Vitamin D_3 analogue EB1089 inhibits the proliferation of human laryngeal squamous carcinoma cells via p57

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Abstract

The objective of this study is to evaluate the role of the cyclin-dependent kinase inhibitor p57 in EB1089-inhibited proliferation of human laryngeal squamous carcinoma cells (HEp-2). HEp-2 cells were treated with the vitamin D_3 analogue EB1089 for 48 h and total RNA was extracted for reverse transcription-PCR amplification using primers for the p57 coding sequence. Proteins were detected by Western blot analysis. For interference using silencing RNA (siRNA), HEp-2 cells were transfected with siRNA specific for p57 (siRNA-p57) or a negative control sequence (siRNA-con) followed by treatment with 10 nmol/L EB1089. The effects of EB1089 on cell proliferation were evaluated by 5-bromo-2′-deoxyuridine incorporation and '3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay. Cell death and cell cycle dynamics were monitored using flow cytometry. EB1089 significantly inhibited HEp-2 cell proliferation and increased p57 mRNA and protein levels; this was blocked by siRNA-p57 but not by siRNA-con. The EB1089-induced suppression of HEp-2 cell proliferation recovered to near-normal levels with siRNA-p57 transfection. EB1089 inhibits the proliferation of HEp-2 cells and p57 plays an important role in this. [Mol Cancer Ther 2008;7(5):1268–74]

Introduction

1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the active form of vitamin D_3, inhibits the proliferation of cultured cells derived from a number of tumors (1, 2). Analogues of $1,25(OH)_2D_3$ inhibit cell proliferation *in vitro* and models of myeloid leukemia and carcinomas of the breast, prostate, and colon *in vivo* (3–6). The main barrier to the clinical use of $1,25(OH)_2D_3$ has been its hypercalcemic effects; more than 800 analogues have been developed in an attempt to maintain the inhibitory effect on tumor cell proliferation while reducing hypercalcemia (7, 8). One such analogue is EB1089, a derivative with a side chain modified to render it less susceptible to catabolic degradation. EB1089 was 60 times more potent than $1,25(OH)_2D_3$ in inhibiting the growth of MCF-7 breast cancer cells *in vitro* and 100 times more potent than $1,25(OH)_2D_3$ in inhibiting tumor growth in animal models of breast cancer, with only half the hypercalcemic activity (5). These potent growth-inhibitory properties and the potentially limited toxicity of EB1089 suggest that it might be effective for chemotherapy or for the chemoprevention of human cancers, especially in human laryngeal squamous carcinoma cells (SCC).

The actions of EB1089 are thought to be mediated by the vitamin D receptor, a transcription factor and member of the steroid/thyroid/retinoid nuclear receptor superfamily (9). However, the antiproliferative activity of $1,25(OH)_2D_3$ is caused by expression of the cell cycle inhibitory proteins p21 and p27. These arrest the cell cycle at G_0-G_1 in SCCs from the head and neck. This ability of the vitamin D analogue EB1089 to act via the same molecular mechanism as the natural hormone but with less hypercalcemic activity could have therapeutic implications for patients with such cancers (10). However, the effects of EB1089 on proliferation and cell cycle regulation in human laryngeal SCCs remain unclear.

Cyclin-dependent kinase (CDK) inhibitors play important roles in proliferation and cell cycle regulation. Among these, p57, a protein with 316 amino acids, acts as a potent inhibitor of G_1-S-phase CDKs (11) and causes cell cycle arrest at G_1. Complete knockout of the gene for p57 in mice results in embryonic/neonatal lethality, multiple developmental defects such as altered differentiation and apoptosis, and altered cellular proliferation (12–14). Thus, p57 has an important regulatory role in cell proliferation and differentiation. p57 expression is also decreased in patients with laryngeal SCCs showing advanced tumor size, worse clinical stage, and the recurrence of disease. The overall 5-year disease-free survival rate of patients with laryngeal SCCs was significantly higher in p57-positive than in p57-negative groups. Thus, reduction of p57 expression is related to carcinogenesis in the laryngeal mucosa. Assessment of p57 expression may be a valid biomarker in patients with laryngeal SCCs. However, it remains unclear how EB1089 exerts its antiproliferative effects in such cells.

In this study, we examined whether EB1089 could inhibit the growth of human laryngeal SCC lines *in vitro* and *in vivo*. In addition, we analyzed the regulation of EB1089 target genes encoding the CDK inhibitor p57, whose action

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has been associated with growth arrest. We used silencing RNA (siRNA) against p57 to knock down the EB1089-stimulated expression of p57. We also investigated whether this technique could reverse EB1089-induced inhibition of cell proliferation in human laryngeal SCCs. We conclude that p57 is an important cellular target for the action of EB1089 on such cells.

Materials and Methods

Cell Culture

The human larynx carcinoma cell line HEp-2 was donated by the Department of Pathology, Fourth Military Medical University. SCC25 (head and neck squamous cell carcinoma) and FaDu (pharynx squamous cell carcinoma) cells were obtained from the American Type Culture Collection. Cells and transfectants were cultured at 37°C in a 5% CO_2 incubator in DMEM (Calbiochem) supplemented with 10% fetal bovine serum (FBS; Nova-Tech), 1% penicillin/streptomycin, and 2 mmol/L L-glutamine (Life Technologies).

Reagents and Antibodies

EB1089 (22,24-diene-24a,26a,27a-trihomo-1,25-dihydroxyvitamin D_3) was donated by LEO Pharmaceutical Products and was used as described (15). Antibodies against p57, p21, and p27 were from Santa Cruz Biotechnology. Anti-β-actin antibody was obtained from Sigma-Aldrich.

DNA Synthesis Assay

DNA was measured using ELISA according to the manufacturer's protocol (Amersham Biosciences) based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis of proliferating cells. Briefly, 10,000 cells were seeded in each well of a 96-well plate and left for 24 h. Cells were then synchronized *in situ* by incubation with serum-depleted medium for 24 h. They were then treated with EB1089 (0, 1, or 10 nmol/L) for the indicated times. BrdU incorporation was measured after 4 h. The colorimetric change was measured at 450 nm on a microplate reader (Quant, BioTek Instruments).

3-(4,5-Dimethylthiazol-2-yl)2,5-Diphenyltetrazolium Bromide Assay

Cellular toxicity and proliferation were further measured by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay, which can detect cell cytotoxicity or proliferation (16, 17). Briefly, HEp-2 cells (1×10^4) were seeded into each well of 96-well plates. The cells ($n = 6$ wells in each group) were cultured in a 5% CO_2 incubator. After 24 h, the cells were treated with EB1089 (1 or 10 nmol/L) or with a mock control for the indicated times. All culture media were then replaced with 100 μL fresh 10% FBS/DMEM plus 20 μL MTT reagent at 37°C for 4 h; 10% DMSO (25 μL) was added to stop the reaction. Optical absorbance was determined using a microplate reader (Quant, BioTek Instruments) at 590 nm.

Detection of *In vitro* Cell Death and Cell Cycle by Flow Cytometry

To analyze the cell cycle by propidium iodide staining and apoptotic cells by Annexin V staining, cells were placed in six-well plates at 3×10^5 per well and cultured in 10% FBS until 80% confluence. After exposure to 10 nmol/L EB1089 for the indicated time, the cells were collected and fixed in ice-cold 75% ethanol at -20°C overnight. Fixed cells were stained in buffer containing 100 mmol/L sodium citrate, 0.1% Triton X-100, 0.2 mg/mL RNase A, and 50 μg/mL propidium iodide at 4°C for 1 h and then analyzed using an Epics XL FACS cytometer (Beckman Coulter) as described (18).

Inhibition of HEp-2 Cell Tumorigenesis by EB1089 in Nude Mice

BALB/c athymic nude mice (5 weeks old) were purchased from Chinese Academy of Science Shanghai SLAC Laboratory Animal and acclimated for 1 week. Mice were used when they reached 6 weeks old (~20 g). Implants of 7.5×10^6 HEp-2 cells in 0.2 mL growth medium without serum were placed in the flank of each mouse ($n = 32$). Tumors were allowed to grow and volumes were measured in two dimensions with calipers. Volumes were calculated using the formula: $(L \times W^2) \times 0.5$, where L is the length and W is the width in cm (19). When tumor volumes reached 0.05 cm^3, 0.20 μg/kg EB1089 was administered i.p. daily for 5 days each week for 5 weeks. Another 16 nude mice were injected with 0.9% saline and ethanol (9:1) vehicle alone as mock controls. Tumor volumes were measured and compared with those of the controls.

Cell Transfection

Commercially available siRNA-p57 (sc-35125) duplexes and control siRNA-A (sc-37007) were from Santa Cruz Biotechnology. siRNA-p57 is a pool of three target-specific 20- to 25-nucleotide siRNAs designed to knock down p57 gene expression. Negative control siRNA (siRNA-con) consisted of a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA. The HEp-2 cells were transferred using siRNA duplexes according to the manufacturer's protocol. After 24 h, the transfected cells were reseeded per well of a 96-well plate (1×10^4) or 6-well plate (3×10^5) and left for 24 h. The cells were treated with EB1089 (0 or 10 nmol/L) for 96 h. BrdU incorporation, MTT assay, cell death, and protein expression were then measured.

RNA Extraction and Reverse Transcription-PCR

HEp-2 cells suspended in DMEM with 10% FBS were added to each well of six-well plates. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. After the cell confluence reached 80%, the cell culture medium was replaced with DMEM with 0.1% FBS and incubated for 24 h, and the cells were treated with a final concentration of 0, 1, or 10 nmol/L EB1089 in DMEM with 0.1% FBS. Cells were collected 2 days after exposure to EB1089, and total RNA was extracted from the cells using Trizol reagent (Invitrogen). cDNAs were synthesized using a ThermoScript RT-PCR system according to the manufacturer's instructions (Invitrogen). Two oligonucleotides (5′-agatcagcgcctgagaagtc-3′ and 5′-gggaccagtgtaccttctcg-3′) were used as specific primers to amplify the human p57 sequence. The human β-actin cDNA fragments were amplified by the primers 5′-gctcgtcgtcgacaacggct-3′ and

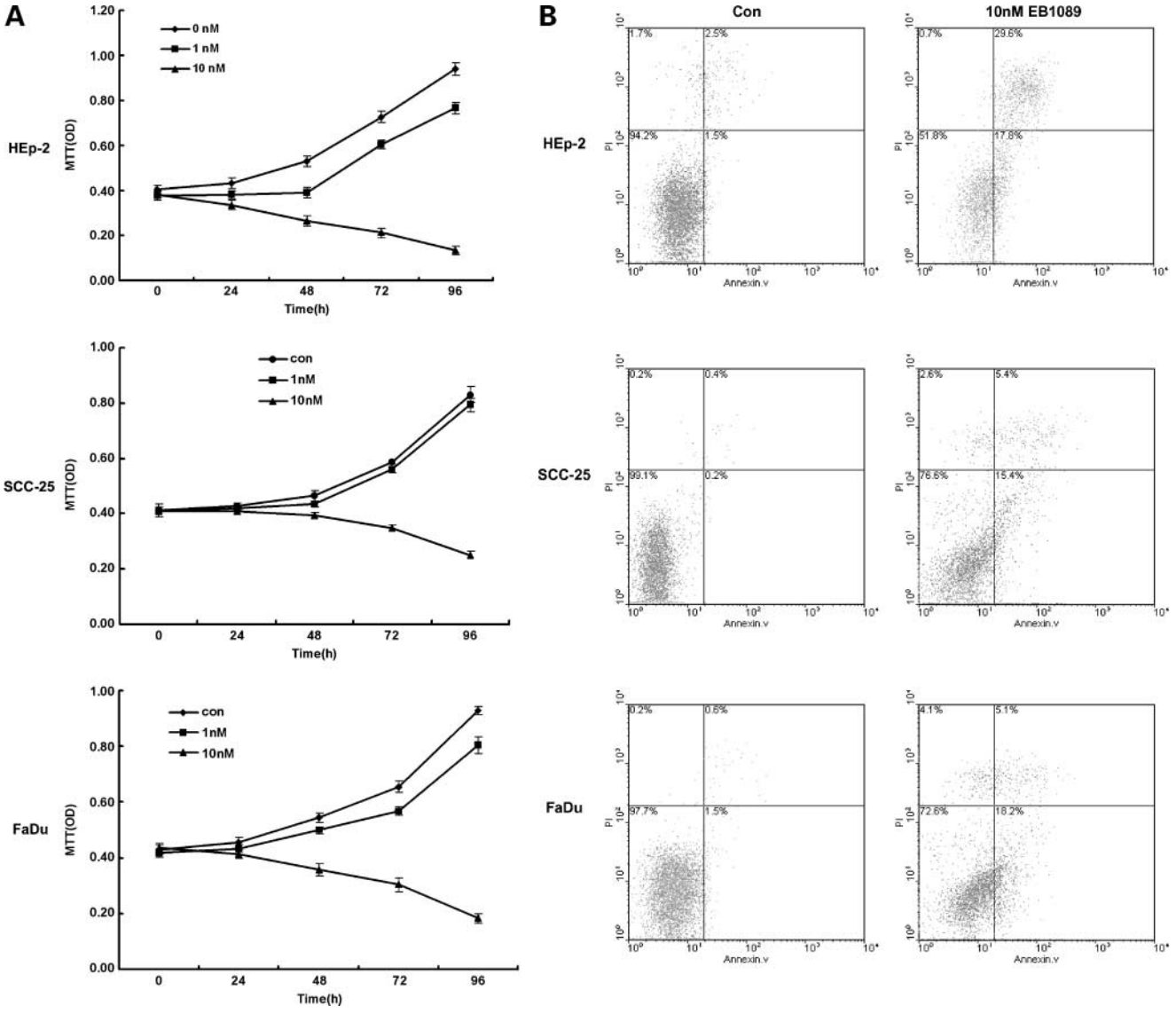

Figure 1. Cell proliferation is inhibited in human SCCs (HEp-2, SCC25, and FaDu) *in vitro*. The cells were grown as described in Materials and Methods and were treated with vehicle (control) or EB1089 (1 or 10 nmol/L) for the indicated times. **A,** cell growth was measured by MTT assay. Three separate experiments were done in duplicate. *Points,* mean of three experiments; *bars,* SE. **B,** cell death was determined using Annexin V staining and detected by flow cytometry (three independent experiments).

5′-caaacatgatctgggtcatcttctc-3′ (14, 20). The predicted sizes of the amplified genes were 329 bp for p57 and 353 bp for β-actin. The PCR products were separated on 2% agarose gels and the density of each product was measured.

Western Blotting

HEp-2 cells (3×10^5) were cultured in each well of six-well plates to 80% to 90% confluence. The cell culture medium was replaced with DMEM plus 0.1% FBS and incubated for 24 h. The cells were exposed to EB1089 (0 or 10 nmol/L) for 4 days, washed once with ice-cold PBS, and then lysed with lysis buffer. The cell lysates were quantified and separated on SDS-polyacrylamide gels, transferred, and probed with primary antibodies as indicated. The protein bands specifically bound to primary antibodies were detected using an anti-rabbit alkaline phosphatase–linked IgG and by an enhanced chemiluminescence Western blotting system (Amersham Biosciences; ref. 21).

Statistical Analysis

Differences between measures for treatment groups were determined using Dunnett's T3 test and were considered significant at $P < 0.05$.

Results

EB1089 Inhibited the Proliferation of Human SCCs

As shown in Fig. 1A, EB1089 completely inhibited HEp-2 cell growth determined by MTT assay at nanomolar

concentrations over 6 days. EB1089 showed a significantly suppression of cell growth. EB1089-induced cell death was further confirmed by the flow cytometry (Fig. 1B). EB1089-induced antiproliferative effects were also observed in SCC25 and FaDu cells (Fig. 1), consistent with the effects of EB1089 on other human tumors (15, 22, 23).

EB1089 Inhibited Human Laryngeal SCC Tumor Growth in Nude Mice

As shown in Fig. 2A and B, tumors were significantly smaller in the EB1089-treated group than in controls. Thus, EB1089 treatment inhibited tumor growth in athymic nude mice; this was consistent with the cell culture results.

EB1089 Up-regulated p57 Gene Expression in Human SCCs

As shown in Fig. 3A, EB1089 stimulated p57 mRNA synthesis in a dose-dependent manner after 2 days. As shown in Fig. 3B, treatment of cells with 10 nmol/L EB1089 for 4 days led to a significant accumulation of p57 protein in HEp-2 cells and in SCC25 and FaDu cells (Fig. 3C). The expression of p27 was slightly induced by EB1089 in HEp-2 cells, but there was no induction of p21 expression (Fig. 3D). As shown in Fig. 3E, EB1089 treatment caused cell cycle arrest at G_1, which is a typical effect of p57 as a potent inhibitor of cyclin A/CDK2 and cyclin E/CDK2

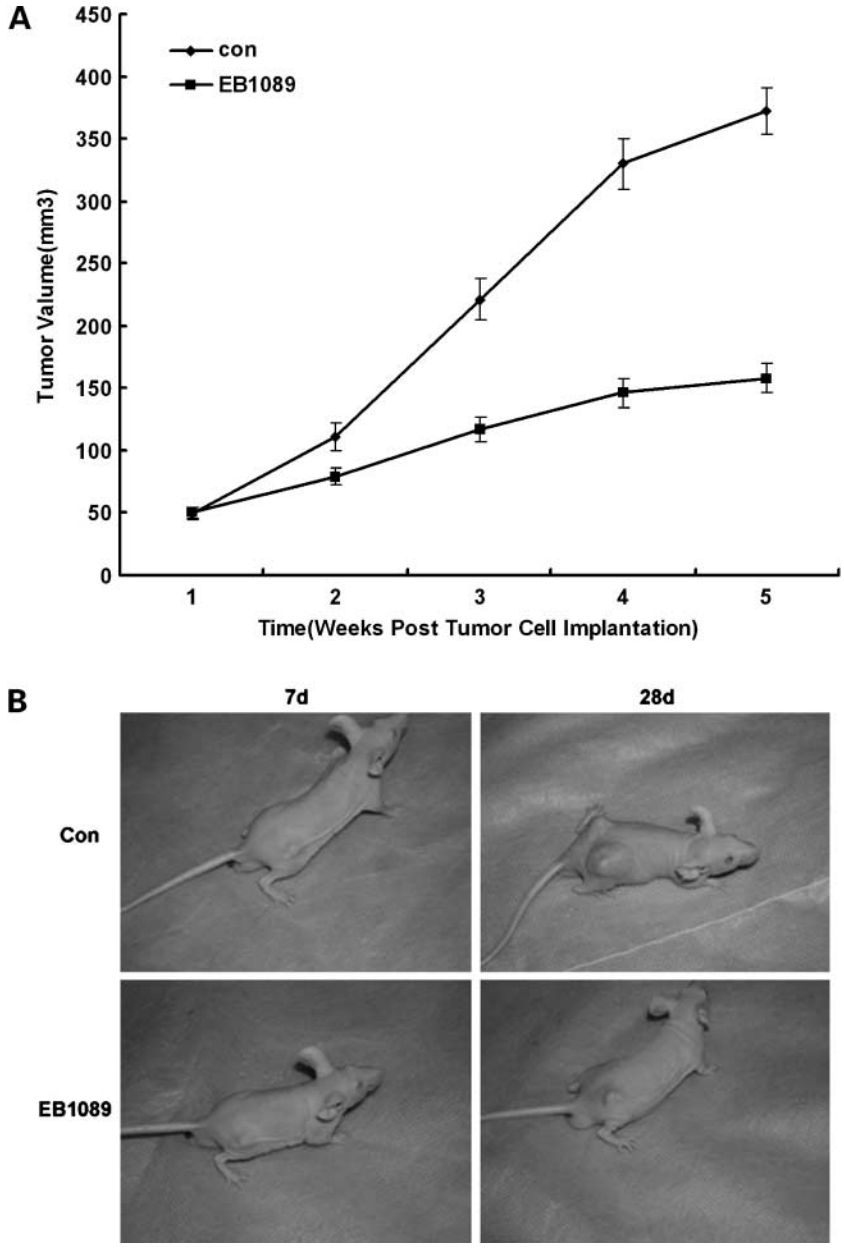

Figure 2. Effects of EB1089 on the growth of HEp-2 cells *in vivo* in an early tumor model. Nude mice aged 6 wk were used. HEp-2 cells were injected s.c. into the flanks of each mouse to initiate tumor growth. Animals were treated i.p. with either saline and ethanol vehicle or EB1089 (0.20 μg/kg) for 5 d each week for 5 wk as indicated in Materials and Methods. Tumor sizes were measured weekly in two dimensions throughout the study. The experiments were conducted with 16 mice in each group. The growth kinetics of the inoculated tumors are shown. **A,** mean ± SE. *, $P < 0.05$, control group compared with EB1089 group. **B,** external appearance of tumors from the control group (*top*) and the EB1089 group (*bottom*).

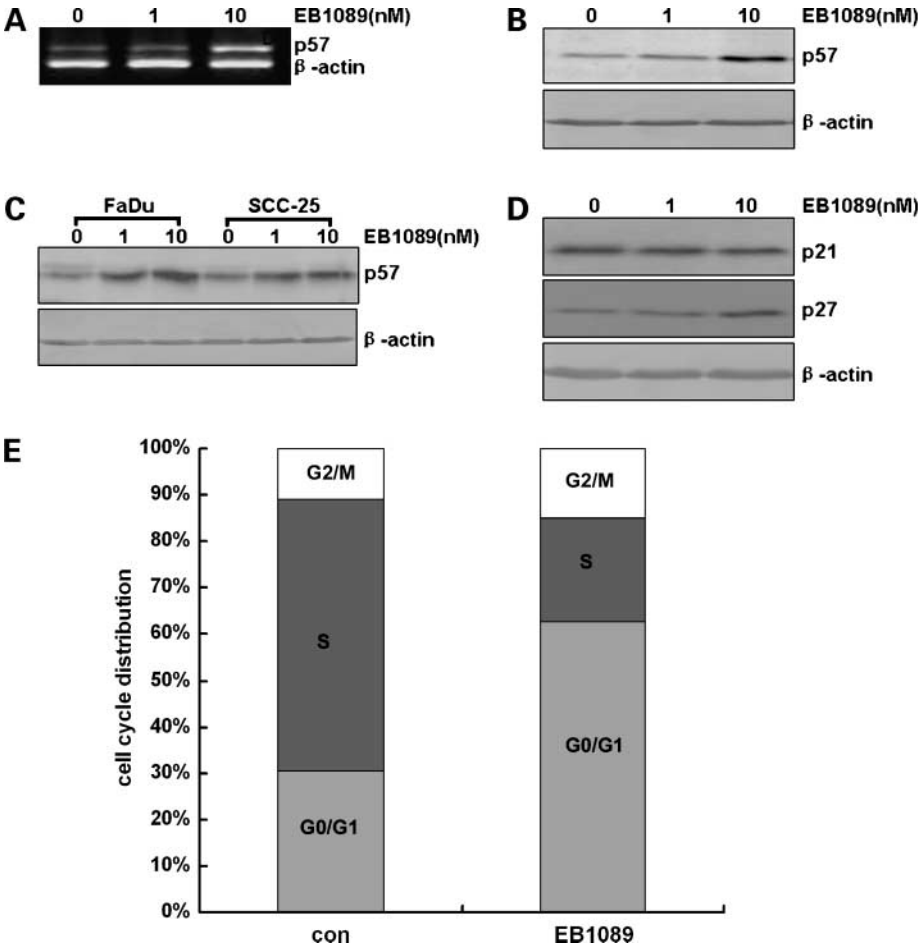

Figure 3. EB1089 induced the expression of p57 in human laryngeal SCCs. **A,** HEp-2 cells in culture were treated with different doses of EB1089 for 2 days and expression levels of p57 mRNA were analyzed by semiquantitative reverse transcription-PCR. *Bottom,* a control for β-actin expression (loading control). **B,** HEp-2 cells were treated with 1 or 10 nmol/L EB1089 or vehicle for 4 d. The amounts of p57 protein were determined by Western blotting. **C,** SCC25 and FaDu cells were treated with 1 or 10 nmol/L EB1089 or vehicle for 4 d. The amounts of p57 protein were determined by Western blotting. **D,** HEp-2 cells were treated with 1 or 10 nmol/L EB1089 or vehicle for 4 d. The amounts of p21 and p27 proteins were determined by Western blotting. **E,** cell cycle was determined using propidium iodide staining and detected by flow cytometry (three independent experiments).

complexes (12–14). Thus, the antiproliferative effects of EB1089 appear to be via increased p57 gene expression.

siRNA-p57 Blocked the EB1089-Induced Expression of p57 in Human SCCs

As shown in Fig. 4A, the levels of p57 measured by Western blotting were reduced dramatically after transfection with siRNA-p57 following EB1089 treatment (Fig. 4B). These results indicate that p57 is a downstream target of EB1089.

p57 siRNA Blocked the Inhibitory Effect of EB1089 on Proliferation of Human SCCs

EB1089 treatment reduced MTT absorbance from 0.802 ± 0.023 to 0.302 ± 0.015. In the siRNA-p57 groups, MTT recovered to 0.555 ± 0.019 with EB1089 (Fig. 4C). EB1089 significantly reduced BrdU incorporation from 0.747 ± 0.023 to 0.248 ± 0.015 in the control siRNA-con group. In the siRNA-p57 groups, BrdU incorporation recovered to 0.510 ± 0.018 following EB1089 treatment compared with 0.538 ± 0.017 in siRNA-p57-treated cells without EB1089 treatment (Fig. 4D). EB1089-induced death in HEp-2 cells could also be reversed by blocking p57 expression (Fig. 4E). Thus, p57 is required for the inhibition of proliferation and induction of cell death of human laryngeal SCCs by EB1089.

Discussion

We found here that EB1089 completely inhibited the growth *in vitro* of human laryngeal SCCs. We observed similar inhibitory effects of EB1089 on tumors growing *in vivo* in immunodeficient nude mice. We also found that siRNA-p57 markedly blocked the up-regulation of p57 and the antiproliferative effects of EB1089. Thus, EB1089 appears to inhibit the proliferation of human laryngeal SCCs via p57.

The active metabolite of vitamin D_3 has potent antitumor activities *in vitro* and *in vivo* in multiple cancers. Concerns about the induction of hypercalcemia by calcitriol and the desire for more potent agents have prompted development of less calcemic vitamin D analogues. EB1089 has been shown to regulate the differentiation and proliferation of multiple human cancer cell lines and thus is a potential therapeutic agent for cancers (23, 24). In the present study, we have shown for the first time that EB1089 significantly suppresses the growth of human laryngeal SCCs *in vitro*; this might reflect the general antitumor activity of EB1089.

Previous studies have shown that the vitamin D analogue, 1-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitamin D_3, can modulate cell cycle regulators, including increasing mRNA and protein levels of p21 and

CDK2 and increasing p27 protein expression in SCCs (24). p57 is a potent inhibitor of several G_1 cyclin/CDK complexes and its overexpression leads to cell cycle arrest in G_1 (25). Human p57, like p21, contains a proliferating cell nuclear antigen-binding domain within its COOH terminus that, when separated from its NH_2-terminal CDK-cyclin binding domain, can prevent DNA replication (26). Moreover, p57, but not p21 or p27, is indispensable for the inhibition of cyclin A/CDK2 and cyclin E/CDK2 complexes (27). p57 also controls cell cycle exit and the differentiation of lens fiber cells, placental trophoblasts, chondrocytes, and human skeletal myoblasts (28–30). Although p57 appears to be a critical terminal effector of signal transduction pathways that control cell differentiation and proliferation, its precise involvement in differentiation and proliferation in response to cytotoxic drugs or during tumorigenesis is unknown. Interestingly, p57 gene expression is also reduced in human laryngeal cancers (16), reinforcing a possible function for this protein in tumorigenesis. In the present study, semiquantitative assays and Western blots showed that EB1089 stimulated the expression of p57 at the mRNA and protein levels in human laryngeal SCCs. Thus, the up-regulation of p57 protein and mRNA levels by EB1089 may contribute to its antitumor effects.

siRNAs inhibit gene expression through the specific degradation of mRNAs (31). We used this to block the production of endogenous p57 protein. Silencing p57 gene expression completely inhibited the induction of p57 by

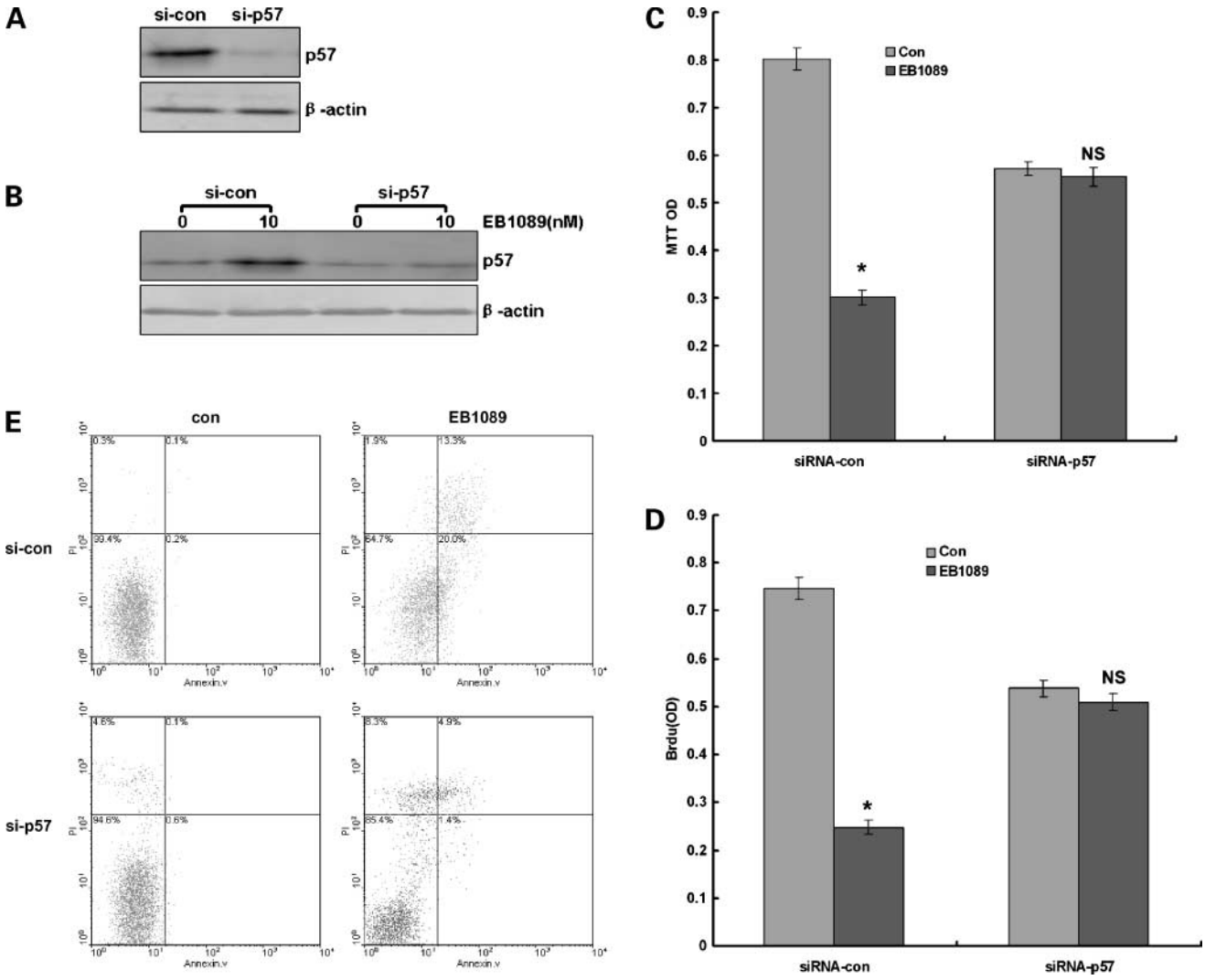

Figure 4. siRNA-p57 knocked down p57 expression and reversed the cell proliferation of HEp-2 cells induced by EB1089. Cells were transfected transiently with siRNA-p57 or siRNA-con (control). At 24 h after transfection, the cells were reseeded at the proper density and cultured with fresh medium containing 0 or 10 nmol/L EB1089. **A,** expression levels of p57 protein. **B,** cell lysates were prepared at 4 d after EB1089 treatment and subjected to antibody probes for p57 and β-actin. MTT assay (**C**) and BrdU incorporation (**D**) showing the effects of siRNA-con and siRNA-p57 on cell proliferation induced by EB1089 treatment in HEp-2 cells. NS, not significant. *, $P < 0.05$. **E,** cell death was determined using Annexin V staining and detected by flow cytometry (three independent experiments).

EB1089. Moreover, we found that the antiproliferative role of p57 was associated with its mediation of DNA synthesis, as shown by the suppression of DNA synthesis in HEp-2 cells, and the restoration of a low sensitivity to EB1089 response by the introduction of siRNA-p57 into HEp-2 cells. Thus, p57 mediated the antiproliferative role via suppression of DNA synthesis. However, how p57 interacts with apoptosis is unclear. In cancer cells, it primarily promotes intrinsic apoptotic pathways, favoring Bax activation, consequent release of cytochrome *c* into cytosol, and caspase-3 activation, leading to the mitochondrial apoptotic pathway (32). We hypothesize that EB1089 influences these pathways in cancer cells via p57, but this needs further study.

These data indicate that p57 plays an important role in the inhibition of proliferation caused by EB1089 in human laryngeal SCCs, as this can be reversed by knockdown of p57 gene expression. It is likely that other cell signaling pathways affected by EB1089, such as MAPK, p27, and mitochondrial pathways, play a role in this process. Further investigations are needed to dissect the biological response to EB1089 at the molecular level. Our results also underscore the potential utility of EB1089 and siRNA in regulating human laryngeal SCC proliferation in human laryngeal cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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