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# Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction

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In a recent study on head and neck squamous cell carcinoma (HNSCC) cells we found that epigallocatechin-3-gallate (EGCG), a major biologically active component of green tea, inhibited activation of the epidermal growth factor receptor (EGFR) and related signaling pathways. Since activation of EGFR signaling pathways is associated with angiogenesis, we examined the effects of EGCG on vascular endothelial growth factor (VEGF) production by YCU-H891 HNSCC and MDA-MB-231 breast carcinoma cell lines, because we found that both of these cell lines display autocrine activation of transforming growth factor-$\alpha$ (TGF-$\alpha$)/EGFR signaling and produce high levels of VEGF. Treatment with EGCG inhibited the constitutive activation of the EGFR, Stat3, and Akt in both cell lines. These changes were associated with inhibition of VEGF promoter activity and cellular production of VEGF. Mechanistic studies indicated that inhibition of Stat3, but not mitogen-activated protein kinase kinase (MEK)1 or phosphatidylinositol 3′-kinase (PI3K), significantly decreased VEGF promoter activity. However, the inhibitory effects of a dominant negative Stat3 on VEGF expression was not as strong as that produced by EGCG. An analysis of alternative pathways indicated that EGCG strongly inhibited the constitutive activation of NF-$\kappa$B in both cell lines, and an NF-$\kappa$B inhibitor strongly inhibited VEGF production. These results suggest that EGCG inhibits VEGF production by inhibiting both the constitutive activation of Stat3 and NF-$\kappa$B, but not extracellular-signal-regulated kinase (ERK) or Akt, in these cells. Therefore, EGCG may be useful in treating HNSCC and breast carcinoma because it can exert both antiproliferative and antiangiogenic activities.

**Keywords:** EGCG, VEGF, Stat3, NF-$\kappa$B, PI3K-Akt, angiogenesis.

## INTRODUCTION

In a recent study on HNSCC[3] cell lines (1) we found that epigallocatechin-3-gallate (EGCG), a major biologically active component of green tea, inhibited phosphorylation of the epidermal growth factor receptor (EGFR) and thereby blocked EGFR-related downstream signaling pathways, including the activation of Stat3 and extracellular-signal-regulated kinase (ERK). Autocrine activation of transforming growth factor-$\alpha$ (TGF-$\alpha$)/EGFR signaling frequently occurs in both head and neck squamous cell carcinoma (HNSCC) and breast carcinoma and is strongly associated with tumor progression and a poor prognosis (2). In addition, tumors with this abnormality possess angiogenic properties including increased production of angiogenic vascular endothelial growth factor (VEGF) (2,3). In view of these findings, it was of interest to examine the effects of EGCG on VEGF production and the possible roles of Stat3, ERK, Akt, and NF-$\kappa$B, which are target molecules of EGFR signaling (4–7). For this purpose we utilized the YCU-H891 HNSCC cell line and the MDA-MB-231 breast cancer cell line, since both cell lines display autocrine activation of TGF-$\alpha$/EGFR signaling and produce relatively high levels of VEGF, even under normoxic

 

conditions. In this study, we also examined the roles of Stat3, ERK, Akt, and NF-κB in the production of VEGF, since the precise molecular mechanisms by which TGF-α/EGFR signaling pathways enhance VEGF production in HNSCC and breast carcinoma are not known.

## MATERIALS AND METHODS

### *Cell Lines and Cell Culture*

The human HNSCC cell line YCU-H891 (originally derived from a carcinoma of the hypopharynx) was provided by Dr. M. Tsukuda and is described in our previous studies (1,8). The derivatives of the YCU-H891 cell line Stat3DN66 and Stat3DN99, which stably express dominant negative Stat3 proteins, were established and described in our previous study (8). The human breast carcinoma cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD). Derivatives of this cell line that stably express a dominant negative Stat3 protein were established using the same procedure (1) that we previously used to develop the above-mentioned derivatives of YCU-H891 cells. All cell lines were maintained in a 5% $CO_2$ and 21% $O_2$ atmosphere at 37°C in RPMI-1640 (YCU-H891) or DMEM (MDA-MB-231) medium with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY), unless specified otherwise. The medium for the derivatives that stably express dominant negative Stat3 protein also contained 400 μg/ml of G418.

### *Chemicals and Drugs*

TGF-α, PD98059, LY294002 (Life Technologies), EGCG (Sigma, St. Louis, MO), and Go6976 (Calbiochem, La Jolla, CA) were obtained from commercial sources, as indicated. PD98059, LY294002, and Go6976 were dissolved in dimethyl sulfoxide (DMSO), and EGCG was dissolved in water. The final concentration of DMSO in the growth medium was always less than 0.1%.

### *ELISA Assays for TGF-α and VEGF*

The levels of TGF-α and VEGF in the conditioned medium were determined by using a TGF-α (Oncogene, Boston, MA) and a VEGF (R&D Systems, Minneapolis, MN) ELISA kit. In brief, 60–70% confluent cells were cultured for 24 h in fresh medium containing 1% calf serum. The cell-free conditioned medium was then collected and the TGF-α and VEGF concentrations were determined according to the manufacturer's instructions. These concentrations were normalized with respect to the number of cells per plate. All assays were performed on triplicate plates, and data are presented as mean values±SD.

### *Protein Extraction and Immunoblotting*

Total cellular protein was extracted and analyzed by the Western blot method, as described previously (1,8). The following primary antibodies were used for detection of the related proteins: EGFR (clone-74) from Transduction Laboratories, Lexington, KY; Stat3 (F-2) and phospho (Y705)-Stat3 (B-7) from Santa Cruz Biotechnology, Santa Cruz, CA; phospho (Y1173)-EGFR, Akt, phospho (Ser473)-Akt, ERK 1/2, and phospho (Thr202/Thr204)-ERK1/2 antibody (9106S) from Cell Signaling, Beverly, MA.

### *Cell Proliferation Assays*

Cell proliferation assays were done essentially as described previously (8), using a PreMix WST-1 cell proliferation assay system (Takara, Tokyo, Japan), according to the manufacturer's instructions. Cells were treated with increasing concentrations of EGCG for 72 h. Each point represents the mean±SD of triplicate wells.

### *Plasmids*

The VEGF promoter-luciferase reporter plasmid phVEGF1, which contains the 5′-flanking region (−2279 to +54) of the VEGF promoter, was originally constructed by Dr. Minchenko (9) and provided by Dr. M. Esumi (10). The dominant negative hemagglutinin peptide (HA)-tagged Stat3D and HA-tagged Stat3F plasmids were provided by Dr. T. Hirano (11) and are described in our previous study (8). The *c-fos* promoter-luciferase reporter plasmid p-FOS-wt-luc, which contains a sis-inducible element (SIE) sequence, was described and used in our previous studies (1,8). The pNF-κB-Luc plasmid, which contains the luciferase reporter gene driven by five tandem repeats of NF-κB responsive elements, was obtained from Stratagene, La Jolla, CA.

### *Luciferase Reporter Assays*

These assays were also done essentially as previously described (1). Briefly, triplicate samples of $1\times10^5$ cells in 35-mm plates were transfected using lipofectin (Life Technologies). One microgram of the reporter plasmid and 10 ng of the pCMV-$\beta$-gal plasmid DNA were cotransfected in opti-MEM®I medium (Life Technologies). After 16 h, the medium was changed to fresh serum-free medium. One-half of the cultures were stimulated with 50 ng/ml of TGF-$\alpha$. The cells were then incubated for 24 h in the presence or absence of the indicated drugs and luciferase activity was determined with the luciferase assay system (Promega, Madison, WI). $\beta$-Gal activities were also determined with the $\beta$-galactosidase enzyme assay system (Promega). Luciferase activities were then normalized with respect to $\beta$-gal activities.

### *RNA extraction and semiquantitative RT-PCR*

Total RNA was extracted with a single step method using the Trizol reagent (Life Technologies). RT-PCR was conducted using a SUPERSCRIPT One-Step RT-PCR system (Life Technologies) in a total volume of a 25 $\mu$l reaction mixture containing 12.5 $\mu$l of 2$\times$ reaction mix, 2 $\mu$l (1 $\mu$g) of template RNA, 0.25 $\mu$l of sense primer (20 $\mu$M), 0.25 $\mu$l of antisense primer (20 $\mu$M), 0.4 $\mu$l of RT/Platinum *Taq* mix, and 9.6 $\mu$l distilled water. Initially, cDNA was generated at 50°C for 30 min. PCR was then conducted for 15, 20, 25, and 30 cycles in a thermal controller (Programmable Thermal Controller; MJ Research, Watertown, MA); optimal PCR cycle number for quantification was determined and found to be 20 to 25 cycles. We adopted 25 cycles for YCU-H891 cells and 20 cycles for MDA-MB-231 cells, respectively. Each amplification cycle consisted of 0.5 min at 94°C for denaturation, 0.5 min at 55°C for primer annealing, and 1 min at 72°C for extension. The sequences of PCR primers were as follows: VEGF sense primer, 5′-CCT GGT GGA CAT CTT CCA GGA GTA CC-3′; VEGF antisense primer, 5′-GAA GCT CAT CTC TCC TAT GTG CTG GC (12); $\beta$-actin sense primer, 5′-CCA GGC ACC AGG GCG TGA TG-3′; and $\beta$-actin antisense primer, 5′-CGG CCA GCC AGG TCC AGA CG-3′. The sizes of the amplicons for VEGF and $\beta$-actin were 196 and 436 bp, respectively. Twelve microliters of each PCR product was then electrophoresed on 2% agarose gels and the intensities of the specific bands were analyzed.

## RESULTS AND DISCUSSION

### *YCU-H891 and MDA-MB-231 Cells Constitutively Express High Levels of Both TGF-$\alpha$ and VEGF*

We initially analyzed the levels of TGF-$\alpha$ and VEGF in the conditioned media obtained from the YCU-H891 HNSCC and MDA-MB-231 breast carcinoma cell lines. We found that the media from both YCU-H891 and MDA-MB-231 cells contained relatively high levels of TGF-$\alpha$ (Fig. 1A) and VEGF (Fig. 1B).

### *EGFR, Stat3, ERK, and Akt Are Constitutively Activated in Both YCU-H 891 and MDA-MB-231 Cells*

We then examined the total cellular levels and the levels of phosphorylated forms of the following proteins, EGFR, ERK, Stat3, and Akt, by Western blot analyses, in both of these cell lines. The cells were grown in medium containing 10% FBS; total cellular proteins were extracted and then examined by Western blot analysis using the respective specific antibodies (Fig. 1C). Relatively high levels of the phosphorylated form of the EGFR, ERK, Stat3, and Akt proteins were detected in both cell lines (Fig. 1C), indicating these proteins are constitutively activated in both cell lines, probably due to autocrine stimulation of the EGFR by TGF-$\alpha$ (Fig. 1A).

### *EGCG Inhibits the Growth of MDA-MB-231 Cells*

We previously demonstrated that EGCG inhibits the growth of YCU-H891 cells (1). Therefore, we also examined the growth-inhibitory effects of EGCG on MDA-MB-231 cells, using cell proliferation assays (Fig. 1D). Cells were treated with various concentrations of EGCG for 72 h and cellular viability was determined. EGCG markedly inhibited the growth of MDA-MB-231 cells, which is consistent with our previous findings with YCU-H891 cells (1). However, MDA-MB-231 cells were somewhat more resistant to EGCG than YCU-H891 cells, because with the MDA-MB-231 cells the $IC_{50}$ concentration for EGCG was about 30 $\mu$g/ml

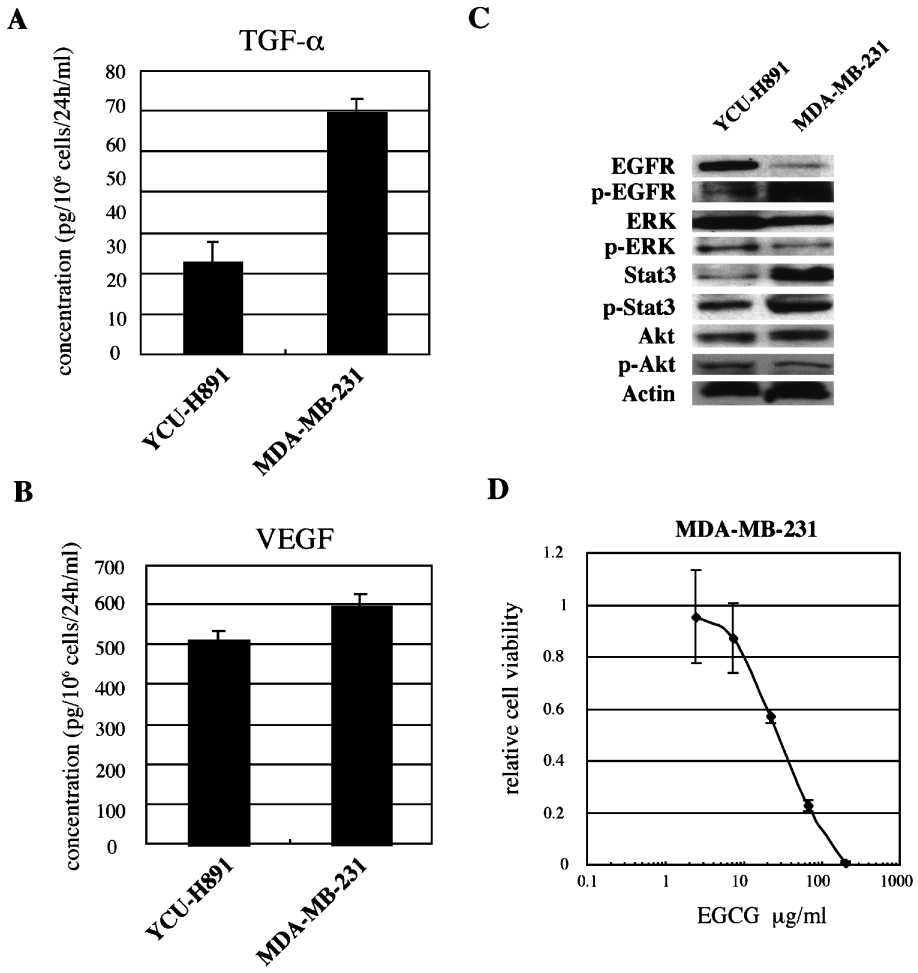


***Figure 1.*** **(A)** Levels of TGF-$\alpha$ and **(B)** levels of VEGF proteins in the conditioned media of YCU-H891 and MDA-MB-231 cells. For details, see *Material and Methods*. **(C)** Western blot analysis of cellular levels of the EGFR, phosphorylated-EGFR (p-EGFR), ERK, phosphorylated-ERK (p-ERK), Stat3, phosphorylated Stat3 (p-Stat3), Akt, and phosphorylated-Akt (p-Akt) proteins in exponentially growing cultures of the indicated cell lines. **(D)** Growth-inhibitory effects of EGCG on MDA-MB-231 cells. Cells were treated with the indicated concentrations of EGCG, and cell viability was determined by cell proliferation assays. The values indicate the means of triplicate plates and the bars the standard deviations.

(Fig. 1D), whereas with YCU-H891 cells the $IC_{50}$ concentration was about 7 $\mu$g/ml (1).

### *EGCG Inhibits the Constitutive Activation of EGFR Related Signaling in Both YCU-H891 and MDA-MB-231 Cells*

In our previous study we found that EGCG inhibits the activation of EGFR and thereby the activation of Stat3 and ERK in YCU-H891 cells (1). Therefore, we examined the effects of EGCG on these proteins in MDA-MB-231 cells. We found that treatment of MDA-MB-231 cells with 30 $\mu$g/ml of EGCG (the $IC_{50}$ concentration) inhibited the phosphorylation, i.e., the activation, of EGFR and Stat3, in both the absence and presence of TGF-$\alpha$. However, activation of the ERK protein was not inhibited by EGCG in the absence or presence of TGF-$\alpha$ (Fig. 2B). We also examined the effects of EGCG on the activation of the Akt protein, since we found that this protein is constitutively activated in both cell lines (Fig. 1C). We found that treatment of YCU-H891 cells with 10 $\mu$g/ml of EGCG (Fig. 2A) and treatment of MDA-MB-231 cells with 30 $\mu$g/ml of EGCG (Fig. 2B) inhibited the activation of Akt, both in the absence and presence of TGF-$\alpha$.

### *EGCG Inhibits Both VEGF Promoter Activity And VEGF Production*

As discussed in the *Introduction*, activation of EGFR is associated with angiogenesis,

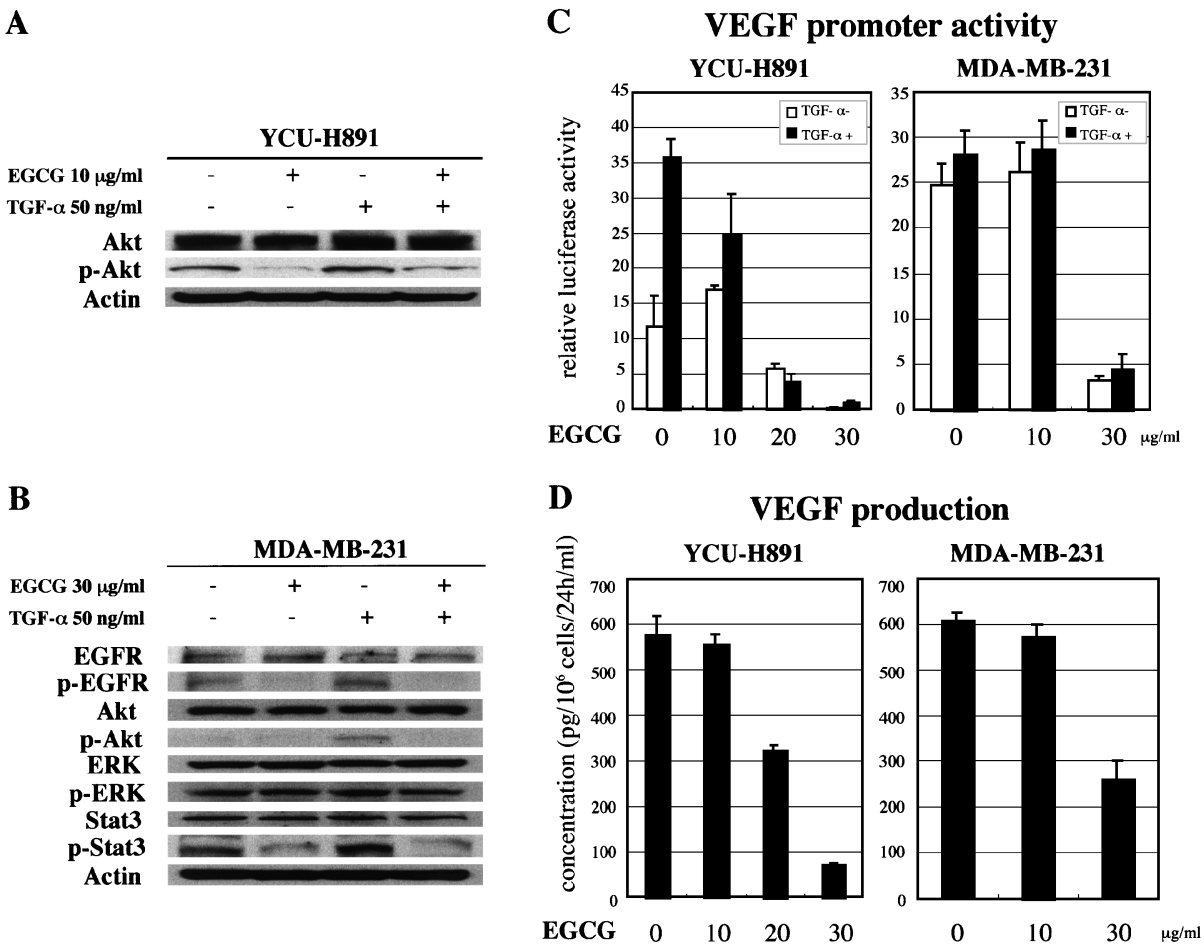


***Figure 2.*** **(A)** and **(B)** Effects of EGCG on activation of the EGFR, Akt, ERK and Stat3 proteins. Cells were cultured in serum-free medium for 24 h and then incubated with or without 10 (YCU-H891) or 30 $\mu$g/ml (MDA-MB-231) of EGCG for an additional 24 hours, and then harvested. For TGF-$\alpha$ stimulation, cells were treated with 50 ng/ml of TGF-$\alpha$ for 3 h before harvesting. Extracts were examined by Western blot analysis with the respective antibodies. An antibody for actin was used as a loading control. **(C)** Effcts of EGCG on VEGF promoter activity. Cells were transfected in opti-MEM® I medium with a VEGF promoter-luciferase reporter plasmid for 16 h and were then cultured in serum-free medium with the indicated concentrations of EGCG for 24 h. For growth factor stimulation assays, 50 ng/ml of TGF-$\alpha$ was added 30 min after EGCG. Luciferase activities were normalized by doing parallel assays for $\beta$-gal activity. The values indicate the means of triplicate plates and the bars the standard deviations. **(D)** Effects of EGCG on VEGF production. The indicated cells were grown in fresh medium containing 1% calf serum for 24 h. The cell-free conditioned media were then collected and the concentrations of TGF-$\alpha$ and VEGF were determined and normalized with respect to the total number of cells in the corresponding plates. The values indicate the means of triplicate plates and the bars the standard deviations.

including increased expression of VEGF (2,3). Because treatment with EGCG inhibited activation of EGFR and downstream signaling pathways (1) (Figs. 2A and 2B), we examined the effects of EGCG on the transcriptional activity of the promoter of the VEGF gene and on production of the VEGF protein in these two lines. In both cell lines EGCG strongly inhibited the activity of a VEGF promoter-luciferase reporter, both in the absence and presence of TGF-$\alpha$ (Fig. 2C). It is of interest that the stimulatory effect by TGF-$\alpha$ on the VEGF promoter was stronger with the YCU-H891 cells than with the MDA-MB-231 cells (Fig. 2C). This may reflect the fact that MDA-MB-231 cells produce higher endogenous amount of TGF-$\alpha$ (Fig. 1A) than YCU-H891 cells. Therefore, the stimulatory effect of TGF-$\alpha$ on VEGF promoter activity may be nearly saturated in the former cell line. Inhibition of VEGF promoter activity occurred at a lower concentration of EGCG in YCU-H891 cells than in MDA-MB-231 cells (Fig. 2C), which is consistent with the greater sensitivity of the former cells to growth inhibition by EGCG (1) (Fig. 1D). Assays of

conditioned media indicated that EGCG also inhibited the production of the VEGF protein in both cell lines (Fig. 2D). Again the inhibitory effect was greater in the YCU-H891 cells. Thus, treatments with 30 $\mu$g/ml of EGCG caused an 85% inhibition of VEGF production in YCU-H891 cells, but the same concentration of EGCG caused only a 55% inhibition in MDA-MB-231 cells (Fig. 2D).

### *VEGF Promoter Activity Is Inhibited by a Dominant Negative Stat3, but Not by MEK or PI3K Inhibitors*

In view of the above results we specifically examined the effects of Stat3, ERK, and phosphatidylinositol 3′-kinase (PI3K)-Akt–related signaling pathways on VEGF promoter activity by utilizing dominant negative Stat3 constructs, a mitogen-activated protein kinase kinase (MEK)1 inhibitor (PD98059), and a PI3K inhibitor (LY294002) in transient transfection assays employing the VEGF promoter-luciferase reporter (Fig. 3). The activities of the two dominant negative mutants of Stat3 were first validated in these two cell lines by using a *c-fos* promoter-luciferase reporter that contains an SIE element which has a high affinity for Stat3. In both cell lines, cotransfection of the cells with two dominant negative Stat3 constructs

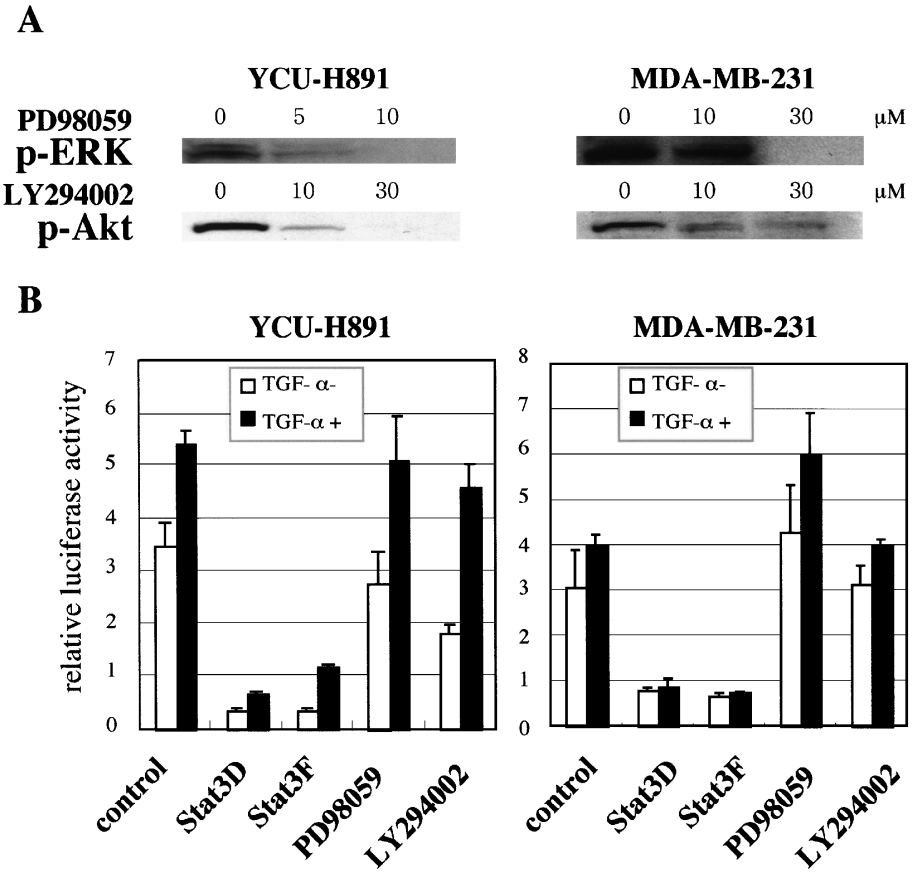


***Figure 3.*** **(A)** Effects of specific kinase inhibitors on the phosphorylation status of the ERK and Akt proteins. Cells were grown in serum-free medium for 24 h, treated with the indicated concentrations of PD98059 or LY294002 for 30 min, and then stimulated with 50 ng/ml of TGF-$\alpha$ for 3 h in the continued presence of the indicated inhibitor. Proteins were then extracted and examined by Western blot analysis with the respective antibodies. **(B)** Effects of two dominant negative Stat3 mutants and specific kinase inhibitors on VEGF promoter activity. Cells were transfected with a VEGF promoter-luciferase reporter plasmid, either alone or together with the dominant negative Stat3 construct Stat3D or the dominant negative Stat3 construct Stat3F, in serum-free opti-MEM, for 16 h. Then these media were replaced with either serum-free RPMI-1640 medium (YCU-H891) or DMEM (MDA-MB-231), in the absence or presence of PD98058 (10 $\mu$M for YCU-H891 cells and 30 $\mu$M for MDA-MB-231 cells), or in the absence or presence of LY294002 (10 $\mu$M for both cell lines). The cells were then incubated in these media for 30 min and TGF-$\alpha$ (50 ng/ml) was added to half of the cultures, then an additional 24 h extracts were prepared and luciferase activities were determined; bars, standard deviation, on triplicate assays.

(Stat3D and Stat3F) markedly inhibited *c-fos* promoter-luciferase reporter activity, in both serum-starved and TGF-$\alpha$–stimulated cells (data not shown). These results indicated that these two constructs function effectively as dominant negative mutants in both of these cell lines. We then determined the optimal concentrations of PD980590 and LY294002 required to inhibit phosphorylation of the ERK and Akt proteins, respectively, in both cell lines (Fig. 3A). Cells were grown in serum-free medium for 16 h, pretreated with the indicated concentration of PD98059 or LY294002 for 30 min, and then stimulated with 50 ng/ml of TGF-$\alpha$ for an additional 3 h. In YCU-H891 and MDA-MB-231 cells, 10 $\mu$M PD9805930 and 30 $\mu$M PD98059, respectively, markedly inhibited ERK phosphorylation. In both cell lines, 10 $\mu$M of LY294002 markedly inhibited Akt phosphorylation (Fig. 3A).

We then carried out a transient transfection assay with the VEGF promoter-luciferase reporter. The optimal concentrations of PD98059 and LY294002 determined above were added to the cells 16 h after transfection of the reporter, the cells were grown with the inhibitors for an additional 24 h, and extracts were then prepared and assayed for luciferase activity. We found that neither PD98059 or LY294002 significantly inhibited VEGF promoter activity, either with or without stimulation of the cells with exogenous TGF-$\alpha$. However, when cells were cotransfected with either the Stat3D or Stat3F dominant negative mutants of Stat3, there was marked inhibition of VEGF promoter activity, both in the absence and presence of exogenous TGF-$\alpha$ (Fig. 3B).

### *Stable Expression of a Dominant Negative Mutant of Stat3 Inhibits VEGF Expression in Derivatives of Both YCU-H891 and MDA-MB-231 Cells*

In view of the above results, we examined the effects of a dominant negative Stat3 protein on the expression of endogenous VEGF by using derivatives of YCU-H891 and MDA-MB-231 cells that stably express an HA-tagged dominant negative Stat3 protein (Figs. 4A and 4B). The establishment and characteristics of two clones of YCU-H891 cells (Stat3DN66 and Stat3DN99) that stably express relatively high levels of a dominant negative HA-tagged Stat3D protein are described in our previous study (8). In the present study, we developed derivatives of MDA-MB-231 cells that stably expressed an HA-tagged Stat3F protein, using our previously described procedure (8).

We examined the levels of VEGF mRNA in the above-described cells and in vector control cells by semiquantitative RT-PCR (Fig. 4A), utilizing $\beta$-actin mRNA as an internal control. We found that the derivatives of YCU-H891 and MDA-MB-231 that expressed dominant negative Stat3 proteins displayed a decrease in the levels of VEGF mRNA, when compared to the parental or vector control cells (Fig. 4A).

We then examined the levels of the VEGF protein in the conditioned media obtained from these cells (Fig. 4B). In the YCU-H891 cells that expressed the dominant negative Stat3 there was a 40% to 60% reduction, and in the MDA-MB-231 derivative there was a 25% reduction in VEGF when compared to the parental cells (Fig. 4B). These results, taken together with the results obtained in the transient transfection VEGF promoter assays (Fig. 3B) and the semiquantitative RT-PCR assays (Fig. 4A), provide evidence that activated wild-type Stat3 transcriptionally up-regulates VEGF expression in both YCU-H891 and MDA-MB-231 cells.

However, the dominant negative Stat3 protein only partially inhibited endogenous VEGF production (Fig. 4B), and although in our previous study 10 $\mu$g/ml of EGCG markedly inhibited Stat3 activity in YCU-H891 cells (1), in the present study a higher concentration was required to produce significant inhibition of VEGF production in these cells (Fig. 2D). These results suggest that signaling molecules downstream of the EGFR, in addition to Stat3, play important roles in stimulating the production of VEGF.

### *EGCG Inhibits Constitutive Activation of NF-$\kappa$B in Both Cell Lines*

Since recent studies provide evidence that NF-$\kappa$B is also an important target of EGFR signaling in MDA-MB-231 (6) and A431 epidermal carcinoma cell lines (7), we examined the effects of EGCG on this transcription factor by using an NF-$\kappa$B luciferase reporter in transient transfection assays. Both cell

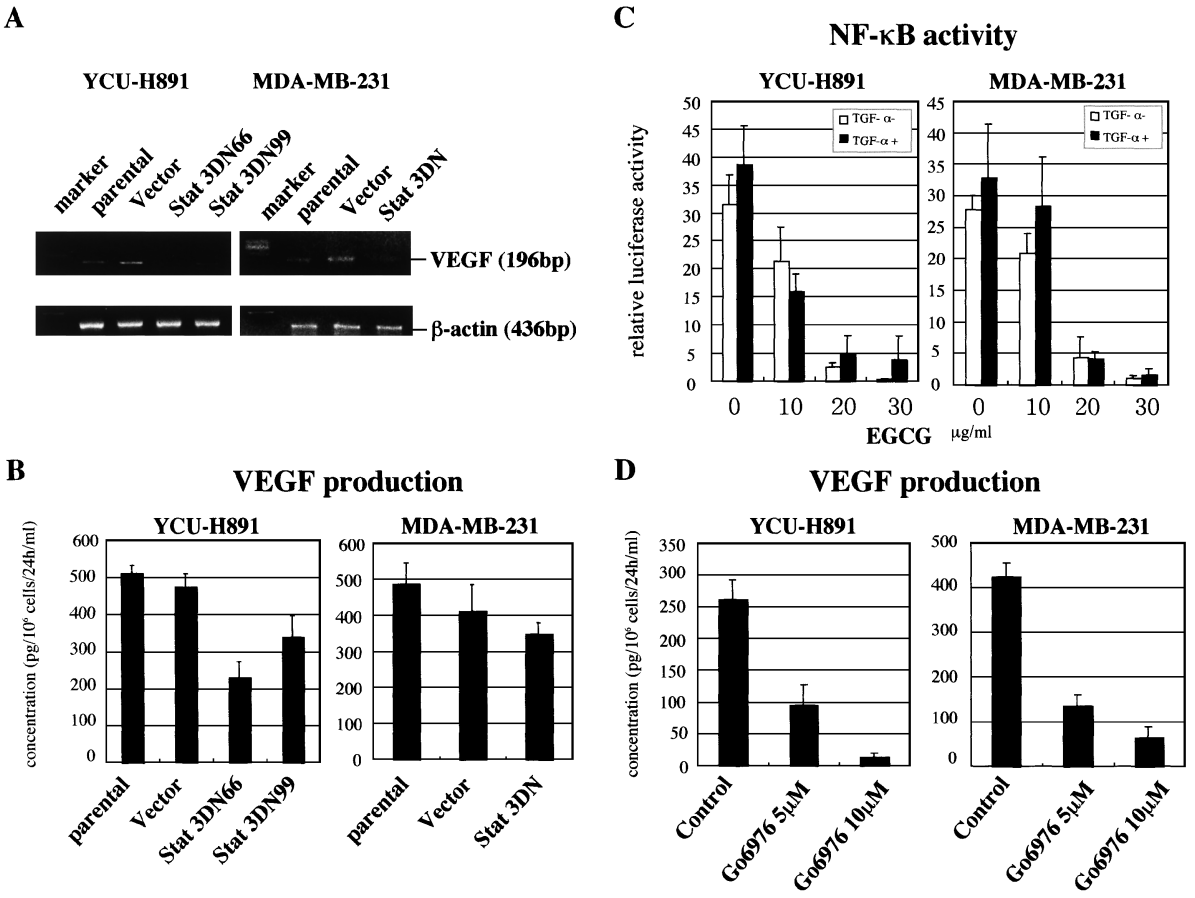


***Figure 4.*** **(A)** Semiquantitative RT-PCR assays for VEGF mRNA in extracts from YCU-H891 cells (parental), vector control (vector) and two stably transfected clones (Stat3D66 and Stat3D99) **(left)**, and in extracts from MDA-MB-231 cells (parental), vector control (vector) and stably transfected cell pool (Stat3DN) **(right)**. $\beta$-Actin mRNA was used as an internal control. **(B)** Levels of the VEGF protein in the conditioned medium from YCU-H891 cells (parental), vector control (vector) and two stably transfected clones (Stat3D66 and Stat3D99) **(left)**, and in extracts from MDA-MB-231 cells (parental), vector control (vector) and stably transfected cells pool (Stat3DN) **(right)**. Cells were grown in medium containing 1% calf serum for 24 h, the conditioned medium was collected, and the concentrations of VEGF protein were determined and normalized with respect to the total number of cells in the respective cell culture plates. The values indicate the mean for triplicate plates, and the bars indicate standard deviations. **(C)** Effects of EGCG on NF-$\kappa$B activity. Cells were transfected in opti-MEM®I medium with the NF-$\kappa$B luciferase reporter plasmid for 16 h and were then cultured in serum-free medium with the indicated concentrations of EGCG for 24 h. For the growth factor stimulation assays, 50 ng/ml of TGF-$\alpha$ was added 30 min after EGCG. Luciferase activities were normalized to parallel assays for $\beta$-gal activities. **(D)** Effects of the NF-$\kappa$B inhibitor (Go6976) on VEGF production. Cells were grown in medium containing 1% calf serum for 24 h in the absence or presence of the indicated concentrations of EGCG, the conditioned medium was collected, and the concentration of VEGF protein was determined and normalized with respect to the total number of cells in the respective cell culture plates. The values indicate the mean for triplicate plates, and the bars indicate standard deviations.

lines showed relatively high levels of NF-$\kappa$B activity even when studied in serum-free medium, and this activity was somewhat further stimulated by the addition of TGF-$\alpha$ (Fig. 4C). Treatment with EGCG strongly inhibited NF-$\kappa$B activity, both in the presence and absence of exogenous TGF-$\alpha$, in a dose-dependent manner. Again, YCU-H891 cells were more sensitive than MDA-MB-231 cells. These results are consistent with the recent finding that EGCG inhibits tumor necrosis factor-$\alpha$ (TNF-$\alpha$)-induced activation of NF-$\kappa$B in IEC-6 cells (13).

### *An NF-κB Inhibitor Strongly Inhibits VEGF Production in Both Cell Lines*

To verify that NF-$\kappa$B plays an important role in enhancing VEGF production in our two cell lines, we treated these cell lines with the drug Go6976, which was previously shown to strongly inhibit EGF-driven NF-$\kappa$B activity in MDA-MB-231 cells (6). Assays of the condi-

tioned media obtained from the treated cells indicated that 5 and 10 $\mu$M of Go6976 markedly inhibited VEGF production in both cell lines (Fig. 4D), thus implicating NF-$\kappa$B in the production of VEGF in these cell lines.

Taken together, the results of this study suggest that EGCG can inhibit the expression of VEGF in both HNSCC and breast carcinoma cells by inhibiting the autocrine activation of EGFR and, thereby, inhibiting downstream signaling pathways that control transcription of the VEGF gene. Our results are consistent with evidence that inhibition of EGFR or erbB2 by a specific antibody or tyrosine kinase inhibitor also down-regulates VEGF production (14). Furthermore, our findings with EGCG are not restricted to the two cell lines examined in the present study, since a very recent study demonstrated that EGCG also inhibits an increase of VEGF promoter activity and VEGF production induced by serum starvation in a colon cancer cell line (15).

Our findings also suggest that inhibition of signaling through Stat3 and NF-$\kappa$B, but not through Akt or ERK, plays a critical role in the inhibitory effects of EGCG on VEGF expression. Our studies implicating Stat3 are consistent with a recent report demonstrating that activation of Stat3 is required for glycoprotein 130–mediated induction of VEGF in cardiac myocytes (16). We note that there is an SIE-like sequence in the 5′-flanking region of the VEGF promoter between residues −1073 to 1063, but it remains to be determined whether activated Stat3 directly binds to this region or whether activated Stat3 acts through an indirect mechanism to enhance activity of the VEGF promoter. Our evidence that NF-$\kappa$B may also play a critical role in enhancing VEGF expression in these cells is consistent with evidence that activation of NF-$\kappa$B is involved in oxidative-stress-induced VEGF production in rat myocardium (17), and that an NF-$\kappa$B inhibitor suppresses retinal neovascularization in mice (18). In addition, the VEGF promoter contains NF-$\kappa$B–like sequences (10). However, as with Stat3, the precise mechanisms by which activation of NF-$\kappa$B enhances VEGF expression also remain to be determined. In glioblastoma cells, the PI3K pathway has been implicated in EGFR-induced VEGF expression (19), but in the latter study the possible roles of Stat3 or NF-$\kappa$B were not examined. In a derivative of NIH3T cells, which overexpresses an exogenous erbB2 protein, and in heregulin-stimulated MCF-7 cells, the levels of hypoxia-inducible factor (HIF) proteins were increased via activation of the PI3K-Akt pathway, thus resulting in increased levels of VEGF mRNA (20). Therefore, the mechanisms by which activation of EGFR leads to increased expression of VEGF may differ in different types of cells.

It is of interest that in vivo EGCG can inhibit VEGF-induced angiogenesis (21) and that EGCG can directly inhibit VEGF-induced activation of VEGFR-2 (KDR) (22). Thus, it appears that EGCG can inhibit angiogenesis by multiple mechanisms, including inhibition of VEGF production by tumor cells, as well as inhibition of VEGF activity and VEGF-receptor tyrosine kinase activity in endothelial cells. Therefore, EGCG may be a clinically useful antitumor agent because it directly inhibits the growth of tumor cells and also the process of angiogenesis. The present studies suggest that EGCG may be particularly effective in carcinoma cells in which there is autocrine activation of the EGFR.

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