HSulf-1 Modulates FGF2- and Hypoxia-Mediated Migration and Invasion of Breast Cancer Cells

Ashwani Khurana, Peng Liu, Pasquale Mellone, Laura Lorenzon, Bruno Vincenzi, Kaustubh Datta, Bruno Vincenzi, Wilma Lingle, Jeremy Chien, Alfonso Baldi, and Viji Shridhar

Abstract

HSulf-1 modulates the sulfation states of heparan sulfate proteoglycans critical for heparin binding growth factor signaling. In the present study, we show that HSulf-1 is transcriptionally deregulated under hypoxia in breast cancer cell lines. Knockdown of HIF-1α rescued HSulf-1 downregulation imposed by hypoxia, both at the RNA and protein levels. Chromatin immunoprecipitation with HIF-1α and HIF-2α antibodies confirmed recruitment of HIF-α proteins to the two functional hypoxia-responsive elements on the native HSulf-1 promoter. HSulf-1 depletion in breast cancer cells resulted in an increased and sustained bFGF2 (basic fibroblast growth factor) signaling and promoted cell migration and invasion under hypoxic conditions. In addition, FGFR2 (fibroblast growth factor receptor 2) depletion in HSulf-1–silenced breast cancer cells attenuated hypoxia-mediated cell invasion. Immunohistochemical analysis of 53 invasive ductal carcinomas and their autologous metastatic lesions revealed an inverse correlation for the expression of HSulf-1 to CAIX in both the primary tumors (P > 0.0198) and metastatic lesions (P ≥ 0.0067), respectively, by χ² test. Finally, HSulf-1 expression levels in breast tumors by RNA in situ hybridization showed that high HSulf-1 expression is associated with increased disease-free and overall survival (P ≥ 0.03 and P ≥ 0.0001, respectively). Collectively, these results reveal an important link between loss of HSulf-1 under hypoxic microenvironment and increased growth factor signaling, cell migration, and invasion. Cancer Res; 71(6): 2152–61. ©2011 AACR.

Introduction

Heparan sulfate proteoglycans (HSPG) are integral components of the extracellular matrix that surround all mammalian cells and also exist as membrane-bound glycoproteins that are sulfated (1). In addition to providing structural integrity, they act as a storage depot for a variety of heparin binding factors and chemokines (2), and as coreceptors of heparin binding growth factors and thus can modulate signaling (3). Recently, endosulfatases 1 and 2 (HSulf-1 and -2) were identified which function to remove sulfate moieties at 6-O positions of glucosamine (4). Recent report suggests that HSulf-1 and -2 knockout could lead to upregulation of HS biosynthetic enzymes culminating in differences in 2-O and N-O sulfation in mouse embryonic fibroblasts derived from knockout mice (5). It is now well recognized that sulfation status of HSPGs is critical for the interaction with several heparin binding growth factors such as bFGF2 (basic fibroblast growth factor 2), VEGF165, Wnts, HGF (hepatocyte growth factor), amphiregulin, GDNF, and SDF-1 (6–13). Gene knockout studies clearly indicate that loss of both HSulfs affect embryonic development leading to embryonic lethality (14). Several biological functions have been shown to be regulated by Sulfs, namely, Wnt-dependent myogenic specification (15), Sonic hedgehog (Shh)-regulated oligodendroglial specification (16), and esophageal and skeletal defects (6, 17).

We previously identified HSulf-1 as a downregulated gene in several tumor types including ovarian, breast, and hepatocellular carcinomas (8, 9, 12). Loss of HSulf-1, upregulates heparin-binding growth factor signaling and confers resistance to chemotherapy-induced apoptosis (18).

Transcription factor, hypoxia-inducible factor-1α (HIF-1α), is a well-established regulator of tumor angiogenesis (19). Inhibition of proline hydroxylation in the oxygen-dependent degradation domain of HIF-1α with prolyl hydroxylases stabilizes HIF-1α, under hypoxia (20). Under normoxic conditions, HIF-1α is actively degraded by E3 ligase VHL in a proteosome-dependent manner (21). High levels of HIF-1α have been correlated with poor prognosis and increased degree of metastasis in several cancer types including breast cancer (22).
In the present study, we show for the first time that hypoxia regulates HSulf-1 expression in HIF-1α-dependent manner and that HSulf-1 depletion promotes breast cancer cell migration induced by both hypoxic conditions and bFGF2 signaling.

Materials and Methods

Cell lines and cell culture
Breast cancer and HEK293 cells were grown as previously described (8, 12, 23). Cells were exposed to 3% oxygen for 16 hours or for indicated time intervals in a hypoxia incubator (Thermo Electron Corporation). Antibodies used in this study are listed in the Supplementary Materials and Methods section. MCF10DCIS cells were obtained from Dr. Fred Miller (Wayne State University, Detroit, MI) in 2008 and were tested and authenticated by genotyping with microsatellite markers in October 2010.

Plasmid constructs
Human HIF-1α (CEP4/HIF-1α) and dominant-negative form of HIF-1α (pCEP4/dnHIF-1α) plasmids were purchased from American Type Culture Collection. Plasmid encoding HIF-2α was a gift from Dr. Celeste Simon (University of Pennsylvania, School of Medicine, PA). Human HSulf-1 promoter constructs 1 and 2 (−966 to −2,145 and −19 to −989) were cloned to pGL3 basic vector (Promega Corp.), using primers (Supplementary Table S1). Mutation of the putative hypoxia-responsive element (HRE) sequences in the HSulf-1 promoter constructs were generated by site-directed mutagenesis with the indicated primers (Supplementary Table S1). All mutations were confirmed by DNA sequencing.

Quantitative real-time PCR
Quantitative real-time PCR (qRT-PCR) was carried out on the SYBR-Green PCR Master Mix (Applied Biosystems), using specific primers for human HSulf-1, HSulf-2, HIF-1α, β-actin, and ribosomal 18S subunit (Applied Biosystems) in a Light Cycler kit (BioRad Chromo 4). Normalization across samples was carried out using the average of the constitutive human gene 18S and/or β-actin primers and calculated by the method (24). Binding efficiencies of primer sets for both target and reference genes were similar.

Chromatin immunoprecipitation and luciferase reporter assays
Chromatin immunoprecipitation (ChIP) assays were carried out as previously described (25), using rabbit anti-HIF-1α antibody or rabbit immunoglobulin (Ig) G as a negative control. PCR was carried out using gene-specific primers (Supplementary Table S1). Individual ChIP assays were repeated 3 times to confirm reproducibility. Luciferase activity was measured 24 hours posttransfection with Promega’s Dual-Luciferase Reporter as described earlier (25).

Transwell migration assays
Transwell migration and invasion assays were carried out as previously described (9). bFGF2 was added only to bottom chamber containing serum-free medium for both migration and invasion assays. For hypoxia-mediated cell migration and invasion assays, growth medium was used in bottom chambers. All samples were analyzed in triplicate wells. Results were analyzed using Student’s t test.

siRNA transfections and shRNAs
A total of 100 nmol/L of siRNA of each oligo was transfected using Oligofectamine (Invitrogen). Cells were analyzed 48 hours after transfection or stated otherwise. siRNA oligos for HIF-1α (26) are shown in Supplementary Table S1. Short-hairpin RNAs (shRNA) cloned into the lentivirus vector pLKO.1-puro were chosen from the human library (MISSION TRC-Hs 1.0) and purchased as glycerol stock from Sigma. Detailed protocol is provided in Supplementary Materials.

Cell surface biotinylation
Cells were grown in 10-cm culture dishes. After treatment (either hypoxia or bFGF2), cells were washed in ice-cold PBS and incubated with 0.5 mg/mL sulfo-NHS-LC-biotin in PBS for 30 minutes at 4°C as described (27). Further details are provided in Supplementary Materials. Western immunoblot analysis was carried out using equal amounts of protein with indicated antibodies as previously described (28). Patient characteristics used for immunohistochemical and RNA in situ analysis are provided in the Supplemental section (Supplementary Tables S2 and S6).

Statistical analyses
Pearson’s correlation test was used to assess relationship between clinical parameters and immunohistochemical data. The values of P ≤ 0.05 were regarded as statistically significant in χ² tests at α = 0.05. SPSS software (Version 13.0; SPSS) and JMP software (Version 6.0; SAS Institute, Inc.) were used for statistical analyses.

Results

Hypoxic conditions diminish HSulf-1 levels in an HIF-1α-dependent manner in breast cancer cells
Immunoblot analysis of a panel of breast cancer cell lines showed low or near absent levels of HSulf-1 expression in highly invasive MDA231 and MDA468 cell lines and expressed at variable levels in other cell lines, such as HMEC, MCF10DCIS.com (referred to from now on as MCF10DCIS cells), MCF7, and Hs578T, showing higher levels of expression (Fig. 1A) and that HSulf-1 expression in MCF10DCIS cells was downregulated on exposure to hypoxia with concomitant accumulation of HIF-1α (Fig. 1B). Consistent with this observation, qRT-PCR analysis of MCF10DCIS cells subjected to hypoxic conditions (3% oxygen) for 16 hours revealed that HSulf-1 mRNA levels were downregulated (Fig. 1C). Collectively, these data indicate that low-oxygen conditions lead to downregulation of HSulf-1 expression.

Treatment of MCF10DCIS cells with 100 μmol/L DFO, an agent that stabilizes HIF-1α (29), resulted in reduced HSulf-1 protein levels with increasing accumulation of HIF-1α (Fig. 1D), implicating hypoxia in HSulf-1 regulation. Similarly, hypoxic exposure of MCF-7, SKBR3, and MDA157 cells
resulted in downregulation of HSulf-1 protein expression and mRNA (Supplementary Fig. S1A and B). Collectively, these results indicate that HIF-1α may act as a regulator of HSulf-1 in breast cancer cells.

To test the specific involvement of HIF-1α in hypoxia-mediated HSulf-1 downregulation, we knockdown HIF-1α in MCF10DCIS cells with siRNA before exposing the cells to 3% hypoxia for 16 hours. Scrambled RNA served as controls. HIF-1α knockdown in MCF10DCIS cells significantly relieved hypoxia-mediated HSulf-1 suppression (Fig. 1E, top). To determine whether HIF-1α also regulated the expression of HSulf-2, a close member of HSulf-1, we determined the levels of both HSulf-1 and HSulf-2 by RT-PCR in MCF7 cells that express both HSulf-1 and HSulf-2. HIF-1α levels were efficiently downregulated by siRNA to HIF-1α as determined by quantitative PCR (Supplementary Fig. S2). HSulf-1 mRNA was downregulated in MCF7 cells on exposure to hypoxia as early as 2 hours and was sustained for 4 hours (Supplementary Fig. S2B). In contrast, there was slight upregulation HSulf-2 levels at 2 hours but no change at 4 hours (Supplementary Fig. S2C). HIF-1α knockdown restored HSulf-1 mRNA under hypoxia but not HSulf-2 (Supplementary Fig. S2). Collectively, these results show that HIF-1α is essential for hypoxia-mediated specific suppression of HSulf-1 RNA in MCF7 cells. Similarly, transient overexpression of HIF-1α under normoxic conditions in MCF10DCIS cells resulted in decreased HSulf-1 protein expression (Fig. 1F) and a dose-dependent accumulation of HIF-1α (Fig. 1F, middle).

HIF-2α is closely related to HIF-1α and stabilized under hypoxia (30). We next determined the effect of HIF-2α in regulating HSulf-1 expression. Similar to HIF-1α,
overexpression of HIF-2α alone also resulted in a similar degree of HSulf-1 downregulation in MCF10DCIS cells (Fig. 2A). Furthermore, we utilized 786-O renal cell carcinoma cell line that preferentially expresses HIF-2α but not HIF-1α (20) and lacks expression of VHL, a component of E3 ligase complex responsible for degradation of HIF-α proteins. HSulf-1 expression was analyzed by RT-PCR in 786-O cells with and without stable VHL expression. There was minimal expression of HSulf-1 in 786-O cells. However, VHL-expressing 786-O cells showed increased levels of HSulf-1 mRNA (Fig. 2B). Exposure to hypoxia or hypoxia mimetic (DFO) did not alter the low levels of HSulf-1 in 786-O cells, whereas it caused significant downregulation of HSulf-1 levels in 786-O cells supplemented with VHL (Fig. 2B). Despite our best efforts, we could not detect HSulf-1 protein in 786-O VHL cells.

To directly assess the role of HIF-2α–mediated HSulf-1 regulation, we stably knocked down HIF-2α in 786-O cells by using lentivirus-mediated shRNA against HIF-2α. Immunoblot analysis in these clones showed substantial downregulation of HIF-2α level in 786-O clones HB17 and HB19 compared with 786-O nontargeted control (NTC) clones (Fig. 2C). RT-PCR analysis showed that HIF-2α knockdown resulted in enhanced HSulf-1 mRNA levels in HB17 and HB19 clones (Fig. 2D). These data confirm that the expression of

**Figure 3.** Recruitment of HIF-1α to functional HREs in HSulf-1 promoter. Immunoblot analysis following transient transfection of control and dnHIF-1α in MCF10DCIS cells (A) and/or (B) cells were exposed to hypoxia for 16 hours and were subjected to qRT-PCR analysis using HSulf-1 and 18S primers. *, P ≤ 0.05. C, schematic representation of potential HIF-1α binding sites in HSulf-1 promoter (A, inset). Firefly/luciferase activity of 293T cells either left untreated or treated with DFO (100 μmol/L) for 16 hours by dual luciferase reporter assay following cotransfection with either HSulf-1 wild-type luciferase reporter constructs 1 and 2, respectively, with Renilla luciferase vector and (O) with plasmids containing mutations in the putative HIF-1α binding sites. Results are shown as mean ± SEM of triplicate samples. *, P ≤ 0.002. E, HIF-1α was subjected to ChIP analysis using anti-HIF-1α antibody or rabbit IgG in MCF10DCIS cells untreated or treated with 100 μmol/L DFO for 16 hours. ChIP assays using P1, P2, and P3, P4 primers, encompassing the HSulf-1 HRE1 and HRE2 regions, respectively, was carried out to show in vivo recruitment of HIF-1α to HSulf-1 promoter. F, 786-O and 786-O VHL cells were harvested and HIF-2α was subjected to ChIP analysis by using anti-HIF-2α antibody or rabbit IgG. Input control DNA was diluted 5-fold prior to PCR amplification by using P1 and P2 primers, encompassing the HSulf-1 HRE1 region, to show in vivo recruitment of HIF-2α to HSulf-1 promoter.
HSulf-1 was significantly modulated by HIF-α proteins even under normoxic conditions.

We next tested whether dnHIF-1α (lacking the basic and the carboxy terminus of HIF-1α ref. 31) can rescue hypoxia-mediated HSulf-1 depletion. Immunoblot analysis showed that hypoxia-mediated HSulf-1 downregulation was recovered to some extent in MCF10DCIS cells transfected with dnHIF-1α (Fig. 3A). Consistent with the protein data, RT-PCR analysis showed increasing amounts of dnHIF-1α rescued HSulf-1 mRNA levels under hypoxia (Fig. 3B).

**Presence of functional HREs in HSulf-1 promoter**

To further understand the underlying mechanisms involved in HIF-1α-mediated HSulf-1 repression, we examined HSulf-1 promoter and found 3 putative HREs (Fig. 3C, inset). HSulf-1 promoter sequences upstream of the transcriptional start (TS) site containing putative HRE sites were cloned into 2 separate luciferase reporter plasmids (Fig. 3D). Construct 1 contained 2 HREs from −996 to −2,148 bases relative to the TS site (HRE1) and construct 2 contained 1 HRE element between bases −19 and −989 relative to the TS site (HRE2). 293T cells transfected with HSulf-1 luciferase reporter constructs were treated with 100 μmol/L DFO for 16 hours, and luciferase activities were determined using dual luciferase assay. DFO treatment resulted in decreased luciferase activities in construct harboring 2 HRE sites (HRE1) whereas luciferase activities in HRE2 with 1 HRE (Fig. 3C) were not altered. Therefore, it is likely that HRE1 with 2 HREs is functional, whereas HRE2 with 1 HRE is not (Fig. 3C). To determine which of the 2 HREs within HRE1 construct is required for HSulf-1 suppression, the 2 HRE elements within HRE1 were individually mutated using site-directed mutagenesis. Luciferase activities were determined before and after exposure to hypoxia and hypoxia mimic, DFO, as indicated (Fig. 3D). DFO-mediated decrease in HSulf-1 luciferase reporter activities was rescued individually by each of the mutated HRE constructs, implicating that both HRE sites within the HRE1 construct are essential for HIF-1α-mediated suppression of HSulf-1 expression.

**Figure 4.** Downregulation of HSulf-1 leads to increased cell migration and invasion. A, immunoblot analysis of NTC shRNA or HSulf-1 shRNA–targeted stable batch clones HL-55 and HL-58 were exposed to normoxia and/or hypoxia treatment for 16 hours with indicated antibodies. Values below top panel indicate densitometric analysis of the blot. Middle, stabilization of HIF-1α under hypoxia. B, NTC or HSulf-1 shRNA–targeted stable batch clones, HL-55 and HL-58, were subjected to Transwell cell migration assay (B) or Transwell invasion assay (C) under both normoxic and hypoxic conditions for 24 hours. *, P < 0.03 (compare NTC with HL-55 and HL-58, normoxia); **, P < 0.02 (compare NTC with HL-55 and HL-58, hypoxia). D, HIF-1α expression was downregulated in MCF10DCIS cells with shRNA (HL-718) or NTC shRNA (batch clones), followed by hypoxia treatment. Immunoblot analysis with anti-HSulf-1 antibodies shows that downregulation of HIF-1α rescues HSulf-1 expression under hypoxia (compare lanes 2 and 4, top). E and F, Transwell migration assay of HL-718 and NTC MCF10DCIS cells against HIF-1α. *, P < 0.04 (D) or Transwell invasion assay; **, P < 0.06 (E) under both normoxic and hypoxic conditions. The cells in 8 different fields were counted.
HIF-1α is recruited to HSulf-1 promoters

To determine whether HIF-1α was specifically recruited to these elements within HSulf-1 promoter, we next carried out ChIP in DFO-treated MCF10DCIS cells with anti-HIF-1α antibody. PCR amplification using primers flanking these HRE sites specifically amplified DNA sequences with 2 HRE elements (corresponding to HRE1 construct) but not in HRE2 region with 1 HRE element (Fig. 3E). These data are consistent with our reporter analysis (Fig. 3C) and also show that HIF-1α is recruited to the HREs within HRE1 of the HSulf-1 promoter. ChIP assay using anti-HIF-2α antibody revealed that HIF-2α was also recruited to HRE1 of the HSulf-1 promoters (Fig. 3F) but not in 786-O VHL cells. These data suggest that both HIF-1α and HIF-2α can function as repressors of their common target, HSulf-1.

HSulf-1 depletion resulted in increased cell migration and invasion

It is well established that hypoxic microenvironment promotes invasiveness of various breast cancer cells in vitro and in vivo (32–34). Our finding that HSulf-1 is regulated by HIF-1α prompted us to determine the role of HSulf-1 in hypoxia-induced cell migration and invasion. We generated down-regulated HSulf-1 stable batch clones in MCF10DCIS cells, using 2 different shRNAs (HL-55, HL-58). Batch clones of NTC shRNAs served as controls (Fig. 4A, top). More important, HSulf-1 knockdown resulted in enhanced cell migration even under normoxic conditions, which was further increased under hypoxic conditions (Fig. 4B). Our invasion assays using Matrigel-coated Boyden chamber also showed that all 3 clonal lines (NTC, HL-55, and HL-58 transduced cells) exposed to...
hypoxia showed significant invasion compared with normoxic conditions (Fig. 4C). However, hypoxia-exposed HSulf-1–depleted cells exhibited higher degree (>1.6-fold) of invasion over HSulf-1–proficient cells. Collectively, these results suggest that hypoxia-mediated HSulf-1 depletion might contribute to increased cell migration and invasion. To determine the contribution of HIF-1α in hypoxia-induced cell migration and invasion, we downregulated HIF-1α expression by using lentiviral shRNA, HL-718, and generated batch clones with NTC batch clones serving as controls. Downregulation of HIF-1α under hypoxic conditions (Fig. 4D, lane 4, panel 2) restored HSulf-1 levels as observed in normoxic conditions (Fig. 4D, lane 4, panel 1). Although hypoxia increased cell migration and invasion, HIF-1α knockdown significantly attenuated hypoxia-mediated cell migration (Fig. 4E) and invasion (Fig. 4F), respectively, compared with NTC shRNA control. No significant change in cell proliferation was observed with either hypoxia or bFGF2 treatment or knockdown of HSulf-1, FGFR2 (fibroblast growth factor receptor 2), and HIF-1α in MCF10DCIS cells (Supplementary Fig. S3).

To determine whether overexpression of exogenous HSulf-1, expressed under hypoxic conditions, leads to change in hypoxia-mediated cell migration and invasion, MCF10DCIS cells were transiently transfected with HSulf-1–expressing plasmid. Although hypoxia enhanced cell migration and invasion, expression of HSulf-1 resulted in diminished cell migration and invasion (Supplementary Fig. S4B). Similar data were obtained when cells were subjected to cell migration and invasion in response to bFGF2 (Supplementary Fig. S4C and D). Collectively, these data suggest that overexpression of HSulf-1 attenuated hypoxia- and bFGF2-mediated cell migration and invasion.

**HSulf-1 depletion leads to increased FGF2 signaling under both normoxic and hypoxic conditions**

We next examined the effect of hypoxic conditions on FGF2 signaling in MCF10DCIS cells with stable downregulation of HSulf-1. HSulf-1 depletion (HL-55 and -58) resulted in sustained activation of extracellular signal regulated kinase (ERK) up to 60 minutes on stimulation with bFGF2 (Fig. 5A). In contrast, HSulf-1–proficient cells displayed diminished phosphorylation of ERK at 30 and 60 minutes under normoxic conditions (Fig. 5A; compare lanes 1–4 with lanes 9–12). Consistently, bFGF2 treatment in cells preexposed to hypoxia resulted in a sustained activation of ERK (Fig. 5A, compare lanes 1–4 with lanes 5–8). However, in cells depleted of HSulf-1 and preexposed to hypoxia, bFGF2 treatment resulted in robust sustained activation of ERK-1/2 phosphorylation (Fig. 5A, compare lanes 9–12 with lanes 13–16). Collectively, these suggest that downregulation of HSulf-1 expression leads to increased bFGF2 signaling under hypoxic conditions. To determine whether exogenously expressed HSulf-1 under hypoxic conditions altered FGFR2 signaling, we transfected HSulf-1 in MCF10DCIS cells and subjected them to hypoxia and bFGF2 treatment, followed by cell surface biotinylation of proteins. Biotinylated proteins were precipitated with FGFR2 antibody, and proteins were subjected to immunoblot analysis with indicated antibodies (Fig. 6A, inset) and to cell invasion assay (Fig. 6A) in the presence of bFGF2 (10 ng/mL); *, P < 0.05 (compare NTC to sh1 and sh2, control); **, P < 0.05 (compare NTC to sh1 and sh2, bFGF2) or under hypoxic conditions for 24 hours [B; *, P ≤ 0.05 (compare NTC to sh1 and sh2, normoxia), **, P < 0.05 (compare NTC to sh1 and sh2, hypoxia)]. NTC or HSulf-1 shRNA–targeted stable batch clones, HL-55, were transduced with FGFR2 shRNA1 and subjected to (C) immunoblot analysis with anti-FGFR2 and anti-α-tubulin antibodies and (D) cell invasion assay. *, P < 0.05 (compare NTC to NTC-FGFR2 shRNA1, normoxia); **, P < 0.05 (compare NTC to NTC-FGFR2 shRNA1, hypoxia); #, P < 0.05 (compare HL-55 to HL-55–FGFR2 shRNA1, normoxia); ##, P < 0.02 (compare HL-55 to HL-55–FGFR2 shRNA1, hypoxia).
streptavidin beads as described in the Materials and Methods section. Hypoxia exposure resulted in increased degree of FGFR2 phosphorylation (Fig. 5B, lane 2), which was significantly downregulated by overexpression of HSulf-1 (Fig. 5B, lane 4). Similarly, bFGF2 treatment for 30 minutes activated FGFR2 phosphorylation (Fig. 5C, lane 2), which was also decreased by overexpression of HSulf-1 (Fig. 5C, lane 4). Similar levels of biotinylated proteins were detected in all the samples. These data show that overexpression of HSulf-1 inhibited FGFR2 phosphorylation under hypoxia and growth factor–stimulated conditions.

Because bFGF2 signaling was enhanced in HSulf-1–depleted cells, we generated FGFR2-downregulated batch clones in MCF10DCIS cells by using shRNA against FGFR2 to test whether bFGF2–FGFR2 cascade is implicated in cell invasion. Efficient FGFR2 knockdown with 2 different shRNAs, shRNA1 and shRNA2 (Fig. 6A, inset), in MCF10DCIS clones with shRNA1 and shRNA2 resulted in reduced bFGF2 and hypoxia-mediated cell invasion (Fig. 6A and B). We next determined whether higher degree of migration and invasion due to HSulf-1 silencing result from upregulation of FGFR2 signaling. FGFR2 expression was transiently downregulated using shRNA against FGFR2 in HSulf-1–depleted clone HL-55. Immunoblot analysis showed significant FGFR2 knockdown (Fig. 6C). NTC and HL-55 clones lacking FGFR2 were subjected to migration and invasion assays in the absence or presence of hypoxia. Although HSulf-1–downregulated HL-55 clone showed enhanced invasion, FGFR2-depleted HL-55 showed considerable reduced cell migration and invasion (data not shown), suggesting that FGFR2 plays a critical role in hypoxia-mediated cell invasion (Fig. 6D).

**Inverse correlation of HSulf-1 and CAIX expression in metastatic lesions and its corresponding autologous primary ductal carcinomas**

To determine whether downregulation of HSulf-1 in primary ductal carcinomas is associated with increased CAIX (a surrogate marker of HIF-1α) expression and metastatic phenotype, we determined HSulf-1 and CAIX expression by immunohistochemistry in 53 breast ductal carcinomas and their matched nodal metastatic lesions from the same patients. Patient characteristics are described in Supplementary Table S2. There was an inverse correlation in the expression of CAIX and HSulf-1 in both the primary tumors (P = 0.0198) and their associated metastatic lesions (P = 0.0067; Supplementary Table S3). There was no correlation with the grade of the tumor either in the primary tumors or in the metastatic lesions. Given that follow-up information was available only for a subset of samples (<10%), the numbers were insufficient to determine whether loss of HSulf-1 correlated with poor prognosis. Figure 7A shows different levels of HSulf-1 expression in the primary tumors. Figure 7B and C shows representative sections with different levels of HSulf-1 and CAIX expression in the same primary tumors and metastatic lesions. Taken together, these data suggest that increased HIF-α levels are associated with downregulation of HSulf-1 both in the primary lesions and their associated metastatic lesions.

We further evaluated correlation between hypoxia and HSulf-1 by using breast cancer microarray data with defined hypoxia gene signatures. Using the hypoxia gene signature for breast cancer data from Chi and colleagues (35), we classified tumor samples as hypoxia-positive and hypoxia-negative cohorts in the publicly available microarray data (36) by using K-means cluster analysis. The χ² analysis of HSulf-1 and hypoxic gene cluster clearly showed that low HSulf-1–expressing tumors exhibited higher percentage of hypoxia gene signature (61%; Supplementary Fig. S5). Conversely, high HSulf-1–expressing tumors were associated with lower percentage of tumors with hypoxia signature (21%). This inverse association between HSulf-1 and hypoxic signature was statistically significant (P < 0.0001; χ² test; Supplementary Fig. S5).
In addition, we also determined the prognostic significance of HSulf-1 loss in breast cancer, using breast tumors with known clinical outcome by RNA in situ on tissue microarrays as described previously (11). Kaplan–Meier survival analysis showed that tumors with high HSulf-1 expression had longer disease-free survival (in months) and overall survival (in years) than in patients whose tumors had low levels of HSulf-1 expression (Supplementary Fig. S6). This association was statistically significant (P = 0.0351 and 0.0001, respectively), using the log-rank test (Supplementary Fig. S6).

Discussion

This is the first report on the negative regulation of the putative tumor suppressor HSulf-1 by HIF-1α in breast cancer cells. Hypoxia-mediated HSulf-1 downregulation enhanced cell migration, invasion, and bFGF2 signaling. Precedent for negative regulation of target genes as a result of HIF-1α binding to their promoters suggesting a direct repressor effect has been reported for α-fetoprotein (37), carbamoyl-phosphate synthetase 2-aspartate transcarbamylase-dihydroorotase (38), CEB/P (39), and hepcidin (25). Alternative mechanism of HIF-1α downregulation of specific gene targets has been explained by displacement of an activator such as c-Myc from gene promoters by HIF-1α as seen for MSH2, MSH6, and NBS1 (40, 41). HIF-1α counteraction of transcriptional activity of c-Myc for regulating the cycle protein CDKN1A (41) was found to be independent of HIF-1α DNA binding ability but required its N-terminal PAS-B domain. Thus, these studies show various mechanisms by which HIF-1α can suppress target genes and lend support to our notion that HIF-1α is required for HSulf-1 repression.

Our study implicates HSulf-1 in negative regulation of cell migration in response to both growth factor and hypoxia. Increased cell migration was evident in HSulf-1–depleted clones under both growth factor–stimulated and hypoxic environment, indicating that HSulf-1 expression modulates hypoxia-mediated cell migration. This is conceivable as in vivo environment within the tumors harbors both growth factors and hypoxic conditions. Therefore, it is expected that HSulf-1 presence will result in loss of sulfation of HSPGs, thereby limiting heparin binding growth factor signaling. Conversely, hypoxia-mediated HSulf-1 downregulation should decrease the levels of desulfation of HSPGs and promote growth factor signaling. Our results with hypoxia- and growth factor–mediated activation of FGFR2 signaling are in agreement with increased migration of HSulf-1–depleted clones, suggesting that HSulf-1–FGFR2 pathway could be a critical determinant of cell migration and invasion. In summary, Supplementary Figure S7 shows the model of hypoxia/HIF-1α–mediated regulation of HSulf-1 expression, which alters sulfation states of HSPGs leading to enhanced FGFR2 signaling and cell invasion.

More important, using patient-derived tumor samples, we have determined that there is an inverse correlation in the expression of HSulf-1 with CAIX, in both the primary and metastatic lesions. In addition, we found statistically significant inverse correlation between hypoxia gene signature and HSulf-1 levels in the publicly available breast cancer microarray data (35, 36). Also of significance is the determination that patient tumors with high HSulf-1 mRNA had better prognosis in terms of disease-free survival and overall survival. These findings are consistent with our previous finding in ovarian cancer in which serous tumors with moderate to high levels of HSulf-1 showed a trend toward improved survival (42). Collectively, this study has identified HIF-1α as a novel negative regulator of HSulf-1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

The authors thank Dr. Mukhopadhyay (Mayo Clinic) for providing 786-O cells with and without VIII.

Grant Support

This work was supported by grants from the National Cancer Institute, NIH (CA106954-04) and the Mayo Clinic College of Medicine (to V. Shridhar) and by grants from Second University of Naples and Futura-onlus (to A. Baldi). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 20, 2010; revised November 5, 2010; accepted December 3, 2010; published OnlineFirst January 25, 2011.

References


HSulf-1 Modulates FGF2- and Hypoxia-Mediated Migration and Invasion of Breast Cancer Cells

Ashwani Khurana, Peng Liu, Pasquale Mellone, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-3059

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/01/25/0008-5472.CAN-10-3059.DC1
http://cancerres.aacrjournals.org/content/suppl/2016/08/11/0008-5472.CAN-10-3059.DC2

Cited articles
This article cites 42 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/6/2152.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/71/6/2152.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link:
http://cancerres.aacrjournals.org/content/71/6/2152.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.