differentiation is(are) unclear but is probably dependent on cell cycle stage, presence of other factors, and levels of expression of various oncogenes and tumor suppressor genes. These variables contribute to what appears to be cell-specific actions of vitamin D to induce apoptosis. An interesting phenomenon to be studied concerning vitamin D and apoptosis is calbindin 28K. Calbindin 28K is a well known vitamin D-induced protein, which has been shown to inhibit apoptosis [326]. It is tempting to speculate that calbindin 28K plays

a role in the decision of whether vitamin D induces cells to differentiate or to go into apoptosis or that it is involved when $1,25(OH)_2D_3$ protects against apoptosis. Additionally, EB1089 induces lysosomal changes and autophagic cell death in human MCF-7 breast cancer cells [327,328].

Differentiation

In addition to proliferation and apoptosis, the third major cellular process in the array of vitamin D anticancer actions is differentiation. As described above for the classic actions of $1,25(OH)_2D_3$ related to calcium homeostasis, effects on cell differentiation and proliferation are involved. There is a considerable body of evidence that the principal human cancer cells can be suitable candidates for chemoprevention or differentiation therapy with vitamin D. However, different mechanisms of $1,25(OH)_2D_3$ induced differentiation are cell-type and cell-context specific [329,330]. The coupling between proliferation and differentiation has been most widely studied for cells of the hematopoietic system and keratinocytes. In general, $1,25(OH)_2D_3$ inhibits proliferation and induces differentiation along the monocyte-macrophage lineage. Rapidly proliferating and poorly differentiated keratinocytes can be induced to differentiate by $1,25(OH)_2D_3$. A further relationship between the vitamin D_3 system and differentiation is demonstrated by the fact that in poorly differentiated keratinocytes $1,25(OH)_2D_3$ production and VDR levels are high, whereas after induction of differentiation these levels decrease [331]. In melanoma cells, in addition to growth inhibition [96], $1,25(OH)_2D_3$ stimulates melanin production [332]. Effects on differentiation have also been reported for other cell types. Inhibition of prostate cancer cell proliferation is paralleled by an increased production of PSA per cell, a sign of differentiation [333,334]. In the BT-20 breast cancer cells $1,25(OH)_2D_3$ induced morphological changes indicative for differentiation [335]. In several breast cancer cell lines the stimulation of differentiation has been established by determining lipid production by the cells [256]. In this study, Elstner et al. demonstrated an uncoupling between effects on proliferation and differentiation. In two breast cancer cell lines $1,25(OH)_2D_3$ and various analogs induced differentiation even though the cells were resistant to cell cycle and antiproliferative effects. This together with data obtained with human myelogenous leukemia cells [249] suggest a dissociation between the cellular vitamin D_3 pathways involved in regulation of differentiation and proliferation (see also section Resistance and Vitamin D Metabolism). For an HL-60 subclone a similar observation was made [231], and in another HL-60 subclone the induction of differentiation was found to precede the G_0/G_1 cell cycle blockade. In contrast to the above-mentioned observations on stimulation of differentiation, $1,25(OH)_2D_3$ inhibits erythroid differentiation of the erythroleukemia cell line K562 [336] and $1,25(OH)_2D_3$ inhibits Activin A-induced differentiation of murine erythroleukemic F5-5 cells [337]. Paracalcitol, a vitamin D_2 analogs, converted committed myeloid hematopoietic stem cells from wild-type but not from VDR-knockout mice to differentiate into macrophages [338].

In an early paper Shabahang et al.found that the level of VDR correlated with the degree of differentiation in human colon cancer cell lines and suggested it might serve as a useful biological marker in predicting clinical outcome in patients [2]. Differentiation of rapidly dividing HT-29 colon cancer cells to differentiated slowly proliferating cells was associated with decreased VDR abundance, loss of VDR homologous upregulation, and the development of hormone unresponsiveness to $1,25(OH)_2D_3$ [311]. $1,25(OH)_2D_3$ induces an adhesive phenotype typical of the differentiated epithelial cells that is mostly based on the upregulation of E-cadherin and other plasma membrane adhesion proteins of adherens junctions (α-catenin) and tight junctions (occludin, claudins, ZO-1) [235,339]. In addition, $1,25(OH)_2D_3$ regulates the phenotype of human breast cancer cells, Thus, it increases the expression of E-cadherin, claudin-7 and occludin and of proteins such as paxillin, focal adhesion kinase and αv and β5 integrins that are involved in adhesion to the substratum [340]. Moreover, $1,25(OH)_2D_3$ represses several markers of the basal/myoepithelial phenotype (P-cadherin, smooth muscle α-actin and α6 and β4-integrins), the proinvasive and proangiogenic protein tenascin-C protein, and the mesenchymal marker N-cadherin that are associated with aggressiveness and poor prognosis in breast cancer [341,342]. Another prodifferentiation action of vitamin D, that may be beneficial in breast cancer, is the differentiation of preadipocytes that express high levels of aromatase, to differentiated adipocytes that express much lower levels of aromatase [294]. Although precise relationships among growth inhibition, cell cycle effects, and apoptosis are not entirely clear, it can be concluded that an important effect of vitamin D_3 on both normal and malignant cells is induction of differentiation.

Growth Factors and Growth Factor Receptors

Besides regulation of cell cycle-related oncogenes and tumor suppressor genes, interaction with tumor- or stroma-derived growth factors is important for growth inhibition. Stimulation of breast cancer cell proliferation by coculture with fibroblasts is inhibited by $1,25(OH)_2D_3$ [343]. A good candidate to interact with the $1,25(OH)_2D_3$ action is transforming growth factor-β (TGFβ). TGFβ is involved in cell cycle control and apoptosis [344,345]. TGFβ can interfere with the cascade of events in the G1 phase described above and inhibit the ability of cells to enter S phase when it is present during the G1 phase. TGFβ has been shown to suppress *c-myc*, cyclin A, cyclin E, and cdk2 and cdk4 expression [345]. In line with this, TGFβ has been reported to inhibit phosphorylation of $p110^{RB}$ [346]. Vitamin D_3 compounds induce dephosphorylation of the retinoblastoma gene product, and vitamin D_3 growth inhibition of MCF-7 breast cancer cells is inhibited by a TGFβ neutralizing antibody [347]. $1,25(OH)_2D_3$ and several analogs stimulated the expression of TGFβ mRNA and secretion of active and latent $TGFβ_1$ by the breast cancer cell line BT-20 [174]. $1,25(OH)_2D_3$ enhanced $TGβ_1$ gene expression in human keratinocytes [348] and the secretion of TGFβ in murine keratinocytes [349]. In both studies antibodies against TGFβ inhibited the growth inhibitory effect

of vitamin D_3. Further evidence for a vitamin D_3-TGFβ interaction is that bone matrix of vitamin D-deficient rats contains substantially less TGFβ than controls [350]. It has been shown for the interaction between TGFβ signaling pathways and vitamin D that the cross talk may be mediated by Smad3. Smad3, one of the SMAD proteins downstream in the TGFβ signaling pathway, was found in mammalian cells to act as a coactivator specific for ligand-induced transactivation of VDR by forming a complex with a member of the steroid receptor coactivator-1 protein family in the nucleus [351]. However, Smad3 is not of itself sufficient to coactivate VDR in TGFβ/vitamin D3 resistant MCF7L cells and other factors are required. It was found that the PI 3-kinase pathway inhibitor LY29004 inhibited the synergy of TGFβ and EB1089 on VDR-dependent transactivation activity. This indicates that the cross talk between TGFβ and vitamin D signaling is also PI 3-kinase pathway dependent [352]. Therefore, on the basis of these consistent findings, TGFβ is a likely candidate to play a role in the $1,25(OH)_2D_3$-induced growth inhibition [352].

Interactions with the insulin-like growth factor [258] system have also been described. IGFs are potent growth stimulators of various cells, and their effect is regulated via a series of IGF-binding proteins (IGFBPs). The IGFBPs, especially IGFBP-3 have potent antiproliferative and proapoptotic actions [353]. These effects include both IGF-dependent actions, by sequestering the potent growth factor, and IGF-independent, having direct actions via its own receptor [354,355]. Among the many ways vitamin D inhibits prostate cancer growth, stimulation of IGFBP-3 may be a major contributor [356].

$1,25(OH)_2D_3$ and the analogs EB1089 inhibit the IGFI-stimulated growth of MCF-7 breast cancer cells [357]. In prostate cancer cell lines, $1,25(OH)_2D_3$ induced expression of IGFBP-6 but not IGFBP-4 [358]. In human osteosarcoma cell lines, $1,25(OH)_2D_3$ and the analogs 1α-dihydroxy-16-ene-23-yne-26,27-hexafluorochole-calciferol potently stimulated the expression and secretion of IGFBP-3 [359–361]. In one study an association has been made between increased IGFBP-3 levels and $1,25(OH)_2D_3$ growth inhibition [359]. Recent observations that antisense oligonucleotides to IGFBP-3 prevented growth inhibition of prostate cancer cells by $1,25(OH)_2D_3$ [303] provided further evidence for an interplay between $1,25(OH)_2D_3$ and IGFBP-3. Interestingly, in the human osteosarcoma cell line MG-63, $1,25(OH)_2D_3$ and TGFβ synergistically increased IGFBP-3 secretion [361]. IGF-II is also a growth and survival factor for colorectal cancer cells and $1,25(OH)_2D_3$ and several analogs interfere with IGF-II signaling. They upregulate IGFBP-6, which inhibits IGF-II signaling, and type II IGF receptor (IGF-R-II) that also blocks this pathway and accelerates IGF-II degradation [362,363]. An example of growth factor receptor regulation by $1,25(OH)_2D_3$ concerns the epidermal growth factor receptor (EGFR). This receptor is downregulated in T47-D breast cancer cells and upregulated in BT-20 breast cancer cells. Nevertheless, $1,25(OH)_2D_3$ inhibits the growth of both cell lines [364,365]. These data provide evidence that interactions with growth factors are part of the $1,25(OH)_2D_3$ action on tumor cells. In primary colon adenocarcinoma cells as well as in the colon cancer Caco-2 cell line $1,25(OH)_2D_3$ inhibits EGF mitogenic signaling and a mutual modulation of receptor expression between $1,25(OH)_2D_3$ and EGF has been proposed [366,367]. In A431 epidermoid cells $1,25(OH)_2D_3$ alters EGFR membrane trafficking and inhibits EGFR signaling [368].

It was found that TCF-4, a transcriptional regulator and beta-catenin binding partner is an indirect target of the VDR pathway. TCF-4 functions as a transcriptional repressor that restricts breast and colorectal cancer cell growth. $1,25(OH)_2D_3$ increases TCF-4 at the RNA and protein levels in several human colorectal cancer cell lines, the effect of which is completely dependent on the VDR. This $1,25(OH)_2D_3$/VDR-mediated increase in TCF-4 may have a protective role in colon cancer as well as other diseases [369]. In an in vivo model of liver tumor formation, vitamin D deprivation caused tumor growth in the context of TGFβ/Smad3 disruption. This via regulation of toll-like receptor 7 expression and β-catenin activation [370].

As described above, it is clear that $1,25(OH)_2D_3$ has effects on the expression of various oncogenes and tumor suppressor genes and that multiple interactions with various growth factors exist. However, the data on these aspects separately as well as in combination are still too limited to define the total mechanism of action for the $1,25(OH)_2D_3$ anticancer effects. However, with respect to growth inhibition, at this time two models of action can be postulated. In the first one $1,25(OH)_2D_3$ directly interferes with a crucial gene(s) involved in the control of the cell cycle. In this case, in view of the general pattern of the genes involved in cell cycle control, this mechanism of action will be similar in all types of cancer cells. However, the effect on cell cycle genes will be dependent on the presence or absence of additional growth factors. This will eventually determine, depending on which growth factors are present, the differences in $1,25(OH)_2D_3$ action not only between cancer types of different origin but also within cancer types of similar origin. In the second model $1,25(OH)_2D_3$ may regulate cell cycle indirectly via changing the production of growth factors, growth factor signaling, growth factor-binding protein levels, or receptor regulation. It is conceivable that a combination of both models forms the basis of $1,25(OH)_2D_3$ regulation of tumor cell growth.

COMBINATION THERAPY

The data obtained with $1,25(OH)_2D_3$ and its analogs on growth inhibition and stimulation of differentiation offer promise for their use as an endocrine anticancer treatment. Single agent treatment with low calcemic $1,25(OH)_2D_3$ analogs could be useful; however, combination therapy with other tumor effective drugs may provide an even more beneficial effect [157]. Up to now several in vitro and in vivo studies have focused on possible future combination therapies with $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ analogs.

For breast cancer cells, the combination of one of the most widely used endocrine therapies, the antiestrogen tamoxifen, with $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ analogs resulted in a greater growth inhibition of MCF-7 and ZR-75-1 cells than treatment

with either compound alone [133,371]. In combination with tamoxifen, the cells were more sensitive to the antiproliferative action of $1,25(OH)_2D_3$ and the analogs; that is, the EC_{50} values of the vitamin D_3 compounds in the presence of tamoxifen were lower than those in the absence of tamoxifen. Studies with MCF-7 cells suggested a synergistic effect of $1,25(OH)_2D_3$ and tamoxifen on apoptosis [372]. In addition, in in vivo breast cancer models, a synergistic effect of the tamoxifen-l,$25(OH)_2D_3$ analogs combination was observed [133,134].

Another interesting interaction relevant to breast cancer is that vitamin D inhibits aromatase thus reducing the estrogenic stimulus for proliferation [294]. Combination of $1,25(OH)_2D_3$ and aromatase inhibitors also showed synergistic activity in breast cancer cells. $1,25(OH)_2D_3$ also downregulates the ER, again reducing the ability of estrogens to stimulate breast cancer growth [373]. Additional data on the interaction between the estrogen/antiestrogen system and vitamin D comes from studies showing the presence of an estrogen responsive element in the VDR promoter and regulation of VDR by estradiol in breast cancer cells [374]. It is intriguing that the stimulator of breast cancer cell growth induces the expression of the receptor for a growth inhibitor. VDR upregulation in breast cancer cells and increased transcriptional activity was mimicked by the phytoestrogens resveratrol and genistein and blocked by tamoxifen [375]. Estradiol induces metastasis-associated protein (MTA)-3, a component of the Mi-2/NuRD transcriptional corepressor complex that inhibits Snail1, which is in turn a repressor of VDR gene expression [376,377]. In this way, estradiol may increase VDR levels in breast cells. In colon cancer also VDR upregulation by estradiol has been reported, however, in colon it was hypothesized to contribute to the protective effect of estradiol on chemically induced colon carcinogenesis [378]. These important and complex interactions between the vitamin D and estrogen endocrine systems in the regulation of cancer [293] are promising and warrant further detailed analyses, e.g., regarding tissue (cancer)-specific effects. In addition, the estrogen endocrine system may regulate the metabolism of $1,25(OH)_2D_3$ in cancer cells and thereby affect its action (see section Resistance and Vitamin D Metabolism). Interaction with another sex steroid, testosterone, has been described for ovarian cancer. Vitamin D inhibits dihydrotestosterone (not convertible to estradiol) growth stimulation of ovarian cancer cells [379]. Intriguingly, also here the growth stimulator and growth inhibitor mutually upregulate their receptors.

Interestingly, triple-negative breast cancer can be targeted with androgen receptor (AR) and/or VDR agonists to reduce viability of cancer cells and to change in cancer stem cell phenotype. The combination of AR and VDR agonists with chemotherapy was additive [380]. In prostate cancer cells it has been shown that $1,25(OH)_2D_3$, while inhibiting androgen stimulated growth upregulates the androgen receptor [381].

Interaction with another steroid in regulating cancer cells had already been reported in 1983. The synthetic glucocorticoid, dexamethasone and $1,25(OH)_2D_3$ synergistically induced differentiation of murine myeloid leukemia cells [382]. This was supported by in vitro and in vivo data that dexamethasone enhanced the effect of vitamin D on growth inhibition, cell cycle arrest and apoptosis of squamous carcinoma cells [383,384]. A possible mechanism is the upregulation of VDR by dexamethasone [383]. An interesting aspect of this combination is not only the direct interaction at cancer cell level but also in the control of the calcemic action of $1,25(OH)_2D_3$. Glucocorticoids inhibit intestinal calcium absorption and increase renal calcium excretion and in this way it may limit the hypercalcemic action of $1,25(OH)_2D_3$ [385].

Combination of vitamin D_3 and retinoids has been examined in various systems. A combination of retinoic acid and $1,25(OH)_2D_3$ resulted in a more profound growth inhibition of both T47-D breast cancer cells [386] and LA-N-5 human neuroblastoma cells [387]. 9-cis-Retinoic acid augmented l,25-$(OH)_2D_3$-induced growth inhibition and differentiation of HL-60 cells [388]. Besides growth inhibition and differentiation effects, the combination of $1,25(OH)_2D_3$ and various isomers of retinoic acid were more potent in reducing angiogenesis than either compound alone [167,389,390]. The background of the interaction between retinoids and $1,25(OH)_2D_3$ may be attributed to heterodimer formation of the respective receptors [391].

For several cytokines, interactions with $1,25(OH)_2D_3$ have been described, stressing the importance of the antiinflammatory actions of vitamin D in cancer [392,393]. Interferon-γ and $1,25(OH)_2D_3$ synergistically inhibited the proliferation and stimulated the differentiation of myeloid leukemia cells [394]. Treatment of LLC-LN7 tumor cells with $1,25(OH)_2D_3$ and IFN-γ synergistically reduced tumor granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion and a blockage in the capacity of the tumor cells to induce granulocyte-macrophage-suppressor cells [116]. In the mouse myeloid leukemia cells interleukin-4 enhanced $1,25(OH)_2$ D_3-induced differentiation [395]. Also with interleukin-1β, interleukin-3, interleukin-6, and interleukin-12 interactions with $1,25(OH)_2D_3$ have been reported [396–398]. $1,25(OH)_2D_3$ and tumor necrosis factor synergistically induced growth inhibition and differentiation of HL-60 [399]. For MCF-7 cells an interaction between $1,25(OH)_2D_3$ and tumor necrosis factor has also been reported [398,400]. In the presence of GM-CSF lower concentrations of $1,25(OH)_2D_3$ could be used to achieve a similar antiproliferative effect in MCF-7 cells [401] and to induce differentiation of U937 myeloid leukemic cells [402]. Other factors shown to interact with $1,25(OH)_2D_3$ are butyrate [403,404], melatonin [405], and factors described in Section Differentiation.

Furthermore, combinations of vitamin D_3 compounds with cytotoxic drugs, antioxidants and radiation have been studied. In vivo adriamycin and in vitro carboplatin and cisplatin, doxorubicin interacted synergistically with $1,25(OH)_2D_3$ to inhibit breast cancer cell growth [128,406–408].

In a carcinogen-induced rat mammary tumor model, treatment with $1α$-$(OH)D_3$ and 5-fluorouracil, however, did not result in enhanced antitumor effects [113]. Recently interactions with a plant-derived polyphenolic antioxidant, carnosic acid were demonstrated in the differentiation of HL-60 cells, which was related to a decrease in the intracellular levels of reactive oxygen species [409,410]. Also interaction with radiation therapy in breast cancer has been described [411–413]. In

a murine skin cancer model, brief oral administration of cholecalciferol before photodynamic therapy enhanced tumor cell death [414].

The data on combinations of $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ analogs with various other anticancer compounds are promising and merit further analyses. The development of effective combination therapies may result in better response rates and lower required dosages, thereby reducing the risk of negative side effects. An additional benefit is that some direct actions of $1,25(OH)_2D_3$ may reduce side effects of toxic chemotherapy drugs when given in combination [415]. An overview and possibilities of combined cancer treatments of vitamin D and other compounds is given by Gocek and Studzinski [416].

RESISTANCE AND VITAMIN D METABOLISM

Classic vitamin D resistance concerns the disease hereditary vitamin D-resistant rickets, which is characterized by the presence of a nonfunctional VDR and consequently aberrations in calcium and bone metabolism (see Chapter 72). For cancer cells the presence of a functional VDR is also a prerequisite for a growth regulatory response, and a relationship between VDR level and growth inhibition has been suggested for osteosarcoma, colon carcinoma, breast cancer, prostate cancer cells, and rat glioma [255,417–420]. Cell lines established from DMBA-induced breast tumors in VDR-knockout mice are insensitive to growth arrest and apoptosis by $1,25(OH)_2D_3$, EB1089 and CB1093 [421]. Albeit that VDR is a prerequisite for tumor cell growth regulation, the presence of the VDR is not always coupled to a growth inhibitory response of $1,25(OH)_2D_3$. Results from studies with transformed fibroblasts [238], myelogenous leukemia cells [231,249,422], transformed keratinocytes [423], and various breast cancer cell lines [256,424] demonstrated a lack of growth inhibition by $1,25(OH)_2D_3$ even in the presence of VDR. In this situation the designation "resistant" is based on the lack of growth inhibition, even though, as discussed earlier in Differentiation section, some of these cells are still capable of being induced to differentiate [249,256]. This points to a specific defect in the growth inhibitory pathway. In the resistant MCF-7 cells this defect is not located at a very common site in the growth inhibitory pathway of the cell, because the growth could still be inhibited with the antiestrogen tamoxifen [424]. For myelogenous leukemia cells similar observations have been made [425]. Human VDR gene is transcriptionally repressed by SNAIL1 and SNAIL2/SLUG in human colon cancer cells leading to decreased levels of VDR RNA and protein and unresponsiveness to $1,25(OH)_2D_3$ effects [426–428]. SNAIL1 also causes a decrease in VDR RNA stability [426], while Snail1 represses VDR in mouse osteoblasts and SNAIL2/SLUG in human breast cancer cells [429]. In addition, Snail1 is probably mediating the decrease in VDR mRNA stability induced by oncogenic Ha-ras in mouse NIH-3T3 cells [430,431].

For VDR-independent resistance to growth inhibition and in general to $1,25(OH)_2D_3$ effects several the underlying mechanism(s) have been proposed: increased levels of VDR corepressors, reduced bioavailability of $1,25(OH)_2D_3$ due to either or both 24-hydroxylase (CYP24) upregulation and 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1) downregulation, and disruption or phosphorylation of VDR-RXR dimers. Resistance to $1,25(OH)_2D_3$ in breast and prostate cancer cells has also been found to be a consequence of increased levels of the VDR corepressors NCoR or SMRT [432,433]. This is in line with the reported synergistic effect on the proliferation of prostate cancer cells of combined treatment with $1,25(OH)_2D_3$ and the histone deacetylase inhibitor trichostatin [403].

The resistant MCF-7 clone described by Welsh and colleagues is not related to upregulation of the P-glycoprotein [424]. Interestingly, these vitamin D resistant MCF-7 clones can be sensitized to vitamin D by activation of protein kinase C, resulting in induction of apoptosis and transcriptional activation, suggesting that alterations in phosphorylation may affect vitamin D sensitivity [434]. Hansen et al. described a different interesting growth inhibition resistant MCF-7 cell clone. This clone was not growth inhibited, while VDR was still present and CYP24 could still be induced [435].

Recurrent tumors are often resistant to therapy. Adding to the complexity of this phenomenon is the presence of a specific subset of cancer cells: the cancer stem cells. These cells are highly resistant to therapies and effective in repopulating the tumor [436]. Mammospheres, an indicator of stem cell activity, generated from breast cancer cell lines showed suppressed VDR signaling, but combined treatment with $1,25(OH)_2D_3$ and a nitric oxide (NO)-donor caused a significant decrease in mammosphere size and smaller tumor volume in nude mice [437]. Inhibition of breast cancer stem cell spheroid (mammosphere) formation by $1,25(OH)_2D_3$ was also found by Jeong et al. [438]. Effects of vitamin D on prostate progenitor/stem cells resulted in cell-cycle arrest, senescence, and differentiation that were mediated by IL-1α [439]. These strategies may lead the way to find new concepts to overcome therapy resistance. Targeting cancer stem cells by vitamin D is reviewed by So and Suh [440].

Another example of vitamin D resistance are HL60 cells that have been cultured for 4 years in the presence of $1,25(OH)_2D_3$. This resulted in clones that are resistant to differentiation induction and growth inhibition. They became not only resistant to vitamin D but also to 5-beta-D-arabinocytosine suggesting a common metabolic pathway being responsible [441]. Whether this relates to the upregulation of the multidrug resistance proteins is not clear. In the resistant leukemia $JMRD_3$ cell line, altered regulation and DNA-binding activity of *junD* as part of the AP-1 complex has been reported [244]. Resistance to growth inhibition in the presence of VDR has also been linked to disruption of the VDR-RXR complex [442] and increased RXR degradation [443]. In addition, other factors, like the acute myeloid leukemia translocation products (e.g., PLZF) may contribute to resistance to vitamin D by sequestering the VDR [280,281]. More recently it was shown that alterated

corepressor and coactivator interaction with VDR and that epigenetic preferential suppression of antiproliferative gene promoters can explain the resistance to growth inhibition [444]. Resistance has also been linked to epigenetic changes in the VDR promoter leading to suppressed or absent expression of VDR [445].

A unique mechanism for vitamin D resistance in immortalized cells has recently been uncovered. Epstein–Barr virus (EBV) has been used to transform and immortalize lymphoblasts that can grow as cell lines in vitro. EBNA-3 is an EBV encoded protein that can regulate transcription of cellular and viral genes. EBNA3 binds the VDR and blocks the activation of VDR-dependent genes and protects transformed cell lines against vitamin-D3-induced growth arrest and/or apoptosis [446]. The $1,25(OH)_2D_3$ sensitive and resistant cell clones provide interesting models to examine the molecular mechanisms of $1,25(OH)_2D_3$-induced growth inhibition. For example, lack of p21 results in no cell cycle block [447] and no apoptosis was detected with a mutated p53 [256]. Finally, the identification of cellular proteins that are involved in the vitamin D resistance in new world primates might add to the understanding of tumor cell resistance to vitamin D [448,449].

At the moment the major mechanism for vitamin D resistance or reduced sensitivity in VDR containing tumor and cancer cells is $1,25(OH)_2D_3$ catabolism via the C24-hydroxylation pathway. An inverse relationship between cellular metabolism of $1,25(OH)_2D_3$ via 24-hydroxylation and growth inhibition of prostate cancer cells has been suggested [418]. The latter observation is intriguing, the more so as an inverse relationship between VDR level and induction of CYP24 activity was reported. In general, there may exist a direct relationship between VDR level and induction of CYP24 activity [419,450].

An important role in the control of $1,25(OH)_2D_3$ action on cancer cells was provided by studies with the $1,25(OH)_2D_3$ resistant prostate cancer cell line DU145. It was shown that $1,25(OH)_2D_3$ did inhibit the growth of these cells when it was combined with the 24-hydroxylase inhibitor Liazorole [451]. $1,25(OH)_2D_3$ activity was likewise enhanced by combination with ketoconazole, a drug commonly used to treat prostate cancer that inhibits CYP24 activity [452,453]. Inhibition of CYP24 activity in HL-60 cells also altered the effect of $1,25(OH)_2D_3$ and 20-epi analogs [454]. Recently, epigenetic silencing of the CYP24 gene modulates the growth response of tumor-derived endothelial cells [455]. The action of the analogs EB1089 was also limited by hydroxylation at the C24 position [456]. However, it was suggested that the increased potency of EB1089 is at least partly due to resistance to CYP24 [302]. Alternatively, 24-hydroxylation of the analogs KH1060 has been implicated as one of the mechanisms to explain the potency of this analogs. The 24-hydroxylated metabolites of this analogs are very stable and remain biologically active [457,458]. It has been shown that the naturally occurring 24-hydroxylated metabolite of vitamin D_3 ($24R,25\text{-}(OH)_2D_3$) also has a preventive effect on chemically induced colon cancer [459].

Interaction between the estrogen system and CYP24 is also of importance. Data have shown that the phytoestrogen genistein inhibits CYP24 activity in prostate cancer cells and thereby increases the responsiveness to $1,25(OH)_2D_3$ [460,461]. A role for CYP24 as oncogene is suggested by data showing amplification of the CYP24 locus on chromosome 20q13.2 [462], and increased copy-number causing overexpression in colorectal cancer [463]. CYP24A1 has been mentioned as a new prognostic biomarker for colorectal cancer patients [464].

In contrast to degradation of $1,25(OH)_2D_3$ by CYP24 in cancer cells recently it has become clear that tumor cells contain CYP27B1 activity and thereby are able to locally generate $1,25(OH)_2D_3$. Expression of 1α-hydroxylase has been demonstrated in colorectal cancer [465]. It was postulated that in early stages tumor cells respond by upregulating 1α-hydroxylase activity to counteract neoplastic growth, while at later stages of tumor development this is lost [465]. Also in prostate cancer [466] and inflammatory myofibroblastic tumor [467] CYP27B1 has been detected, albeit that in the latter case the tumor contains large numbers of macrophages. It can be anticipated that in the coming years investigation of the expression of both CYP24A1, CYP27B1 in tumors will add to the understanding the role of vitamin D in inhibiting the initiation and progression of cancer. An overview of the signaling pathways of vitamin D in cancer and their role in therapeutic involvement is shown by Deeb et al. [468], a review of molecular mechanisms underlying the positive effects of vitamin D in cancer is given by Fleet et al. [469].

STIMULATION OF PROLIFERATION

Over the years a limited number of studies have demonstrated that, in contrast to growth inhibition, $1,25(OH)_2D_3$ can also stimulate tumor cell growth and tumor development. In several cells $1,25(OH)_2D_3$ has been reported to have a biphasic effect, that is, at lower concentrations ($<10^{-9}$ M) it stimulates proliferation and at higher concentrations (10^{-9} to 10^{-7} M) it inhibits proliferation. However, clear growth stimulation can sometimes be observed not only at low concentrations but also at the concentrations generally found to inhibit tumor cell proliferation and tumor development. $1,25(OH)_2D_3$ has been shown to stimulate the growth of a human medullary thyroid carcinoma cell line [470]. Not only cancer cells but also several normal cells, for example, human monocytes [471], smooth muscle cells [472], and alveolar type II cells [473], are stimulated to grow by $1,25(OH)_2D_3$. Skin is another organ in which different effects of $1,25(OH)_2D_3$ have been observed. In vivo studies demonstrated that $1,25(OH)_2D_3$ and analogs stimulate keratinocyte proliferation in normal mice [474–477] and enhance anchorage-independent growth of preneoplastic epidermal cells [478]. In contrast, other studies showed $1,25(OH)_2D_3$ inhibition of proliferation of mouse and human keratinocytes [479,480], and $1,25(OH)_2D_3$ is also effective in the treatment of the hyperproliferative disorder psoriasis [481]. Moreover, in vivo studies demonstrated

that, depending on the carcinogen, $1,25(OH)_2D_3$ can either reduce [105] or enhance the induction and development of skin tumors in mice [482,483]. In addition, $1,25(OH)_2D_3$ enhances the chemically induced transformation of BALB 3T3 cells and hamster embryo cells [484,485]. $1,25(OH)_2D_3$ also enhanced 12-O-tetradecanoylphorbol-13-acetate-induced tumorigenic transformation of mouse epidermal JB6 Cl41.5a cells [486,487].

Another example comes from research on osteosarcoma cells. In 1986 it was shown that $1,25(OH)_2D_3$ stimulated the growth of tumors in athymic mice inoculated with the ROS 17/2.8 osteosarcoma cell line [488]. Earlier the same group reported growth stimulation in vitro of these osteosarcoma cells at low concentrations but growth inhibition by 10^{-8} M [417]. They speculated that this discrepancy resulted from limited in vivo availability of $1,25(OH)_2D_3$ for the tumor cells, resulting in concentrations shown to be growth stimulatory in vitro. However, in other experiments with nude mice the availability of $1,25(OH)_2D_3$ did not seem to be a factor, as growth inhibition was observed (see Table 94.2). In particular, in nude mice implanted with human osteosarcoma cells (MG-63), growth inhibition and tumor suppression by $1,25(OH)_2D_3$ were observed [115]. In two different in vitro studies, growth inhibition of MG-63 and growth stimulation of ROS 17/2.8 cells was reported [489,490]. For smooth muscle cells it has been demonstrated, for example, that growth inhibition or stimulation can depend on the presence of additional growth factors in the culture medium [472]. We followed up on this concept by comparing the effects of $1,25(OH)_2D_3$ and analogs on the growth and osteoblastic characteristics of the two osteosarcoma cell lines under identical culture conditions. At concentrations 10^{-10} to 10^{-7} M $1,25(OH)_2D_3$ caused an increase in cell proliferation by 100% in ROS 17/2.8 cells, whereas the proliferation of MG-63 cells was inhibited [241]. In contrast, in both cell lines $1,25(OH)_2D_3$ stimulated osteoblastic differentiation characteristics such as production of osteocalcin and alkaline phosphatase activity [241,489]. Analyses with another steroid hormone demonstrated that glucocorticoids inhibited the growth of both osteosarcoma cell lines [491,492]. These data indicate specific differences between these cell lines, especially with respect to the $1,25(OH)_2D_3$ growth regulatory mechanisms.

In addition to these biological data in cells, an epidemiological study also showed an increased risk of aggressive prostate cancer with higher levels of 25-hydroxyvitamin D_3 [57]. Taken together, the data on growth stimulation and tumor development, although detected in only a small minority of cancer cells, demonstrate that treatment with $1,25(OH)_2D_3$ or analogs may not always cause growth inhibition and tumor size reduction. It is therefore of utmost importance to identify the mechanism(s) by which $1,25(OH)_2D_3$ exerts its inhibitory and stimulatory effects on cell growth. This may provide tools to assess whether treatment of a particular tumor will be beneficial. Moreover, purely from a mechanistic point of view, further study of growth-stimulated and growth-inhibited cells, like the $1,25(OH)_2D_3$ sensitive and resistant cells, may provide tools to examine the $1,25(OH)_2D_3$ mechanism of growth regulation.

CONCLUSIONS

The data obtained so far, on (1) the distribution of the VDR in a broad range of tumors and (2) the inhibition of cancer cell growth, angiogenesis, metastasis, inflammation and PTHrP synthesis as well as the stimulation of differentiation and apoptosis by $1,25(OH)_2D_3$, all hold promise for the development of treatment strategies based on the avoidance of vitamin D deficiency and the adjunctive use of vitamin D_3 in a wide range of cancers in combination with other antitumor drugs as an important therapeutic option. Throughout the previous decade data have accumulated on the cellular targets and mechanism of action of $1,25(OH)_2D_3$-induced cancer growth inhibition. The clinical application is enhanced by the development of $1,25(OH)_2D_3$ analogs with potent growth inhibitory actions and reduced hypercalcemic activity. Nevertheless it is crucial for the coming years to deliver strong randomized controlled clinical trials in humans to support the potential of vitamin D in cancer treatment uncovered by investigation of cultured cells, animal models and epidemiological studies. In the meantime, continuing research to understand the mechanisms by which vitamin D_3 exerts its effects on tumor cell growth is needed so that therapeutic modalities may be employed more effectively.

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