Activation of Epidermal Growth Factor Receptor/p38/Hypoxia-inducible Factor-1α Is Pivotal for Angiogenesis and Tumorigenesis of Malignantly Transformed Cells Induced by Hexavalent Chromium*

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HEXAVALENT CHROMIUM (Cr(VI))-CONTAINING COMPOUNDS ARE CLASSIFIED AS GROUP 1 HUMAN CARCINOGENS IN ENVIRONMENTAL SETTINGS. HUMAN EXPOSURE TO CR(VI) HAS BEEN RECORDED FOR SEVERAL MILLENNIA AND IS A WELL-ESTABLISHED ENVIRONMENTAL CARCINOGEN. MALIGNANCY CAN OCCUR SECONDARY TO THE DEVELOPMENT OF ANGIOGENESIS. THIS STUDY INVESTIGATES THE PARTIAL ROLE OF HYPOXIA-INDUCIBLE FACTOR-1α (HIF-1α) AND THE ETIOLOGY OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) SIGNALING IN ANGIOGENESIS AND TUMORIGENESIS OF MALIGNANTLY TRANSFORMED CELLS. HIF-1α IS AN HSF-RELATED TRANSCRIPTION FACTOR THAT IS STABILIZED UNDER HYPOXIC CONDITIONS. IT STIMULATES THE EXPRESSION OF SELECTED GENES IMMEDIATELY UPON STRESS AND REGULATES THE EXPRESSION OF PRO-INVASIVE PROTEINS AND ANGIOGENIC FACTORS. EGFR IS AN INTRACELLULAR SIGNALING PATHWAY THAT REGULATES THE EXPRESSION OF MANY TUMOR PROMOTING FACTORS, INCLUDING VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND METALLOPROTEINASES. IT CO-LOCALIZES WITH HIF-1α IN MALIGNANTLY TRANSFORMED CELLS AND IN HUMAN LUNG TUMOR TISSUE.

Hypoxia-inducible Factor-1α is a transcription factor that is stabilize under hypoxic conditions. It stimulates the expression of selected genes immediately upon stress and regulates the expression of pro-invasive proteins and angiogenic factors. EGFR is an intracellular signaling pathway that regulates the expression of many tumor promoting factors, including vascular endothelial growth factor (VEGF) and metalloproteinases. It co-localizes with HIF-1α in malignantly transformed cells and in human lung tumor tissue.

This article has been retracted by the publisher. Young-Ok Son agrees with the retraction. Youn-hee Park and Leonard Yenwong Fai could not be reached. The University of Kentucky identified that figure components in Figs. 2 (H and I) and 6B had incomplete metadata, such as camera system, date, metering mode, and subject distance. Exposure times were not associated with the data. Without recorded exposure times, the validity of comparing protein expression in panels b and e for Fig. 2H; panels b, e, and h for Fig. 2I; and panel b for Fig. 6B cannot be confirmed. In addition, the Journal determined the following. The image of HUVECs treated with conditioned medium from BEAS-2B-Cr cells in Fig. 1A was reused in Figs. 3B and 5B, representing different experimental conditions. The image from Fig. 1B showing a chick embryo exposed to BEAS-2B-Cr cells was reused in Fig. 3C, representing different experimental conditions. The image of the Matrigel plug containing BEAS-2B-Cr cells in Fig. 1C was reused in Figs. 3D and 5C, representing different experimental conditions. In Fig. 4C, the DAPI image from the upper Scramble panel was reused as shEGFR in the lower panel. In addition, the DAPI image from the upper shEGFR panel was reused as Scramble in the lower panel.

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The abbreviations used are: Cr(VI), hexavalent chromium; EGFR, EGF receptor; HIF-1α, hypoxia-inducible factor-1α; MMP, matrix metalloproteinase; HUVEC, human umbilical vein endothelial cell; CAM, chorioallantoic membrane.
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(22–24). It has been reported that high MMP-1 level in patient lung tumor tissue is associated with metastasis of non-small cell lung cancer (25, 26). MMP-1 knock-out mice exhibit reduced growth and angiogenesis of lung tumors (27). A previous study has observed that treatment of human U937 macrophages with Cr(III) elevates MMP-1 expression (28). Recent studies have indicated that EGFR mediates synthesis and function of MMPs in epithelial cells (29–31). However, the question concerning how EGFR regulates these pro-angiogenic proteins remains to be answered. The present study investigated the role of constitutive activation of EGFR in angiogenesis of Cr(VI)-transformed cells and mechanisms concerning how EGFR regulates angiogenesis by focusing on the p38 MAPK/hypoxia-inducible factor-1α (HIF-1α) pathway.

Results

Increased Angiogenesis of Cr(VI)-transformed Cells—Our previous study has shown that chronic exposure of human bronchial epithelial BEAS-2B cells to Cr(VI) is able to cause malignant cell transformation. Those transformed cells are tumorigenic (12). Angiogenesis is a vital pathological process during tumorigenesis. To investigate whether Cr(VI)-transformed cells exhibit elevated angiogenesis, three approaches were used: in vitro tube formation of HUVECs, ex ovo chicken chorioallantoic membrane (CAM) blood vessel formation, and in vivo Matrigel plug angiogenesis assay. As shown in Fig. 1A, tube formation was observed in Cr(VI)-transformed cells (right panel) but not in passage-matched normal cells (left panel). The results from quantitation of tube formation in Cr(VI)-transformed cells were also compared with that in passage-matched normal cells (middle panel). The results from tube formation assay indicated blood vessels and tube branches were formed in Cr(VI)-transformed cells but not in the passage-matched normal cells (left and middle panels). Quantitative analysis showed that the number of newly formed blood vessels in Cr(VI)-transformed cells increased 3-fold compared with that in passage-matched normal cells (Fig. 1B, right panel). Another way to further verify elevated angiogenesis in Cr(VI)-transformed cells, in vivo Matrigel plug assay was performed. The plugs containing Cr(VI)-transformed cells exhibit a red color, indicating new blood vessel formation (angiogenesis) in the plugs (Fig. 1C, middle panel). The plugs containing passage-matched normal BEAS-2B cells were light red or pale pink, indicating less blood vessel formation (Fig. 1C, left panel). The extent of angiogenesis was also quantified by measuring hemoglobin content in the plugs. The results show that the hemoglobin concentration in the plugs containing Cr(VI)-transformed cells was 16.9 μg/mg Matrigel (Fig. 1C, right panel). It was 1.2 μg/mg Matrigel in the plugs containing passage-matched normal cells (Fig. 1C, right panel). These results demonstrate increased angiogenesis in Cr(VI)-transformed cells.

Up-regulation of Pro-angiogenic Genes in Cr(VI)-transformed Cells—Given the observation of increased angiogenesis in Cr(VI)-transformed cells, a total of 43 angiogenic proteins were examined using angiogenesis antibody array. Fig. 2A shows that expression of angiogenin (dot 3), IL-6 (dot 4), and MMP-1 (dot 2) was increased in Cr(VI)-transformed cells compared those in passage-matched normal cells. mRNA levels of these genes were also increased in these cells (Fig. 2B). Angiogenic factors are responsible for increased angiogenesis. Among those factors, VEGF and MMP-1 are key proteins to induce angiogenesis. Our results show that expression of VEGF and MMP-1 was markedly increased in Cr(VI)-transformed cells (Fig. 2C). VEGF concentrations secreted from BEAS-2B and Cr(VI)-transformed cells were also measured. The results show that VEGF concentration was increased 4-fold in Cr(VI)-transformed cells compared with that in normal ones (Fig. 2D). The HIF-1α level was dramatically increased in Cr(VI)-transformed cells (Fig. 2E). Expression of these angiogenic proteins in lung tissues from animals exposed to Cr(VI) for 12 weeks was examined. High expressions of MMP-1, HIF-1α, and VEGF were observed in Cr(VI)-exposed lung, but not in the control without Cr(VI) exposure (Fig. 2, F–H). Expression of MMP-1, HIF-1α, and VEGF in normal lung tissue from a non-smoking healthy patient and in lung tumor and its adjacent normal tissues from a non-smoking worker exposed to Cr(VI) for 19 years with diagnosis of stage 1 squamous lung carcinoma were detected. The results show that MMP-1 was highly expressed in lung tumor tissue and that it was much less expressed in adjacent normal tissue (Fig. 2F). This protein was not detectable in normal tissue from a healthy patient (Fig. 2F). Similarly, HIF-1α (green fluorescence) and VEGF (red fluorescence) were highly expressed
in parenchyma of tumor lung tissue, and these proteins were slightly expressed in the adjacent normal tissue (Fig. 2). These proteins were not expressed in normal lung tissue from a healthy patient (Fig. 2).

EGFR Is a Positive Regulator of Angiogenesis in Cr(VI)-transformed Cells—To determine whether EGFR plays an important role in angiogenesis of Cr(VI)-transformed cells, expression of EGFR was inhibited by its shRNA (Fig. 3A). Results from tube formation assay show that knockdown of EGFR markedly reduced tube formation compared with that in scramble Cr(VI)-transformed cells (Fig. 3B). Knockdown of EGFR in Cr(VI)-transformed cells significantly reduced numbers of new blood vessels (Fig. 3C). Similarly, results from in vivo Matrigel plug angiogenesis assay show that both new blood vessel formation and hemoglobin concentration in the plugs were suppressed in Cr(VI)-transformed cells with EGFR knockdown (Fig. 3D). To determine whether elevated angiogenic proteins in Cr(VI)-transformed cells are regulated by EGFR, expression of these angiogenic proteins was measured. The results show that knockdown of EGFR in Cr(VI)-transformed cells decreased MMP-1 expression at both mRNA and protein levels (Fig. 4, A, C, and D). Knockdown of EGFR also reduced expression and secretion of VEGF (Fig. 4, A–C). However, inhibition of EGFR did not influence mRNA level of angiogenin, IL-6, or GM-CSF (Fig. 4D), indicating that the increased level of angiogenin, IL-6, or GM-CSF is independent of EGFR. The above results demonstrate that EGFR plays an important role in angiogenesis of Cr(VI)-transformed cells and that MMP-1 and VEGF are downstream targets of EGFR.

Inhibition of MMP-1 Reduces Angiogenesis and Tumorigenesis of Cr(VI)-transformed Cells—Next, to investigate whether activation of MMP-1 contributes to elevated angiogenesis of
Cr(VI)-transformed cells, MMP-1 stable knockdown cells were established (Fig. 5A). Knockdown of MMP-1 markedly reduced tube formation (Fig. 5B), new blood vessel formation, and hemoglobin concentration in the plugs (Fig. 5C) compared with scramble cells. Thus, it is expected that MMP-1 plays a positive role in tumorigenesis of Cr(VI)-transformed cells.

Results from xenograft tumor growth assay show that all animals injected with Cr(VI)-transformed cells grew tumors and that none of animals grew any tumor in MMP-1 knockdown cells (Fig. 5D). These results indicate that MMP-1 plays a major role in angiogenesis and tumorigenesis of Cr(VI)-transformed cells.

**EGFR-dependent Activation of p38/HIF-1α Signaling and Its Role in Angiogenesis of Cr(VI)-transformed Cells**—To understand mechanism of angiogenesis of Cr(VI)-transformed cells, p38/HIF-1α signaling was examined. The results show that phos-
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**FIGURE 5. Knockdown of MMP-1 inhibits the angiogenesis in Cr(VI)-transformed cells.** A, immunoblotting analysis was used to confirm MMP-1 expression in Cr(VI)-transformed cells (BEAS-2B-Cr) with (shMMP-1) or without (Scramble). B, representative images of tube formation in HUVECs (left panel) and quantitation of tube branches (right panel) induced by conditioned medium from BEAS-2B-Cr cells with (shMMP-1) or without (Scramble) stable MMP-1 knockdown. *p < 0.05, compared with scramble cells. C, in vivo Matrigel plug angiogenesis assay. BEAS-2B-Cr cells with (shMMP-1) or without (Scramble) stable MMP-1 knockdown. *p < 0.05, compared with scramble cells. D, xenograft tumor growth assay. BEAS-2B-Cr cells with (shMMP-1) or without (Scramble) stable MMP-1 knockdown. **p < 0.01, compared with scramble cells. Top panel, hemoglobin level of Matrigel plugs. The data are means ± S.D. (n = 6). Bottom panel, tumor volume (■, scramble cells; ▲, shMMP-1 cells). The data are means ± S.D. (n = 6). In all groups, six animals in each group. E, hemoglobin level of Matrigel plugs. The data are means ± S.D. (n = 6). F, tumor volume (■, scramble cells; ▲, shMMP-1 cells). The data are means ± S.D. (n = 6). G, tumor weight (■, scramble cells; ▲, shMMP-1 cells). The data are means ± S.D. (n = 6).

**Discussion**

Changes from normal cells to malignantly transformed cells are considered the first stage of metal-induced carcinogenesis (12, 13). The development of malignantly transformed cells into tumor is the second stage. Angiogenesis is required for tumor growth and is very important in this second stage of metal or Cr(VI) carcino genesis (Fig. 7). Angiogenesis occurs by sprouting new vessels from pre-existing blood vessels or by inserting interstitial tissue columns into the lumen of pre-existing vessels (14–16, 19). Tumor angiogenesis is a key mechanism for tumor growth and metastasis. Thus, studying the mechanism of

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angiogenesis of Cr(VI)-transformed cells is very much needed for understanding the progression of Cr(VI)-transformed cells to tumor.

EGFR is frequently overexpressed and mutated in various types of cancer, including lung (32). Anti-EGFR therapeutic approaches, specifically receptor blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors, prolong tumor stabilization (33, 34). EGFR signaling is involved in blood vessel development and angiogenesis by directly or indirectly regulating pro-angiogenic factors (35–37). Ligand-induced EGFR dimerization triggers downstream signaling cascades including MAPK, PI3K-AKT, STAT, and PKC pathways. Our previous study has shown that constitutive activation of EGFR in Cr(VI)-transformed BEAS-2B cells is dependent on its ligand amphiregulin (12). Knockdown of EGFR reduced both tumor incidence and volume of Cr(VI)-transformed cells (12). The present study has found that knockdown of EGFR in Cr(VI)-transformed cells inhibited angiogenesis in vitro and in vivo, suggesting the essential role of EGFR in angiogenesis of Cr(VI)-transformed cells.

Our previous study has also demonstrated angiogenesis of Cr(VI)-transformed cells is very much needed for understanding the progression of Cr(VI)-transformed cells to tumor.

EGFR is frequently overexpressed and mutated in various types of cancer, including lung (32). Anti-EGFR therapeutic approaches, specifically receptor blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors, prolong tumor stabilization (33, 34). EGFR signaling is involved in blood vessel development and angiogenesis by directly or indirectly regulating pro-angiogenic factors (35–37). Ligand-induced EGFR dimerization triggers downstream signaling cascades including MAPK, PI3K-AKT, STAT, and PKC pathways. Our previous study has shown that constitutive activation of EGFR in Cr(VI)-transformed BEAS-2B cells is dependent on its ligand amphiregulin (12). Knockdown of EGFR reduced both tumor incidence and volume of Cr(VI)-transformed cells (12). The present study has found that knockdown of EGFR in Cr(VI)-transformed cells inhibited angiogenesis in vitro and in vivo, suggesting the essential role of EGFR in angiogenesis of Cr(VI)-transformed cells. Our previous study has also demonstrated
that activation of PI3K-AKT is dependent on EGFR in Cr(VI)-transformed cells (12). Inhibition of EGFR by its tyrosine kinase inhibitor or shRNA abolished expression of PI3K and AKT, leading to an increase in apoptosis and a decrease in tumorogenesis of Cr(VI)-transformed cells (12). It has been reported that EGFR also activates JNK and p38 (38). The observations in the present study indicate that activation of p38 is EGFR-dependent in Cr(VI)-transformed cells. Activation of p38 has been shown to correlate with increased tumor malignancy and poor prognosis of cancer patients (39, 40). Although hypoxia up-regulates HIF-1α through p38 in various cells (41), it has been reported that activation of p38 by hypoxia is mediated by HIF-1α in human hepatoma BEL-7405 cells (41). Our previous study has shown that short term exposure of Cr(VI) to DU145 human prostate cancer cells activated p38, which in turn up-regulated HIF-1α (42). Our previous study has also demonstrated that reactive oxygen species are responsible for Cr(VI)-induced malignant cell transformation (43). p38 is an oxidative stress-sensitive MAPK. It appears that Cr(VI)-induced ROS generation caused phosphorylation of p38 and subsequently activation of HIF-1α. The present study has found that inhibition of p38 decreased expression of HIF-1α and MMP-1, resulting in reduction of tube formation of human umbilical vein endothelial cells (HUVECs) stimulated by Cr(VI)-transformed cells, demonstrating that p38 is an upstream regulator of HIF-1α and that p38 is indeed involved in angiogenesis of Cr(VI)-transformed cells.

In the angiogenesis process, MMPs degrade basement membrane and other ECM components helping cell migration and by releasing growth factors such as basic FGF, VEGF, and TGF-β (44). MMP-1, one of the MMP family, is involved in angiogenesis of lung tumors (27), melanoma (28), and prostate (29) Cr(VI)-treated cells exhibit an increased microvessel density via tyrosine kinases (28). MMP-1 cooperates with autocrine signaling cascade for bone metastasis by autocrine release of membrane-bound EGF-like growth factors including amphiregulin, heparin-binding EGF, and TGF-α (47). EGFR ligand epiregulin, COX2, and MMP-1 collectively facilitate sequential steps of pulmonary metastasis (48). The present study has shown that MMP-1 expression was elevated in Cr(VI)-transformed cells, in lung tissue from Cr(VI)-exposed animals, and in lung tumor tissue from Cr(VI)-exposed worker. Knockdown of EGFR suppressed MMP-1 expression, suggesting that activation of MMP-1 in Cr(VI)-transformed cells is dependent on EGFR. Importantly, knockdown of MMP-1 reduced angiogenesis and tumorogenesis of Cr(VI)-transformed cells, indicating an important role of MMP-1 in up-regulating angiogenesis and tumorogenesis induced by Cr(VI).

HIF-1α signaling plays a crucial role in tumor metastasis, invasion, metabolism, and angiogenesis (49, 50). HIF-1α activation in tumor cells is one of the key elements orchestrating their adaptation mechanisms to the hypoxia environment. Activated HIF-1α is crucial in adaptive responses of tumor cells to changes in oxygen concentration through transcriptional activation of over 100 downstream genes that regulate biological processes required for tumor survival and progression. During hypoxia, activation of HIF-1α/VEGF plays a critical role in initiating angiogenesis. In addition to hypoxia, HIF-1α protein synthesis, stability, and activity can also be regulated by other mechanisms, such as growth factor-induced signaling (51). Signaling via the HER2/neu or IGF-1 receptor tyrosine kinase induces HIF-1α expression by an oxygen-independent mechanism that increases the rate of HIF-1α protein synthesis (52, 53). HIF-1α overexpression is associated with increased microvessel density and/or VEGF expression in non-small cell lung cancers (54). HIF-1α protein is subjected to rapid degradation at normoxia via the pVHL-mediated ubiquitin-proteasome pathway, whereas hypoxia blocks degradation of HIF-1α protein, leading to its accumulation. The association of HIF-1α with pVHL is triggered by post-translational hydroxylation of a proline residue that is mediated by prolyl hydroxylase. In the present study, reduced prolyl hydroxylation in Cr(VI)-transformed cells led to stabilization of HIF-1α. HIF-1α activates transcription of genes encoding proteases that degrade the extracellular matrix (ECM) and several MMPs, such as MMP-2, MMP-9, and MMP-1. MMP-1 is also up-regulated when HIF-1α is activated in A549 epithelial cell line (56). Hypoxia promotes tumor cells into distant and more oxygen-rich metastatic sites by deregulation of HIF-1α. The response to hypoxia is due to oxygen sensing through the HIF-1α pathway. EGFR/p38/HIF-1α signaling is involved in the regulation of tumor growth. EGFR signaling via the HER2/neu or IGF-1 receptor tyrosine kinase mechanisms, such as growth factor-induced signaling (51). Signaling via the HER2/neu or IGF-1 receptor tyrosine kinase induces HIF-1α expression by an oxygen-independent mechanism that increases the rate of HIF-1α protein synthesis (52, 53). HIF-1α overexpression is associated with increased microvessel density and/or VEGF expression in non-small cell lung cancers (54). HIF-1α protein is subjected to rapid degradation at normoxia via the pVHL-mediated ubiquitin-proteasome pathway, whereas hypoxia blocks degradation of HIF-1α protein, leading to its accumulation. The association of HIF-1α with pVHL is triggered by post-translational hydroxylation of a proline residue that is mediated by prolyl hydroxylase. In the present study, reduced prolyl hydroxylation in Cr(VI)-transformed cells led to stabilization of HIF-1α. HIF-1α activates transcription of genes encoding proteases that degrade the extracellular matrix (ECM) and several MMPs, such as MMP-2, MMP-9, and MMP-1. MMP-1 is also up-regulated when HIF-1α is activated in A549 epithelial cell line (56). Hypoxia promotes tumor cells into distant and more oxygen-rich metastatic sites by deregulation of HIF-1α. The response to hypoxia is due to oxygen sensing through the HIF-1α pathway. EGFR/p38/HIF-1α signaling is involved in the regulation of tumor growth.


**EGFR/p38/HIF-1α in Cr(VI) Angiogenesis**

qScript cDNA synthesis kit and Perfecta Sybr Green Fastmix were from Quanta Biosciences (Gaithersburg, MD). shRNAs of EGFR, MMP-1, and HIF-1α were from Origene (Rockville, MD). p3XFLAG-CMV/EGFR and its control plasmids were kindly provided by Dr. Qiou Wei (Department of Toxicology and Cancer Biology, University of Kentucky) (58). Bradford protein assay reagent was from Bio-Rad. Enhanced chemiluminescence reagent was from GE Healthcare. Human VEGF Quantikine ELISA kit was from R&D Systems. Collagen type I was from BD Biosciences. Antibodies against VEGF, GAPDH, and β-actin were from Santa Cruz. Antibody against HIF-1α was from Millipore (Billerica, MA). Antibodies against EGFR, p-EGFR, MMP-1, p38, p-p38, p44/22, p-p44/44, and PH2/EGL-9 and p38 inhibitor SB203580 were from Cell Signaling Technology (Danvers, MA). Matrigel basement membrane matrix was from BD Biosciences. Vectashield mounting medium containing DAPI was from Vector Laboratories (Burlingame, CA).

**Cell Culture**—Both human bronchial epithelial BEAS-2B cells and HUVECs were from American Type Culture Collection (Rockville, MD). BEAS-2B cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. HUVECs were maintained in endothelial cell basal medium-2 supplemented with 20% FBS and 1% penicillin-streptomycin.

**Cell Transformation Assay**—BEAS-2B cells were chronically exposed to 0.25 μM Cr(VI). Fresh medium containing Cr(VI) was added every 3 days. After 6 months of Cr(VI) exposure, these cells were subjected for soft agar assay. A single colony was picked up and continued to grow. Cells from the single colony were considered as Cr(VI)-transformed cells. Passage-1 cells and HUVECs were from American Type Culture Collection (Rockville, MD). BEAS-2B cells were maintained in endothelial cell basal medium-2 supplemented with 20% FBS and 1% penicillin-streptomycin.

**Chronic Exposure of Animals to Cr(VI) Particles**—The 6–8-week-old, female BALB/c mice were from The Jackson Laboratory. Endothelial cell basal medium-2 was added every 3 days. After 6 months of Cr(VI) exposure, these cells were subjected for soft agar assay. A single colony was picked up and continued to grow. Cells from the single colony were considered as Cr(VI)-transformed cells. Passage-1 cells and HUVECs were from American Type Culture Collection (Rockville, MD). BEAS-2B cells were maintained in endothelial cell basal medium-2 supplemented with 20% FBS and 1% penicillin-streptomycin.

**Plasmid Transfection**—Briefly, the cells were seeded in 6-well culture plates. After 70% confluency, the cells were transfected with 4 μg of plasmid using Lipofectamine. To establish stable transfecting cells, the cells were selected using puromycin for 3 months followed by immunoblotting analysis for verification of expression.

**Human Angiogenesis Array**—Cr(VI)-transformed cells and passage-matched normal cells were cultured in serum-free medium for 24 h. Conditioned medium was collected. Proteins in the conditioned medium were hybridized with a human angiogenesis antibody array dotted with 43 human angiogenesis-related antibodies. The assay was performed according to the manufacturer’s manual. Briefly, conditioned medium was incubated with human angiogenesis array membranes. The membranes were washed with specific buffer and treated with biotin-conjugated antibodies followed by treatment with diluted horseradish peroxidase-conjugated streptavidin. X-ray film and a chemiluminescence imaging system were used to detect an angiogenesis array signal.

**Real Time PCR**—cDNA was extracted and purified using a Qiagen RNA extraction kit of RNA was reversely transcribed using a qScript cDNA synthesis kit. Primers were designed using Primer3 for: angigenin, 5′-CGT ACT TAC GGC GTC GCT A-3′ and 5′-GCA CGA AGA GAA C-3′; IL-6, 5′-AAA TGC CAG CCT GCT GAC A-3′ and 5′-GTC TGT CTC GGC TGC CG-3′; Collagen type I, 5′-GGG TGC CCA TGC TAC GCC ACT GCC AAA TG-3′ and 5′-GCA CGA AGA GAA C-3′; Collagen type II, 5′-TGC CCT GCT GCC TCC TGC TGC CTA-3′; HIF-1α, 5′-CAG ATC TCG GCG GCA AG-3′ and 5′-TCA AGG CAG CCT TATCAA TTA G-3′; and β-actin, 5′-CTG GAA TGA TAA ATG CA-3′ and 5′-AAG GGA TGT CTT GAA TCA ATG CA-3′. All primers were tested using standard curves in 10-fold serial dilutions. Quantitative PCR was performed using Perfecta Sybr Green Fastmix in the CFX96 real time PCR detection system (Bio-Rad). The data were analyzed using CFX manager software (Bio-Rad).

**Immunoblotting Analysis**—The cell lysates were prepared in radioimmune precipitation assay buffer. Protein concentration was measured using Bradford protein assay. 30 μg of proteins were separated by SDS-PAGE followed by incubation with primary antibody for overnight. The blots were then reprobed with secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were detected using enhanced chemiluminescence reagent.

**Immunofluorescence Assay**—The cells were cultured on chamber slides. After washing with PBS, the cells were fixed with 4% paraformaldehyde for followed by incubation with PBS containing 1% Triton X-100. After washing with PBS-Tween 20 solution (containing 0.02% Tween 20), the cells were incubated with PBS containing 0.1% Triton X-100 and 10% normal serum. The cells were incubated with primary antibody for overnight followed by reprobed with secondary antibody. The slides were mounted with Vectashield mounting medium containing DAPI. Finally, the cells were visualized using Olympus BX53 fluorescence microscope (Pittsburgh, PA).
**HUCHE Tube Formation Assay**—Tube formation assay was performed as previously described (60). HUCHECs were seeded into wells coated with 50 μl of growth factor-reduced Matrigel matrix and then incubated with 100 μl of conditioned medium for 12 h to allow formation of tube-like structures. Tube formation was analyzed using Olympus IX-73 microscope (Pittsburgh, PA). The tube forming ability was determined by measuring the total tube length of HUCHECs.

**Human VEGF Immunostaining**—Production of VEGF was detected using a human VEGF Quantikine ELISA kit according to the manufacturer’s manual. Briefly, 200 μl of cell supernatant was added in each well and incubated for 2 h followed by adding 200 μl of conjugate for 2 h. After aspirating and washing, 200 μl of substrate was added and incubated for 20 min. The absorbance was measured using Labsystems Multiskan MS plate reader (Thermo Scientific) at 450-nm wavelength.

**In Vivo Matrigel Plug Angiogenesis Assay**—A Matrigel plug assay was performed as described previously (96); female athymic nu/nu mice (Charles River Laboratories, Wilmington, MA) were individually kept in filter-topped cages and maintained at the animal facility at the Chandler Medical Center Laboratory at the University of Kentucky. All animals were on the same institutional animal care and use committee protocol. A total of 1 × 10⁶ cells in 50 μl of DMEM mixed with 10% charcoal-cold Matrigel were subcutaneously implanted on the right flank of each mouse. Two weeks post-transplantation, the animals were euthanized using CO₂, and plugs were isolated. The plugs were homogenized, and supernatant was collected to measure hemoglobin content using Drabkin reagent according to the manufacturer’s protocol. The absorbance was measured at 540 nm wavelength using Spectra Max GEMINI microplate reader (Molecular Devices).

**ChIP Assay**—The cells were fixed with formaldehyde and cross-linked. The chromatin was sheared and immunoprecipitated with anti-HIF1α antibody or control IgG. Binding of HIF-1α to the hypoxia response element region of VEGF promoter was measured by quantitative RT-PCR using custom-made primers. The primers were as follows: forward, 5′-CAG GAA CAA GGG CCT CTG TCT-3′; and reverse, 5′-TGT CCC TCT GAC AAT GTG CCA TC-3′. The mixture was run in the RT-PCR detection system according to the manufacturer’s protocol. The amounts of immunoprecipitated DNA were normalized to the inputs and plotted.

**Statistical Analysis**—The data were expressed as means ± S.D. Statistical significance of differences among treatment groups was determined by Student’s t test. A p < 0.05 was considered as statistical significance.

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