Identification of Insulin-Like Growth Factor Binding Protein-3 as a Farnesyl Transferase Inhibitor SCH66336-Induced Negative Regulator of Angiogenesis in Head and Neck Squamous Cell Carcinoma

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Abstract The farnesyl transferase inhibitor (FTI) SCH66336 has been shown to have antitumor activities in head and neck squamous cell carcinoma (HNSCC) in vitro and in vivo. However, its mechanism of action has not been well defined. Here, we report that the insulin-like growth factor (IGF) binding protein (IGFBP)-3 mediates antitumor activities of SCH66336 in HNSCC by inhibiting angiogenesis. SCH66336 significantly suppressed HNSCC tumor growth and angiogenesis via mechanisms that are independent of H-Ras and RhoB. By inducing IGFBP-3 secretion from HNSCC cells, this compound suppresses angiogenic activities of endothelial cells, including vessel formation in chorioallantoic membranes of chick, endothelial cell sprouting from chick aorta, and capillary tube formation of human umbilical vascular endothelial cells (HUVEC). Knockdown of IGFBP-3 expression in HNSCC cells by RNA interference or depletion of IGFBP-3 in HUVECs by neutralizing antibody effectively blocked the effects of IGFBP-3 secreted from SCH66336-treated HNSCC cells on HUVECs. These findings suggest that IGFBP-3 could be a primary target for antitumor activities of FTIs and that IGFBP-3 is an effective therapeutic approach against angiogenesis in HNSCC.

Despite advances in therapy, including surgery, chemotherapy, and radiation, the survival rate of patients with head and neck squamous cell carcinoma (HNSCC) has not improved substantially (1). Conventional treatments have targeted tumor cells alone. In patients with HNSCC, however, the primary tumor has metastasized to regional lymph nodes, distant organs, or both by the time the diagnosis is made (2, 3). Angiogenesis is an essential step in the transition of a tumor from a small cluster of mutated cells to a large, malignant growth and subsequent metastasis to other organs throughout the body (4–6). It has been hypothesized, therefore, that the development of agents targeting tumor angiogenesis could be an effective strategy to control and treat various malignancies.

Increasing number of evidence implicate insulin-like growth factor (IGF) binding proteins (IGFBP) in regulating angiogenesis. IGFBPs modulate the bioactivity of IGFs by sequestering IGFs away from their receptors in the extracellular milieu, thereby regulating the stimulating action of IGF on angiogenesis and invasion (7). Some IGFBPs, however, in particular IGFBP-3, also have exhibited more active, IGF-independent antitumor activities, which are probably mediated by other cell surface receptors, such as the type V transforming growth factor-β receptor (8–11). IGFBP-3 has recently been identified as an IGF-independent inhibitor of vascular endothelial growth factor–induced endothelial cell proliferation (12).

While searching for agents that have antiangiogenic activities in HNSCC cells, we found that farnesyl transferase inhibitors (FTI), especially SCH66336 (Lonafarnib, Sarasar) that has shown to induce tumor regression in vitro and in vivo, inhibit angiogenic activities of HNSCC cells by inducing IGFBP-3 expression. FTIs were originally designed to inhibit posttranslational activation of Ras by blocking farnesylation (13). Recent studies suggest, however, that the cytotoxic actions of FTIs are due not to the inhibition of Ras proteins exclusively but to the modulation of other targets, including RhoB, a G protein that regulates receptor trafficking; the centromere-binding proteins CENP-E and CENP-F; and/or other proteins that have not yet been identified (14). Our results show that SCH66336...
induced expression of IGFBP-3 protein, which in turn blocked the PI3K/Akt pathway, resulting in the induction of apoptosis in vascular endothelial cells and regression of HNSCC tumor growth and angiogenesis.

**Materials and Methods**

**Cells, animals, and materials.** Human HNSCC cell lines UMSCC38, UMSCC22B, and SqCC/Y1, established originally by Dr. Michael Reiss (Yale University, New Haven, CT) and Dr. Thomas Carey (University of Michigan, Ann Arbor, MI), respectively, were obtained from Dr. Reuben Lotan (M.D. Anderson Cancer Center, Houston, TX). (15) Human non–small cell lung cancer (NSCLC) cell lines H460, Calu1, and H358 were purchased from the American Type Culture Collection (Manassas, VA). These cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. Human umbilical vascular endothelial cells (HUVEC; Cambrex BioScience, Walkersville, MD) were maintained in a gelatin-coated dish in endothelial cell basal medium (Cambrex BioScience) containing endothelial cell growth supplement at 37°C in a humidified environment with 5% CO2. HUVECs used in this study were from passages 2 to 7. Female nude mice, 6 weeks of age, were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). Chick eggs were obtained from Charles River Laboratories (Wilmington, MA).

Expression vectors containing pH-ras-V12 or pRhoB-PP were kindly given by Dr. George C. Prendergast (Thomas Jefferson University, Philadelphia, PA). Bovine serum albumin, gelatin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were obtained from Sigma-Aldrich (St. Louis, MO). IGF-1 and Des(1-3)IGF were purchased from R&D Systems (Minneapolis, MN). Amicon Ultra-4 was obtained from Millipore Co. (Bedford, MA). Cell culture inserts containing PET membranes (6.4 mm diameter, 8-µm pore size) and 24-well plates were from Costar (Cambridge, MA). Anti-IGF-1R antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against IGFBP-2, IGFBP-6, IGF-1R, H-Ras, RhoB, α-tubulin, and anti-β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-CD31 antibody was obtained from BD PharMingen (San Diego, CA). Anti-IGFBP-3 antibody was from Diagnostic Systems Laboratories, Inc. (Webster, TX). SCH66336 was provided by Schering-Plough Research Institute (Kenilworth, NJ). FITC-277 was purchased from Calbiochem (San Diego, CA). SCH6636 and FITC-277 were dissolved in DMSO at various concentrations to establish dose responses. Synthetic small interfering RNAs (siRNA) targeting H-Ras or RhoB were purchased from Ambion (Austin, TX), and IGFBP-3 and nonspecific control siRNA were from Dharmaco (Lafayette, CO).

To test the effects of conditioned medium on proliferation of vascular endothelial cells, HUVECs were plated at 3 × 10^3 per well in 96-well culture plates, untreated or preincubated with IgG or IGFBP-3 neutralizing antibody, and then treated with conditioned medium (10 µg) from the HNSCC cells. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as previously described (16).

In experiments assessing the effects of SCH66336 on protein and mRNA expression, HNSCC and NSCLC cell lines (1 × 10^5 in 100-mm^2 dishes) were treated with different concentrations of SCH66336 (0.5, 1, or 5 µmol/L) for indicated time periods in complete medium. When the effects of SCH66336 on pIGF-1R were tested, cells were serum starved for 1 day and stimulated with IGF-1 (100 ng/mL, for 30 minutes) before harvest. For siRNA transfection, UMSCC38 cells were plated at concentrations of 1 × 10^4 per well in 10-cm plates. The next day, the cells were transfected with siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) and cultured in growth medium with or without SCH66336 (5 µmol/L). One day after transfection, cells were changed to serum-free medium containing the same concentration of SCH66336. After 2 days of incubation, cells and conditioned media were collected. When HUVECs were treated with conditioned media from UMSCC38 cells, 1 × 10^5 per well in six-well plates were treated with conditioned media in endothelial cell basal medium in the absence or presence of IGFBP-3 neutralizing antibody for 12 hours.

**Conditioned medium.** To collect conditioned media from UMSCC38 cells, UMSCC38 cells (1 × 10^6 per plate) in 10-cm plates were incubated in growth medium containing SCH66336 (0.5, 1, or 5 µmol/L) for 1 day, washed with PBS, and then resupplied with serum-free medium containing the same concentrations of SCH66336. After 2 days of incubation, conditioned medium was collected and subjected to centrifugation through an Amicon Ultra-4 filter to remove any traces of SCH66336. Concentration of the conditioned medium was measured by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). The molecular mass cutoff of the filter was 5 kDa; the molecular mass of SCH66336 was 0.56 kDa, thus the flow-through containing excess SCH66336 was discarded, and the retentate was collected. Removal of SCH66336 from the conditioned medium was determined as previously described (17). The final filter retentate was concentrated 40-fold for several analyses, including Western blot, HUVEC proliferation and tube formation, chick aortic arch, and chorioallantoic membrane assays.

**Animal care and injections.** All animal procedures were done in accordance with a protocol approved by the Institutional Animal Care and Usage Committee. Orthotopic sublingual injections of tumor cells were described elsewhere (18). Female nude mice (6-week-old) under anesthesia were injected with UMSCC38 cells (2 × 10^6) into the lateral tongue (n = 5). One week later, when tumors started to develop, 40 mg/kg of SCH66336 or 20% hydroxyl-propyl-β-cyclodextrin control vehicle was given orally twice a day for 3 weeks. Thirteen days after cell injection, when tumors reached at least 50 mm^3 in volume, tumor size was measured twice a week for 15 days. Tumor growth was quantified by measuring the tumors in two dimensions and calculating volume as described elsewhere (16). One week later, when tumors started to develop, the mouse food was replaced by commercially available soft food (transgenic mice dough; Bio-serv, Frenchtown, NJ), which the mice can ingest even when the oral cavity was blocked by tumor. The mice were humanely killed by CO2 inhalation when they had lost >20% of their preinjection body weight (average 18.92 g). The tongues were removed and separated into two parts. One part was fixed, embedded in paraffin, and sectioned for massive CD31 staining. The other part was frozen and sectioned for CD31 staining.

**Western blot analysis.** Preparation of whole-cell lysates, quantification of the proteins, gel electrophoresis, and Western blotting were done as described elsewhere (19). Equal amounts of proteins in conditioned medium samples were confirmed by Coomassie blue staining of the duplicate gels and Ponceau staining of membrane.

**Immunohistochemistry.** Immunohistochemical analysis on IGFBP-3 was done using tongue tumor tissues from mice as described elsewhere (17). For CD31 staining, frozen sections of tumor tissues were stained with anti-CD31 antibody (BD Pharmingen; 1:100 dilution) and then detected by 3,3′-diaminobenzidine.

**Chick aortic arch assay.** Chick aortic arch assay was done as described elsewhere (20). Briefly, conditioned media collected from UMSCC38 cells were added to aortic rings from 14-day-old chick embryos. The plates were incubated for 48 hours at 37°C to allow microvessel sprouting from the adventitial layer. Average sprouting was measured with the Image program (NIH) after the plates were photographed under the stereomicroscope (Zeiss, Göttingen, Germany). Each condition was tested in six wells. The experiment was repeated thrice with similar results.

**Tube formation assay.** Tube formation assay was done as described elsewhere (21). HUVECs (5 × 10^3) were seeded on Matrigel surfaces and grown in the absence or presence of conditioned media from different treatment groups. After 18 hours, images were photographed at ×40 magnification, and tube formation was scored by blinded
observer as follows: a three-branch point event was scored as one tube, as described elsewhere (21). Each condition was tested in six wells. The experiment was repeated thrice with similar results.

**Chorioallantoic membrane assay.** Chorioallantoic membrane assay was conducted on 9-day-old chick embryos as described elsewhere (21). A coverslip loaded with 50 μg of conditioned medium was applied onto the surface of the chorioallantoic membrane. After 2 days of incubation, a fat emulsion was injected into the chorioallantoic membrane to allow visualization of the blood vessels, and chorioallantoic membranes were photographed with a stereomicroscope.

Reverse transcription-PCR. Total RNA was isolated from cells with the use of Trizol reagent (Invitrogen). cDNA was synthesized as previously described (17). Reverse transcription-PCR was done by amplification of genomic DNA, the primers of each gene were chosen from different exons. Reverse transcription-PCR was done in a total volume of 25 μL containing 1 μL of cDNA solution and 0.2 μmol/L of sense and antisense primers. The reverse transcription-PCR exponential phase was determined on 28 to 38 cycles using cDNAs developed from identical primer sets. The primer sequences were as follows: 5′-GAAGGCGGACACTGTTTTC-3′ (sense) and 5′-CCAGGCTCCAGAAAATGCTAG-3′ (antisense) for IGFBP-3; 5′-CAAGATGCCGTGACCATCC-3′ (sense) and 5′-CCGGATCCTCAGCCCAACAC-3′ (antisense) for H-ras; 5′-GGGTTCCGACAGGACCTGTG-3′ (sense) and 5′-TCTACGACCTTG-CAGCAGTT-3′ (antisense) for rho-B; 5′-GTTGAAAGTCCGG-GTGTAACCGATT-3′ (sense) and 5′-AATGCCAAGGATTGTGTAGATGGATACC-3′(antisense) for GAPDH. To avoid amplification of genomic DNA, the primers of each gene were chosen from different exons. Reverse transcription-PCR was done in a total volume of 25 μL containing 1 μL of cDNA solution and 0.2 μmol/L of sense and antisense primers. The reverse transcription-PCR exponential phase was determined on 28 to 38 cycles using cDNAs developed from identical reactions. The thermocycler condition used for amplification was as follows: 6 minutes at 94°C (one cycle), 6 minutes hot start at 94°C, 45 seconds at 56°C to 60°C (25-35 cycles), and 1 minute at 72°C (one cycle). Amplification products (8 μL) were resolved in 2% agarose gel, stained with ethidium bromide, and visualized in a transilluminator and photographed.

**Immunoprecipitation.** Equal amounts of protein samples were incubated with anti-pTyr antibody and protein A-Sepharose overnight and washed with lysis buffer thrice and 2× PBS twice. Proteins were eluted with 4× SDS loading buffer. Samples were boiled and subjected to electrophoresis on SDS-polyacrylamide gels, and binding was detected by using an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Piscataway, NJ).

**Northern blot analysis.** Total cellular RNA was prepared, as previously described (22). Twenty micrograms of total RNA were subjected to electrophoresis on SDS-polyacrylamide gels, and binding was detected by using an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Piscataway, NJ).

**Inhibition of Angiogenesis by FTI-Induced IGFBP-3**

**Results**

**SCH66336 inhibits angiogenic activities of HNSCC.** We tested the effects of SCH66336 on HNSCC tumor growth by establishing orthotopic tongue tumors of UMSCC38 HNSCC cells in nude mice and treating the mice with SCH66336. Representative tongue tissues from a healthy mouse (Normal), and tongue tumors from SCH66336-untreated (Con) and SCH66336-treated mice (SCH66336) are shown (Fig. 1A). Oral treatment with SCH66336 (40 mg/kg) almost completely suppressed tumor growth (P < 0.005). On day 25, the average tumor volume for untreated control mice had increased to 287.86 ± 53.93% (mean ± SD) of the volume on day 13, whereas that for SCH66336-treated mice showed 123.20 ± 14.67% of the volume before the SCH66336 treatment (Fig. 1B, left). The average body weight of SCH66336-treated mice was not remarkably changed during the treatment (Fig. 1B, right) compared with that of control mice. These findings suggest that FTI SCH66336 is an efficient therapeutic agent in head and neck tumors.

We studied the mechanism of the antitumor activities of SCH66336 in HNSCC. Several HNSCC cell lines treated with as much as 5 μmol/L SCH66336 yielded neither a terminal deoxynucleotidyl transferase–mediated nick end labeling–positive cell population nor changes in expression of caspase-3 and poly (ADP-ribose) polymerase (data not shown), indicating that SCH66336 induces little apoptotic activities in HNSCC cells. Given this finding and the critical role of angiogenesis in tumor growth, we evaluated the effects of SCH66336 on angiogenic activities of HNSCC cells. As shown in Fig. 1C, SCH66336 significantly decreased tumor vascularization (P < 0.01) as determined by microvessel density in anti-CDS1-stained tongue tumor tissues from control and SCH66336-treated nude mice. We then did a series of in vitro and in vivo experiments to test the angiogenic activities of SCH66336. We directly applied conditioned media from UMSCC38 cells to HUVECs. The proliferation (Fig. 1D) and capillary tube formation (Fig. 1E) of HUVECs were significantly inhibited by conditioned media from the untreated UMSCC38 cells but not by conditioned media from SCH66336-treated cells (P < 0.01). The ex vivo chick aortic ring assay revealed that conditioned media from untreated UMSCC38 cells also stimulated endothelial cell sprouts (P < 0.01), whereas conditioned media from SCH66336-treated cells did not exhibit stimulating effects on endothelial cell sprouting (Fig. 1F).

The chorioallantoic membrane assay, an established in vivo angiogenesis model, revealed that treatment with conditioned media from untreated UMSCC38 cells but not by conditioned media from SCH66336 (5 μmol/L)–treated cells (P < 0.01). The ex vivo chick aortic ring arch assay revealed that conditioned media from untreated UMSCC38 cells also stimulated endothelial cell sprouts (P < 0.01), whereas conditioned media from SCH66336-treated cells did not exhibit stimulating effects on endothelial cell sprouting (Fig. 1F). The chorioallantoic membrane assay, an established in vivo angiogenesis model, revealed that treatment with conditioned media from untreated UMSCC38 cells but not by conditioned media from SCH66336-treated cells significantly induced new vessel formation in chorioallantoic membranes of chick embryos (P < 0.05; Fig. 1G). We did not find any signs of toxicity, such as thrombosis, hemorrhage, or egg lethality, in the chorioallantoic membrane assay. Together, this data indicate that SCH66336 can elicit antiangiogenic activities in HNSCC.

**Antiangiogenic activities of SCH66336 in HNSCC cells are independent of H-Ras and RhoB.** We investigated the mechanisms by which SCH66336 elicits antiangiogenic activities in HNSCC cells. Because FTIs are designed to inhibit Ras farnesylation, the most important step in ras activation (13), we first tested the effects of SCH66336 on Ras in HNSCC cells. Western blot analysis revealed that SCH66336 induced
dose-dependent decreases in farnesylated H-Ras (H-Ras-F) in UMSCC38, UMSCC22B, and SqCC/Y1 cells (Fig. 2A). None of these cells have a ras mutation (data not shown), suggesting that the antiangiogenic activities of SCH66336 could be traced to proteins other than Ras. One potential target is RhoB, a 21-kDa G-protein, that is both farnesylated and geranylgeranylated by FTase and GGTase I (23), respectively. Recent evidence indicates that RhoB is a mediator of the anti-tumor activities of FTIs in mouse and rodent model systems and in human cancer cells (24, 25). Indeed, treatment with SCH66336 elicited elevations in the levels of RhoB (Fig. 2A), presumably due to the increased expression of the geranylgeranylated isoform of RhoB (RhoB-GG), as was observed previously (26).

To understand whether decreases in H-Ras-F or induction of RhoB mediated the antiangiogenic activity of SCH66336, we determined whether silencing H-Ras or RhoB expression by siRNA could abolish the activities of SCH66336 in UMSCC38 cells. Transfection with siRNAs targeting H-ras or RhoB specifically inhibited H-Ras or RhoB protein expression in UMSCC38 cells, whereas control scrambled (scr) siRNA did not affect the expression of these genes (Fig. 2B). Two different siRNAs induced similar degrees of gene silencing. These siRNAs also specifically inhibited mRNA expression of target genes regardless of the presence of SCH66336 (Fig. 2C). Cell viability was not specifically altered in any of these cells (data not shown). According to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 2D) and
In a chorioallantoic membrane assay, in vivo gene and in vivo promoter activity of IGFBP-3 promoter (pGL2-BP3; ref. 32) were repeated three times. Columns, mean of 30 eggs; bars, SD. Representative group from each condition. Bar, 200 μm. **, *P < 0.01 compared with negative control.

In vivo angiogenesis stimulated by conditioned media from UMSCC38 cells was photographed (left) or quantitatively evaluated (right). Independent experiments were repeated three times. Columns, mean of 30 eggs; bars, SD/95% confidence intervals. *, *P < 0.05; **, ***P < 0.01; ****P < 0.001 compared with negative control.

tube formation assays (Fig. 2E), HUVEC proliferation and morphogenesis were stimulated by conditioned media from control UMSCC 38 cells transected with sc. Conditioned media from SCH66336-treated UMSCC38 cells did not show these angiogenic activities. The effects of SCH66336 were still consistent in UMSCC38 cells, which had been transected with siRNA targeting H-ras or RhoB. Two different siRNAs for H-Ras and RhoB showed similar results (data not shown). All of these findings indicate that SCH66336 suppressed the angiogenic activities of HNSCC cells via H-Ras-independent and RhoB-independent mechanisms.

**FTI induces IGFBP-3 expression in vitro and in vivo via mechanisms that are independent of H-Ras or RhoB.** Because the IGF system has an important role in regulating proliferation and angiogenesis (27–29), we determined whether SCH66336 treatment stimulates the expression of IGFBPs. An obvious increase in IGFBP-3 protein level was observed in UMSCC38, UMSCC22B, and SqCC/Y1 cells treated with SCH66336 or FTI-277, another FTI (Fig. 3A). Increased levels of the IGFBP-3 protein were also observed in conditioned media from these cells (data not shown). No change was detected in the expression of other IGFBP subfamily members, including IGFBP-2 and IGFBP-6, after SCH66336 treatment (Fig. 3A). Because IGFBP-3 is supposed to control IGF-1–induced IGF-1R activation (30), we next determined the effects of SCH66336 on the levels of phosphorylated IGF-1R (pIGF-1R) in UMSCC38 cells. Western blot analysis revealed that treatment with SCH66336 caused increases in pIGF-1R, which is inhibited by SCH66336 in UMSCC38 cells (Fig. 3B, left). Whereas phosphorylation of IGF-1R stimulated by Des(1-3) (10 nmol/L), a mutant IGF-I that has a vastly diminished affinity for the IGFBPs but retains high avidity for IGF-1R (31), was marginally affected by SCH66336 in UMSCC38 cells (Fig. 3B, middle), indicating that secretion of functional IGFBP-3 by SCH66336-treated cells down-regulated pIGF-1R. Immunoprecipitation analysis using an antiphosphotyrosine antibody followed by Western blot analysis with an anti-IGF-1Rβ antibody confirmed that SCH66336 inhibited IGF-stimulated IGF-1R phosphorylation (Fig. 3B, right). Induction of IGFBP-3 by SCH66336 also found in H460, Calu1, and H358 NSCLC cell lines (Fig. 3C).

We further studied the mechanism by which SCH66336 activates IGFBP-3 expression. Northern and Western blot analyses of UMSCC38 cells revealed that IGFBP-3 gene expression is induced within 1.5 hours after the SCH66336 treatment, respectively (Fig. 3D). Moreover, a transient transfection experiment done with a luciferase reporter plasmid containing 1.8-kb IGFBP-3 promoter (pGL2-BP3; ref. 32) indicated that SCH66336 increased IGFBP-3 promoter activity in UMSCC38 and H460 cells in a dose-dependent manner (Fig. 3E). IGFBP-3 promoter activity was also increased in UMSCC38 cells by the treatment with FTI-277. These findings indicate that SCH66336 induced IGFBP-3 expression at a transcriptional level and that the induction of IGFBP-3 expression is a generic response to FTIs. Blockade of H-Ras or RhoB expression by siRNA (Fig. 3F and G) or overexpression of p-H-Ras-V12 or pRhoB-GG (Fig. 3H) did not affect the ability of SCH66336 to induce IGFBP-3 promoter activity and protein expression, indicating that induction of IGFBP-3 expression by SCH66336 is independent of H-Ras or RhoB.

**Oral administration of FTI SCH66336 induces IGFBP-3 expression in vivo in tongue tumor tissues.** We tested whether SCH66336 could increase IGFBP-3 expression in vivo. We found obviously higher levels of IGFBP-3 in orthotopic tongue tumor tissues removed from SCH66336-treated mice than in tongue tumor tissues from untreated control mice (Fig. 4A). IGFBP-3 expression was also analyzed in the tissue samples from patients who had received SCH66336. Results are shown from the pretreatment and/or posttreatment matched tumor tissues from three patients. Prominent IGFBP-3 expression was observed in the first patient (33) in histologically normal cells of the anterior tongue (Fig. 4B, left), but only focal or no IGFBP-3 expression was found in their squamous carcinoma tumor cells (SCC, Fig. 4B, right). Tumor tissues from this patient were not available after the SCH66336 treatment. Very low or undetectable levels of IGFBP-3 were also observed in squamous carcinoma tumor cells of the other two patients (patients 2 and 3) who had not been treated with SCH66336 (Fig. 4C, left). Three months after...
these patients’ treatment with SCH66336, however, IGFBP-3 expression was strongly induced in the membranes of the cancer cells undergoing karyolysis (Fig. 4C, right). These findings indicate that oral administration of SCH66336 was sufficient to induce IGFBP-3 expression in vivo.

IGFBP-3 secreted from SCH66336-treated HNSCC cells inhibits angiogenic activities in HUVECs by blocking Akt activation. To investigate whether IGFBP-3 secreted from SCH66336-treated UMSCC38 cells could restore the angiogenic effects of conditioned media from these cells. To this end, UMSCC38 cells were transfected with IGFBP-3 siRNA, and the conditioned medium from these cells was applied to HUVECs. IGFBP-3 siRNA specifically inhibited IGFBP-3 mRNA expression (Fig. 5A, left) as well as protein expression in the untreated and SCH66336-treated UMSCC38 cells (Fig. 5A, right). Conditioned media from UMSCC38 cells transfected with IGFBP-3 siRNA before the SCH66336 treatment (SCH) did not show significant effects on tube formation (Fig. 5B) and proliferation (Fig. 5C) of HUVECs compared with conditioned media from **scr**-transfected cells. To mask the effect of IGFBP-3 secreted from UMSCC38 cells on HUVECs, HUVECs were preincubated with IGFBP-3 neutralizing antibody (aBP3) before conditioned medium treatment. Stimulation of HUVECs proliferation by the conditioned media from control UMSCC38 cells was not affected with aBP3 (Fig. 5D). In contrast, proliferation-stimulating activities of conditioned media from SCH66336-treated UMSCC38 cells were almost completely blocked. These findings indicate that IGFBP-3 secreted from SCH66336-treated cells played a major role in inhibiting HUVECs proliferation and morphogenesis.

It has been shown that IGFBP-3 induces apoptosis in endothelial cells by inhibiting activation of Akt, a key enzyme for cell survival (12). Thus, we next tested whether IGFBP-3 secreted from SCH66336-treated HNSCC cells could inhibit activation of Akt. Western blot assays showed that conditioned media from control UMSCC38 cells stimulated phosphorylation of Akt (pAkt, Ser473) in HUVECs (Fig. 5E), which was not affected by the aBP3 treatment. Conditioned media from SCH66336-treated UMSCC38 cells marginally stimulated phosphorylation of Akt; however, pAkt levels were obviously induced by the incubation of HUVECs with aBP3. Phosphorylation of extracellular signal-regulated kinase (pERK) was stimulated in HUVECs by conditioned media from control or SCH66336-treated UMSCC cells but was not affected by the aBP3 (Fig. 5E). These findings suggest that IGFBP-3 secreted from SCH66336-treated HNSCC cells inhibited proliferation of HUVECs by inhibiting Akt activation.

**Discussion**

In this article, we have shown for the first time that the FTI SCH66336 has antitumor activities, especially those related to angiogenesis, by inducing IGFBP-3 expression. The increased IGFBP-3 in turn induces tumor regression by inhibiting tumor angiogenesis via a mechanism that is independent of H-Ras and RhoB. We were encouraged to pursue this line of research by early observations of tumor regression in a clinical trial in which patients with advanced HNSCC were randomized to receive a short 8- to 14-day course of SCH66336 in the preoperative setting. Our in vivo results also clearly show that SCH66336 induces efficient antitumor activities; daily oral administration of SCH66336 (40 mg/kg) was sufficient to suppress growth of implanted UMSCC38 tumors in the tongues of nude mice. SCH66336 was active at a concentration of 1 μmol/L, which is well below the concentration reported to be achievable in vivo (about 8 μmol/L) in mice given a single oral dose of 25 mg/kg SCH66336 (34). SCH66336 inhibits proliferation of HNSCC cells (15); similar results have been observed in cell lines derived from breast, colon, pancreas, brain, and lung cancers (35–37).

Despite these promising results, the mechanism of action of FTIs in tumors is still incompletely understood. We did not find evidence of SCH66336-mediated apoptotic activities in most of the HNSCC cell lines used in our study, results which are consistent with previous findings that SCH66336 as a single agent cannot induce apoptosis at doses similar to those we used (38). This led us to investigate the angiogenic activities of SCH66336 in HNSCC cells. SCH66336 inhibited the angiogenic activities of HNSCC cells in vitro and in vivo: (a)

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Unpublished data.
administration of SCH6636 significantly reduced tumor vascularization in HNSCC orthotopic tongue tumors; (b) pretreatment with SCH6636 effectively suppressed angiogenesis-stimulating effects of the conditioned media from HNSCC38 cells on HUVECs. The antiangiogenic activities of SCH6636 in HNSCC cells did not correlate with mutation and expression of Ras and RhoB in our study, suggesting that FTIs have mechanisms of action other than inhibiting Ras or RhoB.

We found that FTIs, including SCH6636 and FTI-277, induced expression of IGFBP-3, a major IGFBP in serum (39), in several different cancer cell lines, orthotopic tongue tumor tissues from mice, and a subset of patients with HNSCC. These findings revealed that the effect of FTIs on IGFBP-3 expression is a generic response to FTI treatment, and that oral administration of SCH6636 is sufficient to induce IGFBP-3 expression in vivo. Because FTIs up-regulated promoter activities and transcription of the IGFBP-3 gene, FTIs seemed to induce IGFBP-3 gene expression at transcription level. The effects of FTIs on IGFBP-3 expression could be mediated by novel farnesylated proteins (37) or by farnesyl transferase–independent off-target activity of FTIs. It is also possible that FTIs may activate various transcription factors that stimulate the IGFBP-3 promoter, such as Sp-1/Sp-3, p53, vitamin D receptor, and retinoid X receptors. A detailed mechanism that is critical for the induction of IGFBP-3 expression by SCH6636 is currently under active investigation in our laboratory.

Perhaps most strikingly, our findings presented here show the role of IGFBP-3 as the functional basis for the use of FTIs in HNSCC targeting tumor angiogenesis; inhibition of
the secretion of SCH66336-induced IGFBP-3 from UMSCC38 cells by RNA interference or depletion of IGFBP-3 in HUVECs by neutralizing antibody significantly restored angiogenic effects of conditioned media from SCH66336-treated UMSCC38 cells. Therefore, it is plausible to say that FTIs induce IGFBP-3 expression, which in turn regulate tumor angiogenesis by inhibiting Akt, a key enzyme for cell survival, and thus inducing apoptosis in endothelial cells.

In conclusion, our results reveal, for the first time, that the FTIs have potent antiangiogenic activities in HNSCC cells through the induction of IGFBP-3 expression. Our results could explain the antitumor activities of FTIs in the cancer cells that do not harbor activated Ras oncogene (40–43). IGFBP-3 has been also identified as having antitumor activities in a variety of cancers (16, 44, 45). Several case control studies have shown that serum IGFBP-3 levels inversely correlate with the risk of numerous cancers, including prostate (46), bladder (46), and colon (46). Smoking reduces IGFBP-3 levels (46) and low

Fig. 4. Immunohistochemical analysis of IGFBP-3 in tongue tumor tissues. A, IGFBP-3 expression in orthotopic tongue tumor tissues from control nude mice (left) and SCH66336-treated mice (right) at 28 days. B, IGFBP-3 expression in normal and SCC tissues from a patient with HNSCC. C, IGFBP-3 expression was also tested in two patients before (left) or after 3 months of treatment with SCH66336 (right), ×400.

Fig. 5. IGFBP-3 secreted from SCH66336-treated HNSCC cells inhibits angiogenic activities in HUVECs by blocking Akt activation. A-C, effects of knock down of IGFBP-3 expression on antiangiogenic activities of SCH66336. A, semiquantitative reverse transcription-PCR analysis for IGFBP-3, H-ras, and RhoB mRNA expression (left) and Western blot analyses for IGFBP-3 protein expression (right) were done in UMSCC38 cells transfected with control (scr) or IGFBP-3 siRNAs for 1 day before the SCH66336 (5 μmol/L) treatment. GAPDH mRNA and β-actin protein expression were also detected as controls for reverse transcription-PCR and Western blot analyses, respectively. Effects of conditioned media (CM) from these cells on HUVEC morphogenesis (E) and proliferation (C) were tested by tube formation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analyses, respectively. Independent experiments were repeated three times. Columns, percentages of eight samples; bars, SD/95% confidence intervals. *, P < 0.05; **, P < 0.01 compared with control. D–E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (D) and Western blot analyses (E) were performed in HUVECs, which were untreated or preincubated with IGFBP-3-neutralizing antibody (αBP-3) for 2 hours and then treated with EB (–) or conditioned media from control UMSCC38 cells (Con) or from SCH66336-treated UMSCC38 cells (SCH). Independent experiments on cell proliferation were repeated three times. Columns, percentages of eight samples; bars, SD. αBP-3, antibody neutralizing IGFBP-3. **, P < 0.01 compared with HUVECs treated with control conditioned media.
IGFBP-3 concentrations are associated with increased risk of head and neck (46) as well as lung cancers (24). Furthermore, a decrease in IGFBP-3 expression due to the methylation or polymorphisms of the IGFBP-3 promoter is associated with an increased risk of lung, breast, and prostate cancers (46). Considering all of these promising results, IGFBP-3 should be critically evaluated in translational clinical trials against pathologic angiogenesis.

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