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Cellular Adaptation to VEGF-Targeted Antiangiogenic Therapy Induces Evasive Resistance by Overproduction of Alternative Endothelial Cell Growth Factors in Renal Cell Carcinoma[1,2]

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Abstract

Vascular endothelial growth factor (VEGF)–targeted antiangiogenic therapy significantly inhibits the growth of clear cell renal cell carcinoma (RCC). Eventually, therapy resistance develops in even the most responsive cases, but the mechanisms of resistance remain unclear. Herein, we developed two tumor models derived from an RCC cell line by conditioning the parental cells to two different stresses caused by VEGF-targeted therapy (sunitinib exposure and hypoxia) to investigate the mechanism of resistance to such therapy in RCC. Sunitinib-conditioned Caki-1 cells *in vitro* did not show resistance to sunitinib compared with parental cells, but when tested *in vivo*, these cells appeared to be highly resistant to sunitinib treatment. Hypoxia-conditioned Caki-1 cells are more resistant to hypoxia and have increased vascularity due to the upregulation of VEGF production; however, they did not develop sunitinib resistance either *in vitro* or *in vivo*. Human endothelial cells were more proliferative and showed increased tube formation in conditioned media from sunitinib-conditioned Caki-1 cells compared with parental cells. Gene expression profiling using RNA microarrays revealed that several genes related to tissue development and remodeling, including the development and migration of endothelial cells, were upregulated in sunitinib-conditioned Caki-1 cells compared with parental and hypoxia-conditioned cells. These findings suggest that evasive resistance to VEGF-targeted therapy is acquired by activation of VEGF-independent angiogenesis pathways induced through interactions with VEGF-targeted drugs, but not by hypoxia. These results emphasize that increased inhibition of tumor angiogenesis is required to delay the development of resistance to antiangiogenic therapy and maintain the therapeutic response in RCC.

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Introduction

Angiogenesis is an essential process for the growth and spread of tumors, and blocking tumor angiogenesis has been used as a therapeutic strategy against many types of cancer [1]. Because vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis, therapeutic approaches have focused on inhibition of the VEGF pathway [2]. Renal cell carcinoma (RCC) is generally resistant to conventional chemo- and radiotherapy and is a highly vascular tumor. VEGF is highly upregulated in RCC cells, likely because of mutations in the Von Hippel-Lindau gene, which induces degradation of hypoxia-inducible factor-1α through ubiquitination and is mutated in 75% of sporadic clear cell type RCCs [3]. VEGF is the key element in the pathogenesis of RCC, and VEGF receptor tyrosine kinase inhibitors have been successfully applied to treat RCC and have shown clinical benefits [4]. One multityrosine kinase inhibitor (TKI) that targets the VEGF and platelet derived growth

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factor (PDGF) receptor tyrosine kinases, sunitinib, has improved the outcomes of RCC patients compared with other therapies, such as cytokine therapy, and is currently considered a first-line option in patients with advanced RCC [5].

Unfortunately, durable and complete responses to TKIs are rare, and treatment has typically been associated with the development of resistance after short-term disease stabilization. There are several hypotheses regarding the development of evasive resistance to antiangiogenic therapy, but the exact mechanisms remain unknown. Because TKIs act on the endothelium's angiogenic cascade, therapy resistance cannot be explained simply by mutations within the important mediators in the angiogenic cascade [5]. Antiangiogenic therapy induces several stresses in cancer cells either directly or indirectly by inhibiting tumor endothelial cells. One of the initial responses to antiangiogenic therapies is the decrease in vessel number and function and the consequently increased intratumor hypoxia. Also, single-agent antiangiogenic therapy-induced tumor hypoxia may increase the expression of other proangiogenic factors, causing resistance to the initial drug [6]. Evidence for the existence of evasive resistance originating from alternative proangiogenic signaling was revealed during preclinical trials in a genetically engineered mouse model of pancreatic neuroendocrine (islet cell) cancer, Rip1-Tag2 [7,8]. In this study, the resistant tumors expressed higher levels of the mRNAs for the proangiogenic factors fibroblast growth factor (FGF) 1 and 2, ephrin A1 and A2, and angiopoietin 1, and the addition of FGF inhibitor suppressed revascularization and tumor growth. These studies suggested that antiangiogenic therapy-induced hypoxic stress may induce overexpression of different proangiogenic factors, resulting in resistance to VEGF-targeted therapy.

Although antiangiogenic therapy targets genetically stable endothelial cells in the tumor vasculature, genetic alterations that decrease the vascular dependence of tumor cells can influence the therapeutic response of tumors [9]. It has been reported that the hypoxia generated by angiogenesis inhibition triggers pathways resulting in more aggressive and metastatic tumors. In glioblastoma multiforme, cancer cells can migrate as diffuse colonies into the surrounding brain without initiating angiogenesis [8,10]. In addition, cancer cells can adapt to a hypoxic environment when tumor angiogenesis is inhibited by selection of clones that are p53 negative, and therefore become hypoxia resistant [9]. These studies proposed various methods by which cancer cells can grow without the recruitment of new blood vessels, and expansion of hypoxia-resistant clones selected by chronic hypoxic stress through angiogenesis inhibition could be a cause of resistance to antiangiogenic therapy.

In this study, we show that chronic hypoxic stress induces overproduction of VEGF and hypoxia resistance in RCC cells, but tumor cells adapted to hypoxia remain dependent on VEGF, and hypoxic stress does not induce *in vivo* resistance to VEGF-targeted antiangiogenic therapy in RCC xenografts. In contrast, we show that VEGF-targeted antiangiogenic drugs directly affect RCC cells and induce tumor cell production of several VEGF-independent proangiogenic factors, thereby inducing evasive resistance in RCC xenografts.

Materials and Methods

Reagents

Sunitinib was used as a VEGF-targeted tyrosine kinase inhibitor. Free-base sunitinib and sunitinib malate were purchased from LC Laboratories. Mass spectrometry was used to ensure the quality of sunitinib compared with its pharmaceutical-grade counterpart (Pfizer, NY). Free-base sunitinib was preserved as aliquots at a concentration of 10 mM in DMSO (Sigma) for *in vitro* experiments, and sunitinib malate was mixed with citrate-buffered solution (pH 3.5) for *in vivo* studies.

Cell Culture

Human clear cell type RCC cell lines Caki-1 and Caki-2 were purchased from the American Type Culture Collection. Caki-1 and Caki-2 were maintained with McCoy's 5A medium (Invitrogen) containing 10% fetal bovine serum (FBS). The other human RCC cell lines, UMRC-3 and UMRC-6, were kindly gifted by Dr. P. Black (Vancouver Prostate Centre, UBC). UMRC-3 and UMRC-6 were maintained in MEM medium (Invitrogen) containing 10% FBS and L-glutamine. The human renal proximal tubular epithelial cell line HK-2 was kindly provided by Dr. C. Du (Vancouver Prostate Centre, UBC). HK-2 cells were cultured in DMEM/Ham's F12 (Invitrogen) supplemented with 10% FBS and glutamine. Immortalized human umbilical vascular endothelial cells (HUVECs) were obtained from Dr. C. Du and maintained with EBM-2 medium (Lonza) containing EGM-2 SingleQuots (Lonza). All cells were cultured at 37°C in a humid atmosphere with 5% CO_2. Mycoplasma contamination was tested. For all *in vitro* studies, cell lines were passaged for a maximum of 2 months.

Establishment of Sunitinib-Conditioned Tumor Cells

Caki-1 cells were plated in 15-cm plates with McCoy's 5A medium with 10% FBS, grown to 50% confluence, and incubated overnight for attachment. Cells were then exposed to sunitinib by replacing media with fresh sunitinib-containing media. The sunitinib concentration and exposure time were adjusted depending on the tolerance of the cells. Cells were exposed to sunitinib for 3 to 5 days, and media were replaced with fresh media without sunitinib for 24 to 48 hours. Cells that showed proliferation at a specific sunitinib concentration were replated and exposed to a higher concentration (<25% higher than the previous concentration). If the increased concentration was not tolerated, the cells were maintained in media with an identical or lower sunitinib concentration. The sunitinib on-off exposure cycle was maintained until the cells could proliferate in the presence of the target concentration (approximately 14-20 cycles). The final tolerated concentration of sunitinib was 15 μmol/l. Cells at all steps were frozen after use. Mycoplasma contamination was evaluated every five cycles.

Establishment of Hypoxia-Conditioned Tumor Cells

Caki-1 cells were plated onto a 15-cm plate with McCoy's 5A medium with 10% FBS, grown to 50% confluence, and incubated overnight for attachment. Cells were then exposed to hypoxia (1% oxygen) until they became confluent. Cells were maintained in the hypoxia chamber in same manner, excluding passaging. When the cells became confluent, they were replated in normoxic conditions and incubated in a normoxic tissue culture incubator for 24 hours. Hypoxic conditions (1% oxygen) were induced using a hypoxia chamber (Coy Corporation). During each hypoxic exposure cycle, development of resistance to hypoxia was tested by examining the growth of hypoxia-conditioned cells and parental cells under hypoxic conditions. For all *in vitro* experiments, cells were maintained under hypoxic conditions before use. For *in vivo* studies, cells were maintained under hypoxic conditions until injection.

Cell Viability Assay

Cells were seeded onto 48-well plates at a density of 1.5×10^4 per well in media with 10% FBS and allowed to attach for 24 hours.

DMSO or sunitinib was added at different concentrations under normoxic or hypoxic (1% O_2) conditions. After 48 hours of treatment, cells were fixed with 10% glutaraldehyde and stained with 0.5% crystal violet solution. Cells were then washed with water and dried. Stained crystal violets were resolved in Sorensen's solution, and absorbance was measured at 520 nm using a spectrophotometer. All experiments were performed in triplicate and repeated a minimum of three times.

Anchorage-Independent Cell Growth Assay

Culture plates (48 wells) were coated with 0.5 ml of the bottom agar mixture (1× RPMI, 10% FBS, and 0.6% agar) and solidified at 4°C. Cells (1.5×10^3) were trypsinized and resuspended in 0.5 ml of top agar mixture (1× RPMI, 10% FBS, and 0.3% agar) containing various doses of sunitinib. The top agar mixtures containing cells were added to the prepared bottom agar mixture. Plates were incubated at 37°C for 14 days, and medium was added once per week. Colonies were stained overnight at 37°C with 0.05% crystal violet in ethanol, and the colonies were visualized at 50× magnification; five different fields were counted manually using Axiovision software, and the average number of colonies per well was calculated. All experiments were performed in triplicate and repeated three times.

Cell Cycle Analysis

At 30% to 40% confluency, cells were synchronized at G0/G1 phase by eliminating serum from the culture medium for 24 hours. After release from the G1 block by substitution with fresh media containing 10% FBS, cells were maintained for 24 hours. Cells were fixed with 70% ethanol at −20°C overnight and then treated with 10 μg/ml of RNAase and stained with 100 μg/ml of propidium iodide (PI). Cell cycle phase was determined by flow cytometry. All experiments were performed in triplicate and repeated three times.

Quantitative Reverse Transcription Polymerase Chain Reaction (PCR)

RNA was extracted from cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified by the ratio of absorbance at 260/280 nm. First-strand cDNA was synthesized from 2 μg of RNA by reverse transcriptase (Invitrogen) with a random hexamer primer. Primers for PCR were as follows: VEGF-A, basic FGF, PlGF, IL-8, MMP-2, TSP-1, and 18S rRNA. All experiments were performed in triplicate and repeated a minimum of three times.

Tumor Xenografts

All animal studies were performed in accordance to the guidelines of the Canadian Council on Animal Care with institutional certifications (University of British Columbia). Parental Caki-1 cells (Caki-1-WT), hypoxia-conditioned Caki-1 cells (Caki-1-HC), and sunitinib-conditioned Caki-1 cells (Caki-1-SC) (5×10^6 cells) were injected subcutaneously (s.c.) in the flank region of 8- to 10-week-old nude mice. When tumors reached a volume of 100 to 200 mm^3, mice were randomized and divided into two groups (sunitinib versus vehicle treatment). Each treatment group consisted of eight mice. Sunitinib malate was suspended in citrate-buffered solution (pH 3.5). Tumor-bearing mice were orally administered sunitinib malate (40 mg/kg) or vehicle (citrate-buffered solution) once daily. Tumor volume was calculated using the equation tumor volume (mm^3) = length × width × height × 0.5 and measured every 3 days using calipers. Tumors were harvested at various time points including the start of treatment and during treatment, according to responses. Tumors were fixed in formaldehyde, paraffin embedded for immunohistochemical staining, and frozen in liquid nitrogen for protein and mRNA analyses. For hypoxia staining, pomonidazole was injected intravenously 30 minutes before euthanization. Mice were anesthetized, and tumor tissues were extracted immediately so that the tissue hypoxia status was not affected. All animals at the completion of the study were euthanized by inhalation of CO_2 according to the animal ethics protocol.

Tumor Reimplantation

Caki-1-WT and Caki-1-SC xenografts were used for reimplantation. Xenograft tumors, treated with either sunitinib or vehicle, were obtained before sacrifice at the end of the previous experiment. Immediately after sacrifice, tumors were excised and cut into 3 × 3 × 3–mm cubic pieces and implanted s.c. into 6- to 8-week-old female nude mice. When tumors reached 200 to 300 mm^3, they were randomly divided into two groups followed by treatment with sunitinib or vehicle.

In vivo Angiogenesis Assay

Caki-1-WT and Caki-1-SC cells in exponential growth were harvested, and 1×10^6 cells in 300 μl of Matrigel were implanted s.c. into the flanks of 6- to 8-week-old female nude mice. Ten days postimplantation, mice were divided into two groups; one group was treated with sunitinib (40 mg/kg per day) and the other with vehicle. After 5 days of treatment, all mice were sacrificed, and the implanted plugs were excised and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA). After being frozen at −80°C for 1 hour, 5-μm sections were cut from the embedded tissues using a CM1850 cryostat (Leica Microsystems, Richmond Hill, ON, Canada) and applied to glass slides. Immunofluorescence staining was performed using a rat anti-mouse CD31 antibody.

Tube Formation Assay

Low-passage HUVECs were cultured in M199 media with 20% FBS and starved for 4 hours before the tube formation assay. HUVECs were mixed with media containing 1% FBS and then seeded on Matrigel-coated 24-well plates at a density of 10^4 cells per well. VEGF and various doses of sunitinib were added to the media for VEGF-dependent tube formation assays. Conditioned media from tumor cells were used for the tube formation assay as a validation study of the *in vivo* angiogenesis effect. Caki-1 cells and sunitinib-conditioned Caki-1 cells were cultured in complete media. Media were replaced by media containing 1% FBS at 50% confluency. After 24 hours, the conditioned media were filtered and directly added to HUVECs for the tube formation assay. Tube formation was evaluated using a microscope, and photographs were taken 3 to 18 hours after seeding. The area covered by the tube network was determined with an optical imaging technique. Images of the tubes were scanned into Adobe Photoshop and quantified using Image J software.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded 4-μm tumor sections using the appropriate primary antibody (mouse anti-CD31 and anti-pimonidazole antibodies) and the Ventana autostainer Discover XT (Ventana Medical Systems) using an enzyme-labeled biotin streptavidin system and

solvent-resistant 3,3'-diaminobennyidine map kit. All staining intensity comparisons were made at 200× magnification and scored independently by two histopathologists (Dr. L.F. and Dr. K.H.).

mRNA Microarray

Total RNA from the parental Caki-1 cells, sunitinib-conditioned Caki-1 cells, and hypoxia-conditioned Caki-1 cells were obtained using the TRIzol method. Three independent RNA samples were obtained from the cells to minimize experimental variation. Microarrays of 21,000 (70-mer) human oligonucleotides representing 21,000 genes (Operon Technologies) printed in duplicate in 3× SSC onto aminosilane-coated slides (ERIE C28) were supplied by the Array Facility of The Prostate Center at Vancouver General Hospital. Arrays were hybridized with 3DNA DNA Dendrimer Probes generated from 10 μg of total RNA according to the manufacturer's protocol (Genisphere). Briefly, reverse transcription incorporated a specific sequence present on the 5′ end of the reverse transcription primer supplied with the kit. The complementary DNA was hybridized to the array overnight at 42°C. After stringent washes, the fluorescent 3DNA reagent, which included a "capture sequence" complementary to the sequence at the 5′ end of the reverse transcription primer, was hybridized to the complementary DNA (47°C for 2-3 hours). After washing, the arrays were immediately scanned on a ScanArrayExpress Microarray Scanner (PerkinElmer). Signal quality and quantity were assessed using Imagene 5.6 (BioDiscovery). Data from Imagene were analyzed in GeneSpring 7.2 (Silicon Genetics) for profiling of changes in gene expression. Analyses performed in GeneSpring included background correction, LOWESS normalization, and hierarchical clustering using standard correlation. Differentially expressed genes were identified by two-sample *t* tests, and *P* values were adjusted for multiple comparisons using the false-discovery rate method of Benjamini and Hochberg. The Ingenuity Pathway Analysis (Mountain View, CA) was used to examine functional and network associations between differentially expressed genes. Significances for biological functions were compared with the whole Ingenuity Pathway Knowledge Base as a reference set.

Results

Effect of Sunitinib on Angiogenesis in Human RCC Cells

We examined the direct effect of the VEGF-targeted tyrosine kinase inhibitor, sunitinib, on cell proliferation in both RCC and endothelial cells using anchorage-dependent and -independent cell proliferation assays. Crystal violet assays after sunitinib treatment demonstrated that tumor cell growth was inhibited by sunitinib in all RCC cells (Caki-1, Caki-2, UMRC-3, and UMRC-6) and normal renal epithelial cells (HK-2) in a dose-dependent manner (Figure 1*A*). The IC_{50} was 4 to 8 μM for RCC cells and 15 μM for normal renal cells. Although sunitinib suppressed tumor cell proliferation at higher doses, these are not considered pharmacologically relevant doses (<1 μM). To further evaluate the effect of sunitinib on tumor growth, we performed anchorage-independent colony formation assays using Caki-1 and Caki-2 cells. These assays confirmed that tumor cell growth was not inhibited by sunitinib at pharmacologically relevant doses (Figure 1*B*). We also examined the effect of sunitinib on cell cycle progression in RCC using FACS cytometry with PI staining. Cell cycle analysis showed that sunitinib induced G0/G1 phase arrest at high doses but had no effect on cell cycle progression at low doses (Figure 1*C*). In contrast, cell growth assays with HUVECs revealed that proliferation was effectively inhibited by sunitinib at low doses (IC50) (Figure 1*D*). We next performed a tube formation assay to corroborate this finding. Tube formation was also inhibited by low doses of sunitinib (<1 μM), confirming that sunitinib suppressed endothelial cell growth and angiogenesis but not RCC cell proliferation.

Chronic Hypoxia Increases the Hypoxia Resistance of RCC Cells

To evaluate whether chronic hypoxic stress increased the resistance to hypoxia of RCC cells, Caki-1 cells were chronically exposed to 1% O_2 hypoxic conditions using a hypoxia culture chamber. Cell growth assays showed that hypoxia reduced tumor cell growth when the cells were initially exposed to hypoxia; however, after several passages, the growth rate was restored (Figure 2*A*). The resistance increased depending on the hypoxic exposure time, and cell proliferation was increased in normoxia compared with parental cells, but there was no increase in resistance after 15 passages under hypoxic conditions. Next, we tested hypoxia-conditioned cells with sunitinib to evaluate the effects of chronic hypoxia on sunitinib response. Cell growth assays using hypoxia-conditioned Caki-1 cells (Caki-1-HC) showed the same pattern as did those using parental Caki-1 cells (Caki-1-WT), suggesting that hypoxia does not affect the response to sunitinib (Figure 2*B*).

Chronic Exposure to Sunitinib Does Not Induce In Vitro Resistance to Sunitinib in RCC Cells

To evaluate whether chronic adaptation to sunitinib induces resistance to sunitinib in RCC, Caki-1 cells were chronically exposed to sunitinib. Cells were initially exposed to low doses of sunitinib, which were then increased sequentially. Caki-1 cells stably adapted to 10 μM sunitinib (Caki-1-SC). The cell growth assay revealed that sunitinib-conditioned Caki-1 cells (Caki-1-SC) were more resistant to sunitinib, and the IC_{50} increased from 4 to 8 μM to 10 to 15 μM (Figure 2*B*). However, there was no significant difference in sunitinib sensitivity at pharmacologically relevant doses between Caki-1-WT and Caki-1-SC cells. We next tested the hypoxia resistance of Caki-SC cells to evaluate the effect of chronic sunitinib stress on tolerance to hypoxia in renal carcinoma cells. Caki-1-SC cells were more tolerant to hypoxic stress than were parental cells but less tolerant than hypoxia-conditioned cells (Figure 2*A*).

Chronic Adaptation to Sunitinib or Hypoxia Changes Expression Patterns of Proangiogenic Factors in RCC Cells

Changes in proangiogenic properties of RCC cells after exposure to hypoxia or sunitinib were assessed using reverse transcription PCR. VEGF mRNA expression levels were significantly increased in Caki-1-SC cells and even higher in Caki-1-HC cells compared with Caki-1-WT cells (Figure 3*A*). ELISA confirmed these differences in VEGF expression (Figure 3*B*). We also examined the expression of the proangiogenic factors FGF2 and IL-8, which may play a role in the resistance to VEGF-targeted antiangiogenic therapy [7,11]. FGF2 expression was not significantly increased by chronic adaptation to sunitinib or hypoxia (Figure 3*C*). IL-8 was increased only by chronic adaptation to Caki-1-HC cells, but its expression remained relatively low (Figure 3*D*). These results suggest that cellular adaptation to hypoxia makes RCC cells more angiogenic, which is dependent on overproduction of VEGF. We also examined the expression of other proangiogenic factors including PlGF, angiopoietin, TGFβ1,

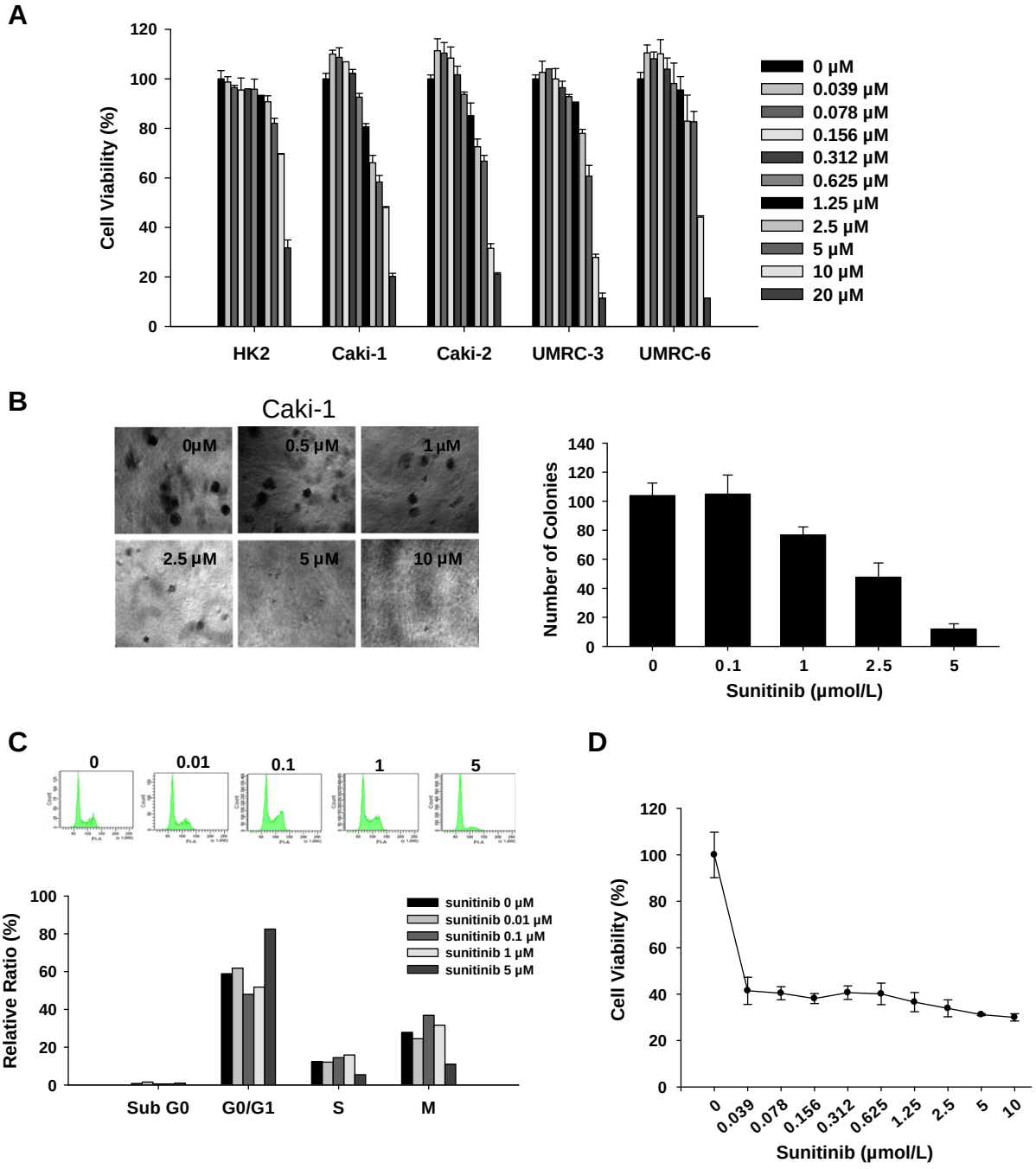


Figure 1. *In vitro* effects of sunitinib on cell proliferation in RCC and endothelial cells. (A) Cell viability was evaluated using a crystal violet assay following treatment with sunitinib at the indicated concentrations (0020 μM) on a panel of RCC cells. (B) Caki-1 cells were resuspended in an agar mixture containing various doses of sunitinib, and colonies were evaluated after 14 days of incubation at 37°C. (C) Caki-1 cells were maintained for 24 hours after serum starvation and analyzed by flow cytometry with PI staining. (D) HUVECs were maintained with VEGF, and sunitinib was added at the indicated concentrations.

antiangiogenic factors, and TSP-1; their expression levels did not differ significantly (data not shown).

Effects of Chronic Sunitinib and Hypoxia In Vivo

We investigated the effects of chronic adaptation to sunitinib and hypoxia on sunitinib resistance *in vivo* using a subcutaneous xenograft model with both Caki-1-WT and conditioned Caki-1 cells. Daily oral sunitinib treatment inhibited tumor growth by up to 75% in xenografts developed by parental cells (Figure 4*A*). The growth of Caki-1-HC tumors was suppressed by sunitinib treatment, and their growth was identical to that of Caki-1-WT tumors (Figure 4*B*). In comparison, Caki-1-SC tumors were more resistant to sunitinib treatment compared with Caki-1-WT tumors (Figure 4, *C* and *D*). To confirm these findings, we reimplanted tumors into sunitinib-treated naïve mice following treatment. The reimplanted xenografts from Caki-1-WT tumors responded to sunitinib, whereas Caki-1-SC tumors remained resistant to sunitinib (Figure S1). Therefore, resistant tumors remain resistant to sunitinib even when placed in new environments.

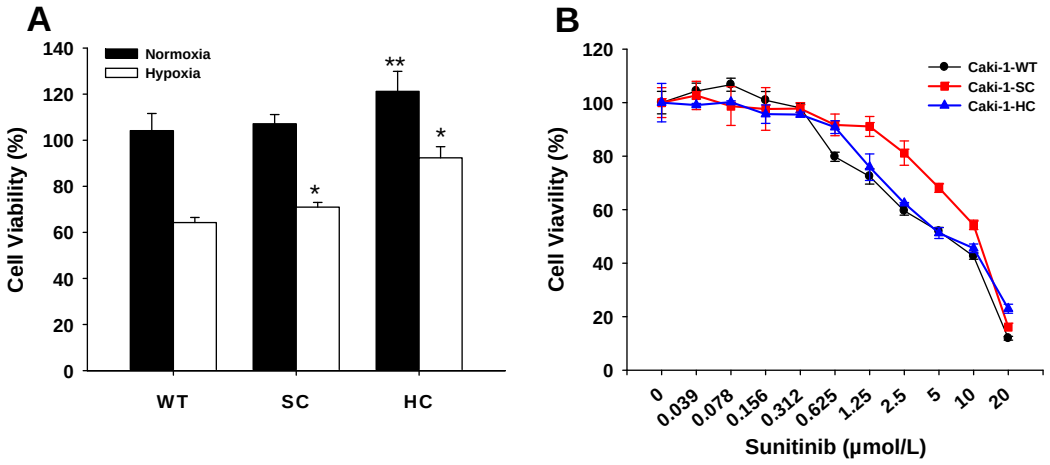


Figure 2. *In vitro* phenotypic changes in Caki-1 cells following chronic exposure to sunitinib or hypoxia. Caki-1 cells were chronically exposed and adapted to sunitinib or hypoxia. Cell viability was then tested following sunitinib treatment (A) or under hypoxic conditions (B). *$P < .001$ versus WT in hypoxia, **$P < .001$ versus WT in normoxia.

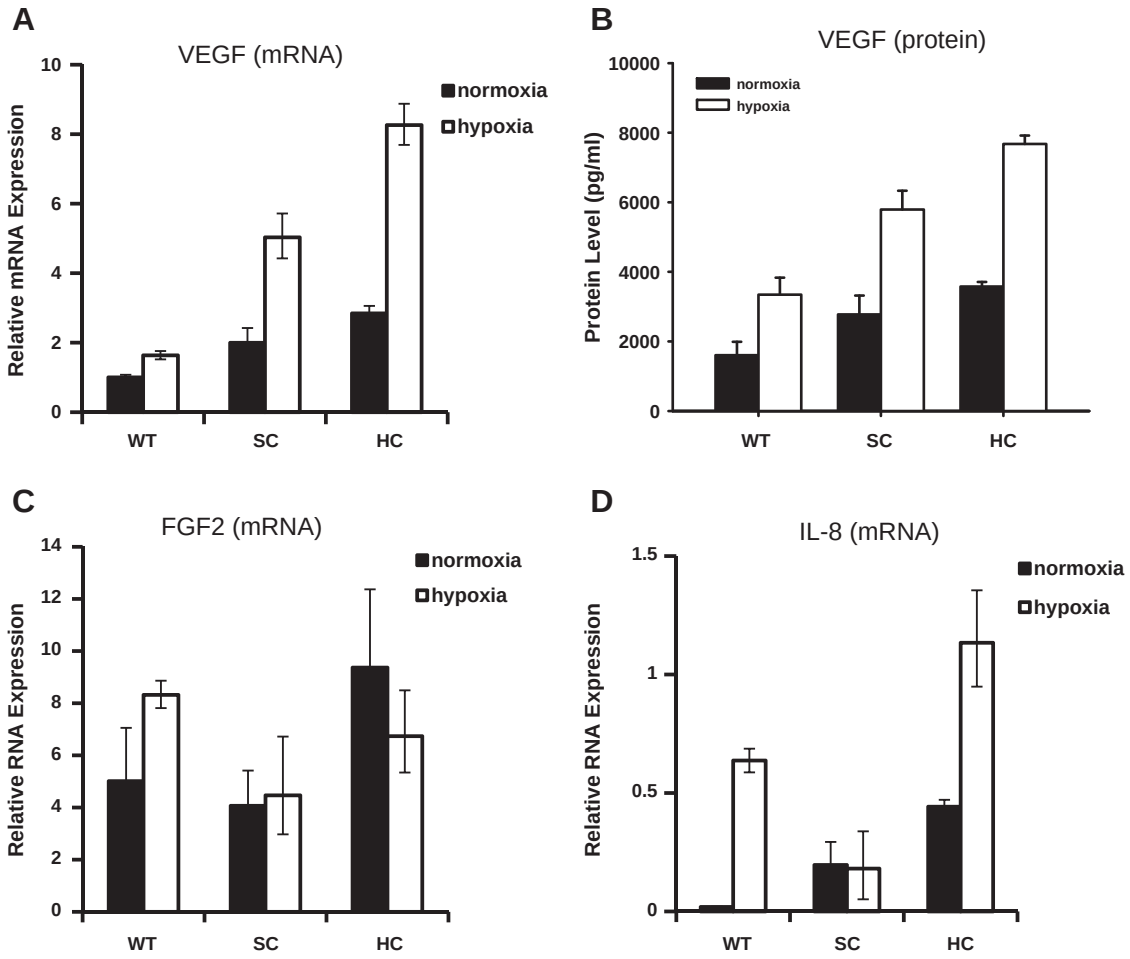


Figure 3. Effects of chronic sunitinib exposure and hypoxia on the expression patterns of proangiogenic factors in Caki-1 cells. (A and B) Expression and production of VEGF were evaluated using qPCR and ELISA in parental Caki-1 cells, sunitinib-conditioned cells (Caki-1-SC), and hypoxia-conditioned cells (Caki-1-HC). (C and D) Expression of FGF2, a previously studied proangiogenic factor (C), and IL-8, another known proangiogenic factor (D), was evaluated using qPCR in Caki-1-WT, Caki-1-SC, and Caki-1-HC cells. *$P < .001$ versus WT in normoxia, ** $P < .001$ versus WT in hypoxia.

These data suggest that resistance to sunitinib is developed primarily by tumor cells but not by tumor endothelial cells and that these changes are persistent.

Effect of Chronic Suppression of VEGF in Tumor Cells on Other Angiogenic Factors

Immunohistochemical staining for the expression of vascular marker CD31 was performed on xenografts to assess the effect of sunitinib on angiogenesis. Microvessel density was markedly reduced in Caki-1-WT and Caki-1-HC xenografts. Conversely, similar to vehicle-treated controls, Caki-1-SC tumors exhibited a microvessel density that was relatively intact (Figure 5*A*). We also examined hypoxia in tumor xenografts using immunohistochemical staining with an antipimonidazole antibody. Consistent with the results described above, pimonidazole expression was high in Caki-1-WT and Caki-1-HC tumors treated with sunitinib but low in Cali-1-SC tumors (Figure 5*B*). To investigate whether this difference originates at the beginning of treatment or occurs at a later stage, xenografts were retreated with sunitinib (40 mg/kg) for 5 days and harvested to assess tumor vaculature using CD31 (Figure 5*C*). Taken together, these results suggest that sunitinib successfully induced extensive hypoxia in tumor cells by inhibiting tumor angiogenesis in Caki-1-WT and Caki-1-HC tumors but not in Caki-1-SC tumors.

To confirm these findings, we used *in vivo* Matrigel or tumor xenograft models to assess the effect of sunitinib on different cell lines. Microvessel density after 5 days of sunitinib treatment was highest in the xenografts originating from sunitinib-conditioned cells compared with parental cells and hypoxia-conditioned cells. Matrigel plug assays in nude mice showed that immunofluorescent staining with CD31 revealed a remarkable disappearance of microvessels in the plugs containing Caki-1-WT cells, whereas vessels were relatively intact in the plugs containing Caki-1-SC cells as well as the xenografts (Figure 6, *A* and *B*). To investigate the existence of alternative endothelial cell growth factors escaping the angiogenic suppression of sunitinib, conditioned media from Caki-1-SC and Caki-1-WT cells were applied to HUVECs, which were then assessed for *in vitro* angiogenesis following sunitinib treatment. Tube formation assays also confirmed the findings from the Matrigel plug assay. HUVEC tube formation was completely suppressed by sunitinib at 1.0 and 2.0 μM in conditioned media from the Caki-1-WT cells, whereas

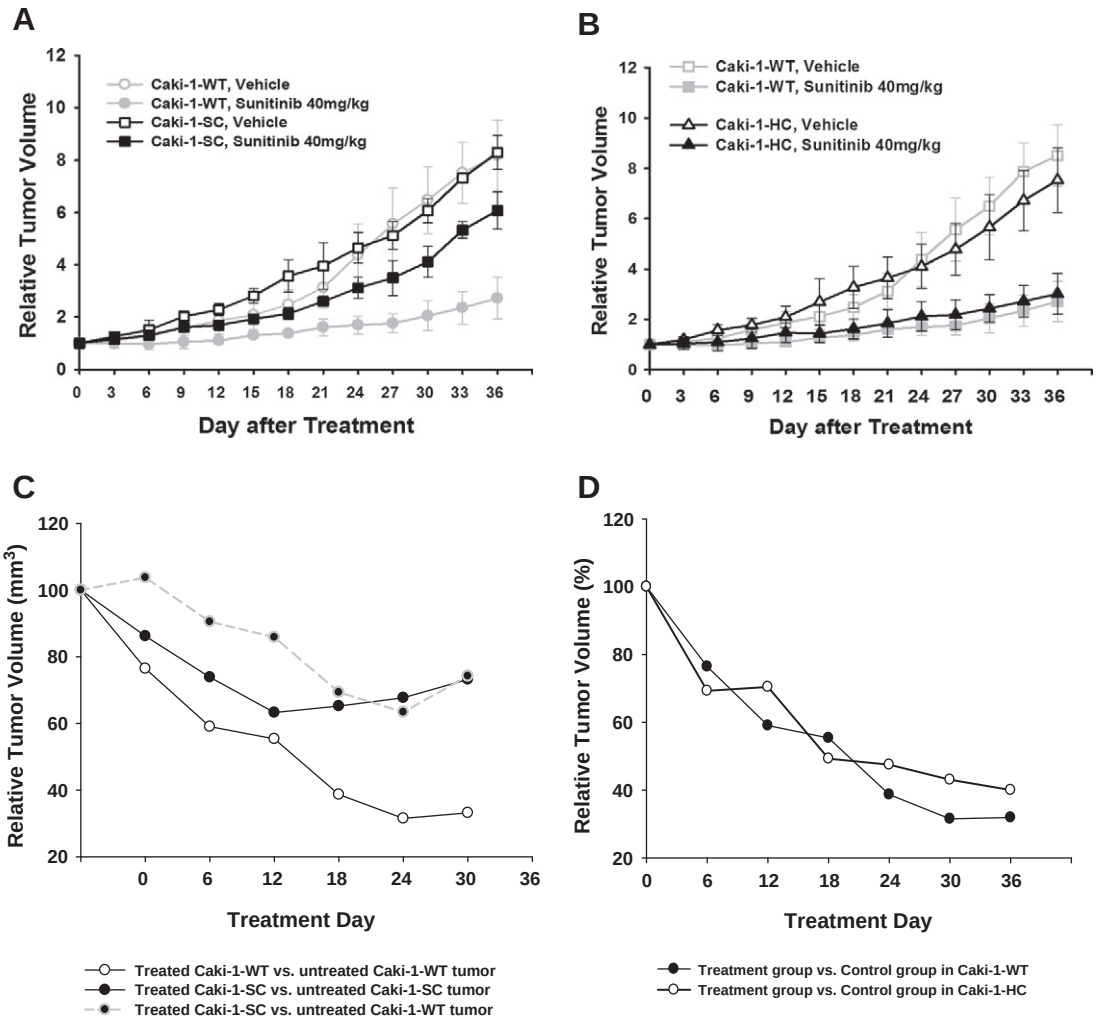


Figure 4. *In vivo* response to sunitinib by sunitinib-conditioned or hypoxia-conditioned Caki-1 cells. (A and B) Subcutaneous xenografts from Caki-1-WT, Caki-1-SC, and Caki-1-HC tumors were developed and treated with sunitinib (40 mg/kg) when the tumors reached 100 to 200 mm^3. *$P < .001$. (C and D) The volume of tumors treated with sunitinib was compared with that of untreated tumors in xenografts from Caki-1-SC (C) and Caki-1-HC mice (D). *$P < .001$ versus untreated tumor.

it was not suppressed in conditioned media from Caki-1-SC cells (Figure 6*C*). These data suggest that tumor cells are able to chronically adapt to sunitinib treatment and continue angiogenesis even with VEGF inhibition.

Effect of Sunitinib on VEGF-Independent Angiogenic Pathways

To identify the factors responsible for sunitinib resistance, the mRNA profiles of parental cells, sunitinib-conditioned cells, and hypoxia-conditioned cells were compared using mRNA microarrays (Table S1). RNA profiling revealed that some neuronal guiding and angiogenic factors were highly expressed in Caki-1-SC cells but not in Caki-1-WT or Caki-1-HC cells (Figure 7*A*). We found that neuronal guidance factors, such as Reelin, were highly upregulated, as were angiogenic factors Notch and BMP-6 in sunitinib-conditioned cells compared with parental and hypoxia-conditioned cells (Figure 7, *B* and *C*). Western blot analysis was performed to corroborate the findings from the mRNA microarray (Figure 7*D*). Taken together, these results suggest that chronic inhibition of VEGF signaling in tumor cells increases the expression of different growth factors, which may induce angiogenesis (Figure 7, *E* and *F*). These growth factors can induce evasive resistance by compensating for the inhibition of VEGF signaling in RCC cells.

Discussion

During antiangiogenic therapy, antitumor effects are achieved primarily by inducing hypoxia secondary to reducing tumor microvessels, which, in turn, leads to apoptotic cell death or necrosis. Anti-VEGF–induced hypoxic stress alters the expression patterns of proangiogenic factors from tumor cells. Unfortunately, tumor cells modify their angiogenic profiles to maintain adequate vasculature to proliferate even during VEGF inhibition. Several proangiogenic factors, such as FGF, ephrins, angiopoietin, and IL-8, are overexpressed in resistant tumors treated with VEGF-targeted therapy, and inhibition of these growth factors inhibits tumor growth [7,11]. However, the development of evasive resistance to antiangiogenic therapy remains unclear. Interestingly, we have shown that simple hypoxic stress was not sufficient to trigger resistance to antiangiogenic therapy. In contrast, VEGF-targeted inhibition induced overexpression of several proangiogenic factors, which was not dependent on VEGF. Thus, tumor cell overexpression of angiogenic growth factors induced *in vivo* resistance to VEGF-targeted therapy in RCC cells.

Reduced reliance on vascular supply is believed to be one mechanism of resistance to antiangiogenic therapy [10]. Hypoxic stress induces several phenotypic changes in tumor cells. After hypoxia, tumor cells become more angiogenic, proliferative, and metastatic [6]. Hypoxic tumor cells induce several factors to stimulate angiogenesis, of which the upregulation of HIF-1α plays a crucial role. HIF-1α modulates cell cycle arrest, has pro- and antiapoptotic effects, induces glycolysis, and stimulates angiogenesis through upregulation of VEGF [12–14]. Tumor cells deficient in p53 display a diminished rate of apoptosis under hypoxic conditions, which may reduce their reliance on vascular supply and their responsiveness to antiangiogenic therapy [10]. Tumor cells resistant to hypoxia survive

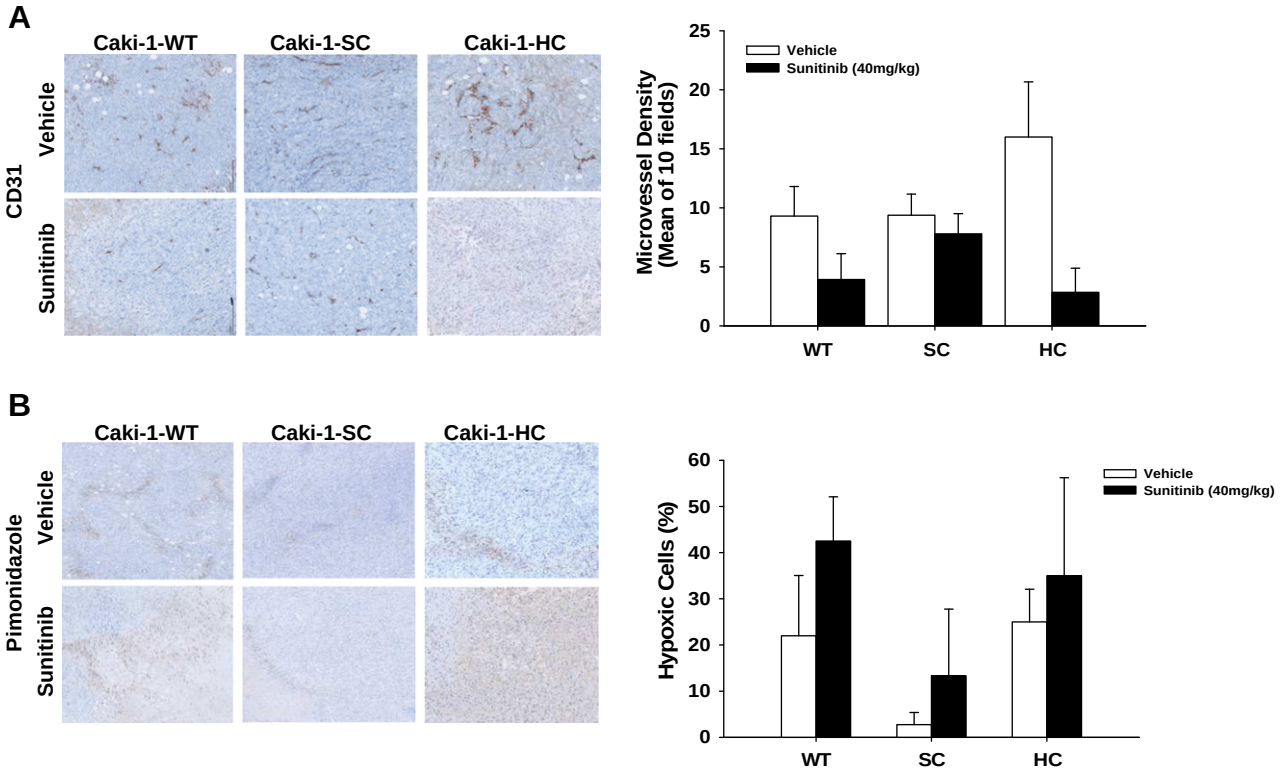


Figure 5. Changes in tumor angiogenesis by cell conditioning with sunitinib or hypoxia. (A) CD31 immunostaining to evaluate tumor vasculature in xenografts treated with sunitinib (40 mg/kg) or vehicle (DMSO). (B) Hypoxic areas were assessed by immunostaining of pimonidazole by injection with pimonidazole 30 minutes before sacrifice. *$P < .001$.

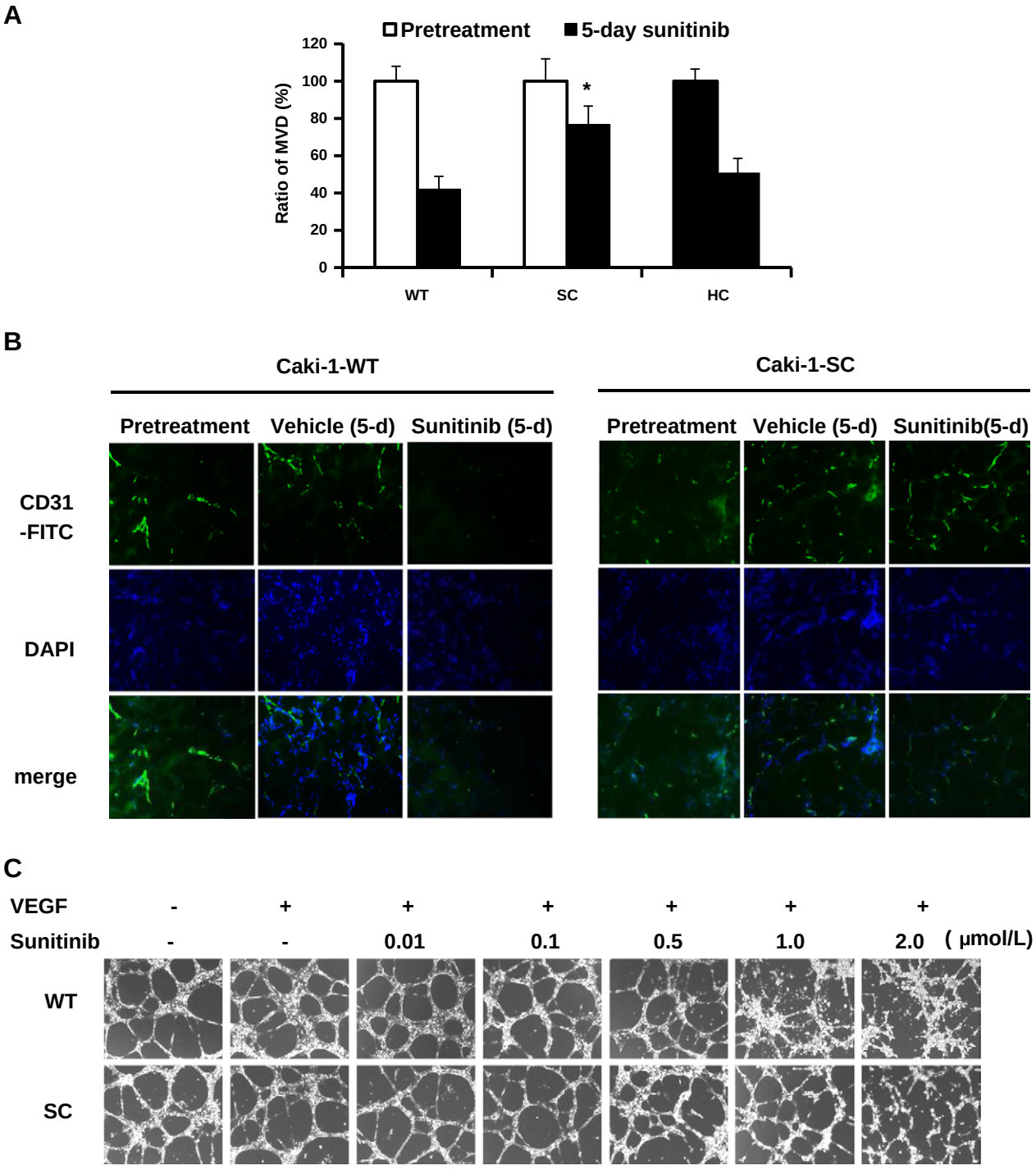


Figure 6. Effect of sunitinib-conditioned Caki-1 cells on tumor angiogenesis. (A and B) Tumor vasculature was assessed using immunofluorescent imaging with a CD31 antibody in tumor xenografts (A) and in Matrigel plugs (B) containing Caki-1-WT and Caki-1-SC cells after 5-day sunitinib treatment (40 mg/kg). (C) VEGF-dependent HUVEC tube formation with supernatants from Caki-1-WT or Caki-1-SC cells was performed with sunitinib at the indicated concentrations. *$P < .001$.

in low-oxygen environments by decreasing their oxygen dependency and increasing their movement toward oxygen. In a previous study, p53-mutated human colorectal cancer cells were less responsive to antiangiogenic combination therapy (DC101/Vbl), and p53-induced hypoxia resistance was thought to play a key role in antiangiogenic therapy resistance. However, p53 mutations induced overexpression of proangiogenic factors such as FGF, IL-8, IGF, and VEGF and could induce hypoxia resistance in tumor cells [15–17]. Therefore, resistance to antiangiogenic therapy after a p53 mutation could be derived from overexpression of several proangiogenic factors, as well as a reduced reliance on oxygen. Our data showed that hypoxia reduced the reliance of tumor cells on oxygen, and chronic exposure to hypoxia increased the general angiogenic potential of tumors. However, VEGF was mostly overexpressed under only hypoxia-induced

stress conditions, suggesting that tumor cells remain dependent on VEGF for their angiogenesis. Thus, *in vivo* resistance in RCC cells was not acquired by simple exposure to hypoxia.

The direct effects of VEGF-targeted therapy on tumor cells have been investigated but remain unclear. Recent studies reported that sunitinib induced cell apoptosis and growth arrest in RCC and medulloblastoma tumor cells by inhibiting signal transducer and activator of transcription 3 [18,19], whereas another study reported no direct cytotoxic effects of sunitinib against tumor cells. Sunitinib showed no significant effects on tumor cell proliferation or apoptosis at pharmacologically relevant doses [20]. However, VEGFR inhibition by sunitinib may affect tumor cells, and chronic inhibition of VEGFR signals on tumor cells could induce molecular and phenotypic changes, even though pharmacologically relevant doses of sunitinib did not inhibit tumor proliferation. Our study suggests that prolonged exposure to sunitinib directly affected tumor cells and altered the proangiogenic profiles therein, despite its lack of cytotoxicity against RCC cells. Chronic exposure of tumor cells to sunitinib protected endothelial cells from sunitinib-mediated inhibition of VEGF signaling, which suggests that production of some proangiogenic factors was induced by sunitinib treatment. These results indicate that tumor cells can compensate for the inhibition of growth factors by overproducing other growth factors. Also, these induced proangiogenic factors play an important role in the evasion of VEGF inhibition in RCC.

Many types of tumor cell depend on VEGF for tumor angiogenesis. However, tumors can produce more than one type of

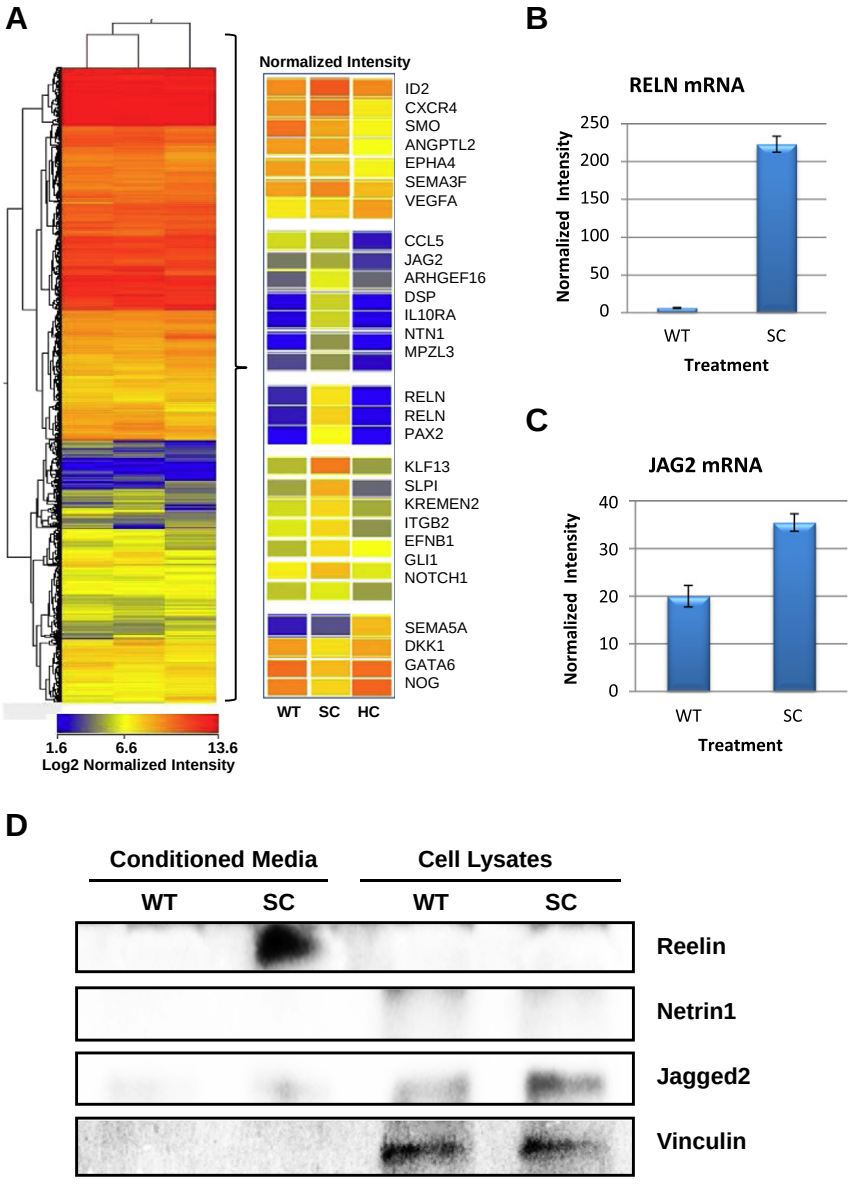
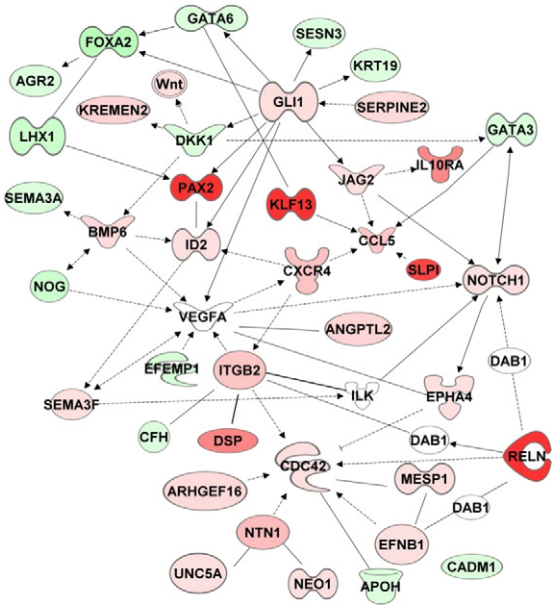


Figure 7. mRNA microarray and validation. (A to C) Differences in mRNA expression were assessed by a microarray of 21,000 (70-mer) human oligonucleotides representing 21,000 genes (Operon Technologies) printed in duplicate in 3× SSC onto aminosilane-coated slides (ERIE C28). *$P < .001$ versus WT. (D) Protein levels of reelin, netrin1, and jagged2, candidate genes that showed the largest number of different expressions in Caki-1-SC cells, were confirmed by Western blot analysis with supernatants and cell lysates from Caki-1-WT and Caki-1-SC cells. (E) Interactions between angiogenic/morphogenic genes differentially expressed in Caki-1-SC versus Caki-1-WT cells. (F) Proposed VEGF-independent pathways of angiogenesis in sunitinib-resistant RCC cells.

E

Interactions of angiogenic/morphogenic genes differentially expressed in SC vs HC cells.



F

Proposed VEGF-independent Pathways of Angiogenesis in Sunitinib-resistant cells.

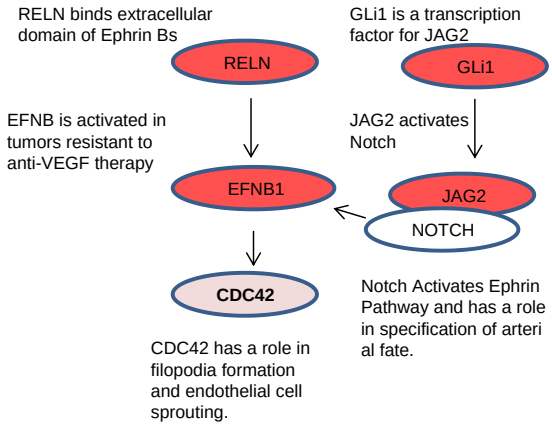


Figure 7. (*continued*)

endothelial growth stimulus or may, by genetic drift, produce different stimulators over time [21]. Recently, FGF and IL-8 were proposed to be proangiogenic by bypassing VEGF signaling inhibition through inducing the growth of vascular endothelial cells [7,11]. In our study, there was no increase in FGF expression in the resistant RCC after exposure to hypoxia or sunitinib. Recently, IL-8 was also reported to be a candidate proangiogenic factor that plays a role in sunitinib resistance. Specific inhibition of these factors also suppressed tumor growth in resistant tumors, but the effects were modest and temporary. Tumor cells adapted to hypoxia exhibited a moderate increase in IL-8 expression but no *in vivo* resistance to antiangiogenic therapy in our study. Instead, we found prominent increases in several growth factors, which may induce endothelial cell growth and angiogenesis in resistant tumor cells. The most overexpressed factors in our resistance model were Reelin and Netrin, which are known to regulate axon guidance [22,23]. However, recent studies revealed that Reelin and Netrin also play a role in lymphatic and vascular formation [24,25]. Neuronal growth factors have been suggested to be related to angiogenesis because their sprouting and growth patterns are similar to the process of angiogenesis. Ephrins are important factors in embryonic patterning and neuronal targeting and function as a neuronal guidance cue. They are also known to play a critical role in vascular development during embryogenesis [26]. Furthermore, ephrin-B2 regulates VEGFR2 function in developmental and tumor angiogenesis [27]. Eph/ephrin-dependent tumor neovascularization is mediated by the interplay of Eph receptors/ephrin ligands expressed by tumor cells and endothelial cells [26]. Recent studies demonstrated that the neuronal guidance cues by ephrin B proteins are essential for Reelin signaling during the development of laminated structures in the brain [28], suggesting that Reelin may play a role in tumor angiogenesis. In addition, resistant cells showed increased Jagged2 expression. Activation of the Notch

system leads to a more mature vasculature, which, in turn, increases tumor perfusion [6]. Tumors intrinsically resistant to anti-VEGF agents appear to be sensitive to inhibition of Notch signaling [29].

Conclusions

Our data suggest that tumor angiogenesis is not fixed to VEGF signaling and that there is redundancy in the angiogenic potential of tumor cells. Tumor cells recognize VEGFR inhibitory signals and change their angiogenic factor profiles according to the inhibitory signals of certain proangiogenic factors. Resistance to VEGF-targeted therapy is induced not by a single but by several proangiogenic factors independent of VEGF. Although novel VEGF-targeted therapies, including axitinib, pazopanib, and tivozanib, have been developed recently and show improved clinical outcomes, broader inhibition of several proangiogenic factors is required to prevent resistance to antiangiogenic therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2015.11.001.

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