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Hypoxia-inducible factor (HIF)-1α directly enhances the transcriptional activity of stem cell factor (SCF) in response to hypoxia and epidermal growth factor (EGF)

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Stem cell factor (SCF) plays important roles in tumor growth and angiogenesis. However, its regulatory mechanism remains largely undefined. Here, we report that hypoxia upregulated the expression of SCF in MCF-7 breast cancer cells in both messenger RNA and protein levels. When hypoxia-inducible factor (HIF)-1α expression was knocked down by RNA interference, the MCF-7 cell expression of SCF was decreased significantly. Furthermore, the SCF receptor, c-kit phosphorylation was significantly strengthened by the condition culture media from hypoxic MCF-7 and MCF-7-c cells. The survival of A549 cells was more dependent on SCF under hypoxia. Analysis of SCF promoter 5′-flanking region revealed a potential hypoxia-response element (HRE; 5′-GCGTG-3′) located at −68 to −64 relative to the transcriptional start site. Chromatin immunoprecipitation assay demonstrated that HIF-1α directly bound to this region under normoxia, and this binding activity was significantly enhanced under hypoxia. Overexpression of HIF-1α significantly upregulated the expression of luciferase reporter gene under control of the SCF promoters in both MCF-7 cells and human embryonic kidney 293 cells, but mutation of the HRE site completely blocked this effect. Epidermal growth factor was also able to enhance the SCF expression under normoxia in MCF-7 cells, which was dependent on HIF-1α. Taken together, our data demonstrated that HIF-1α was a key regulator of SCF expression in breast cancer cells. Hypoxia and epidermal growth factor receptor signal coexisted in the tumor microenvironment and might promote angiogenesis through HIF-1α-mediated upregulation of SCF and other angiogenic factors.

Introduction

Stem cell factor (SCF) is a multifunctional cytokine involving in tumor progression. SCF and its receptor, c-kit, are overexpressed in some human malignancies including gastrointestinal stromal tumors, breast cancer, small-cell lung carcinoma, glioma and acute myelogenous leukemia (1). Binding of SCF to c-kit results in activation of its intrinsic tyrosine kinase activity and promotes tumor growth (2,3). Recent reports suggest that cancer stem cells initiate the tumor development in a variety of cancers (4–6). Cancer stem cells may originate from stem or progenitor cells through a precancerous stage, during which stem cells are hierarchically disturbed in their genetic program of self-renewal by environmental insults (7). The process of the precancerous stem cells to cancer is associated with an upregulation of c-kit (8), suggesting that SCF–c-kit signaling may be involved in the formation and survival of cancer stem cells during the early stage of carcinogenesis. For instance, the SCF–c-kit signaling is specifically involved in the acquisition of autonomous growth and malignant transformation of uveal melanoma cells through extracellular signal-related kinases1/2 activation (9). In advanced phases of tumor development, an angiogenesis process takes place and finally tumor cells acquire the capacity of tissue invasion and metastasize to other organs. SCF has been demonstrated as a new angiogenic factor that directly promotes the survival, migration and capillary tube formation of human umbilical vein endothelial cells (HUVECs) (10) *in vitro* and be involved in glioma-associated angiogenesis *in vivo* (11). It has been shown that blocking SCF–c-kit pathway by antisense oligonucleotides or small molecular inhibitors reduces tumor growth (12) or angiogenesis (13), suggesting that SCF–c-kit signaling can be a potential target for cancer therapy.

Although accumulating data indicate SCF as a mitogen and angiogenic factor in carcinogenesis, the regulation of SCF expression in tumor cells is still poorly understood. It is reported very recently that high mobility group A1α directly upregulates the SCF promoter in MCF-7 breast cancer and OCC1 ovarian cancer cells (14). Since the bioactivity of SCF is associated with tumor progression, signal transduction pathways involved in tumor progression may influence SCF gene expression.

One of the important tumor microenvironmental stimuli is hypoxia. Hypoxia is defined as oxygen deficiency in tissues and is commonplace in solid tumors due to the outgrowth of tumor over supporting vasculature. The homeostatic response to hypoxia is predominantly mediated by the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 transcription factor is a heterodimer that consists of a HIF-1α subunit and a constitutively expressed HIF-1β subunit. The HIF-1α subunit, in contrast to HIF-1β, is highly regulated within cells and is formed in response to hypoxia and growth factor stimulation. This active transcription factor recognizes and binds to the hypoxia-response elements (HREs; 5′-A/GCGTG-3′) in hypoxia-inducible promoters (15). As a result of hypoxia and genetic mutations, HIF-1α is overexpressed in many human cancers (16,17) including breast cancer (18,19).

Hypoxia-induced SCF secretion has been demonstrated in the region of cerebral ischemia (20). HIF-1α is predominant in regulating gene in response to hypoxia in breast cancer cells (21). We postulate that hypoxia might upregulate SCF gene expression in breast cancer cells through HIF-1α.

In addition to hypoxia, activation of epidermal growth factor receptor (EGFR) has been reported to increase the level of HIF-1α through the phosphatidylinositol 3-kinase–serine-threonine protein kinase Akt (PI3K–AKT) pathway under normoxic conditions (22–24). Therefore, we also investigated whether epidermal growth factor (EGF) could induce SCF expression via HIF-1α independent of hypoxia.

Abbreviations: CCM, condition culture media; cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; HEK293, human embryonic kidney; HIF, hypoxia-inducible factor; HRE, hypoxia-response element; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA; PI3K—AKT, phosphatidylinositol 3-kinase–serine-threonine protein kinase Akt; PCR, polymerase chain reaction; RT, reverse transcription; SCF, stem cell factor; SDS, sodium dodecyl sulfate; shRNA, short hairpin RNA; sSCF, secreted stem cell factor; VEGF, vascular endothelial growth factor.

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Materials and methods

Cell culture and hypoxic treatment

Human MCF-7 breast cancer cells, human embryonic kidney (HEK293) cells and A549 lung cancer cells were obtained from the Cell Culture Center, Peking Union Medical College (Beijing, China). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, L-glutamine and 1% penicillin–streptomycin. Cells were grown at 37°C in a humidified atmosphere of 95% air

and 5% CO_2. For hypoxic treatment, cells were placed in a modulator–incubator (Thermo Electron Co., Forma, MA) in an atmosphere consisting of 93.5% N_2, 5% CO_2 and 1.5% O_2.

Knockdown HIF-1α by short hairpin RNAs

To knock down the expression of HIF-1α, the pGE-1 predigested short hairpin RNA (shRNA) expression vector (Stratagene, La Jolla, CA) was used for cloning shRNAs targets to HIF-1α (silencing sequence: GCACAGTTACAGTATTCCA) (25). Transfection into MCF-7 cells was performed using lipofectamine 2000 (Invitrogen) and DMEM following the supplier's protocol. Control cells were produced by using a similar vector containing vehicle complementary DNA (cDNA) sequences (GTCGCGACTATAGAGTAAG). All the cells were selected with G418 (400 μg/ml) for 2 weeks and then maintained in complete medium with G418 (300 μg/ml).

Quantitative real-time reverse transcription–polymerase chain reaction

Total RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction (PCR) analyses for SCF and HIF-1α were performed using ABI 7000 system (PE Applied Biosystems, Foster City, CA) as described (26). PCR cycling conditions were 95°C for 15 min and 40 cycles of 95°C for 15 s, 57°C for 30 s and 72°C for 45 s, followed by the final melting curve program. Each sample was done in triplicate and mean values were used for quantization. β-Actin and vascular endothelial growth factor (VEGF) RNA were used as loading control and positive control. Primers were listed in supplementary Table 1A (available at *Carcinogenesis* Online). For EGF treatment, MCF-7 cells were cultured in the absence or presence of 100 ng/ml EGF, 10 μM of LY294002, PD98059 or AG1478 (Calbiochem, La Jolla, CA) for 12 h. Then, RNA was isolated for quantitative real-time reverse transcription (RT)–PCR.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Waltham, MA) according to the manufacturer's instructions. Briefly, MCF-7 cells were cultured under normoxia or hypoxia for 12 h, washed once in phosphate-buffered saline and then cross-linked by 1% formaldehyde at 37°C for 10 min. After washing with ice-cold phosphate-buffered saline, cells were collected and lysed for 10 min in sodium dodecyl sulfate (SDS) lysis buffer [1% SDS, 10 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM Tris, pH 8.1] containing protease inhibitors. Lysate was sonicated to shear DNA to lengths between 200 and 1000 bp. Then, the cross-linked protein (HIF-1α) was immunoprecipitated by mouse anti-human HIF-1α monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or non-specific IgG antibodies (Sigma-Aldrich, St Louis, MO). After collection in salmon sperm DNA-saturated protein A, immune complexes were washed once (5 min) with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1 and 150 mM NaCl), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.1 and 500 mM NaCl), once with LiCl wash buffer [0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA and 10 mM Tris, pH 8.1] and two times with Tris-EDTA buffer (10 mM Tris–HCL and 1 mM EDTA, pH 8.0). Immune complexes were extracted in elution buffer (1% SDS and 0.1 M $NaHCO_3$); DNA cross-links were reversed by heating at 65°C for 4 h. After protease k digestion, DNA was extracted by phenol–chloroform and precipitated by ethanol. The DNA was used for PCR together with primers flanking the putative HRE within the SCF promoter. Primers flanking the HRE of the VEGF promoter were used as a positive control (27). Primers flanking a SCF promoter region that do not contain HRE were used as a negative control. All the primers were listed in supplementary Table 1B (available at *Carcinogenesis* Online).

Plasmid constructs, transient transfection and luciferase assay

For isolation of full-length cDNA fragments of HIF-1α, we employed a one-step RT–PCR from RNA extracts of HEL cells (a human erythroleukemia cell line). The full-length HIF-1α cDNA (GenBank no. U22431) was ligated into expression vector pcDNA3.1 (+) (Invitrogen) and verified by sequencing. The plasmids (0.5 and 1 μg) were transfected into MCF-7 or HEK293 cells using lipofactamine 2000 (Invitrogen) or calcium phosphate (Promega, Madison, WI) for overexpression of HIF-1α. The primers for expression plasmid of HIF-1α were listed in supplementary Table 1C (available at *Carcinogenesis* Online).

Plasmids containing genomic DNA fragments of the human SCF gene 5′-flanking region, respectively, spanning from +184 to −76, −853 or −2185 relative to the transcription initiation sites (nominated pGL3-SCF1, pGL3-SCF2 and pGL3-SCF3) were generated by PCR and inserted into pGL3-Basic vectors. Mutations were introduced into HIF-1-binding sites by specific primers (nominated pGL3-SCF1m, pGL3-SCF2m and pGL3-SCF3m) (supplementary Table 1D is available at *Carcinogenesis* Online). All constructs were sequenced to confirm their identity. In addition, pGL3-Basic (Promega) and pGL3-VEGF (kind gifts from Dr Z.Fang, Institute of Hematology, Tianjin, China) were chosen as negative and positive control, respectively. Reporter plasmids (500 ng) were cotransfected with a constitutively expressed Renilla luciferase construct (10 ng) (pRL-SV40, Promega) into MCF-7 or HEK293 cells. The cells were incubated in normoxia for 48 h. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). For EGF treatment, after pGL3-SCF1 was transfected into MCF-7 cells for 24 h, cells were cultured in medium containing 2% serum with EGF (100 ng/ml) for 12 h and then subjected to luciferase assay. All data were normalized to Renilla luciferase expression from at least three independent experiments. Cotransfection experiments using plasmids for oxygen-independent HIF-1α were compared with control transfection using the appropriate empty vector (pcDNA3.1) for each construct.

Western blot analysis

Western blot analysis was performed according to previous description with some modifications (28). Briefly, proteins were lysed by SDS lysis buffer (Beyotime, Shanghai, China) supplemented with proteinase inhibitors cocktail (Sigma) and fractioned by 15 or 8% SDS–polyacrylamide gel electrophoresis. Protein samples were then immunoblot assayed using anti-SCF (sc-9132), anti-HIF-1α (sc-13515), anti-actin (sc-8432) (Santa Cruz Biotechnology) and anti HIF-2α antibodies (Novus Biological, Littleton, CO). We used the Protein Detector LumiGLO Reserve Western Blotting Kit (KPL Gaithersburg, MA) to test the immunoblotted bands according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay

Soluble SCF levels were quantified in growth medium using a SCF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Briefly, cells were seeded in six-well plates with a density of 70–80% confluence and incubated overnight. For hypoxic treatment, cells were changed to low-serum medium (DMEM, 0.5% serum) and cultured for 12, 24 and 48 h under hypoxia. For EGF treatment, cells were changed to the medium containing 2% serum and the supernatants were collected and cell numbers were counted. SCF levels were expressed as pg/ml/10^5 cells.

Quantification of phosphorylated c-kit

MCF-7, MCF-7-sh or MCF-7-c cells (5×10^5 per well) were seeded on six-well multiplate in 2 ml complete medium as described previously. After achieving subconfluence (70–80%), cells were changed to fresh medium and consecutively cultured for 24 h at hypoxic (1.5% O_2) or normoxic (21%) conditions. Then, a series of condition culture media (CCM) were collected. HUVECs (3×10^5) were seeded on six-well multiplates at complete medium as described in supplementary Materials and Methods (available at *Carcinogenesis* Online). After achieving subconfluence (70–80%), cells were changed to the CCM and cultured for 5 min to induce tyrosine phosphorylation of c-kit. Then, cells were lysed by SDS lysis buffer (Beyotime) supplemented with proteinase inhibitors cocktail (Sigma). Finally, cell lysates were collected and phospho-c-kit was quantified by human phospho-SCF R–c-kit ELISA kit (R&D Systems) following the manufacturer's protocols. Results were expressed as optimal density values (450 nm) after normalization to the total protein levels (quantified by a bicinchonic acid assay).

Cellular proliferation assay

A549 cells (1×10^5) were seeded on six-well plates in DMEM supplemented with 10% fetal bovine serum and cultured under normoxic and hypoxic conditions for 4 days. A measure of 5 μg/ml anti-SCF rabbit polyclonal antibodies (Santa Cruz) were used to neutralize the bioactivity of SCF in the culture medium. The cells were stained with trypan blue and counted at indicated intervals.

Statistics

The results are displayed as mean ± SD. Statistical analysis was performed using Student's *t*-test. A $P < 0.05$ was considered statistically significant.

Results

Hypoxia-induced SCF expression

Since hypoxia is a common feature of the microenvironment of solid tumors, it is important to detect whether hypoxia promotes the expression of SCF in tumor cells. Human MCF-7 breast cancer cell line was preferentially chosen in current study because it expresses SCF but not its receptor, c-kit. Thus, the expression levels of SCF protein cannot be interfered by its interaction with c-kit. Moreover, MCF-7 cell line has recently been characterized as a good model to reflect molecular events in human breast carcinomas (29). SCF expression was determined by quantitative real-time RT–PCR on MCF-7 breast

cancer cells cultured under normoxia (21% O_2) or hypoxia (1.5% O_2) for 0, 6 and 12 h. The hypoxic condition was defined to mimic the average oxygen tension in tumors reported previously (30,31). As shown in Figure 1A, compared with normoxia, SCF expression was increased ∼1.7-fold by 6 h and ∼4.2-fold by 12 h after hypoxic treatment. As a positive control, VEGF expression was increased ∼10.1-fold after hypoxic treatment for 12 h (Figure 1B). Western blot analysis of MCF-7 cells showed significant induction of both secreted stem cell factor (sSCF, 45 kDa) and membrane-bound SCF (28 kDa) (32), with the stabilization of HIF-1α protein under hypoxia for 12 h, and the level was maintained to 24 h (Figure 1C). To further quantify SCF protein, sSCF in culture supernatants was assessed by ELISA. As shown in Figure 1D, sSCF levels increased ∼2-fold after hypoxia for 12 h. It should be noted that after hypoxia for 48 h, the basal secretion of sSCF appeared rather high and hypoxia-induced expression of SCF had decreased. These data indicate that hypoxia upregulates SCF messenger RNA (mRNA) and protein expression in MCF-7 breast cancer cells. Increased mRNA expression of SCF induced by hypoxia was also validated in other tumor cell lines, such as MDA-MB231 (estrogen receptor-negative breast cancer), A549 (lung cancer) and KB (ovarian cancer) cell lines (supplementary Table 2 is available at *Carcinogenesis* Online), suggesting that this hypoxia-induced effect is not specific to one cell type.

Inhibition of SCF expression by shRNAs targeting HIF-1α

Recent evidence has shown that HIF-1α, other than HIF-2α, dominates the regulation of the genes in response to hypoxia in cancer cells (21). Moreover, VEGF, a classical gene induced by hypoxia, was regulated by HIF-1α only in MCF-7 cells (33). To determine the role of HIF-1α in regulating the expression of SCF, MCF-7 cells were transfected with shRNAs targeting HIF-1α or control vectors (defined as MCF-7-sh and MCF-7-c, respectively) to knock down the HIF-1α expression. The inhibition rate was evaluated by RT–PCR and western blot (Figure 2A). In MCF-7-sh cells, HIF-1α mRNA and protein levels were repressed >80% compared with MCF-7-c cells under normoxia and hypoxia, whereas HIF-2α levels were not inhibited, indicating that this interference effect was specific to HIF-1α. The expression of HIF-1α remained unchanged in the MCF-7-c cells compared with MCF-7 wild-type cells (data not shown). Consistent with the repression of HIF-1α expression, SCF mRNA was markedly decreased in MCF-7-sh cells compared with MCF-7-c groups under normoxic and hypoxic conditions (Figure 2B, both $P < 0.01$). The protein level of SCF was also decreased in MCF-7-sh cells under normoxia and hypoxia (Figure 2C). As a positive control, VEGF protein was also significantly inhibited in MCF-7-sh cells, although the inhibition rate was not as prominent as that in the SCF protein. To further quantify the SCF protein, culture media were collected for ELISA after 48 h of culture. As shown in Figure 2D, sSCF was also significantly reduced in MCF-7-sh cells in comparison with MCF-7-c cells under normoxia and hypoxia (both $P < 0.01$). These data demonstrated that both constitutive and hypoxia-induced SCF expressions in MCF-7 cells were dependent on HIF-1α.

Functional measure of hypoxia-induced upregulation of SCF

It has been reported that c-kit is expressed on HUVECs and can be rapidly activated by SCF (10). For the functional measure of SCF upregulation under hypoxia, the levels of c-kit phosphorylation on HUVECs driven by the CCM from MCF-7 cells were measured. c-kit phosphorylation was significantly strengthened by the CCM from hypoxic MCF-7 and MCF-7-c cells (Figure 3A, both $P < 0.01$). These data further confirmed the increase of SCF under hypoxia. Importantly, the phospho-c-kit levels driven by the CCM from MCF-7-sh cells were significantly decreased on HUVECs compared with those driven by the CCM from MCF-7-c cells ($P < 0.01$), validating the reduction of SCF from MCF-7-sh cells.

It has been shown that SCF and c-kit are coexpressed in A549 lung cancer cells and important for the cell growth (35). To determine whether A549 cells were more sensitive to the growth inhibition by SCF neutralization under hypoxia than cells under normoxia, antibodies to SCF were added into the culture. SCF neutralization

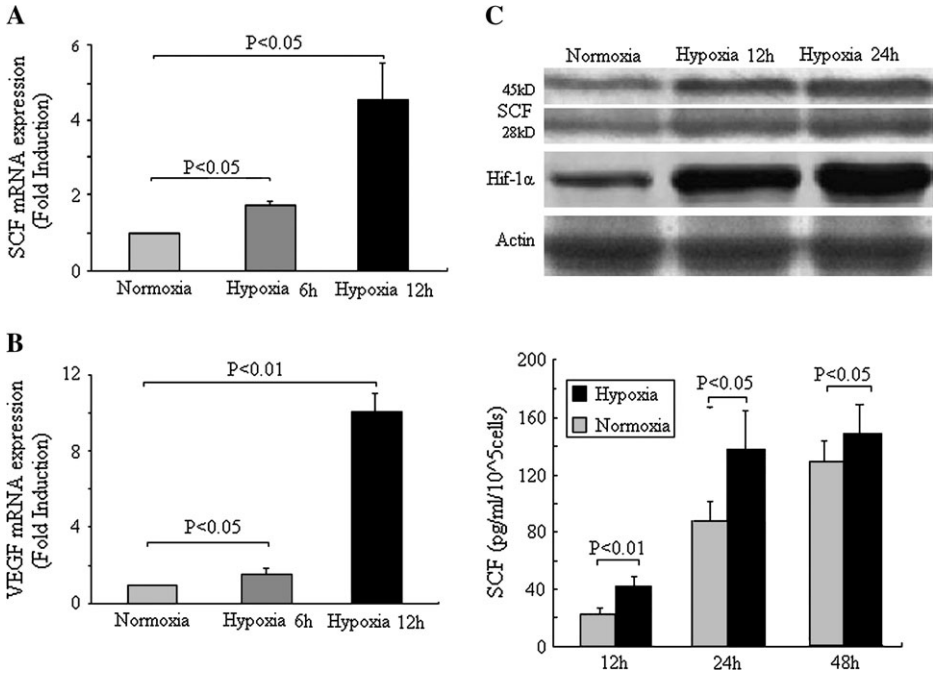

Fig. 1. Hypoxic induction of SCF mRNA and protein expression in MCF-7 cells. (**A**) Quantitative real-time RT–PCR: cells were subjected to 12 h of normoxia (21% O_2) and 6 and 12 h of hypoxia (1.5% O_2), then lysed in trizol for RNA extraction and analyzed for the mRNA expression of SCF and (**B**) VEGF by quantitative real-time RT–PCR: fold induction is relative to cells under normoxia after normalization to the β-actin expression. Columns: mean of three experimental determinations; bars, standard deviation. (**C**) Western blot analysis: cells were incubated under normoxia (24 h) and hypoxia (12 and 24 h), and total protein extracts were processed for immunoblotting by anti-HIF-1α, anti-SCF or anti-actin antibodies. (**D**) ELISA: MCF-7 cells were cultured under normoxia and hypoxia for 12, 24 and 48 h. sSCF in culture medium was quantified by ELISA and normalized for cell numbers.

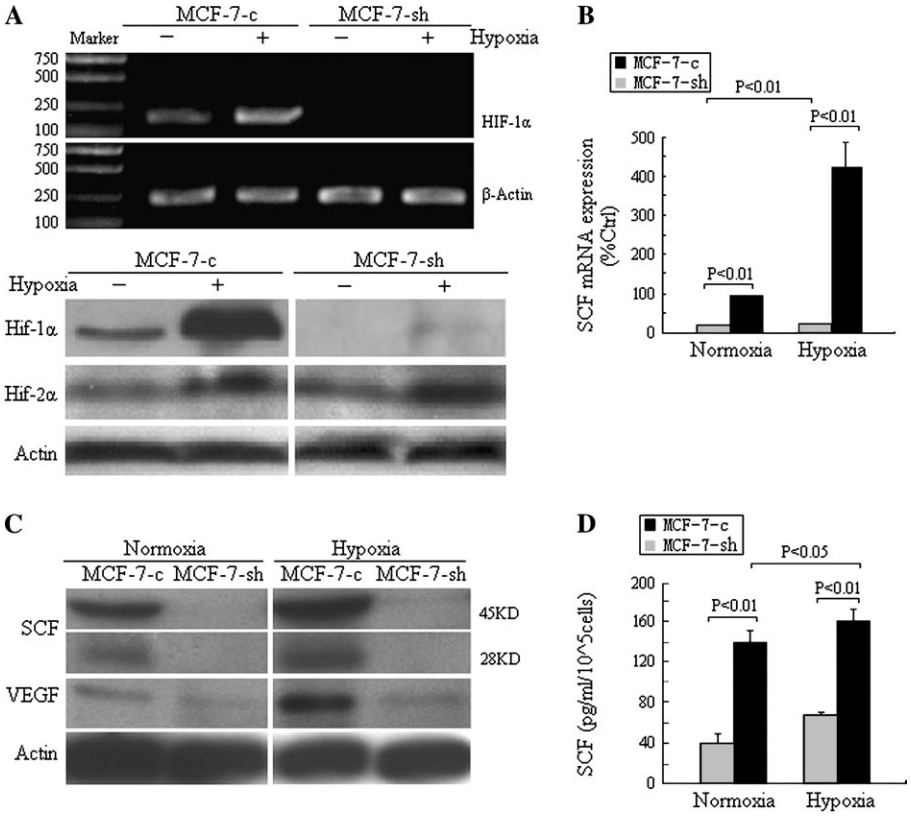

Fig. 2. HIF-1α knockdown by shRNAs reduced the mRNA and protein expression of SCF under normoxia and hypoxia. (**A**) Quantitative real-time RT–PCR (upper panel) and western blot (lower panel) analysis of HIF-1α mRNA, HIF-1α and HIF-2α protein levels in MCF-7 cells stably transected with plasmids encoding shRNAs against HIF-1α mRNA (MCF-7-sh) or a negative control sequence (MCF-7-c). Cells were cultured under normoxia and hypoxia for 12 h before RT–PCR and western blot analysis. (**B**) Quantitative real-time RT–PCR analysis of SCF mRNA levels. MCF-7-c and MCF-7-sh cells were cultured under normoxia and hypoxia for 12 h prior to RNA isolation. Results were expressed as the percentage of SCF levels in MCF-7-c cells under normoxia after normalization to β-actin. Columns, mean of three experimental determinations; bars, standard deviation. (**C**) Western blot analysis of the SCF protein expression. Whole-cell extracts were prepared from MCF-7-c and MCF-7-sh cells after cultured for 12 h under normoxia and hypoxia and analyzed for SCF protein expression using anti-SCF antibodies. Anti-VEGF and anti-actin antibodies were used as positive and loading control, respectively. (**D**) ELISA: MCF-7-c and MCF-7-sh cells were cultured under normoxia and hypoxia for 48 h. sSCF in the culture medium was quantified by ELISA and normalized for cell numbers.

only slightly decreased A549 cell growth by ∼17% after 4 days under normoxia (Figure 3B). In contrast, SCF neutralization reduced A549 cell growth by ∼26% after 2 days and ∼55% after 4 days under hypoxia (Figure 3C). These data suggest that the increase of SCF could play an important role in A549 cell growth under hypoxia.

HIF-1α directly interacted with the SCF promoter

Having shown that HIF-1α plays a critical role in SCF expression, we investigated whether HIF-1α was directly involved in the transcription of SCF gene. Screening of the 5′-flanking region of the SCF gene revealed that a potential HIF-1-binding site near to the start site of transcription was revealed (Figure 4A). To demonstrate the binding of HIF-1α to the SCF promoter in living cells, chromatin immunoprecipitation assay was performed in MCF-7 cells at 1.5% O_2 or 21% O_2. In chromatin fraction pulled down by an anti-HIF-1α antibody, the SCF promoter PCR fragments (−108/+120, with the putative HRE located in this region) under normoxia were detected. These fragments were significantly increased ($P < 0.01$) following 12 h under hypoxia (Figure 4B and C), which was in agreement with previous western blot data (Figure 1C) in that HIF-1α showed a moderate basal expression in normoxia and accumulated after hypoxia in MCF-7 cells. However, SCF promoter PCR fragments were not found in samples pulled down by a control IgG antibody. Furthermore, the SCF promoter region (−1163 to −955) that did not contain HRE could not be pulled down by the anti-HIF-1α antibody, suggesting that HIF-α specifically bound to the HRE region of the SCF promoter *in vivo*.

Overexpression of HIF-1α gene upregulated the SCF promoter activity

In order to determine whether the binding of HIF-1α to the SCF promoter could activate it, we constructed three SCF luciferase promoter vectors containing different lengths of SCF 5′-flanking sequences (pGL3-SCF1: −76/+184; pGL3-SCF2: −853/+184 and pGL3-SCF3: −2185/+184) and HIF-1α cDNA fragments and cotransfected them into pVHL-positive HEK293 cells. HEK293 cells, which express minimal levels of HIF-1α under normoxic conditions, were introduced for excluding the interference of endogenous HIF-1α on SCF promoter activities. In addition, HEK293 cells were easy to be transfected with plasmids and well characterized as a good vehicle for exogenous protein expression. After HEK293 cells were transfected with HIF-1α cDNA, HIF-1α protein levels were augmented with the increase of the transfected plasmids (Figure 5A), confirming that the plasmids did effectively upregulate the HIF-1α protein under normoxia. We transfected this series of 5′ deletion mutants into HEK293 cells with 1 μg HIF-1α expression plasmids or control vectors. After incubation for 48 h, cells were harvested for dual luciferase assay. The full-length SCF promoter activity (pGL3-SCF3) increased ∼2.2-fold in the presence of HIF-1α compared with that treated with plasmids alone ($P < 0.05$) (Figure 5B). Overexpression of HIF-1α also resulted in ∼2.5-fold increase of the pGL3-SCF2 promoter activity ($P < 0.05$). Interestingly, even the activity of the shortest SCF promoter (pGL3-SCF1, which was truncated immediately upstream of the putative HRE) showed ∼1.8-fold induction ($P < 0.05$). To verify whether the HRE site was involved in the HIF-1α-induced expression,

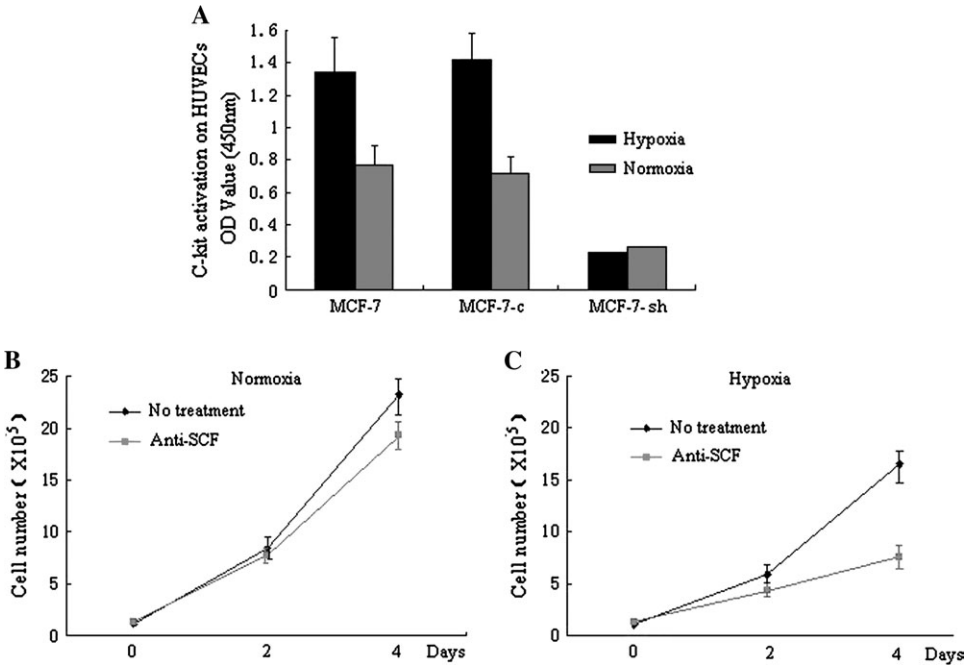

Fig. 3. Functional measure of hypoxia-induced upregulation of SCF. (**A**) Quantification of phospho-c-kit driven by the CCM. HUVECs were cultured in a series of CCM from MCF-7, MCF-7-c and MCF-7-sh cells for 5 min. Then, cells were lysed and the phospho-c-kit levels were quantified by ELISA. Results were expressed as optimal density (OD) values (450 nm) after normalization of the total protein levels. Columns: mean of three experimental determinations; bars, standard deviation. (**B** and **C**) A549 cells are more sensitive to growth inhibition by SCF neutralization. Growth curves of A549 cells treated with SCF antibodies under normoxia (B) and hypoxia (C). Viable cells were counted using trypan blue dye exclusion at the indicated times. Points: mean of three experimental determinations; bars, standard deviation.

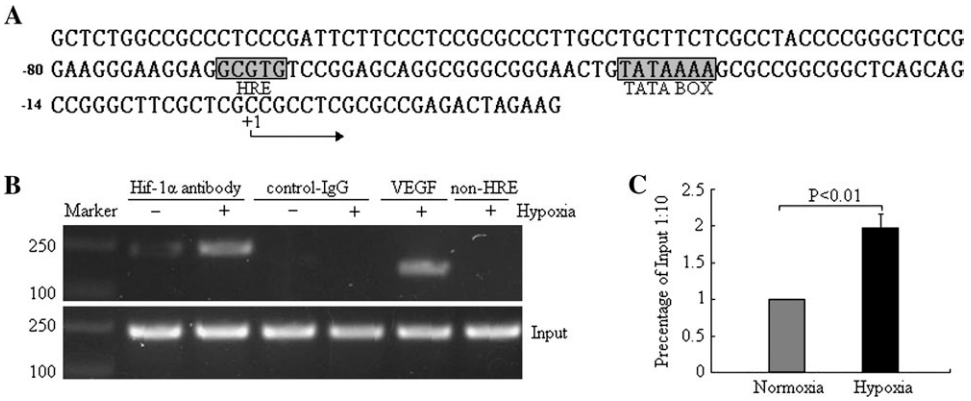

Fig. 4. HIF-1α bound to the SCF promoter *in vivo*. (**A**) The DNA sequence of the SCF promoter. A putative HRE site is located at −68 to −64 nt relative to the start site. (**B**) Chromatin immunoprecipitation analysis. MCF-7 cells were incubated under normoxia and hypoxia for 12 h and then subjected to chromatin immunoprecipitation analysis. The 228 nt PCR products of SCF promoter were only detected in the samples pulled down by HIF-1α antibodies but not in control IgG samples. The SCF promoter region (−1163 to −955) that do not contain HRE (non-HRE) could not be precipitated by HIF-1α antibodies. The 136 nt PCR products of VEGF promoter were used as positive control. (**C**) Semiquantative PCR performed for SCF promoter region. Results were expressed as fold induction relative to the cells under normoxia after normalization to input sample. Column: mean of triplicate PCRs from a representative experiment; bars, standard deviation.

mutant SCF promoters (pGL3-SCF1m, pGL3-SCF2m and pGL3-SCF3m) were constructed. These constructs contained the SCF promoters with the exception of the HRE site, which was mutated from GCGTG to GTAGA. pGL3-SCF1m, pGL3-SCF2m and pGL3-SCF3m activities were no longer increased following HIF-1α overexpression in HEK293 cells. A VEGF luciferase reporter construct (pGL3-VEGF) was used as positive control for the HIF-1α response. Overexpression of HIF-1α induced ∼2.6-fold of the VEGF promoter activity compared with that treated with vector alone. We further cotransfected pGL3-SCF1 and HIF-1α cDNA fragments into MCF-7 cells. Overexpression of HIF-1α (pcDNA-HIF) also markedly increased the SCF promoter activity in MCF-7 cells (Figure 5C, $P < 0.05$) compared with control vectors (pcDNA3.1). Taken together, these data demonstrated that HIF-1α transactivated the SCF promoter through binding to this HRE region.

HIF-1α was involved in EGF-induced upregulation of SCF

Overexpression of EGFR has been found in some breast cancer tissues and is correlated with the poor prognosis for the patients (34,35,36). Interestingly, a recent study has reported that EGF induces the expression of survivin through upregulating HIF-1α in MCF-7 cells

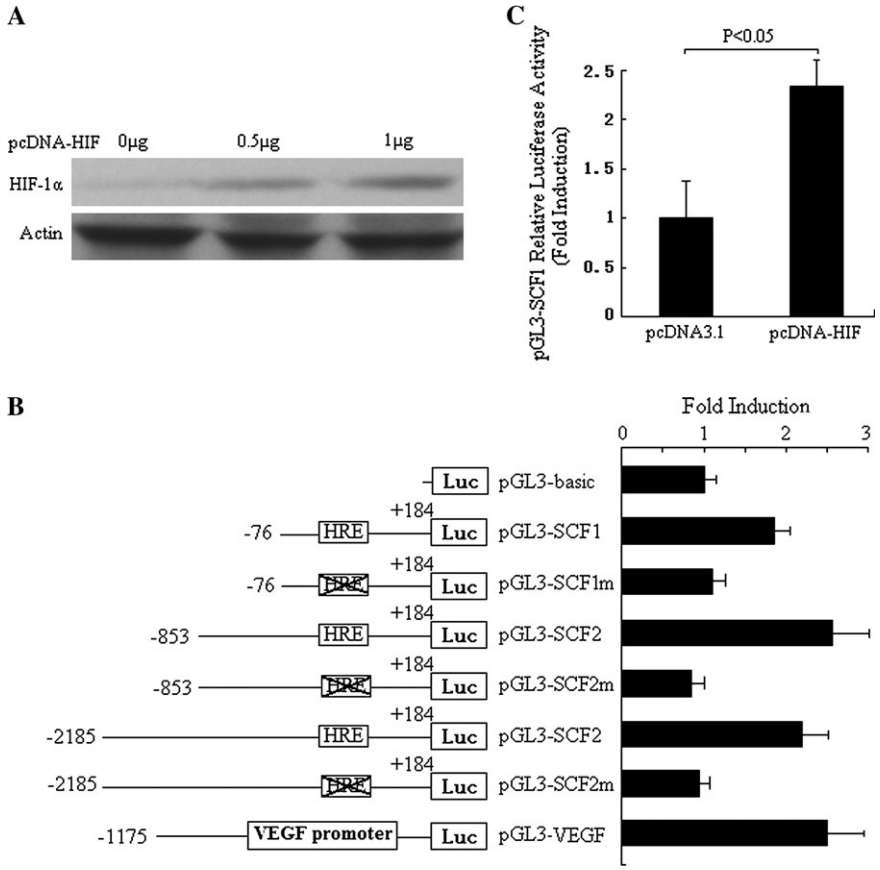

Fig. 5. Overexpression of HIF-1α enhanced the activity of the SCF promoter. (**A**) Western blot analysis: HEK293 cells were transfected with 0.5 or 1 μg HIF-1α expression plasmids. After transfection for 48 h, cells were lysed for immunoblotting by anti-HIF-1α. (**B**) luciferase analysis: pGL3-SCF1, pGL3-SCF2 and pGL3-SCF3: SCF 5′-flanking sequences (–76/+184, –853/+184 and –2185/+184) were fused to pGL3 luciferase coding sequence. pGL3-SCF1m, pGL3-SCF2m and pGL3-SCF3m: equal to pGL3-SCF1, pGL3-SCF2 and pGL3-SCF3, except that HRE site was mutated from GCGTG to GTAGA. pGL3-VEGF: VEGF 5′-flanking region (−1175/+1) was fused to pGL3 luciferase coding sequence. pGL3-Basic: empty pGL3 control vector. HEK293 cells overexpressing 1 μg HIF-1α expression plasmids (pcDNA-HIF) or control vector (pcDNA3.1) were transfected with pGL3-SCF1, pGL3-SCF2, pGL3-SCF3, pGL3-SCF1m, pGL3-SCF2m or pGL3-SCF3m. Transfection with pGL3-Basic or pGL3-VEGF was used as negative or positive control, respectively. After transfection for 48 h, cells were subjected to dual luciferase analysis. Results were expressed as a fold induction relative to the cells transfected with the control vector (pcDNA3.1) after normalization to Renilla activity. Columns, mean of three independent experiments; bars, standard deviation. (**C**) luciferase analysis in MCF-7 cells.

under normoxia (37). To determine whether EGF could induce the expression of SCF through HIF-1α, MCF-7-c or MCF-7-sh cells were transfected with the SCF promoter (pGL3-SCF1) and cultured with or without EGF (100 ng/ml) for 12 h under normoxia. EGF-treated MCF-7-c cells induced ∼1.8-fold in the level of the SCF promoter compared with no-treatment control (Figure 6A, $P < 0.01$). In contrast, EGF-treated MCF-7-sh cells could not upregulate the SCF promoter ($P = 0.5$) when HIF-1α expression was inhibited. Consistent with these results, western blot analysis showed that EGF enhanced the expression of SCF at the protein level in MCF-7-c cells but not in MCF-7-sh cells (Figure 6B). Meanwhile, ELISA data showed that sSCF in the culture supernatants was also increased after EGF treatment in MCF-7-c cells (Figure 6C), but remained undetectable whether EGF was added or not in MCF-7-sh cells.

It is well established that EGFR signaling results in the activation of mitogen-activated protein kinase and PI3K–ATK pathways. To determine the signaling pathways of EGF-induced upregulation of SCF, MCF-7 cells were treated with several inhibitors to block EGFR and its downstream signaling pathways. As shown in Figure 6D, EGF-induced SCF gene transcription was counteracted by AG1478 (the EGFR inhibitor). This result validated the effect of EGF on SCF upregulation. Furthermore, inhibition of PI3K–AKT signaling by LY294002 completely blocked EGF-induced SCF transcription in MCF-7 breast cancer cells. In contrast, inhibition of mitogen-activated protein kinase signaling by PD98059 did not affect such event. Since EGF was reported to stimulate HIF-1 synthesis through PI3K–AKT signaling (22–24,37), those data would suggest that EGF–EGFR–PI3K–AKT–HIF-1 pathway was involved in the SCF upregulation in MCF-7 cells.

Discussion

HIF-1α regulates many genes involved in anaerobic metabolism and angiogenesis and is associated with increased mortality in multiple human tumors (38). Although strictly regulated in normal cells, HIF-1α levels are increased in cancer cells due to intratumoral hypoxia and genetic alteration. In addition, several cytokines contribute to elevating HIF-1α levels such as EGF and insulin-like growth factor-I (39). In this study, we demonstrated that SCF was upregulated by hypoxia and EGF in cancer cells, both of which exerted this effect through a common mediator, HIF-1α.

It should be mentioned that in Figure 2D, levels of sSCF in MCF-7-sh cells were detected when the supernatant was collected after cells were cultured for 48 h. At this time point, the basal secretion of sSCF in MCF-7-c cells under normoxia is rather high. Meanwhile, hypoxia-induced expression of SCF has approached the maximum levels. This figure therefore did not show a prominent upregulation of SCF under hypoxia. When measured in different time points by ELISA, hypoxia-induced expression of SCF is more prominent after treatment for 12 and 24 h (Figure 1D). Under hypoxia for 12 h, the SCF protein

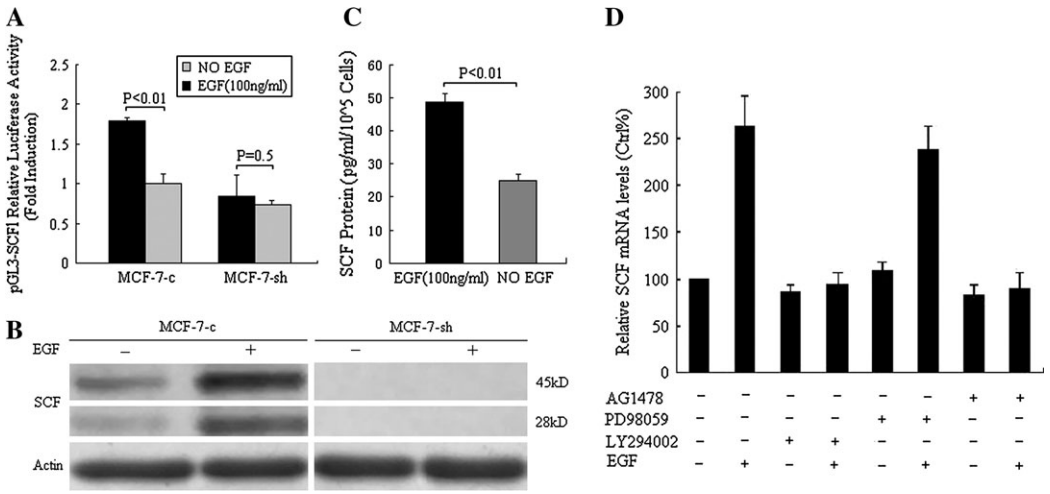

Fig. 6. HIF-1α-dependent regulation of SCF in response to EGF. (**A**) Luciferase analysis: pGL3-SCF1 was transfected into MCF-7-c or MCF-7-sh cells for 24 h. Then, cells were cultured in medium containing 2% serum with or without EGF (100 ng/ml) for 12 h before luciferase analysis. Fold induction is relative to MCF-7-c cells without EGF treatment. Columns, mean of three experimental determinations; bars, standard deviation. (**B**) Western blot analysis: MCF-7-c or MCF-7-sh cells were cultured in medium as described in (A) for 12 h, and then cells were lysed for immunoblotting by anti-SCF or anti-actin antibody. Culture medium was collected for (**C**) ELISA: concentrations of sSCF were normalized to cell numbers. (**D**) Quantitative real-time RT–PCR analysis of SCF mRNA levels. MCF-7 cells were incubated in the absence or presence of 100 ng/ml of EGF, 10 μM of LY294002, PD98059 or AG1478 for 12 h prior to RNA isolation. Results were expressed as the percentage of SCF levels relative to the no-treatment control group, after normalization to β-actin. Columns, mean of three experimental determinations; bars, standard deviation.

increased ∼2-fold, whereas SCF mRNA levels increased ∼4-fold (Figure 2B) at that time point. The discrepancy indicates that hypoxia might also affect the expression of the SCF protein at the posttranscriptional levels.

Importantly, we showed that even the constitutive expression of SCF was strongly dependent on HIF-1α in MCF-7 cells, since the SCF mRNA and protein expression was significantly blunted under normoxia after HIF-1α was silenced. In addition, HIF-1α could bind to the SCF promoter in intact MCF-7 cells under normoxia (Figure 4). The functional expression of HIF-1α under normoxia has been demonstrated in several cell types including macrophages (40), pulmonary artery smooth muscle cells (41) and mesenchymal stem cells (42). In this study, we have also detected the constitutive expression of HIF-1α in MCF-7 cells under normoxia. This finding is in agreement with a previous report (37). Clinical evidence has shown that HIF-1α overexpression is not limited to the hypoxic region but scattered throughout the tissue in breast cancer (43). The constitutive expression of HIF-1α was also detected in other cancer cells (supplementary Figure 1A is available at *Carcinogenesis* Online), suggesting that it is not a rare event. We then transfected a survivin promoter containing a functional HRE (37) into MCF-7, MDA-MB231, KB and HEK293 cells in order to confirm the activated HIF-1 in normoxic MCF-7 cells. The promoter was activated in MCF-7, MDA-MB231, KB but not in HEK293 cells (supplementary Figure 1B is available at *Carcinogenesis* Online). The levels of the survivin promoter are in agreement with those of HIF-1α protein detected under normoxia. It has also been shown that oxygen-independent HIF-1α expression might occur by oncogene activation (44,45), inactivation of tumor suppressor genes (46) and activation of growth factor signaling (22,24,39). In MCF-7 cells, this event may be explained, in part, by the fact that the constitutive expression of EGF and other growth factors can increase HIF-1α synthesis under normoxia. In agreement with this study, Peng *et al.* (37) also reported that HIF-1α could regulate its target gene (survivin) through directly binding to its promoter under normoxia, suggesting that the role of HIF-1α in tumor cells is more complicated than an adaptive mechanism to hypoxia.

SCF plays a critical role in carcinogenesis by autocrine and/or paracrine methods. SCF and its receptor, c-kit, are coexpressed in many tumors such as small-cell lung carcinomas, colorectal carcinomas, neuroblastomas, etc. (47), indicating a presence of an autocrine loop. SCF initiates c-kit activity to support survival and proliferation of cancer cells. For instance, Yasuda *et al.* (48) reported that SCF–c-kit signals promote survival and invasion of pancreatic cancer cells. Interestingly, recent evidence has shown that SCF–c-kit signaling upregulates HIF-1α levels through the PI3K–AKT pathway, and then promotes tumor angiogenesis through VEGF in small-cell lung carcinoma (13). Combined with our data, we postulate that a reciprocal effect may exist between SCF and HIF-1α, thus forming a positive feedback in cancer cells coexpressing SCF and c-kit. Further studies are needed to elucidate this mechanism. In the context of breast cancer, the functions of SCF remain to be defined. Although Hines *et al.* (2) reported that SCF and c-kit were coexpressed in breast cancer and stimulated tumor growth in conjunction with insulin-like growth factor-I or EGF, clinical evidence showed significantly decreased c-kit levels compared with controls in breast cancer (49). Thus, SCF is not likely to function as an autocrine mitogen in breast cancer cells. We reason that SCF is more likely to be an angiogenic factor in breast carcinogenesis.

Angiogenesis is vital for the growth and metastasis of solid tumors. Tumor angiogenesis depends on the interaction of different tumor components such as endothelial cells and mast cells. SCF was reported to promote angiogenesis in an animal model of breast cancer through regulating mast cell migration with a subsequent release of VEGF (50). In addition, SCF has been shown to directly promote proliferation, migration and differentiation of a variety of primary endothelial cells *in vitro* and promote glioma-associated angiogenesis *in vivo* (11). We also detected that blocking SCF bioactivity by its antibody inhibited the migration of HUVECs induced by the culture medium from MCF-7 cells (supplementary Figure 2 is available at *Carcinogenesis* Online), supporting SCF as a paracrine factor to promote angiogenesis in breast cancer. Revealing the role of SCF in angiogenesis and its significance in breast cancer patients is currently under investigation in our laboratory. Previous studies demonstrated the critical role of HIF-1α in the expression of VEGF and other angiogenic cytokines during hypoxia-induced angiogenesis (38). Our data suggest that SCF may also be involved in HIF-1α-associated angiogenesis in tumors.

It should be mentioned that hypoxia regulates many genes in a cell type-specific manner. For example, HIF-1α regulates the expression of VEGFR in mesenchymal stem cells in response to hypoxia but HIF-2α presents this effect in HUVECs (42). Since our studies pertain only to MCF-7 and HEK293 cells, it would be premature to consider HIF-1α as a common mediator for hypoxia-induced SCF expression in other cells. Studies in other tumor or primary cells are required to broaden the role of HIF-1α in the activity of SCF.

In conclusion, both gain-of-function and loss-of-function studies established that HIF-1α is a key regulator of the SCF expression in MCF-7 cells. This effect was mediated by a direct interaction of HIF-1α with a functional HRE located in the SCF promoter region. Hypoxia- and EGF-induced expression of SCF required HIF-1α as a mediator. In breast cancer, the EGF signaling pathway and the hypoxic microenvironment coexist and may synergistically promote angiogenesis through HIF-1α-induced expression of SCF as well as other angiogenic factors (supplementary Figure 3 is available at *Carcinogenesis* Online).

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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