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HSulf-1 Modulates FGF-2 and Hypoxia Mediated Migration and Invasion of Breast Cancer Cells

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Abstract

HSulf-1 modulates the sulfation states of heparan sulfate proteoglycans critical for heparin binding growth factor signaling. In the present study, we demonstrate that HSulf-1 is transcriptionally deregulated under hypoxia in breast cancer cell lines. Knockdown of HIF-1 α rescued HSulf-1 downregulation imposed by hypoxia, both at the RNA and protein levels. Chromatin immunoprecipitation with HIF-1 α and HIF-2 α 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 signaling, promoted cell migration and invasion under hypoxic conditions. Additionally, FGFR2 depletion in HSulf-1 silenced breast cancer cells attenuated hypoxia mediated cell invasion. Immunohistochemical analysis of 53 invasive ductal carcinomas and its autologous metastatic lesions revealed an inverse correlation of expression of HSulf-1 to CAIX in both the primary tumors ($p=0.0198$) and in metastatic lesions ($p=0.0067$) respectively, by χ^2 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.

Introduction

Heparan sulfate proteoglycans (HSPGs) are integral components of the extracellular matrix that surrounds 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 heparan sulfate (HS)-binding proteins, including growth factors and chemokines (2), they act as co-receptors of heparin binding growth factors and thus can modulate signaling (3). Recently endosulfatases 1 and 2 (HSulf-1 and -2) were identified which

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function to remove sulfate moieties at 6-O positions of glucosamine (4). Recent report suggests that HSulf-1 and -2 knock out could lead to upregulation of HS biosynthetic enzymes culminating in differences in 2-O and N-O sulfation in MEFs derived from knock out 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, VEGF₁₆₅, Wnts, HGF, Amphiregulin, GDNF and SDF-1 (6-13). Gene knock out studies clearly indicate that loss of both Sulfs 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), SHH regulated oligodendroglial specification (16), esophageal and skeletal defects (6,17).

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

Transcription factor, hypoxia inducible factor-1 alpha (HIF-1 α), is a well established regulator of tumor angiogenesis (19). Inhibition of proline hydroxylation in the oxygen dependent degradation domain of HIF-1 α by prolyl hydroxylases stabilizes HIF-1 α , under hypoxia (20). Under normoxic conditions, HIF-1 α is actively degraded by E3 ligase VHL in a proteasome dependent manner (21). High levels of HIF-1 α have been correlated with poor prognosis and increased degree of metastasis in several cancer types including breast cancer (22).

In the present study, we demonstrate for the first time that hypoxia regulates HSulf-1 expression in HIF-1 α 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.

Plasmids Constructs

Human HIF-1 α (CEP4/ HIF-1 α) and dominant negative form of HIF-1 α (pCEP4/ HIF-1 α DN) plasmids were purchased from ATCC. Plasmid encoding HIF-2 α was a gift from Dr. Celeste Simon (University of Pennsylvania, School of Medicine, PA). Human HSulf-1 promoter constructs 1 and 2 respectively (-996 to -2145 and -19 to -989) were cloned to pGL3 basic vector (Promega Corp, Madison, WI) using primers (Table S1). Mutation of the putative HRE sequences in the HSulf-1 promoter constructs were generated by site directed mutagenesis with the indicated primers (Table S1). All mutations were confirmed by DNA sequencing.

Quantitative Real Time PCR (QRT-PCR)

QRT-PCR was performed using SYBR-Green PCR Master Mix (Applied Biosystems) using specific primers for human HSulf-1, HSulf-2, HIF-1 α , β -actin and ribosomal 18S subunit (Applied Biosystems) in a Light Cycler (BioRad Chromo 4). Normalization across samples

was performed using the average of the constitutive human gene 18S and/or β -actin primers and calculated by $2^{-\Delta\Delta C_t}$ method (24). Binding efficiencies of primer sets for both target and reference genes were similar.

Chromatin Immunoprecipitation and Luciferase reporter assays

ChIP assays were performed as previously described (25) using rabbit anti-HIF-1 α antibody or rabbit immunoglobulin (Ig) G as a negative control. PCR was performed using gene-specific primers (Table S1). Individual ChIP assays were repeated three times to confirm reproducibility. Luciferase activity was measured 24 hr post-transfection with Promega's Dual-Luciferase Reporter (DLR) as described earlier (25).

Trans-well migration assays

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

Small interfering RNA transfections and shRNAs

100nmol/L of small interfering RNA (siRNA) of each of the oligos were transfected using oligofectamine (Invitrogen, CA). Cells were analyzed 48 hours after transfection or stated otherwise. siRNA oligos for HIF-1 α (26) is shown in Table S1. Short-hairpin RNAs (shRNAs) 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 10cm culture dishes. After treatment (either hypoxia or bFGF2) cells were washed in ice-cold PBS and incubated with 0.5mg/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 performed 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 supplementary section (Table S2 and S6).

Statistical analyses

Pearson's correlation test was used to assess relationship between clinical parameters and immunohistochemical data. P values ≤ 0.05 were regarded as statistically significant in Chi square tests at $\alpha=0.05$. SPSS software (Version 13.0, SPSS, Chicago, IL) JMP software (Version 6.0, SAS Institute, Inc., Cary, NC) were used for statistical analyses.

Results

Hypoxic conditions diminish HSulf-1 levels in a HIF-1 α 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 Hs587T 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-1 α (Fig. 1B). Consistent with this

observation, real-time Q-PCR analysis of MCF10DCIS cells subjected to hypoxic conditions (3% oxygen) for 16h revealed that HSulf-1 mRNA levels were downregulated (Fig. 1C). Collectively, these data indicate that low oxygen conditions lead to down regulation of HSulf-1 expression.

Treatment of MCF10DCIS cells with 100 μ M DFO, an agent that stabilizes HIF-1 α (29) resulted in reduced HSulf-1 protein levels with increasing accumulation of HIF-1 α (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 as well as mRNA (Figs. S1A and B). Collectively, these results indicate that, HIF-1 α may act as a regulator of HSulf-1 in breast cancer cells.

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

HIF-2 α is closely related to HIF-1 α and stabilized under hypoxia (30). We next determined the effect of HIF-2 α in regulating HSulf-1 expression. Similar to HIF-1 α , overexpression of HIF-2 α alone also resulted in a similar degree of HSulf-1 downregulation in MCF10DCIS cells (Fig. 2A). Further, we utilized 786-O renal cell carcinoma cell line that preferentially express HIF-2 α but not HIF-1 α (20) and lack expression of VHL, a component of E3 ligase complex responsible for degradation of HIF- α proteins. HSulf-1 expression was analyzed by real time 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-2 α mediated HSulf-1 regulation, we stably knocked down HIF-2 α in 786-O cells using lentiviral-mediated shRNA against HIF-2 α . Immunoblot analysis in these clones showed substantial downregulation of HIF-2 α level in 786-O clones HB17 and HB19 compared to 786-O NTC clones (Fig. 2C). Real-time PCR analysis showed that HIF-2 α knockdown resulted in enhanced HSulf-1 mRNA levels in HB17 and HB19 clones (Fig. 2D). These data confirm that expression of HSulf-1 was significantly modulated by HIF α proteins even under normoxic conditions.

We next tested whether dominant negative HIF-1 α [lacking the basic and the carboxy terminus of HIF-1 α] (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 dominant negative HIF-1 α (Fig. 3A). Consistent with the protein data, real time PCR analysis showed increasing amounts of dnHIF-1 α rescued HSulf-1 mRNA levels under hypoxia (Fig. 3B).

Presence of functional hypoxia responsive elements (HRE) in HSulf-1 promoter

To further understand the underlying mechanisms involved in HIF-1 α mediated HSulf-1 repression, we examined HSulf-1 promoter and found three putative hypoxia responsive elements (Fig. 3C, inset). HSulf-1 promoter sequences upstream of the transcriptional start site containing putative HRE sites were cloned into two separate luciferase reporter plasmids (Fig. 3D). Construct #1 contained two hypoxia responsive elements (HRE) from -996 to -2148 bases relative to the transcriptional start (TS) site (HRE 1) and construct #2 contained one HRE element between bases -19 to -989 relative to the TS site (HRE 2). 293T cells transfected with HSulf-1 luciferase reporter constructs were treated with 100 μ M DFO for 16 hours and luciferase activities were determined using dual luciferase assay. DFO treatment resulted in decreased luciferase activities in construct harboring two HRE sites (HRE 1) whereas luciferase activities in HRE 2 with one HRE (Fig. 3C) were not altered. Therefore, it is likely that HRE 1 with two HRE's is functional, whereas HRE 2 with one HRE is not (Fig. 3C). To determine which of the two HREs within HRE 1 construct is required for HSulf-1 suppression, the two HRE elements within HRE 1 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 were rescued individually by each of the mutated HRE constructs implicating both HRE sites within the HRE 1 construct are essential for HIF-1 α mediated suppression of HSulf-1 expression.

HIF-1 α is recruited to HSulf-1 promoters

In order to determine if HIF-1 α was specifically recruited to these elements within HSulf-1 promoter, we next performed chromatin immuno-precipitation (ChIP) in MCF10DCIS cells with anti HIF-1 α antibody, treated with DFO. PCR amplification using primers flanking these HRE sites specifically amplified DNA sequences with two HRE elements (corresponding to HRE 1 construct), but not in HRE 2 region with one HRE element (Fig. 3E). These data are consistent with our reporter analysis (Fig. 3C), and also shows that HIF-1 α is recruited to the HRE elements within HRE 1 of the HSulf-1 promoter. ChIP assay using anti-HIF-2 α antibody. HIF-2 α was also recruited to HRE 1 of the HSulf-1 promoters (Fig. 3F) but not in 786-O VHL cells. These data suggest that both HIF-1 α and HIF-2 α is able to 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-1 α prompted us to determine the role of HSulf-1 in hypoxia induced cell migration and invasion. We generated downregulated HSulf-1 stable batch clones in MCF10DCIS cells using two different shRNAs (HL-55, HL-58). Batch clones of nontargeted control (NTC) shRNA served as control (Fig. 4A, top panel). More importantly, 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 all three clonal lines (NTC, HL55 and HL58 transduced cells) exposed to hypoxia showed significant invasion compared to normoxic conditions (Fig. 4C). However, hypoxia exposed HSulf-1 depleted cells exhibited higher degree (over 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-1 α in hypoxia induced cell migration and invasion, we

downregulated HIF-1 α expression using lentiviral shRNA, HL718 and generated batch clones with NTC batch clones serving as controls. Downregulation of HIF-1 α under hypoxic conditions (Fig. 4D, lane 4, panel 2) restored HSulf-1 levels as observed in normoxic conditions (Fig. 4D, lane 4, panel 1). While hypoxia increased cell migration and invasion, HIF-1 α knockdown significantly attenuated hypoxia mediated cell migration (Fig. 4E) and invasion (Fig. 4F) respectively compared to nontargeted control shRNA control (NTC). No significant change in cell proliferation was observed with either hypoxia or bFGF2 treatment or knockdown of HSulf-1, FGFR2 and HIF-1 α in MCF10DCIS cells (Fig S3).

To determine whether over-expression 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. While, hypoxia enhanced cell migration and invasion, expression of HSulf-1 resulted in diminished cell migration and invasion (Fig. S4, B). Similar data was obtained when cells were subjected to cell migration and invasion in response to bFGF2 (Fig S4C, D). Collectively, these data suggest that over-expression 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 ERK upto 60 mins upon stimulation with bFGF2 (Fig. 5A). In contrast, HSulf-1 proficient cells displayed diminished phosphorylation of ERK at 30 and 60 mins under normoxic conditions (Fig. 5A Compare lanes 1-4 with lanes 9-12). Consistently, bFGF2 treatment in cells pre-exposed 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 pre-exposed 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 bFGF treatment, followed by cell surface biotinylation of proteins. Biotinylated proteins were precipitated with streptavidin beads as described in materials and methods. 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) that 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 demonstrate that overexpression of HSulf-1 inhibited FGFR2 phosphorylation under hypoxia as well as growth factor stimulated conditions.

Since bFGF2 signaling was enhanced in HSulf-1 depleted cells, we generated FGFR2 downregulated batch clones in MCF10DCIS cells using shRNA against FGFR2 to test whether bFGF2-FGFR2 cascade is implicated in cell invasion. Efficient FGFR2 knockdown with two different shRNAs -1 and -2 (Inset, Fig.6A) in MCF10DCIS clones with shRNA1 and shRNA2 resulted in reduced bFGF2 and hypoxia mediated cell invasion (Fig. 6A, B). We next determined whether higher degree of migration and invasion due to HSulf-1 silencing is due to upregulation of FGFR2 signaling. FGFR2 expression was transiently downregulated using shRNA against FGFR2 in HSulf-1 depleted clone HL55. Immunoblot analysis showed significant FGFR2 knockdown (Fig. 6C). NTC and HL55 clones lacking FGFR2 were subjected to migration and invasion assay in the absence or presence of hypoxia. While HSulf-1 downregulated HL55 clone showed enhanced invasion, FGFR2

depleted HL55 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 down-regulation of HSulf-1 in primary ductal carcinomas is associated with increased CAIX (a surrogate marker of HIF-1 α) 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 Table S2. There was an inverse correlation in the expression of CAIX and HSulf-1 in both the primary tumors ($p=0.0198$) and its associated metastatic lesions ($p=0.0067$) (Table S3). There was no correlation to 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 if loss of HSulf-1 correlated with poor prognosis. Figure 7A shows different levels of HSulf-1 expression in the primary tumors. Figs. 7B and C show representative sections with different levels of HSulf-1 and CAIX expression in same the primary tumors and metastatic lesions respectively. Taken together, these data suggest that increased HIF- α levels are associated with down-regulation of HSulf-1 both in the primary and its 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 J-T et al (35), we classified tumor samples as hypoxia-positive and hypoxia-negative cohorts in the publically available microarray data (36)- using K means cluster analysis. χ^2 analysis of HSulf-1 and Hypoxic gene cluster clearly showed that low HSulf-1 expressing tumors exhibited higher percentage of hypoxia gene signature (61%)(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 ($p<0.0001$, χ^2) (Fig S5).

Additionally, 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) compared to patients whose tumors had low levels of HSulf-1 expression (Fig S6). This association was statistically significant ($p=.0351$ and 0.0001 respectively) using Log-Rank test (Fig S6).

Discussion

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

various mechanisms by which HIF-1 α can suppress target genes and lend support to our notion that HIF-1 α 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 with 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, figure S7 shows the model of hypoxia/HIF-1 α mediated regulation of HSulf-1 expression, which alters sulfation states of HSPGs leading to enhanced FGFR2 signaling and cell invasion.

More importantly, using patient-derived tumor samples, we have determined that there is an inverse correlation in the expression of HSulf-1 to 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 publically 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 where serous tumors with moderate to high levels of HSulf-1 showed a trend towards improved survival (42). Collectively, this study has identified a HIF-1 α as a novel negative regulator of HSulf-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HSulf-1	Heparan Sulfatase 1
HIF-1α	Hypoxia Inducible factor 1 α
HRE	Hypoxia Response Element
HSPGs	Heparan Sulfate Proteoglycan

Figure 1. Hypoxic conditions, HIF-1 α stabilizing agents diminish HSulf-1 levels in breast cancer cells

(A) Top Panel, Immunoblot analysis of HSulf-1 expression in breast cancer cell lines with β -actin as a loading control (bottom Panel). (B) Immunoblot analysis with indicated antibodies and (C) Quantitative Real-time PCR expression of HSulf-1 in MCF10DCIS cells unexposed or exposed to hypoxia (3% oxygen) for 16 hours. *p value ≤ 0.03 . Immunoblot analysis of MCF10DCIS cells treated with DFO with indicated antibodies (D) and (E) of MCF10DCIS cells exposed to hypoxia (3%) following transient transfection of HIF-1 α siRNA and control scrambled siRNA oligos with indicated antibodies. HSulf-1 levels as determined by densitometry are indicated below. (F) Immunoblot of MCF10DCIS cells following transient transfection of full length HIF-1 α with indicated antibodies.

Figure 2. Regulation of HSulf-1 by HIF-2 α in 786-O cells

(A) Immunoblot analysis of MCF10DCIS cells transfected with increasing amounts of full length myc-tag HIF-2 α plasmid with indicated antibodies. (B) Real time PCR analysis of 786-O RCC cells and 786-O cells stably expressing VHL were either left untreated or exposed to hypoxia for 16 hours or treated with DFO (100 μ M) for 16 hours. (C) Immunoblot analysis of Non-targeted control shRNA (NTC) or HIF-2 α shRNA targeted stable clones HB17 and HB19 in 786-O cells with indicated antibodies and (D) Real time PCR analysis using HSulf-1 and 18S specific primers. *P value <0.05 **p value <0.04.

Figure 3. Recruitment of HIF-1 α to functional hypoxia responsive elements (HRE) in HSulf-1 promoter

Immunoblot analysis following transient transfection of control and/ dominant negative HIF-1 α . 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 value = <0.05. (C) Schematic representation of potential HIF-1 α binding sites in HSulf-1 promoter (A, inset). Firefly/luciferase activity of 293T cells either left untreated or treated with DFO (100 μ M) for 16 hours using dual luciferase reporter assay following co-transfection with either HSulf-1 wild type luciferase reporter constructs 1 and 2 respectively with Renilla luciferase vector and (D) with plasmids containing mutations in the putative HIF-1 α binding sites. Results are shown as mean $\pm$ SEM of triplicate samples. *p values= <0.002. (E) HIF-1 α was subjected to chromatin immunoprecipitation (ChIP) analysis using Anti-HIF-1 α antibody or rabbit IgG in MCF10DCIS cells untreated or treated with 100 μ M DFO for 16 hours. ChIP assays using P1, P2 and P3, P4 primers, encompassing the HSulf-1 HRE 1 and HRE 2 regions respectively was performed to demonstrate *in vivo* recruitment of HIF-1 α to HSulf-1 promoter. (F) 786-O and 786-O VHL cells were harvested and HIF-2 α was subjected to ChIP analysis using Anti- HIF-2 α antibody or rabbit IgG. Input control DNA was diluted 5 fold prior to PCR amplification using P1 and P2 primers, encompassing the HSulf-1 HRE 1 region was performed to demonstrate *in vivo* recruitment of HIF-2 α to HSulf-1 promoter.

Figure 4. Downregulation of HSulf-1 leads to increased cell migration and invasion

(A) Immunoblot analysis of Non-targeted control shRNA (NTC) or HSulf-1 shRNA targeted stable batch clones HL-55 and HL-58 were exposed to normoxia and/or hypoxia treatment for 16 hrs with indicated antibodies. Values below top panel indicate densitometry analysis of the blot. Middle panel shows 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 value = <0.03 (compare NTC to HL-55 and HL58, normoxia) **p value = <0.02 (compare NTC to HL-55 and HL58, hypoxia). (D) HIF-1α expression was downregulated in MCF10DCIS cells with shRNA (HL718) or NTC shRNA (batch clones), followed by hypoxia treatment. Immunoblot analysis with anti-HSulf-1 antibodies shows downregulation of HIF-1α rescues HSulf-1 expression under hypoxia (Compare lanes 2 and 4, Top Panel). (E and F) Transwell migration assay of HL-718 and NTC MCF10DCIS cells against HIF-1α. * p value = <0.04 (D) or transwell invasion assay, * p value = <0.06 (E) under both normoxic and hypoxic conditions. The cells in eight different fields were counted.

Figure 5. Increased bFGF2 signaling following HSulf-1 depletion

(A) NTC or HSulf-1 shRNA targeted stable batch clones HL-55 and HL-58 were exposed to normoxia and/or hypoxia treatment for 16 hrs and were immediately exposed to bFGF2 (10ng/ml) for indicated intervals of time. Immunoblot analysis with indicated antibodies shows sustained activation of p-ERK in HSulf-1 depleted clones exposed to hypoxia (compare lanes 3 and 4 to 7 and 8). (B) MCF10DCIS cells were transfected with control vector or pcDNA3.1 HSulf-1-myc/his plasmids. After 36 hours, cells were exposed to hypoxia. Cell surface proteins on MCF10DCIS cells were labeled with membrane impermeable sulfo-NHS-LC-biotin at 4°C after exposure to hypoxia (16h, 3% oxygen) or left untreated and/or, (C) were serum starved for 16 hours and then subjected to bFGF2 (10ng/ml) for 30 minutes. Biotinylated proteins were immunoprecipitated with streptavidin-beads and subjected to immunoblot analysis with anti-phospho FGFR2, FGFR2 and anti-streptavidin-HRP antibodies. Whole cell lysates were probed with anti-myc (9E10) antibody to detect transfected HSulf-1 and anti-α-tubulin antibody as a loading control.

Figure 6. (A) NTC or FGFR2 shRNA targeted batch clones, shRNA1 and shRNA2 were harvested and subjected to immunoblot analysis with indicated antibodies (A, inset) and to cell invasion assay (A) in the presence of bFGF2 (10ng/ml) [* p value = <0.005 (compare NTC to sh1 and sh2, control), ** p value = <0.05 (compare NTC to sh1 and sh2, bFGF2)] or under hypoxic conditions for 24 hrs (B), [* p value = <0.05 (compare NTC to sh1 and sh2, normoxia), ** p value = <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) to cell invasion assay. * p value = <0.02 (compare NTC to NTC-FGFR2 shRNA1, normoxia), ** p value = <0.05 (compare NTC to NTC-FGFR2 shRNA1, hypoxia), # p value = <0.01 (compare HL55 to HL-55-FGFR2 shRNA1, normoxia), ## p value = <0.02 (compare HL55 to HL-55-FGFR2 shRNA1, hypoxia).

Figure 7.

(A), Immunohistochemical analysis showing different levels of H2Sulf-1 in normal breast and primary tumors (0=absent, 1=low, 2=moderate and 3= high). (B and C) Representative photomicrographs (40x) of H2Sulf-1 and CAIX immunostaining in autologous primary tumors and metastatic lesions. A lower level of H2Sulf-1 expression was associated with higher levels of CAIX staining and vice versa observed in matched primary and metastatic breast tumors.