Kinome Sirna Screen Identifies SMG-1 as a Negative Regulator of Hypoxia-inducible Factor-1α in Hypoxia*

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Hypoxia-inducible factor-1 (HIF-1) plays a central role in tumor progression by regulating genes involved in proliferation, glycolysis, angiogenesis, and metastasis. To improve our understanding of HIF-1 regulation by kinome, we screened a kinase-specific small interference RNA library using a hypoxia-responsive element (HRE) luciferase reporter assay under hypoxic conditions. This screen determined that depletion of cellular SMG-1 kinase most significantly modified cellular HIF-1 activity in hypoxia. SMG-1 is the newest and least studied member of the phosphoinositide 3-kinase-related kinase family, which consists of ATM, ATR, DNA-PKcs, mTOR, and SMG-1. We individually depleted members of the phosphoinositide 3-kinase-related kinase family, and only SMG-1 deficiency significantly augmented HIF-1 activity in hypoxia. We subsequently discovered that SMG-1 kinase activity was activated by hypoxia, and depletion of SMG-1 up-regulated MAPK activity under low oxygen. Suppressing cellular MAPK by silencing ERK1/2 or by treatment with U0126, a MAPK inhibitor, partially blocked the escalation of HIF-1 activity resulting from SMG-1 deficiency in hypoxic cells. Increased expression of SMG-1 but not kinase-dead SMG-1 effectively inhibited the activity of HIF-1α. In addition, cellular SMG-1 deficiency increased secretion of the HIF-1α-regulated angiogenic factor, vascular epidermal growth factor, and survival factor, carbonic anhydrase IX (CA9), as well as promoted the hypoxic cell motility. Taken together, we discovered that SMG-1 negatively regulated HIF-1α activity in hypoxia, in part through blocking MAPK activation.

Solid tumors contain poorly vascularized areas that are hypoxic. Hypoxic tumor cells are resistant to apoptosis, prone to migrate to less hypoxic regions of the body (metastasis), and produce pro-angiogenic factors to stimulate neovascularization leading to tumor oxygenation and tumor growth (1, 2). In fact, hypoxic tumors are typically associated with poor patient prognosis due to the aggressive and pro-angiogenic nature of hypoxic tumor cells, as well as their resistance to radiotherapy and chemotherapy (3, 4).

Hypoxia-induced cancer cell responses are regulated at multiple levels, including gene transcription, protein translation, post-translational modification, and subcellular translocation (5). Hypoxia-inducible factor-1 (HIF-1),2 a heterodimer consisting of a HIF-1α and a HIF-1β subunit, is the master regulator of these cellular responses to low oxygen. HIF-1α activity is regulated by O2-dependent degradation and by the rate of transcription and translation (6). Moreover, oncogenic ERK-dependent phosphorylation of HIF-1α and its coactivator p300 promotes the transcriptional activity of HIF-1α possibly through enhancing the accessibility of RNA polymerase II to the promoters containing hypoxia-responsive elements (HREs) (1).

HIF-1 binds to HREs in the promoters or enhancers of target genes and activates the expression of at least 150 genes encoding proteins that regulate cell metabolism, survival, motility, basement membrane integrity, angiogenesis, hematopoiesis, and other functions (4). In particular, hypoxia-induced HIF-1 activation up-regulates the expression of a metastatic gene (lysyl oxidase), critical angiogenic factors (VEGF-A and Ang-2), and survival factors (carbonic anhydrase IX and XII) (1). Among these molecules, HIF-1α, VEGF, and carbonic anhydrase IX (CA9) proteins are clinical biomarkers for hypoxia (5, 7).

The phosphoinositide 3-kinase-related kinase (PIKK) family of high molecular mass signaling proteins comprises ATM, ATR, DNA-PKcs, mTOR, and SMG-1. PIKKs are serine-threonine kinases and mediate cellular reaction to a variety of stresses, including genome and RNA surveillance and accessibility of nutrients (8–10). Among them, SMG-1 is the newest and least studied member of the PIKKs, and was first recognized for its role in regulating nonsense-mediated mRNA decay, a cellular surveillance mechanism that degrades mRNA transcripts containing premature translation termination codons (11–13). So far, SMG-1 is known for responding to different cellular stresses. For example, like the genotoxic stress-responsive kinases, ATM, ATR, and DNA-PKcs that SMG-1 closely resembles, SMG-1 is activated by DNA damage and

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2 The abbreviations used are: HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-responsive element; siRNA, small interfering RNA; PIKK, phosphoinositide 3-kinase-related kinase; WT, wild type; KD, kinase-dead; CA9, carbonic anhydrase IX; MTT, methylthiazolyldiphenyltetrazolium bromide; VEGF, vascular epidermal growth factor; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; ERK, extra-cellular signal-regulated kinase; MEK, MAPK/ERK kinase; TS, TOR SMG-1.

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phosphorylates p53 during genotoxic stress (14). Moreover, SMG-1 is involved in cell survival during tumor necrosis factor-α-induced stress (15), lifespan regulation (16), as well as in cell cycle checkpoint signaling under oxidative stress (17).

It has been demonstrated that hypoxic stress inhibits mTOR activity (18–20), which may lead to suppression of HIF-1α translation (21–23). ATR and ATM were also indicated in the repair of hypoxia/re-oxygenation-induced DNA damage (5). However, the role of SMG-1 in regulating cellular response to hypoxia is unclear. In this study, we demonstrate that SMG-1 was activated by hypoxia in cancer cells and consequently suppressed HIF-1α activity in part via inhibition of the MAPK pathway, thereby reducing the hypoxia-induced secretion of angiogenic factor, VEGF, and survival factor, CA9, as well as restricting the migration of hypoxic cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human cell lines HeLa, HEK293, and SKOV-3 were obtained from American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (Invitrogen). All cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (Invitrogen). SKOV-3 were obtained from American Type Culture Collection (Manassas, VA).

**Cell Transfections**—The siRNA library contained Smartpool libraries (Dharmacon, Lafayette, CO) of 4 siRNA duplexes per gene targeting 779 protein kinases, lipid kinases, and regulated subunits. For siRNA transfection, HeLa or SKOV-3 cells were plated in 24-well plates in complete medium. After 24 h, cells were transfected with siRNA at the final concentration of 25 nM using the Dharmafect I (Dharmacon). After a further 24 h, cells were transfected with 0.3 μg of HRE luciferase reporter plasmid (a kind gift from Dr. Navdeep S. Chandel (24)) and 0.03 μg of control TK-Renilla reference plasmid DNA using GenJet DNA in vitro transfection reagent (SignaGen Laboratories). After 48 h, cells were transfected with siRNA at the final concentration of 25 nM using the Dharmafect I (Dharmacon). After a further 24 h, cells were transfected with 0.3 μg of HRE luciferase reporter plasmid (a kind gift from Dr. Navdeep S. Chandel (24)) and 0.03 μg of control TK-Renilla reference plasmid DNA using GenJet DNA in vitro transfection reagent (SignaGen Laboratories). After 48 h, cells were transfected with siRNA at the final concentration of 25 nM using the Dharmafect I (Dharmacon). After a further 24 h, cells were transfected with 0.3 μg of HRE luciferase reporter plasmid (a kind gift from Dr. Navdeep S. Chandel (24)) and 0.03 μg of control TK-Renilla reference plasmid DNA using GenJet DNA in vitro transfection reagent (SignaGen Laboratories). After 48 h, cells were transfected with siRNA at the final concentration of 25 nM using the Dharmafect I (Dharmacon). After a further 24 h, cells were transfected with 0.3 μg of HRE luciferase reporter plasmid (a kind gift from Dr. Navdeep S. Chandel (24)) and 0.03 μg of control TK-Renilla reference plasmid DNA using GenJet DNA in vitro transfection reagent (SignaGen Laboratories). After 48 h, cells were transfected with siRNA at the final concentration of 25 nM using the Dharmafect I (Dharmacon).

**Immunoassays**—VEGF and CA9 concentrations in cell culture supernatants were measured using immunoassay kits from Pierce (Rockford, IL) and from R&D Systems (Minneapolis, MN), respectively. In brief, HeLa cells were transfected with SMG-1 siRNA. After 24 h, the cells were starved and incubated under hypoxic conditions (1% oxygen) at 37 °C overnight. Cell culture medium (50 μl) was added to each well of a microwell precoated with anti-human VEGF or CA9 polyclonal antibody and incubated for 2 h at room temperature. Each well was washed with wash buffer. For VEGF, 100 μl of biotinylated antibody was added to each well, and the mixture was incubated for 1 h. For CA9, 200 μl of carbonic anhydrase IX conjugate was added, and the mixture was incubated for 2 h. After washing, streptavidin-horseradish peroxidase was added to each well, and the mixture was incubated for 30 min. Following washes, a substrate solution was added to each well. After incubation for 30 min at room temperature, the enzyme reaction was stopped, and the intensity was measured at 450 nm. The data were normalized using the cell number determined by methylthiazolyl-diphenyltetrazolium bromide (MTT, Sigma).

**Cell Motility Assays**—SKOV-3 cells were harvested with Trypsin/EDTA, washed twice with Dulbecco’s modified Eagle’s medium, and resuspended in migration buffer (Dulbecco’s modified Eagle’s medium, 2 mM MnCl2, 1.8 mM MgCl2, 2 mM CaCl2, 0.5% bovine serum albumin) at a density of 106/ml. Lower chambers (Corning, NY) were filled with 600 μl of warm migration buffer containing 20 μM fibronectin (BD Biosciences) as the attractant, and the upper chambers were filled with 100 μl of the cell suspension. After 5-h incubation in the hypoxic chamber (1% oxygen) at 37 °C, cells were fixed for 30 min at room temperature in 5% glutaraldehyde prepared in phosphate-buffered saline, and stained for 30 min in 0.1% crystal violet and 2% ethanol. Non-migratory cells on the upper surface of the filter were removed by wiping with a cotton swab. The number of migratory cells was measured by counting three
To explore the role of kinases in regulating HIF-1 activity under hypoxia, we depleted kinases in tumor cells using a siRNA kinase library targeting 779 kinases and then analyzed cellular HIF-1 activity under oxygen deprivation using an HRE luciferase reporter assay. In this assay, hypoxia strongly increased HRE luciferase activity and HIF-1α protein stabilization in cells (Fig. 1A). Among the 779 kinases screened by siRNA-mediated silencing, depletion of 18 kinases significantly increased HIF-1 activity and another 10 kinases decreased HIF-1 activity under hypoxic conditions (Fig. 1B). Among these kinases, the depletion of SMG-1 impacted HIF-1 activity most significantly (Fig. 1B). SMG-1 belongs to the PIKK family of protein kinases. We found that siRNA-mediated depletion of SMG-1, but not other members of the PIKK family, ATM, ATR, DNA-PKcs, and mTOR, markedly increased the HIF-1 activity in hypoxic cancer cells (Fig. 2A). To further confirm that the increase in hypoxia-induced HIF-1 activity in SMG-1-depleted cells was directly related to an on-target effect of the siRNA, we repeated this experiment using four different siRNA duplexes to deplete cellular SMG-1 and obtained similar results (Fig. 2B). Next, we forced expression of wild-type (WT) SMG-1 and its kinase-dead (KD) mutant separately in SMG-1-depleted cell and found that forced expression of WT, but not KD SMG-1, could reverse the increased HIF-1 activity observed in hypoxic tumor cells depleted of cellular SMG-1 (Fig. 2C). These results confirmed that SMG-1 deficiency increased HIF-1 activity in hypoxia and suggested that cellular SMG-1 activity is critical in suppressing HIF-1 activation in hypoxic cancer cells.

**SMG-1 Is Activated by Hypoxic Stress and Subsequently Suppresses HIF-1 Activity in Low Oxygen in Part through Repression of MAPK Activation**—Because SMG-1 is known to be activated under various cellular stresses (14), we investigated whether SMG-1 kinase can also be activated under hypoxic stress. We expressed WT and KD SMG-1 in tumor cells followed by hypoxia treatment and found that the kinase activity of WT SMG-1, but not KD SMG-1, was substantially up-regulated for at least 18 h in response to hypoxia (Fig. 3A). To determine whether the activated SMG-1 kinase had an effect on other cellular kinase cascades, we analyzed the ERK1/2, mTOR, AKT, and BMK1 kinase pathways and found that depletion of cellular SMG-1 altered only the intensity of ERK1/2 activation, but not the other pathways, in response to hypoxia (Fig. 3B). Moreover, kinase library screen indicated that depletion of ERK1 significantly decreased the HIF-1 activity. Thus, we suspected that SMG-1 inhibited HIF-1 activity through suppression of ERK1/2 activities under hypoxic conditions. To test this, we depleted ERK1 and/or ERK2 in SMG-1-deficient cells and found that ERK1, but not ERK2, knockdown partially reduced the SMG-1 deficiency-dependent up-regulation of HIF-1 activity in hypoxic cells (Fig. 3C). Similar results were obtained by treating SMG-1-deficient cells with the MEK1 inhibitor, U0126 (Calbiochem), to suppress ERK1/2 activity under hypoxic conditions (Fig. 3D). These results suggested that SMG-1 inhibited the HIF-1 activity through partial repression of MAPK activity in hypoxic tumor cells.

**The Kinase Activity of SMG-1 Is Required to Negatively Regulate HIF-1α Transcriptional Activity**—Because the kinase activity of SMG-1 is up-regulated by hypoxic stress and SMG-1 inhibits HIF-1 activity during hypoxic conditions, we suspected that the kinase activity of SMG-1 played a role in controlling HIF-1-transactivating activity in hypoxic cells. We increased expression of WT or KD SMG-1 in hypoxia cancer cells and found that WT but not KD SMG-1 could block HIF-1 activity in cells under hypoxia (Fig. 4A). Because HIF-1α is a master regulator for hypoxia-induced cellular responses, we next investigated whether SMG-1 had an effect on HIF-1α transcriptional activity. Co-expression of WT SMG-1, but not KD SMG-1, with HIF-1α demonstrated that SMG-1 inhibited HIF-1α-mediated transcription in a dose-dependent manner (Fig. 4B). These results suggested that the kinase activity of SMG-1 was critical in suppressing HIF-1α activity in tumor cells under hypoxia.
SMG-1 Negatively Regulates the Secretion of VEGF and CA9 and the Migratory Potential of Hypoxic Tumor Cells—Increased VEGF and CA9 proteins in epithelial cancers are markers of tumor hypoxia and are associated with a poor prognosis, because expression of VEGF and CA9 promotes tumor-associated angiogenesis and the survival capacity of hypoxic tumors, respectively (5, 7). Because both VEGF and CA9 expression are tightly regulated by HIF-1 activity, we suspected that removal of SMG-1, a negative regulator of HIF-1, would substantially enhance the production of VEGF and CA9 in tumor cells under hypoxia. In Fig. 5 (A and B), we demonstrated that in SMG-1-deficient cells the production of VEGF and CA9 was substantially elevated under hypoxia. As hypoxic conditions significantly enhance tumor metastasis partly by boosting the migratory potential of tumor cells, we tested whether depletion of cellular SMG-1 had any effect on hypoxic tumor cell motility. Indeed, we found that SMG-1 depletion significantly promoted the migratory potential of cancer cells in hypoxia (Fig. 5C). These results indicated that SMG-1 repressed a range of malignant responses induced by oxygen deprivation of tumor cells and suggested that human SMG-1 was a tumor suppressor, particularly for hypoxic tumors.

DISCUSSION

Hypoxia occurs in the majority of tumors, promoting angiogenesis, metastasis, and resistance to therapy (26). HIF-1 is a master transcriptional activator of ~100–200 genes that promote adaptation and survival under hypoxia (27). To identify novel kinase regulators of HIF-1, we screened a siRNA library targeting 779 human kinases and used the HRE reporter assay as readout under hypoxia. Among these 779 human kinases, we found that 18 kinases positively regulated HIF-1 activity. Especially, the depletion of ERK1 and MAPK3K1, two key kinases of the ERK MAPK pathway, significantly decreased HIF-1 activity. It has been reported that treatment of tumor cells with the MEK1/2 inhibitors PD98059 or U0216, or expression of a dominant-negative form of ERK1, blocked HIF-1 activation in hypoxia (28–31). Herein, using specific siRNA targeting of cellular ERK1 or MAPK3K1, we confirmed that the MAPK positively regulated the HIF-1 activity under hypoxia.

On the other hand, we discovered that the depletion of 10 other kinases significantly increased the HIF-1 activity. Among these kinases, it has been reported that suppression of DUSP1 (32) and VRK2 (33) could result in up-regulation of HIF-1 activity under hypoxia. Especially, the depletion of SMG-1 had the strongest enhancing effect on HIF-1 activity. SMG-1, the newest member of the PIKK family, contains a large (>1000 amino acid) insert region between the kinase domain and FATC domain (9, 11, 12, 34). Moreover, SMG-1 is found in both the cytoplasmic and nuclear compartments (14, 35), unlike the related genotoxic stress-responsive kinases, ATM and ATR, which are confined to the nucleus in most cells (36). The cytoplasmic presence of SMG-1 suggests that this PIKK may play additional roles other than stress signaling restricted within the nucleus such as DNA repair. In this study, we found that SMG-1 was activated under hypoxic conditions, the depletion of cellular SMG-1 significantly enhanced HIF-1α activity, and increasing expression of SMG-1 markedly inhibited HIF-1α activity. These results implied that SMG-1 activity
potently suppressed HIF-1-dependent cellular responses in hypoxic cells. However, the activity of mTOR, another member of the PIKK family, was inhibited by hypoxic stress, thereby suppressing HIF-1α protein translation. These observations suggest that hypoxia induces activation of SMG-1 and deactivation of mTOR pathways to contain the HIF-1α activity at post-translational and translational levels, correspondingly, thereby maintaining appropriate cellular responses for numerous physiological and pathological conditions under hypoxia.

HIF-1α activity is regulated in two major ways. The first relies on hydroxylation-dependent degradation/inactivation. The second involves control of HIF-1α protein synthesis via the phosphatidylinositol 3-kinase/AKT and MAPK pathways. To investigate the mechanism of HIF-1α activity in SMG-1-deficient cells, we first studied the effect on the stability of HIF-1α of SMG-1 deficiency under hypoxia and found that SMG-1 knockdown did not affect HIF-1α stability, which suggests that SMG-1 does not involve in the regulation of von Hippel-Lindau ubiquitin ligase complex. HIF-1α activity is also regulated by AKT, mTOR, and MAPK. To determine whether the activated SMG-1 kinase had an effect on other cellular kinase cascades, we analyzed the ERK1/2, mTOR, AKT, and BMK1 kinase pathways and found that depletion of cellular SMG-1 altered only the intensity of ERK1/2 activation. ERK1/2 activity is up-regu-
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FIGURE 4. The kinase activity of SMG-1 was required to negatively regulate HIF-1α transactivation activity. A, HeLa cells were transfected with empty vector (EV), HA-SMG-1-WT (WT), or HA-SMG-1-KD (KD) expression plasmid together with HRE- and pRL-TK luciferase reporter plasmids. After 24 h, cells were incubated in the hypoxic chamber at 37 °C overnight. The luciferase activity of cells transfected with empty vector (EV) was taken as 100%. The expression of HA-SMG-1-WT and HA-SMG-1-KD were detected in Western blot using anti-HA antibody (lower panel). B, HeLa cells were cotransfected with HIF-1α expression plasmid (pcDNA3.0-HA-HIF-1α P402A; P564A, a kind gift from Dr. William G. Kaelin, Jr. [39]) and with an increasing amount of HA-SMG-WT (WT) or HA-SMG-KD (KD) plasmids as indicated. HRE and pRL-TK luciferase reporter plasmids were also included in transfection mixture. 48 h later, the luciferase activities of these cells were analyzed. The luciferase activity of control cells transfected without any HIF-1α, HA-SMG-1-WT, or HA-SMG-1-KD plasmids was taken as 1. The expression of HIF-1α or HA-SMG-1-WT/HA-SMG-1-KD were detected in Western blot using anti-HIF-1α or anti-HA antibody, respectively (lower panel).

FIGURE 5. SMG-1 negatively regulates the secretion of VEGF and CA-9, as well as the migratory potential of hypoxic tumor cells. A and B, HeLa cells were transfected with SMG-1 or control (Cont) siRNAs. After 48 h, the cells were incubated in 20% oxygen or 1% oxygen at 37 °C overnight. VEGF or CA9 protein in cell culture supernatants was measured using immunoassay kits from Pierce or R&D Systems, respectively, and the resultant data were normalized by cell number determined by MTT. The normalized VEGF or CA9 concentration of control cells under 20% oxygen was taken as 1. C, SKOV-3 cells were mock transfected (Mock) or transfected with control siRNA (Cont) or SMG-1 siRNA (SMG-1). 48 h post transfection, the migratory capacity of the cells in hypoxia was analyzed, and the relative rate of cell migration in these cells was normalized to mock transfected cells whose value was taken as 1.

ERK1/2 has been shown to phosphorylate HIF-1α in vitro (28, 37). MAPKs are critical for the transactivation activities of both HIF-1α and p300 through promoting HIF-1α-p300 interaction by mediating the phosphorylation of these two molecules (1, 28). Therefore, it is very likely that the activation of SMG-1 by low oxygen restrains the hypoxia-induced malignant responses in tumor cells partially through inhibition of the MAPK pathway and the consequent HIF-1α activation.

Hypoxia promotes a range of malignant responses in tumor cells such as proliferation, survival, angiogenesis, migration, and metastasis (4). Herein, we demonstrated that SMG-1 activity is critical, in hypoxic tumor cells, in repressing HIF-1α activity and consequent secretion of the angiogenic factor, VEGF, and the survival factor, CA9, and in blocking cell motility under low oxygen conditions as well. There are reports from the COSMIC data base of The Sanger Institute (38) describing mutations of SMG-1 in its kinase, TS, and insertion regions that are found in human breast cancers. It should be interesting to further investigate whether these mutations compromise the ability of SMG-1 to suppress HIF-1α activity in hypoxia and consequently augment the malignant potential of hypoxic breast tumor cells.

Herein, we demonstrated that SMG-1 was a strong negative regulator for hypoxia-induced tumor cell responses such as the secretion of angiogenic factor, VEGF, and survival factor, CA9, as well as for hypoxic tumor cell motility. Thus, our findings have important prognostic and therapeutic implications for the treatment of hypoxic tumors. Analysis of SMG-1 expression levels or mutations in human cancer could be used as a biological marker to predict efficacy of treatment with MAPK or angiogenesis inhibitors. SMG-1 targeted drugs up-regulating SMG-1 protein, mRNA levels, and/or its activity could be used for treating cancer patients, in combination with angiogenesis inhibitors and/or other traditional cancer therapeutic approaches such as chemo- and radiotherapies.
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