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Effect of 20-*epi*-1α,25-dihydroxyvitamin D_3 on the proliferation of human neuroblastoma: role of cell cycle regulators and the Myc–Id2 pathway

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Abstract

The antiproliferative effects of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and its epimer, 20-*epi*-1α,25-dihydroxyvitamin D_3 [20-*epi*-1,25$(OH)_2D_3$], in six human neuroblastoma (NB) cell lines (SH-SY5Y, NB69, SK-N-AS, IMR5, CHP134, and NGP) were investigated. We determined the ability of 1,25$(OH)_2D_3$ and 20-*epi*-1,25$(OH)_2D_3$ to influence cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell proliferation by bromodeoxyuridine (BrdU) incorporation, and their antineoplastic effect on colony formation in a soft agar assay. A concentration-dependent decrease in cell viability, inhibition of DNA synthesis, and suppression of clonal proliferation was observed with both compounds. 20-*epi*-1,25$(OH)_2D_3$ was more potent in suppressing the proliferation of all six NB cell lines. To understand the mechanisms of action, we examined the effect of 20-*epi*-1,25$(OH)_2D_3$ on the Myc–Id2 cell proliferative network and also on key regulators of the cell cycle. For the first time, we show that 20-*epi*-1,25$(OH)_2D_3$ down-regulated Myc and Id2 expression by western blot analysis. Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis revealed that 20-*epi*-1,25$(OH)_2D_3$ induced the expression of retinoic acid receptor-β and $p21^{Cip1}$, and down-regulated the expression of cyclin D1 resulting in decreased phosphorylation of retinoblastoma protein (pRB). In sum, we show that 20-*epi*-1,25$(OH)_2D_3$ exerts strong antiproliferative effects by regulating key growth control networks (Myc–Id2–pRB) in NB cells.

Keywords: Neuroblastoma; Vitamin D analog; Myc; Retinoic acid receptor-β; pRB; $p21^{Cip1}$

1. Introduction

NB is the most common extracranial solid tumor of the peripheral nervous system accounting for more than 15% of cancer-related deaths in children [1,2]. About 40% of children with NB respond to radiation and single agent chemotherapy. These patients are considered the 'low-risk' group characterized by lack of N-*myc* amplification. Patients with metastatic NB are considered the 'high-risk' group. Their tumors contain amplification of the N-*myc* proto-oncogene, contain poorly differentiated cells, and respond poorly to conventional chemo- and radiotherapies [3,4]. At the time of diagnosis, approximately 50% of infants and 70% older NB patients have disseminated disease beyond the primary site to lymph nodes, bone marrow, and liver [5,6]. In spite of intensive multiagent therapy and bone marrow transplantation, most patients with high-risk NB die due to metastatic disease. Therefore, the need exists to develop novel agents to improve treatment

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Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 20-*epi*-1,25$(OH)_2D_3$, 20-*epi*-1α,25-dihydroxyvitamin D_3; AML, acute myelogenous leukemia; APL, acute promyelogenous leukemia; BrdU, bromodeoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NB, neuroblastoma; pRB, retinoblastoma protein; RAR, retinoic acid receptor; RARE, retinoic acid response element; RT–PCR, reverse transcription–polymerase chain reaction; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

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outcomes in this high-risk group. Retinoic acid derivatives (retinoids) function as chemopreventive agents and are used currently in the therapy of AML, APL, and NB [6–8]. Exposure of human NB cell lines to retinoids results in a marked decrease in N-*myc* mRNA expression, a decrease in Id2, cell cycle arrest in the G_0/G_1 phase, and terminal differentiation [9–11]. Therefore, agents that modulate the above pathways potentiate the therapeutic efficacy of retinoids in NB. Since chronic administration of retinoids often results in cytotoxicity and the development of drug resistance, it is essential to develop alternative agents that exhibit low clinical toxicities, which might increase the efficacy of retinoids [3,6].

A combination of vitamin D_3 analogs and retinoids has been shown to exhibit synergistic effects in inhibiting the growth of leukemia, breast, and prostate cancer cells [12–15]. Retinoids and vitamin D_3 analogs regulate the formation of RAR and RXR homodimers, and RAR/RXR/VDR heterodimers, which bind to sequence-specific DNA elements (VDRE or RARE) and modulate the expression of several growth-related genes [16–18]. Despite the antitumor effects of $1,25(OH)_2D_3$, its calcemic effects preclude its development as a therapeutic agent in cancer [19,20]. For this reason, efforts have been directed towards the development of synthetic vitamin D analogs that modulate tumor growth with minimal calcemic activity [20,21]. Several studies have indicated that 20-*epi* analogs of vitamin D_3 cause potent growth inhibition at several fold less concentration than $1,25(OH)_2D_3$ and also exert antiproliferative activity several orders of magnitude higher than $1,25(OH)_2D_3$ [22–24]. NB cells treated with 9-*cis* RA and EB 1089 showed a marked inhibition in proliferation, which was accompanied by a decline in the levels of c-*myc* encoded protein [25,26]. The molecular basis of the therapeutic effects of vitamin D_3 analogs in NB is poorly understood. A greater understanding of the mechanism of apoptosis induction by combinational therapy is likely to aid in our understanding of cell proliferation and in the development of more effective and well-tolerated therapies for NB.

To evaluate the therapeutic potential of $1,25(OH)_2D_3$ and 20-*epi*-$1,25(OH)_2D_3$, we investigated the *in vitro* biological effects and mechanisms of action in a panel of six NB cell lines. Our data indicate that 20-*epi*-$1,25(OH)_2D_3$ exerts antiproliferative effects in human NB cells by regulating the Myc–Id2–pRB signaling pathways.

2. Materials and methods

2.1. Cell culture and compounds

Six human NB cell lines (SH-SY5Y, NB69, SK-N-AS, IMR5, CHP134, and NGP) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% oxaloacetate–pyruvate–insulin, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The SH-SY5Y, NB69, and SK-N-AS cell lines represent N-*myc* single copy, whereas IMR5, CHP134, and NGP cells represent amplification of the N-*myc* locus [27,28]. $1,25(OH)_2D_3$ was provided to us by Dr. Uskokovic, and its 20-epimer was synthesized by Leo Pharmaceuticals; the structures are shown in Fig. 1A. The parental compound $1,25(OH)_2D_3$ and the 20-*epi* analog were dissolved in absolute ethanol at 10^{-3} M as a stock solution, and stored, protected from light, at −20°. The concentration of the analogs was determined by UV absorbance measurements using their molar extinction coefficient at 264 nm. Dilutions were made in the culture medium for various treatments.

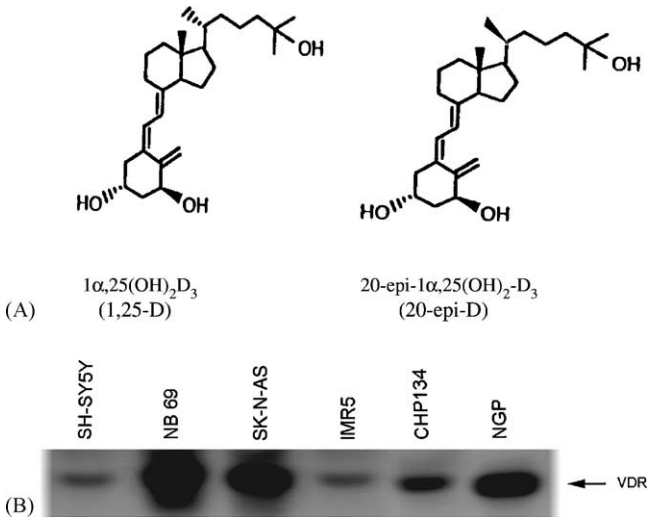

Fig. 1. (A) Structure of vitamin D_3 and its analogs used in the present study. The structures of 1α,25-dihydroxyvitamin D_3 and 20-*epi*-1α,25-dihydroxyvitamin D_3 are indicated. (B) Expression of VDR in NB cell lines. To determine the expression of VDR in NB cell lines, 100 μg of protein was subjected to electrophoresis in SDS–polyacrylamide gels, and the western blots were probed with VDR monoclonal antibodies.

2.2. Western blot analysis

Cells growing in mid-log phase were lysed in lysis buffer (HEPES, pH 7.6, 25 mM; Triton X-100, 0.1%; NaCl, 300 mM; β-glycerophosphate, 20 mM; $MgCl_2$, 1.5 mM; EDTA, 0.2 mM; dithiothreitol, 2 mM; sodium orthovanadate, 0.2 μM; sodium fluoride, 10 μM; benzamidine, 1 μM; leupeptin, 2 μg/mL; aprotinin, 4 μg/mL; and phenylmethylsulfonyl fluoride, 500 μM) and centrifuged at 10,000 *g* for 30 min at 4°. The protein concentration in the supernatant was determined by the bicinchoninic acid method (Pierce). Total protein (100 μg) from the whole cell lysate was electrophoresed in 10% SDS–PAGE gels and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline and probed with respective antibodies. Specific antibody binding was detected by horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies and visualized with ECL reagent (Amersham Pharmacia Biotech). VDR and Id2

antibodies were purchased from Santa Cruz Biotech, pRB antibodies from Pharmingen, and neurofilament-H antibodies from Zymed.

2.3. Cell viability analysis

The effect of vitamin D_3 analogs on the growth rate of NB cells was determined using the MTT method [29] according to the instructions of the manufacturer (Promega). Briefly, 1×10^3 cells were seeded in 96-well plates, incubated at 37° for 24 hr, and treated with either vehicle (ethanol 0.1%) or the indicated concentrations of the compounds for 7 days. The medium was replaced with fresh medium containing the vitamin D_3 compounds after 3 days. MTT reagent (10 μL per well) was added and incubated at 37° for 4 hr. The absorbance of the samples against a background control as blank was measured at 490 nm. Each experiment was repeated three times in triplicate samples. Results are expressed as percent viable cells over control.

2.4. Cell proliferation assay

The ability of $1,25(OH)_2D_3$ and 20-*epi*-$1,25(OH)_2D_3$ to inhibit DNA synthesis was determined by estimating the amount of BrdU incorporation into DNA by a colorimetric immunoassay (Roche Molecular). Briefly, cells were cultured in 48-well plates (1×10^4) in the presence of a 1 μM concentration of the respective analogs for 3 days. BrdU (10 μM final concentration) was added, and the cells were re-incubated for an additional 24 hr. The cells were fixed and incubated with anti-BrdU-POD (100 μL per well) for 2 hr at room temperature. The color was developed by the addition of trimethyl bezidine substrate and measured at 490 nm. The color intensity and the absorbance values directly correlate to the amount of BrdU incorporated into DNA. The results are expressed as percent inhibition of BrdU incorporation by the vitamin D_3 compounds over the control.

2.5. Soft agar colony formation assay

To determine the effect of the two compounds on clonal proliferation, soft agar clonogenic assays were performed. After assessing viability, 1×10^3 cells were seeded in the presence of respective drugs in 35 mm^2 plates in 0.7% agar. After 14 days, the plates were stained with 50 μg/mL of MTT for 4 hr at 37°, and colonies were scored. All experiments were performed using triplicate plates, and the results are the averages of two independent experiments.

2.6. Semi-quantitative RT–PCR

To analyze the mRNA expression of *cyclin D1*, $p21^{Cip1}$, $p27^{Kip1}$, *RAR-β*, and *VDR*, semi-quantitative RT–PCR was performed. Using TRIzol reagent (Invitrogen), total RNA was isolated from the control cells and cells exposed to 1 μM vitamin D compounds for 3 days. Total RNA (1 μg) was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen). Briefly, 1 μg of total RNA was preincubated at 65° for 5 min with an oligo dT, 10 μM dNTP mix. Samples were reverse transcribed for 50 min at 42° with 50 U of SuperScript RT in a total volume of 20 μL. To this mixture, 1 U of Taq DNA polymerase, 1 μM sense primer, 1 μM antisense primer, and 10× PCR reaction buffer were added in a total volume of 50 μL. Amplification was performed using a thermal cycler 480 (28–35 cycles). Each PCR cycle consisted of a denaturing step for 1 min at 94°, an annealing step for 60 s at 50° ($p21^{Cip1}$, *cyclin D1*) or 60° (*RAR-β*), with an extension step for 2 min at 72°. The following PCR primers were used (*RAR-β*: 5′-primer ACCAGCTCTGAGGAACTCGTCCCA, 3′-primer AGGCGGCCTTCAGCAGGGTAATTT; $p21^{Cip1}$: 5′-primer GAGCGATGGAACTTCGACTTT, 3′-primer GGGCTTCCTCTTGGAGAAGAT; $p27^{Kip1}$: 5′-primer CAGCTTGCCCGAGTTATA, 3′-primer TGGGGAACCGTCTGAAAC; *cyclin D1*: 5′-primer TCCTCTCCAAAATGCCAGAG, 3′-primer TGAGGCGGTAGTAGGACAGG; *VDR*: 5′-primer CGCTCCAATGAGTCCTTCACC, 3′-primer GCTTCATGCTGCACTCAGGC; *GAPDH*: 5′-primer ACCACAGTCCATGCCATCAC, 3′-primer TCCACCACCCTGTTGCTGTA.

2.7. Statistical evaluation

Data are expressed as means ± SEM for all experiments. One-way ANOVA was used to assess statistical significance between means. $P < 0.05$ was considered significant.

3. Results

3.1. Expression of vitamin D receptor in human NB cells

To examine the expression levels of VDR in NB, we performed western blot analysis. As shown in Fig. 1B, we observed differential VDR expression between the six NB cell lines. SH-SY5Y and IMR5 cells expressed low levels of VDR when compared with CHP134, NGP, SK-N-AS, and NB69 cells. There was no correlation between the expression of VDR and N-*myc* copy number.

3.2. Effect of $1,25(OH)_2D_3$ and 20-epi-$1,25(OH)_2D_3$ on NB cell viability

To examine the effects of $1,25(OH)_2D_3$ and 20-*epi*-$1,25(OH)_2D_3$, NB cell lines (SH-SY5Y, NB69, SK-N-AS, IMR5, CHP134, and NGP) were exposed to different concentrations (0.3, 1.0, and 3.0 μM) of these compounds for 7 days, and cell viability was determined by MTT assay (Fig. 2A). We consistently observed higher growth inhibition with 20-*epi*-$1,25(OH)_2D_3$ at all concentrations of the compounds used in the study. We calculated the

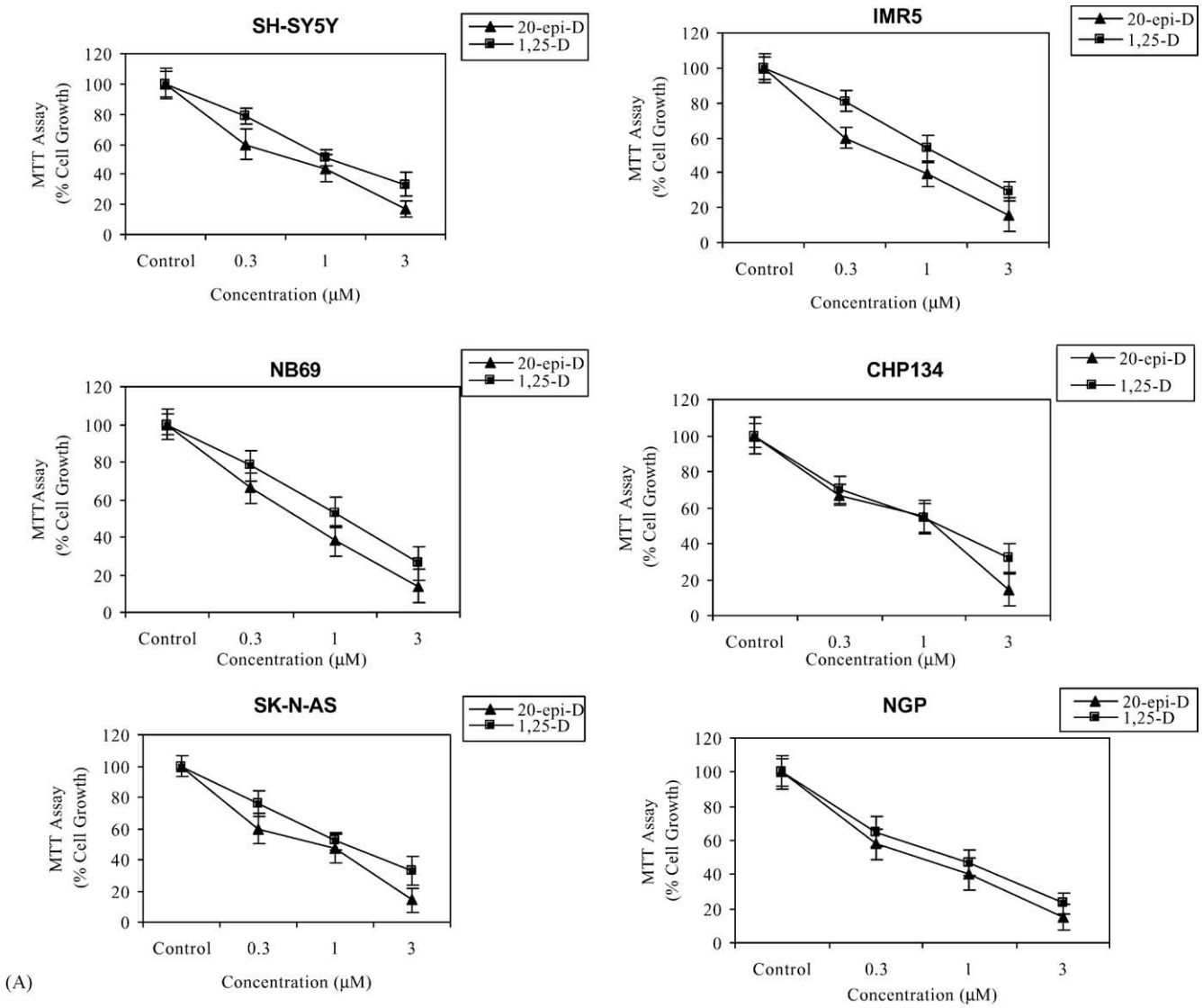

Fig. 2. (A) Effect of 20-*epi*-1,25$(OH)_2D_3$ on the proliferation of NB cells. NB cells (1000 per well) were seeded in a 96-well plate and treated with 0.3, 1.0, and 3 μM concentrations of 1,25$(OH)_2D_3$ and 20-*epi*-1,25$(OH)_2D_3$ for 7 days. Cell viability was determined by MTT assay. The percent of viable cells was calculated in comparison to untreated cells. Data (means ± SEM of triplicate samples) are expressed as percent cell growth. (B) Effect of vitamin D compounds on VDR expression by semi-quantitative RT–PCR. NB cells were treated with 1 μM 1,25$(OH)_2D_3$ or 20-*epi*-1,25$(OH)_2D_3$ for 3 days, and the total RNA was isolated and subjected to semi-quantitative RT–PCR using primers that amplify a 420 bp VDR cDNA.

concentration of vitamin D analogs required to cause 50% inhibition (IC_{50}) of cell proliferation (Table 1). From the IC_{50} values, it is clear that 20-*epi*-1,25$(OH)_2D_3$ is about 2-fold more potent than 1,25$(OH)_2D_3$.

To examine whether the observed effects are due to the induction of VDR by vitamin D_3 compounds, we determined the expression of VDR by the semi-quantitative RT–PCR method. Results shown in Fig. 2B suggest that

Table 1
Effect of $1,25(OH)_2D_3$ and 20-*epi*-$1,25(OH)_2D_3$ on the viability of human NB cells

NB cell lines	IC_{50} (μM)	
	$1,25(OH)_2D_3$	20-*epi*-$1,25(OH)_2D_3$
SH-SY5Y	1.45	0.61
NB69	1.02	0.62
SK-N-AS	1.45	0.62
IMR5	1.20	0.50
CHP134	1.02	0.72
NGP	0.72	0.51

Cells were exposed to different concentrations of $1,25(OH)_2D_3$ and 20-*epi*-$1,25(OH)_2D_3$ for 7 days, and the relative number of viable cells was determined by MTT assay. The concentration of compounds required to clause 50% inhibition of cell proliferation (IC_{50}) was determined from the Sigma plots.

VDR was not induced by either $1,25(OH)_2D_3$ or 20-*epi*-$1,25(OH)_2D_3$ when exposed to these compounds, suggesting that the *VDR* promoter in NB cells may not be responsive to vitamin D.

3.3. Inhibition of NB proliferation by 20-epi-1,25(OH)$_2$D$_3$

To understand whether the decrease in the number of viable cells was due to decreased proliferation, the incorporation of BrdU into DNA was determined in the presence (1 μM) and absence of vitamin D_3 analogs. As shown in Fig. 3, the analysis of BrdU incorporation revealed that treatment with vitamin D_3 analogs resulted in a decrease in the synthesis of DNA, leading to a decrease in the number of proliferating cells. These findings suggest that 20-*epi*-$1,25(OH)_2D_3$ was more effective than $1,25(OH)_2D_3$ in inhibiting BrdU incorporation into DNA (80–90% vs. 20–50% inhibition, respectively) in the six cell lines examined. SK-N-AS and IMR5 cells were relatively resistant as $1,25(OH)_2D_3$ inhibited BrdU incorporation by about 30 and 20%, respectively.

3.4. Effect of 20-epi-1,25(OH)$_2$D$_3$ on clonal proliferation of NB cells

To determine the *in vitro* antineoplastic effect, the ability of vitamin D analogs to suppress the anchorage-independent growth in soft agar was examined. The assays were performed with a 1 μM concentration of $1,25(OH)_2D_3$ and 20-*epi*-$1,25(OH)_2D_3$ in order to achieve significant inhibition with $1,25(OH)_2D_3$. The number of colonies in the control and various treatment groups were counted and are summarized in Fig. 4. From these results it is evident that the 20-epimer caused more than 90% inhibition of colony formation in all the cell lines. In contrast, $1,25(OH)_2D_3$ inhibited colony formation by about 50% in only SH-SY5Y, NB69, SK-N-AS, and NGP cells, in spite of using a high concentration of the compound. The IMR5 and CHP134 cells were the least sensitive to $1,25(OH)_2D_3$ (36 and 25% suppression, respectively). To summarize, our results indicate that 20-*epi*-$1,25(OH)_2D_3$ exhibited a more potent antineoplastic effect than $1,25(OH)_2D_3$.

3.5. Effect of 20-epi-1,25(OH)$_2$D$_3$ on the expression of neurofilament-H

The results of the *in vitro* biological assays revealed that 20-*epi*-$1,25(OH)_2D_3$ was more potent in suppressing the proliferation of the six NB cell lines. To understand the

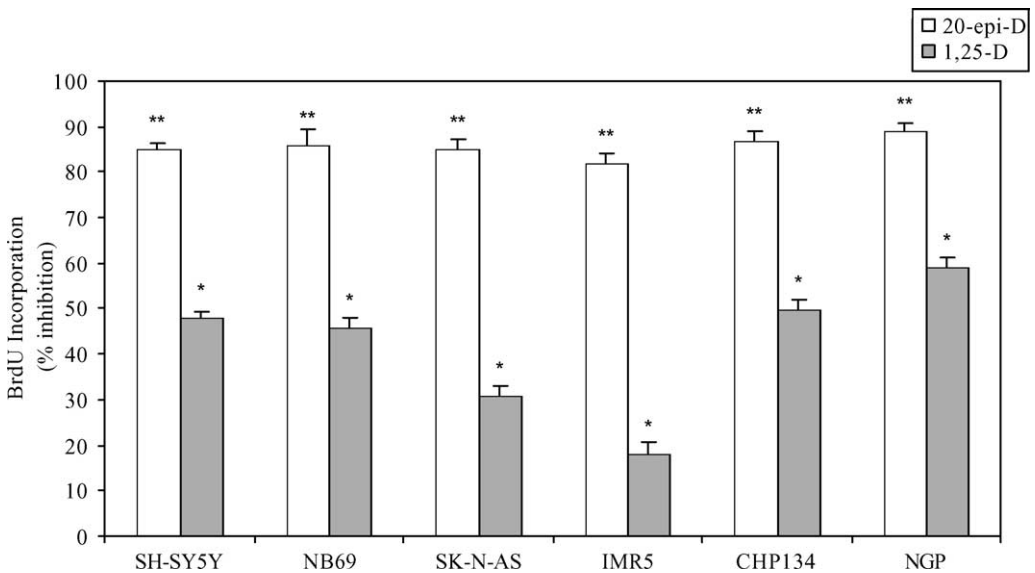

Fig. 3. Inhibition of the synthesis of DNA in NB cells by 20-*epi*-$1,25(OH)_2D_3$. The antiproliferative effects were determined after treating the NB cells for 3 days with a 1 μM concentration of vitamin D analogs and labeling with BrdU (10 μM) for 24 hr. Results are expressed as percentage inhibition of BrdU incorporation relative to control cells. Values are the means (±SEM) of three experiments with triplicate determinations. Key: $^{**}P < 0.0001$ for 20-*epi*-D, and $^{*}P < 0.005$ for 1,25-D.

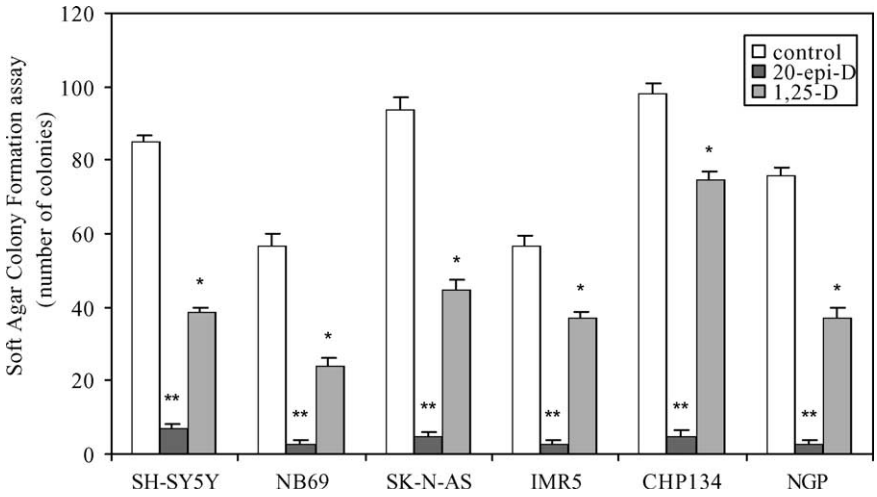

Fig. 4. Suppression of the anchorage-independent growth of NB cells by 20-*epi*-1,25$(OH)_2D_3$. Soft agar colony formation assays were performed in 6-well plates with 1000 cells per well. 1,25$(OH)_2D_3$ and 20-*epi*-1,25$(OH)_2D_3$ (1 μM) were mixed with the cells in the top agar layer and allowed to grow for 14 days. The colonies were stained with MTT reagent and counted manually. Data are shown as means ± range of two experiments. Key: $^{**}P < 0.0008$ for 20-*epi*-D, and $^{*}P < 0.005$ for 1,25-D.

mechanism of cell growth inhibition, we analyzed the critical cellular targets regulated by 20-*epi*-1,25$(OH)_2D_3$ further. It is known that NB cells, when exposed to differentiating agents, induce the expression of many neuronal markers including neurofilament-L, -M, and -H. We did not observe any evidence of morphological differentiation when NB cells were exposed to 1,25$(OH)_2D_3$ and 20-*epi*-1,25$(OH)_2D_3$ for longer periods of time (10 days). To understand whether vitamin D_3 analogs initiate the differentiation of NB cells, the expression of the neuronal marker neurofilament-H was analyzed by western blotting. As shown in Fig. 5, SH-SY5Y and NGP cell lines, when exposed to 1 μM 20-*epi*-1,25$(OH)_2D_3$ for 3 days readily induced the expression of neurofilament-H. We observed a moderate increase in neurofilament-H expression in the CHP134 and SK-N-AS cell lines, whereas there was no change in its expression in NB69 and IMR5 cells. Under identical conditions, 1,25$(OH)_2D_3$ did not cause any alterations in the expression of neurofilament-H in the six NB cells (data not shown).

3.6. Down-regulation of Myc in NB cells by 20-epi-1,25$(OH)_2D_3$

Myc is a key positive regulator of cell proliferation in many cell types [30], and down-regulation of the expression of myc mRNA and protein has been observed in retinoic acid-induced differentiation of NB cells [31]. To test whether the inhibition of cell proliferation was due to a decrease in Myc expression, NB cells were exposed to 1 μM 20-*epi*-1,25$(OH)_2D_3$ for 3 days, and the expression of c-Myc and N-Myc was examined by western blot analysis. Results presented in Fig. 6A show that control SH-SY5Y, NB69, and SK-N-AS cells expressed high levels of c-Myc. Treatment with 20-*epi*-1,25$(OH)_2D_3$ led to a dramatic decrease in c-Myc expression. Similarly, exposure of IMR5 and NGP cells to the 20-epimer resulted in decreased expression of N-Myc. We observed little difference in the expression of N-Myc in CHP134 cells under identical conditions. SH-SY5Y cells expressed low levels of N-Myc, whereas NB69 and SK-N-AS did not express N-Myc. c-Myc expression could not be detected in the IMR5, CHP134, and NGP cell lines (data not shown). It will be interesting to identify why there was no decrease in N-Myc in CHP134 cells, and also to investigate whether the mechanisms of N-*myc* gene regulation are different in this cell line.

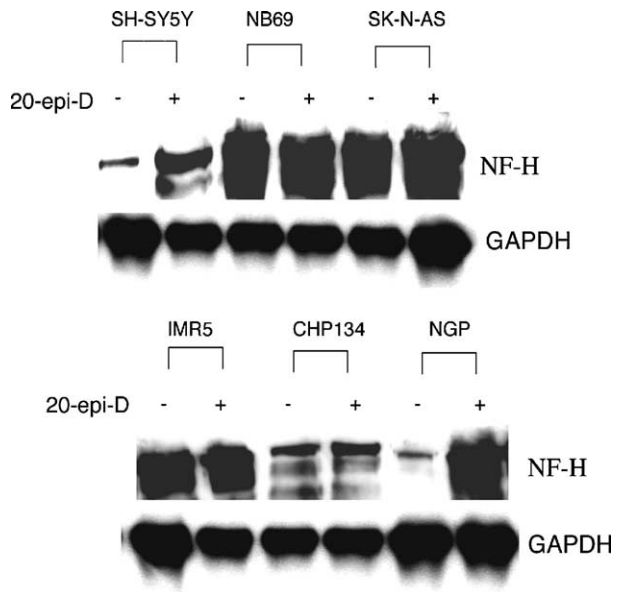

Fig. 5. Induction of neurofilament-H expression in 20-*epi*-1,25$(OH)_2D_3$-treated NB cells. The expression of neurofilament (NF-H) was determined by western blot analysis. NB cells were exposed to 1 μM 20-*epi*-1,25$(OH)_2D_3$ for 3 days. One hundred micrograms of protein was subjected to SDS–PAGE, and the blot was probed with neurofilament-H antibodies.

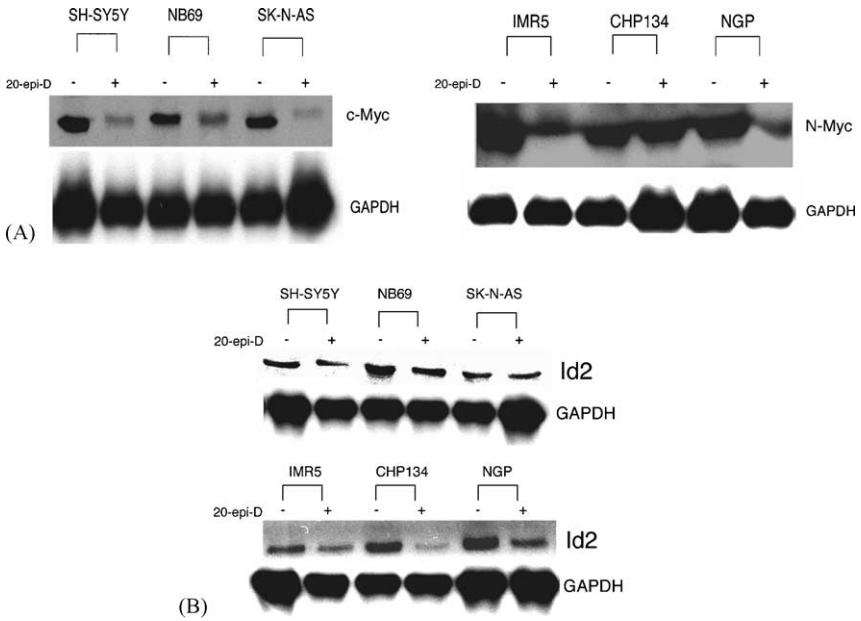

Fig. 6. Modulation of the Myc–Id2 pathway in 20-*epi*-1,25(OH)$_2$D$_3$-treated NB cells. Cells were exposed to a 1 μM concentration of 20-*epi*-1,25(OH)$_2$D$_3$ for 3 days, and protein lysates (50 μg) were subjected to western blot analysis. (A) The blots were probed with mouse monoclonal antibodies to N-Myc (NCM-II/00) and c-Myc (9E10). (B) Rabbit polyclonal antibodies were used to detect Id2 in the western blots.

3.7. Inhibitory effect of 20-epi-1,25(OH)$_2$D$_3$ on Id2 expression

Overexpression of N-*myc* is a hallmark of a majority of NBs [32]. Recently, it was found that Id2 is overexpressed in NB primary tumor tissue and in cell lines that contain N-*myc* amplification [33,34]. In most of the cell lines containing N-*myc* amplification, Id2 levels were elevated. To examine whether the decreased expression of Myc resulted in decreased levels of Id2, we performed western blot analysis of NB cells treated with 1 μM 20-*epi*-1,25(OH)$_2$D$_3$ for 3 days. A moderate decrease in Id2 protein expression (Fig. 6B) was observed, which correlated with a decrease in Myc expression. These results suggest that a decrease in Myc leads to the down-regulation of Id2 expression in NB cells. However, Id2 levels appeared to be unaffected by 20-*epi*-1,25(OH)$_2$D$_3$ in SK-N-AS cells. Interestingly, in the CHP134 cell line Id2 protein expression was down-regulated in 20-*epi*-1,25(OH)$_2$D$_3$-treated cells in spite of unaltered levels of N-Myc expression.

3.8. Induction of RAR-β in 20-epi-1,25(OH)$_2$D$_3$-treated NB cells

Abnormal function or reduced expression of specific retinoid receptors has been shown to regulate retinoid receptor-mediated gene expression in many cancer cells [35,36]. Reduced RAR-β expression plays an important role in breast, esophageal, and lung cancers [36–38]. It is known that RAR-β is undetectable in NB tumors and cell lines, and its expression is induced when cells are exposed to retinoids [39,40]. To investigate whether RAR-β is induced by vitamin D$_3$ analogs, NB cells were exposed to a 1 μM concentration of 20-*epi*-1,25(OH)$_2$D$_3$ for 3 days and total RNA was isolated. Semi-quantitative RT–PCR analysis was performed using RAR-β-specific primers that amplify a 606 bp cDNA. As shown in Fig. 7A, induction of RAR-β was observed in NB69, IMR5, CHP134, and NGP cell lines. Under the experimental conditions used, we could detect very little RAR-β in SH-SY5Y cells, and it was not induced in SK-N-AS cells. The results of the western blot analysis (Fig. 7B) correspond to the alterations observed in RT–PCR, suggesting that 20-*epi*-1,25(OH)$_2$D$_3$ regulates RAR-β expression in NB69, IMR5, CHP134, and NGP cells.

3.9. Modulation of cell cycle regulators by 20-epi-1,25(OH)$_2$D$_3$: effect on the cyclin D1 and CDK inhibitors p21^{Cip1} and p27^{Kip1}

To investigate the effect of 20-*epi*-1,25(OH)$_2$D$_3$ on cell cycle regulators, we performed semi-quantitative RT–PCR analysis of cyclin D1. As shown in Fig. 8A, we observed a decrease in the expression of cyclin D1 in SK-N-AS, IMR5, and NGP cells. However, there was no decrease in the expression of cyclin D1 in SH-SY5Y, NB69, and CHP134 cells (Fig. 8A). Western blot analysis of cyclin D1 shown in the lower portion of this panel indicates that cyclin D1 protein levels decreased in all the cell lines. Consistent with these results, we did not observe any induction of p21^{Cip1} in SH-SY5Y, NB69, and CHP134 cells (Fig. 8B), whereas it was readily induced in SK-N-AS, IMR5 and NGP cells. These results suggest that a decrease in cyclin D1 due to 20-*epi*-1,25(OH)$_2$D$_3$ treatment in combination with an increase in p21^{Cip1} play an

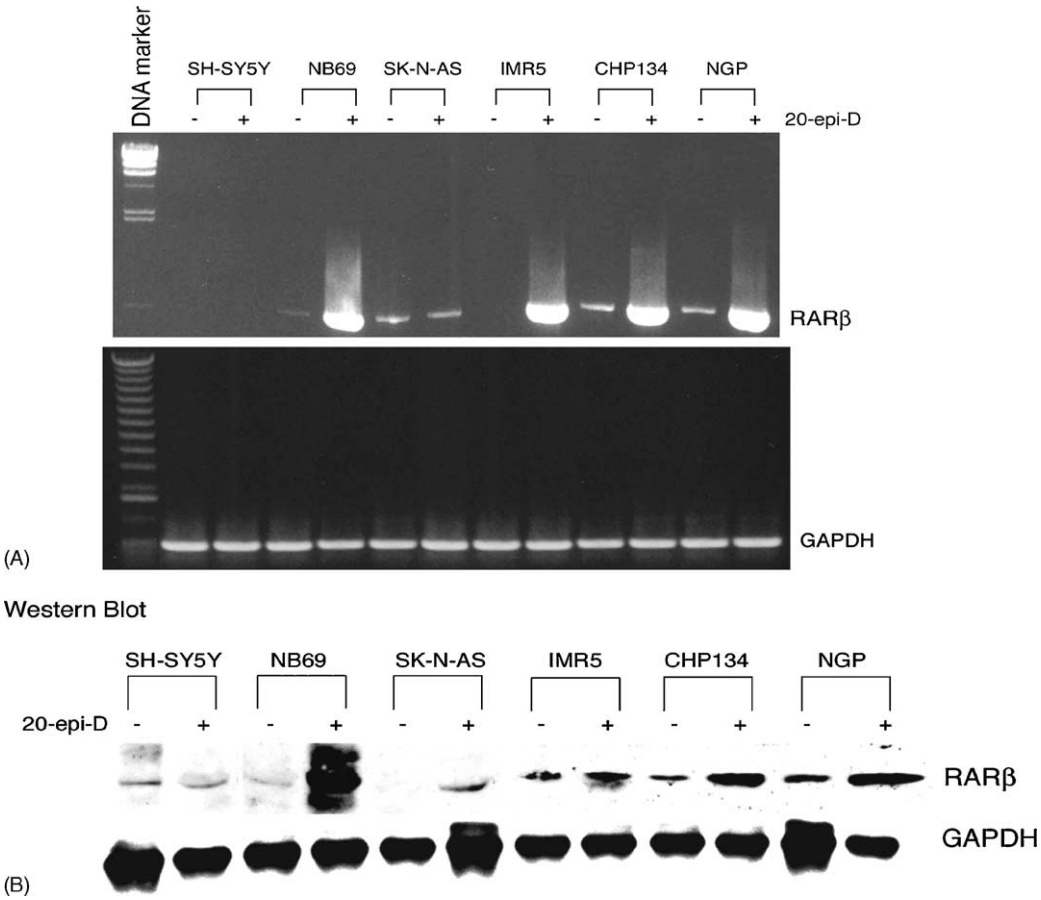

Fig. 7. Effect of 20-*epi*-1,25$(OH)_2D_3$ on RAR-β. (A) Semi-quantitative RT–PCR analysis was performed with RNA isolated from NB cell lines treated with 20-*epi*-1,25$(OH)_2D_3$ (1 μM) for 3 days. One microgram of total RNA was reverse-transcribed in a total volume of 20 μL, and 1 μL of the cDNA was used for RT–PCR. Amplification of the 606 bp PCR product represents RAR-β mRNA. (B) Western blot analysis was performed using 100 μg of protein from NB cell lines treated with 20-*epi*-1,25$(OH)_2D_3$ and probed with RAR-β antibodies (Santa Cruz).

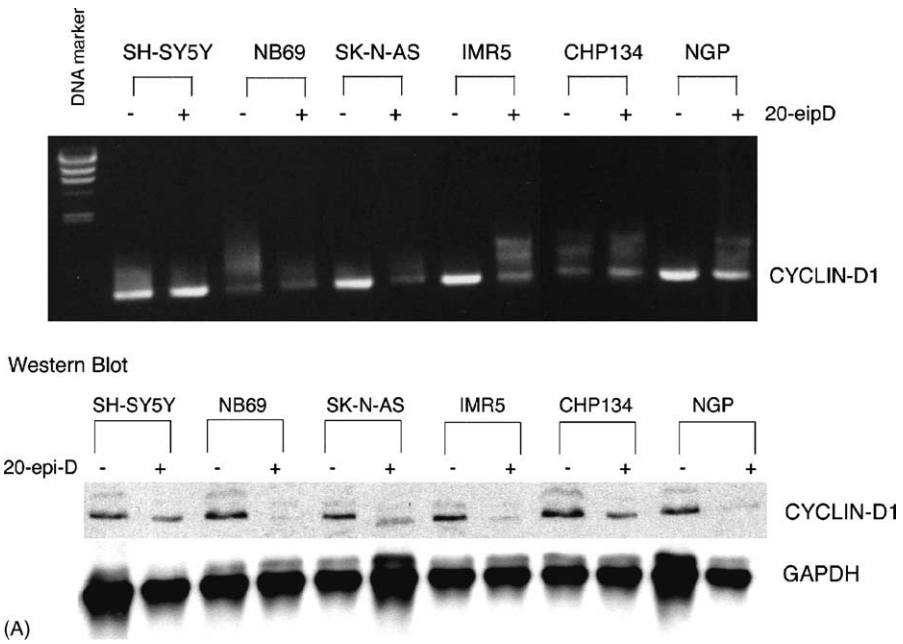

Fig. 8. Effect of 20-*epi*-1,25$(OH)_2D_3$ on cell cycle regulators in human NB cells. (A) Effect on cyclin D1. (B) Effect on $p21^{Cip1}$ and $p27^{Kip1}$. Semi-quantitative RT–PCR analysis was performed with RNA isolated from NB cell lines treated with 20-*epi*-1,25$(OH)_2D_3$ (1 μM) for 3 days. One microgram of total RNA was reverse-transcribed in a total volume of 20 μL, and 1 μL of the cDNA was used for RT–PCR. The blots in the lower portion of each panel indicate the results of western blot analysis for cyclin D1 and $p21^{cip1}$. Appropriate GAPDH controls were included in the PCR and western blots.

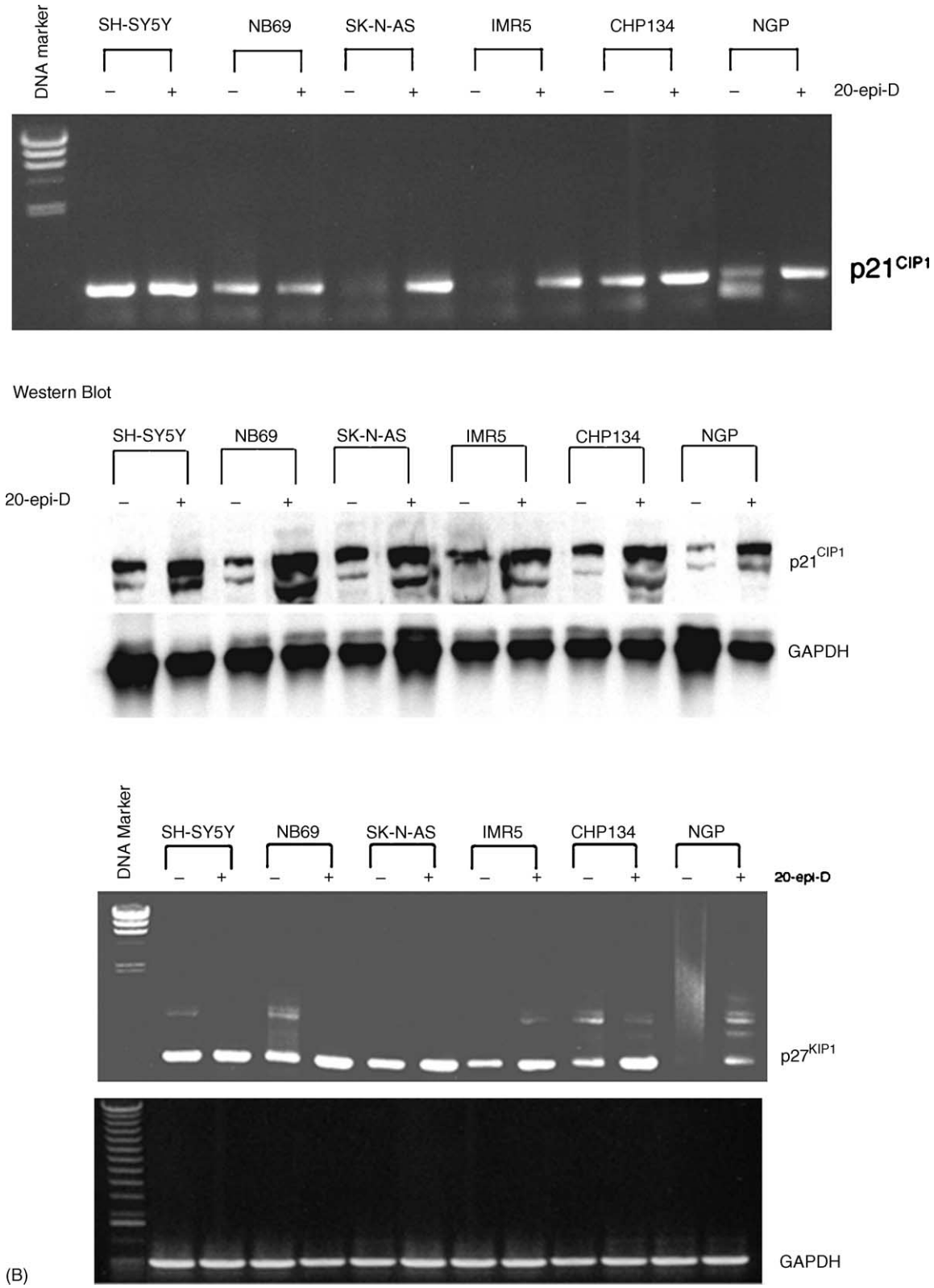

Fig. 8. (*Continued*).

important role in causing NB cell growth arrest. We also demonstrated that these changes in the $p21^{Cip1}$ RNA levels are reflected at the protein level. 20-*epi*-1,25$(OH)_2D_3$ also induced the expression of $p27^{Kip1}$ in NB69, IMR5, CHP134, and NGP cells (Fig. 8B), but no change in $p27^{Kip1}$ expression was observed in SH-SY5Y and SK-N-AS cells. These results suggest that each NB cell line appears to respond differently when exposed to 20-*epi*-1,25$(OH)_2D_3$. However,

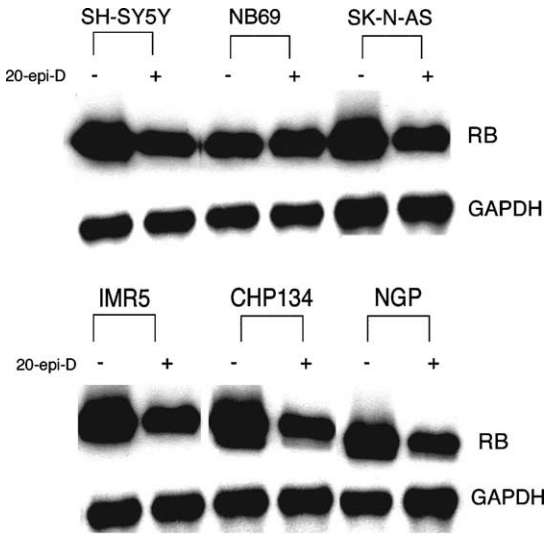

Fig. 9. Alterations in pRB phosphorylation of NB cells exposed to 20-*epi*-1,25$(OH)_2D_3$. Expression and phosphorylation of pRB in NB cell lines treated with 20-*epi*-1,25$(OH)_2D_3$ (1 μM for 3 days) were determined by western blot analysis using 100 μg of protein and probed with pRB monoclonal antibodies.

we did not observe similar alterations in p27 protein by western blot analysis (data not shown), suggesting that post-translational regulatory mechanisms may play a role in modulating p27 in NB cells.

3.10. Modulation of pRB phosphorylation by 20-epi-1,25(OH)$_2$D$_3$

It is known that treatment of NB cells with retinoids induces cell cycle arrest in the G_0/G_1 phase [41]. pRB phosphorylation plays an important role in determining the extent of G_1 arrest [42]. To test whether vitamin D_3 analogs modulate pRB function, we examined the status of pRB phosphorylation in cells exposed to 20-*epi*-1,25$(OH)_2D_3$ by western blot analysis. Exposure of NB cells to 20-*epi*-1,25$(OH)_2D_3$ resulted in hypophosphorylation of pRB (Fig. 9). We did not observe any significant difference in the phosphorylation of pRB in NB69 cells; these cells may be relatively resistant to the actions of 20-*epi*-1,25$(OH)_2D_3$. It is possible that hypophosphorylated pRB in 20-*epi*-1,25$(OH)_2D_3$-treated cells results in sequestering E2F as a pRB–E2F complex, making it unavailable to activate genes that promote the cell cycle at the G_1-S phase.

4. Discussion

Results of cell viability, proliferation, and soft agar colony formation assay indicate that 20-*epi*-1,25$(OH)_2D_3$ is more potent than 1,25$(OH)_2D_3$. Consistent with the results of the MTT assay, 20-*epi*-1,25$(OH)_2D_3$ strongly inhibited the incorporation of BrdU (80–90%) in all of the cell lines. In contrast, 1,25$(OH)_2D_3$ was the least effective (20–50% inhibition) in inhibiting BrdU incorporation into DNA. The potent anticancer activity of 20-*epi*-1,25$(OH)_2D_3$ was also evident in the soft agar colony formation assay. To obtain significant inhibition with 1,25$(OH)_2D_3$, we used a high concentration (1 μM) of the vitamin D_3 compounds in these assays. Our overall objective was to evaluate the effect of vitamin D_3 analogs in several NB cell lines to identify the possible target genes. In this paper, we used 20-*epi*-1,25$(OH)_2D_3$ as a model compound to investigate the possible signaling pathways regulated by vitamin D analogs. By employing 20-*epi*-1,25$(OH)_2D_3$ as a model compound, our studies identified the critical cell pathways modulated in NB cells.

Demonstrating for the first time that these important cell pathways are targets of vitamin D analogs will enable us to evaluate the effect of low calcemic analogs in modulating NB cell growth by altering the function of critical targets (Myc–pRB). Such high concentrations of 1,25$(OH)_2D_3$ have been used by earlier investigators to examine the growth inhibitory effects in leukemia and lung cancer cells [23,43]. We observed about 90% inhibition of colony formation with 20-*epi*-1,25$(OH)_2D_3$, whereas 1,25$(OH)_2D_3$ inhibited colony formation by only 40%. These results indicate that 20-*epi*-1,25 $(OH)_2D_3$ was more potent in inhibiting cell proliferation and also in suppressing colony formation in soft agar. The potent antiproliferative effects of the 20-*epi* class of vitamin D_3 analogs have been attributed to the stereochemistry at carbon 20 [44]. Crystal structure analysis of VDR liganded to 20-*epi*-1,25$(OH)_2D_3$ suggests that ligand–VDR complexes exhibit higher stability and longer half-life contributing to prolonged biological activity [45]. A member of the 20-*epi*-family, KH1060, also exhibits potent growth inhibition at a several fold less concentration than 1,25$(OH)_2D_3$ [15,20]. This could be due to conformational changes induced by 20-*epi*-1,25$(OH)_2D_3$ compounds in the ligand binding domain of VDR. This renders the receptor more resistant to protease digestion thereby increasing the bioavailability of the compounds, leading to potent antiproliferative effects [22,23,45].

The genetic hallmark of NB is amplification of the N-*myc* proto-oncogene [32]. Two recent studies attribute critical function to the N-Myc–Id2 pathway in NB tumors and cell lines [33,34]. Expression of Id2 is essential to regulate the proliferation and differentiation of the neural crest. In humans, neoplastic transformation of neural crest precursors during embryogenesis causes NB. Human NB tumors with N-*myc* amplification are associated with overexpression of Id2. Most of the N-*myc* amplified cell lines express 20- to 30-fold more Id2 than cell lines without N-*myc* amplification. N-Myc proteins directly bind to and activate the *Id2* promoter, and its expression correlates with the N-Myc during development and in NB [34]. High levels of Id2 bind to the hypophosphorylated form of pRB and release E2F, which activates genes associated with cell proliferation. Id2, a protein required to maintain the timing of differentiation during development, is transformed into a potent oncogene in NB. Therefore, disrupting the function

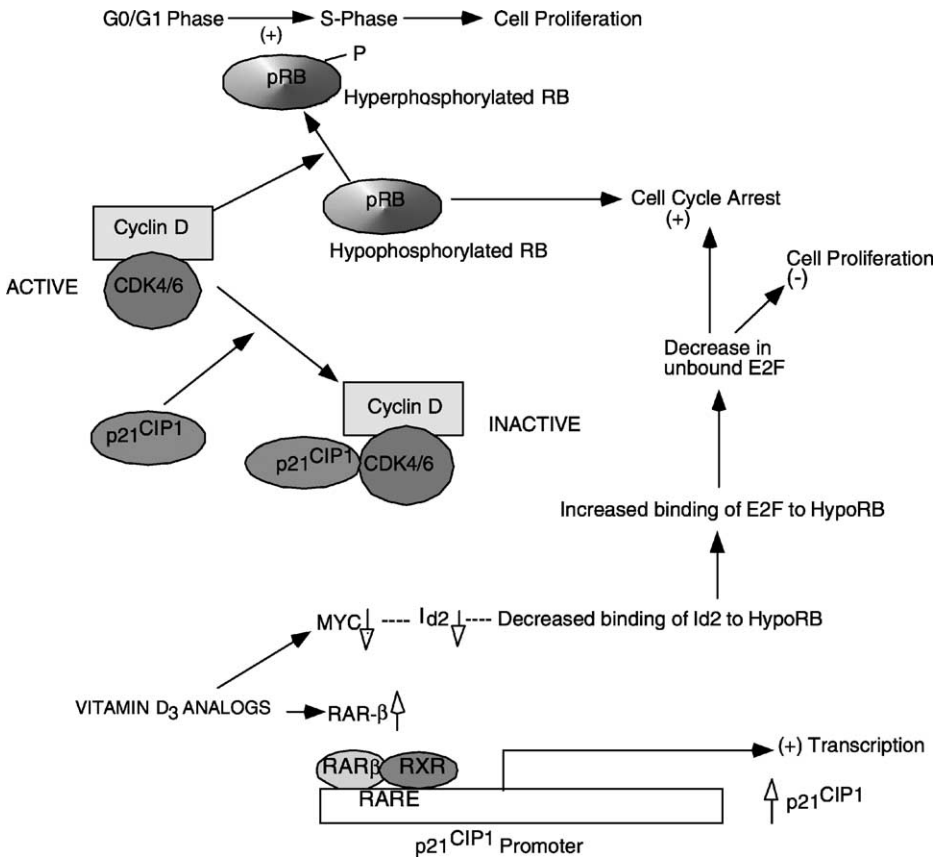

Fig. 10. Schematic representation of vitamin D_3 signaling in neuroblastoma. Based on our results and earlier studies, we postulated a model to explain the cell growth arrest induced by vitamin D_3 analogs. Suppression of the Myc–Id2 network, induction of RAR-β, and modulation of other G_1 signaling proteins are depicted. Apart from its effect on $p21^{cip1}$ expression, other mechanisms of action of RAR-β and the effect of vitamin D_3 on cyclin E, $p16^{INK4}$, and E2F in NB cells are yet to be determined.

of Id2 may lead to new and useful therapeutic strategies in NB therapy. Our results suggest that 20-*epi*-1,25$(OH)_2D_3$ down-regulated c-Myc and N-Myc expression. Decreased levels of Myc result in reduced transcriptional activation of the *Id2* promoter. Decreased levels of Id2 result in decreased binding of Id2 to hypophosphorylated pRB (Fig. 10). Availability of more hypophosphorylated pRB sequesters E2F transcription factors. Thus, the cellular levels of unbound E2F are reduced, resulting in the altered activation of genes that participate in DNA synthesis and cell proliferation. In addition, treatment of NB cells with 20-*epi*-1,25$(OH)_2D_3$ also leads to decreased phosphorylation of pRB due to a decrease in cyclin D1 levels, which might decrease cyclin D1-CDK activities.

Retinoic acid-induced differentiation of NB cells is accompanied by induction of RAR-β expression [40]. Our results indicate that the 20-epimer induces RAR-β in some of the NB cell lines examined (Fig. 7). These results are in agreement with earlier observations indicating that RAR-β is readily induced in NB cells undergoing differentiation [39,40]. Increased RAR-β binds to the *p21*^{Cip1} promoter and increases its transcription in myeloid and NB cell lines [35,39,46]. Increased $p21^{Cip1}$ by associating with cyclin D–CDK complexes might inhibit the kinase activity leading to decreased phosphorylation of pRB. Accumulation of hypophosphorylated pRB sequesters E2F transcription factors and causes cell cycle arrest in the G_1 phase. It is possible that 20-*epi*-1,25$(OH)_2D_3$, apart from indirectly increasing $p21^{Cip1}$ expression by inducing RAR-β, can also have a direct effect on $p21^{Cip1}$ expression due to the presence of a functional VDRE in the *p21*^{Cip1} promoter. In addition, 20-*epi*-1,25$(OH)_2D_3$ also down-regulated the expression of cyclin D1 in the SK-N-AS, IMR5, and NGP cell lines, suggesting that the decrease in phosphorylated pRB could also arise because of a decrease in the expression of cyclin D1 in these cell lines [47]. Further studies are necessary to understand how 20-*epi*-1,25$(OH)_2D_3$ regulates cyclin D1 expression and also the mechanisms regulating $p27^{Kip1}$ expression in NB cells [48]. Thus, the changes in the expression and activity of key regulators of the cell cycle could potentially lead to G_1 arrest. In this study, we have shown that treatment of NB cells with 20-*epi*-1,25$(OH)_2D_3$ induces RAR-β tumor suppressor, down-regulates the Myc–Id2 network, induces $p21^{Cip1}$, and modulates the activities of key G_1 signaling proteins. Fig. 10 shows the possible signaling pathways activated by vitamin D_3 analogs in NB cells. Further studies are necessary to delineate the role of other proteins

involved in controlling G_1 arrest in NB cell lines by vitamin D_3 analogs. In summary, our results indicate that 20-*epi*-1,25$(OH)_2D_3$ exerts strong antiproliferative effects by interfering with critical cell pathways in NB cells. Identification of novel low-calcemic vitamin D_3 analogs that act in combination with retinoids, hence increasing their efficacy, is likely to improve the survival rate in NB. Future investigations in this direction are necessary to examine the mechanisms of action and therapeutic potential of novel vitamin D analogs.

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References

[1] Brodeur GM, Nakagawara A. Molecular basis of clinical heterogeneity in neuroblastoma. Am J Pediatr Hematol Oncol 1992;14: 111–6.

[2] Sidell N. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells. J Natl Cancer Inst 1982;68:589–96.

[3] Jaffe N. Neuroblastoma: review of the literature and an examination of factors contributing to its enigmatic character. Cancer Treat Rev 1976; 3:61–82.

[4] Maris JM, Matthay KK. Molecular biology of neuroblastoma. J Clin Oncol 1999;17:2264–79.

[5] Kushner BH, Cheung NK. Neuroblastoma. Pediatr Ann 1988;17: 269–76.

[6] Reynolds CP, Lemons RS. Retinoid therapy of childhood cancer. Hematol Oncol Clin North Am 2001;15:867–910.

[7] Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, Swift P, Shimada H, Black CT, Brodeur GM, Gerbing RB, Reynolds CP, for the Children's Cancer Group. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-*cis*-retinoic acid. N Engl J Med 1999;341:1165–73.

[8] Evans TR, Kaye SB. Retinoids: present role and future potential. Br J Cancer 1999;80:1–8.

[9] Haussler M, Sidell N, Kelly M, Donaldson C, Altman A, Mangelsdorf D. Specific high affinity binding and biologic action of retinoic acid in human neuroblastoma cell lines. Proc Natl Acad Sci USA 1983; 80:5525–9.

[10] Thiele CJ, Reynolds CP, Israel MA. Decreased expression of N-*myc* precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 1985;313:404–6.

[11] Reynolds CP, Kane DJ, Einhorn PA, Matthay KK, Crouse VL, Wilbur JR, Shurin SB, Seeger RC. Response of neuroblastoma to retinoic acid in vitro and in vivo. Prog Clin Biol Res 1991;366:203–11.

[12] Bollag W, Peck R. Cancer chemotherapy by combination of retinoids with cytokines and vitamin D analogs. Experimental and clinical results. Ann Oncol 1994;5:17–22.

[13] Blutt SE, Allegretto EA, Pike JW, Weigel NL. 1,25-Dihydroxyvitamin D_3 and 9-*cis*-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G_1. Endocrinology 1997;138:1491–7.

[14] Wang Q, Yang W, Uytingco MS, Christakos S, Weider R. 1,25-Dihydroxyvitamin D_3 and all-*trans*-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Res 2000; 60:2040–8.

[15] Elstner E, Linker-Israeli M, Said J, Umiel T, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP. 20-*epi*-vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 1995;55:2822–30.

[16] Freedman LP. Transcriptional targets of the vitamin D_3 receptor-mediating cell cycle arrest and differentiation. J Nutr 1999;129: 581–6.

[17] Schräder M, Bendik I, Becker-André M, Carlberg C. Interaction between retinoic acid and vitamin D signaling pathways. J Biol Chem 1993;268:17830–6.

[18] Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF, Hunziker W. Two nuclear signaling pathways for vitamin D. Nature 1993;361:657–60.

[19] Ettinger RA, DeLuca HF. The vitamin D endocrine system and its therapeutic potential. Adv Drug Res 1996;28:269–312.

[20] Guyton KZ, Kensler TW, Posner GH. Cancer chemoprevention using natural vitamin D and synthetic analogs. Annu Rev Pharmacol Toxicol 2001;41:421–42.

[21] Nishii Y, Okano T. History of the development of new vitamin D analogs: studies on 22-oxacalcitriol (OCT) and 2β-(3-hydroxypropoxy)calcitriol (ED-71). Steroids 2001;66:137–46.

[22] Binderup L, Latini S, Binderup E, Brettin C, Calverley M, Hansen K. 20-*epi*-vitamin D_3 analogues: a novel class of potent regulators of cell growth and immune responses. Biochem Pharmacol 1991;42:1569–75.

[23] Elstner E, Linker-Israeli M, Le J, Umiel T, Michl P, Said JW, Binderup L, Reed JC, Koeffler HP. Synergistic decrease of clonal proliferation, induction of differentiation, and apoptosis of acute promyelocytic leukemia cells after combined treatment with novel 20-*epi*-vitamin D_3 analogs and 9-*cis*-retinoic acid. J Clin Invest 1997;99:349–60.

[24] Scaglione-Sewell BA, Bissonnette M, Skarosi S, Abraham C, Brasitus TA. A vitamin D_3 analog induces a G_1 phase arrest in CaCo-2 cells by inhibiting Cdk2 and Cdk6: roles of cyclin E, $p21^{Waf1}$ and $p27^{Kip1}$. Endocrinology 2000;141:3931–9.

[25] Stio M, Celli A, Treves C. Synergistic antiproliferative effects of vitamin D derivatives and 9-*cis*-retinoic acid in SH-SY5Y human neuroblastoma cells. J Steroid Biochem Mol Biol 2001;77:213–22.

[26] Moore TB, Koeffler HP, Yamashiro JM, Wada RK. Vitamin D_3 analogs inhibit growth and induce differentiation in LA-N-5 human neuroblastoma cells. Clin Exp Metastasis 1996;14:239–45.

[27] Sivak LE, Tai KF, Smith RS, Dillon PA, Brodeur GM, Carroll WL. Autoregulation of the human N-*myc* oncogene is disrupted in amplified but not single-copy neuroblastoma cell lines. Oncogene 1997;15:1937–46.

[28] Reynolds CP, Biedler JL, Spengler BA, Reynolds DA, Ross RA, Frenkel EP, Smith RG. Characterization of human neuroblastoma cell lines established before and after therapy. J Natl Cancer Inst 1986;76:375–87.

[29] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

[30] Grandori C, Cowley SM, James LP, Eisenman RN. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 2000;16:653–99.

[31] Redfern CPF, Lovat PE, Malcolm AJ, Pearson ADJ. Gene expression and neuroblastoma cell differentiation in response to retinoic acid: differential effects of 9-*cis*- and all-*trans*-retinoic acid. Eur J Cancer 1995;31:486–94.

[32] Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D. Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313: 1111–6.

[33] Lasorella A, Noseda M, Beyna M, Lavarone A. Id2 is a retinoblastoma protein target and mediates signaling by Myc oncoproteins. Nature 2000;407:592–8.

[34] Lasorella A, Boldrini R, Dominici C, Donfrancesco A, Yokota Y, Inserra A, Iavarone A. Id2 is critical for cellular proliferation and is the oncogenic effector of N-Myc in human neuroblastoma. Cancer Res 2002;62:301–6.

[35] Defacque H, Commes T, Sevilla C, Rochette-Egly C, Marti J. Synergistic differentiation of U937 cells by all-*trans*-retinoic acid and 1α,25-dihydroxyvitamin D3 is associated with the expression of retinoid X receptor α. Biochem Biophys Res Commun 1994;203: 272–80.

[36] Qiu H, Zhang W, El-Naggar AK, Lippman SM, Lin P, Lotan R, Xu XC. Loss of retinoic acid receptor-β expression is an early event during esophageal carcinogenesis. Am J Pathol 1999;155: 1519–23.

[37] Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG. High frequency of retinoic acid receptor-β abnormalities in human lung cancer. Oncogene 1991;6:1859–68.

[38] Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down-regulation of retinoic acid receptor-β in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. Cell Growth Differ 1994;5:133–41.

[39] Cheung B, Hocker JE, Smith SA, Norris MD, Haber M, Marshall GM. Favorable prognostic significance of high-level retinoic acid receptor-β expression in neuroblastoma mediated by effects on cell cycle regulation. Oncogene 1998;17:751–9.

[40] Clagett-Dame M, Verhalen TJ, Biedler JL, Repa JJ. Identification and characterization of all-*trans*-retinoic acid receptor transcripts and receptor protein in human neuroblastoma cells. Arch Biochem Biophys 1993;300:684–93.

[41] Easton J, Wei T, Lahti JM, Kidd VJ. Disruption of the cyclin D/cyclin-dependent kinase/INK4/retinoblastoma protein regulatory pathway in human neuroblastoma. Cancer Res 1998;58:2624–32.

[42] Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1 phase progression. Genes Dev 1999;13:1501–12.

[43] Güzey M, Sattler C, DeLuca HF. Combinational effects of vitamin D_3 and retinoic acid (all-*trans* and 9-*cis*) on proliferation, differentiation and programmed cell death in two small cell lung carcinoma cell lines. Biochem Biophys Res Commun 1998;249:735–44.

[44] Peleg S, Sastry M, Collins ED, Bishop JE, Norman AW. Distinct conformational changes induced by 20-*epi* analogues of 1α,25-dihydroxyvitamin D_3 are associated with enhanced activation of the vitamin D receptor. J Biol Chem 1995;270:10551–8.

[45] Tocchini-Valentini G, Rochel N, Wurtz JM, Mitschler A, Moras D. Crystal structures of the vitamin D receptor complexed to superagonist 20-*epi* ligands. Proc Natl Acad Sci USA 2001;98:5491–6.

[46] Liu M, Iavarone A, Freedman LP. Transcriptional activation of the human $p21^{WAF1/CIP1}$ gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. J Biol Chem 1996; 271:31723–8.

[47] Wainwright LJ, Lasorella A, Iavarone A. Distinct mechanisms of cell cycle arrest control the decision between differentiation and senescence in human neuroblastoma cells. Proc Natl Acad Sci USA 2001; 98:9396–400.

[48] Matsuo T, Thiele CJ. $p27^{Kip1}$: a key mediator of retinoic acid-induced growth arrest in the SMS-KCNR human neuroblastoma cell line. Oncogene 1998;16:3337–43.