Aspartyl-(asparaginyl) β-hydroxylase, hypoxia-inducible factor-1α and Notch cross-talk in regulating neuronal motility

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Abbreviations: AAH, Aspartyl-(Asparaginyl)-β-Hydroxylase; ALMI, ATP luminescence motility and invasion assay; BCA, bicinchoninic acid; cDNA, complementary DNA to messenger RNA; CNS, central nervous system; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbant assay; FIH, factor inhibiting HIF-1α; GSK-3β, glycogen synthase kinase 3β; HES-1, hairy and enhancer of split 1 transcription factor; HEY-1, hairy/enhancer-of-split related with YRPW motif protein 1; HIF-1α, hypoxia inducible factor-1alpha; HIF-1β, hypoxia inducible factor-1beta; HRE, hypoxia responsive elements; HRP, horse radish peroxidase; IGF-1, 2, insulin-like growth factor, types 1, 2; MAML1, mastermind-like protein 1; pAAH, recombinant plasmid carrying the AAH cDNA; PI3K, phosphoinositol-3-kinase; PNET2, primitive neuroectodermal tumor 2 cells; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; S.E.M., standard error of the mean; si-AAH, si-RNA targeting AAH; siRNA, short interfering RNA duplexes; siScr, scrambled RNA duplexes targeting no specific sequences (negative control)

Aspartyl-(Asparaginyl)-β-Hydroxylase (AAH) promotes cell motility by hydroxylating Notch. Insulin and insulin-like growth factor, type 1 (IGF-I) stimulate AAH through Erk MAPK and phosphoinositol-3-kinase-Akt (PI3K-Akt). However, hypoxia/oxidative stress may also regulate AAH. Hypoxia-inducible factor-1alpha (HIF-1α) regulates cell migration, signals through Notch, and is regulated by hypoxia/oxidative stress, insulin/IGF signaling and factor inhibiting HIF-1α (FIH) hydroxylation. To examine cross-talk between HIF-1α and AAH, we measured AAH, Notch-1, Jagged-1, FIH, HIF-1α, HIF-1β and the hairy and enhancer of split 1 (HES-1) transcription factor expression and directional motility in primitive neuroectodermal tumor 2 (PNET2) human neuronal cells that were exposed to H_2O_2 or transfected with short interfering RNA duplexes (siRNA) targeting AAH, Notch-1 or HIF-1α. We found that: (1) AAH, HIF-1α and neuronal migration were stimulated by H_2O_2; (2) si-HIF-1α reduced AAH expression and cell motility; (3) si-AAH inhibited Notch and cell migration, but not HIF-1α; and (4) si-Notch-1 increased FIH and inhibited HIF-1α. These findings suggest that AAH and HIF-1α cross-talk within a hydroxylation-regulated signaling pathway that may be transiently driven by oxidative stress and chronically regulated by insulin/IGF signaling.

Introduction

Aspartyl-(Asparaginyl)-β-Hydroxylase (AAH) is overexpressed in highly invasive malignant neoplasms,[1,2] including primitive neuroectodermal tumor (PNET) cells of central or peripheral nervous system origin.[3,4] A direct role for AAH in cell motility and invasion is supported by the findings that: (1) overexpression of AAH increases cell motility; (2) inhibition of AAH via gene silencing reduces cell motility; and (3) inhibition of signaling pathways required for AAH expression and function impairs cell motility.[5] AAH catalyzes the hydroxylation of aspartyl and asparaginyl residues in epidermal growth factor (EGF)-like domains[6] of proteins such as Notch and Jagged,[7,8] which have known roles in cell migration.[9,10] Correspondingly, Jagged, the ligand for Notch, is a substrate for AAH hydroxylation,[8] and AAH is capable of physically interacting with both Notch and Jagged.[11] Moreover, overexpression of AAH results in increased nuclear translocation and accumulation of Notch and activation of Notch's downstream target genes, including the transcription factor, hairy and enhancer of split 1 (HES-1).[11]

Hypoxia inducible factor 1 (HIF-1) is an essential mediator of molecular signaling for cellular oxygen sensing and regulates hypoxic responses in nearly all vertebrate cell types by serving as a transcription factor for hypoxia-inducible genes.[12] HIF-1

is composed of two subunits: HIF-1β, which is constitutively expressed, and HIF-1α, which is tightly regulated by oxygen-dependent prolyl hydroxylases.[13] Under normoxic conditions, HIF-1α's transcriptional activity is negatively regulated by the asparaginyl hydroxylase, factor inhibiting HIF (FIH).[14,15] FIH mediates this effect by hydroxylating HIF-1α, thereby enabling HIF-1α's interaction with the von Hippel-Lindau protein complex, resulting in its ubiquitination and degradation via the proteasomal pathway.[16] However, low oxygen tension is rate-limiting for prolyl hydroxylase activity. Consequently, under hypoxic conditions, HIF-1α becomes stabilized due to reduced ubiquitination and proteosomal degradation.[17] In addition, HIF-1α gene expression is stimulated by insulin, IGF-1 and IGF-2.[18] Transcriptionally activated HIF-1α binds to hypoxia-responsive elements (HRE) in the promoter or enhancer regions of hypoxia-inducible genes such as insulin-like growth factor, type 2 (IGF-2), erythropoietin and vascular endothelial growth factor.[13,19]

Previous studies linked activation of HIF-1 signaling to increased cell motility in both malignant neoplastic cells,[20-22] and cellular constituents required for skin wound healing.[23] Moreover, other studies showed that Notch signaling is sensitive to oxygen tension[24] and can be activated by hypoxia.[25-27] Finally, Gustafsson et al.[25] demonstrated that HIF-1α could interact with Notch's intracellular domain and induce Notch-mediated downstream responses. These findings drew our attention because both AAH and HIF-1 belong to hydroxylation signaling networks and mediate their effects through Notch. Moreover, in exploratory studies, we found that AAH expression was stimulated by oxidative stress, and that mild oxidative stress increased cell motility, suggesting potential cross-talk between HIF-1α and AAH signaling pathways. We now characterize the inter-relationships among AAH, HIF-1α, Notch and oxidative stress with respect to motility in human central nervous system (CNS) derived neuronal cells.

Results

Oxidative stress stimulates AAH and HIF-1α protein expression. PNET2 human CNS-derived primitive neuroectodermal tumor 2 (PNET2) neuronal cells were seeded into 96-well plates and treated with 0–45 μM H_2O_2 for 20 h to examine the effects of oxidative stress on AAH, HIF-1α, HIF-1β and FIH expression using a cellular enzyme-linked immunosorbant assay (ELISA). Results were normalized to β-Actin (control) immunoreactivity measured in parallel reactions. All four proteins exhibited H_2O_2 dose-dependent shifts in immunoreactivity, generally at concentrations between 9 and 27 μM (**Figs. 1A–D**). Area under the curve calculations were used to compare the magnitude of H_2O_2-induced increases in protein expression by one-way ANOVA with the post-hoc Tukey test. Those analyses demonstrated significantly greater H_2O_2 stimulated levels of HIF-1α and AAH relative to HIF-1β and FIH immunoreactivity ($p < 0.0001$; **Fig. 1E**).

Oxidative stress promotes cell motility. We next examined the effects of oxidative stress on PNET2 cell directional motility using a more limited range of H_2O_2 treatment, i.e., 13.2 or 22 μM. The 13.2 μM of H_2O_2 dose was below the level associated with increased AAH or HIF-1α expression, whereas 22 μM H_2O_2 was within the optimum concentration range for stimulating these proteins. Using the ATP Luminescence Motility and Invasion (ALMI) assay,[28] we observed that treatment with H_2O_2 significantly altered cell motility (F = 10.9, 3 df, p = 0.004), and that cells treated with 22 μM H_2O_2 had significantly higher mean directional motility indices relative to cells that had been treated with vehicle (p < 0.01) or 13.2 μM (p < 0.05) H_2O_2 (**Fig. 1F**). In addition to total cell motility, the ALMI assay measures the percentages of motile adherent and motile non-adherent cells, and therefore provides information on cell adhesion.[28] The same experiments demonstrated that treatment with 22 μM H_2O_2 mainly increased the percentages of motile-adherent cells (p < 0.01), indicating that the increased motility was not caused by loss of adhesion (**Fig. 1F**).

Inhibition of AAH or HIF-1α expression impairs neuronal motility. PNET2 cells transfected with short interfering RNA duplexes targeting AAH (si-AAH) or HIF-1α (si-HIF-1α) had significantly lower mean total motility indices relative to si-Scr (negative control) transfected cells (**Fig. 2A**). The major inhibitory effects of si-AAH and si-HIF-1α on motility were related to the motile-adherent populations (**Fig. 2B**), whereas the percentages of motile non-adherent cells remained relatively unchanged compared with si-Scr transfected control cells (**Fig. 2C**).

Cross-talk among HIF-1α, AAH and Notch signaling mechanisms. PNET2 cells were transfected with si-AAH, si-HIF-1α, si-Notch-1 or recombinant plasmid expressing AAH cDNA under a CMV promoter. 24–48 hours later, gene expression was measured by qRT-PCR (**Figs. 3 and 4**) and immunoreactivity was measured by ELISA (**Tables 2–5**). Cells transfected with si-HIF-1α had significantly reduced levels of AAH mRNA, whereas si-AAH transfection had no significant effect on HIF-1α mRNA levels (**Figs. 3A and B**). Cells transfected with either si-AAH or si-HIF-1α had significantly reduced levels of Jagged-1 and HES-1 and cells transfected with si-HIF-1α also had reduced levels of FIH mRNA (**Figs. 3C, E and F**). In contrast, Notch-1 mRNA was not inhibited by transfection with si-AAH or si-HIF-1α (**Fig. 3D**). si-RNA silencing of Notch-1 (**Fig. 4A**) significantly reduced the mean mRNA levels of HES-1 (**Fig. 4C**), while increasing expression of HIF-1α (**Fig. 4E**), and not significantly altering expression of Jagged-1, AAH or FIH (**Fig. 4**).

Cellular ELISA studies revealed that si-AAH transfection significantly increased the mean levels of HIF-1α, HIF-1β and Jagged-1 and decreased FIH, β-actin, AAH and Humbug immunoreactivities (**Table 2**). In contrast, overexpression of AAH in cells transfected with recombinant plasmid containing full-length AAH cDNA significantly increased Notch-1, but decreased HIF-1β protein (**Table 3**). Cells transfected with si-Notch had significantly increased levels of HIF-1α and HIF-1β protein expression and reduced levels of Notch-1, Jagged-1, AAH and Humbug (a catalytically inactive homolog of AAH)[7,11,29] immunoreactivity (**Table 4**). Finally, transfection with si-HIF-1α significantly reduced HIF-1α immunoreactivity while significantly increasing HIF-1β, FIH, β-Actin, Notch-1, Jagged-1 and AAH immunoreactivity (**Table 5**). These results are consistent
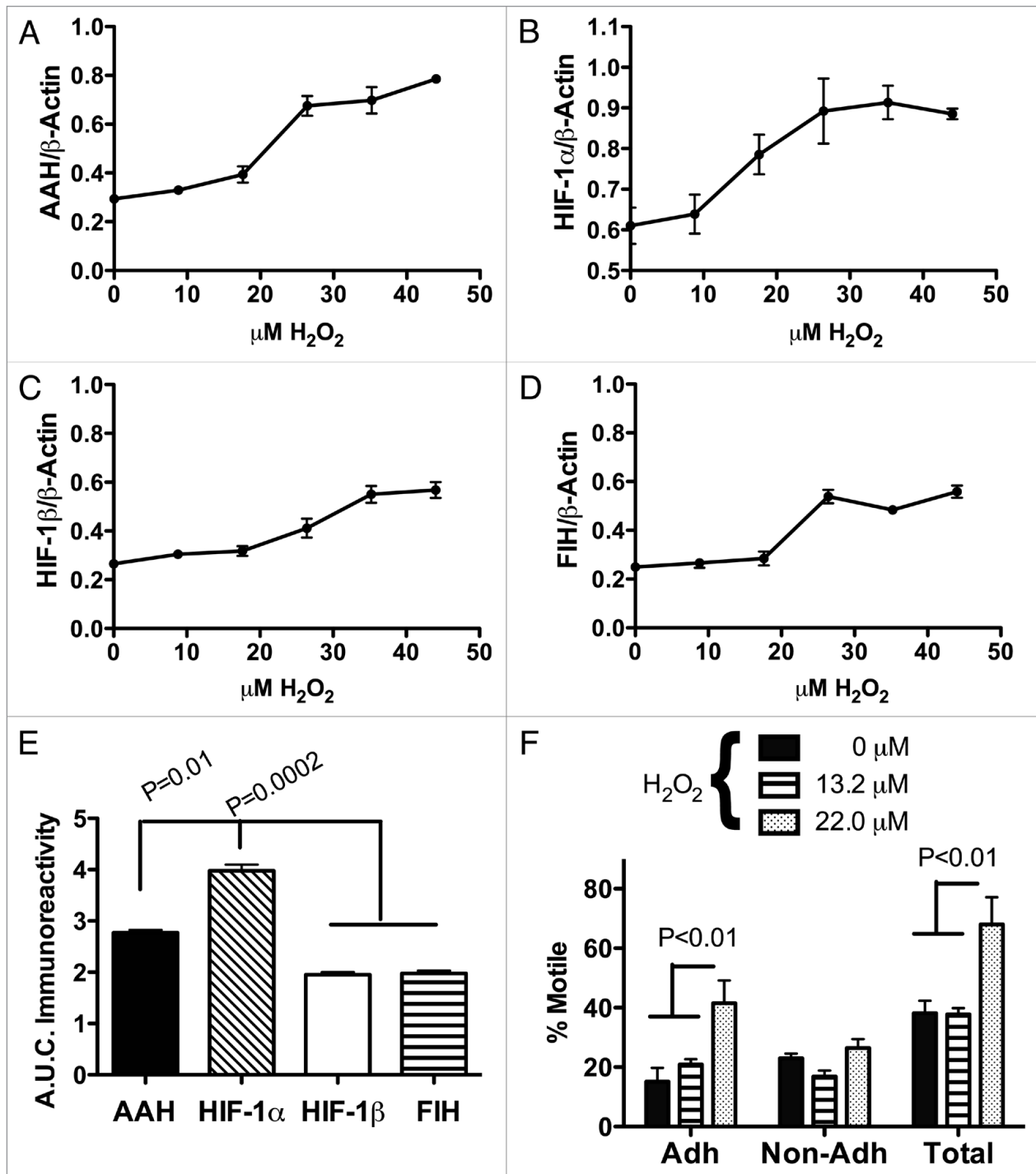
Figure 1. Oxidative stress stimulates AAH and HIF-1α expression and increases directional motility. Human PNET2 neuronal cells were treated with 0 to 44 µM H_2O_2 for 16 h. Cells were harvested to measure immunoreactivity corresponding to (A) AAH, (B) HIF-1α, (C) HIF-1β or (D) FIH by direct binding ELISA. Immunoreactivity was detected with HRP conjugated secondary antibody and Amplex Red fluorophore (Ex 530 nm/Em 590 nm) and quantified in an M = 5 Spectramax microplate reader. Results were normalized to β-actin immunoreactivity measured in replicate assays (see Methods). Results in (A–D) depict changes in mean ± S.E.M. levels of immunoreactivity with increasing H_2O_2 dose in 8 replicate assays. (E) Area under the curve (A.U.C.) was calculated for each dose-response curve and inter-group statistical comparison of the mean A.U.C.s was made using a one-way repeated measures ANOVA with the post-hoc Tukey-Kramer multiple comparisons test for significance. (F) Effect of H_2O_2 treatment (13.2 µM or 22 µM) on directional motility was assessed using the ATP Luminescence-Based Motility and Invasion (ALMI) assay. Graphs depict the mean ± S.E.M. percentages of motile adherent (Adh), motile non-adherent (Non-Adh) and total motile (Total) cells for each H_2O_2 dose. Inter-group comparisons were made using ANOVA and post hoc Tukey Kramer tests. Significant p-values are shown above the bars.
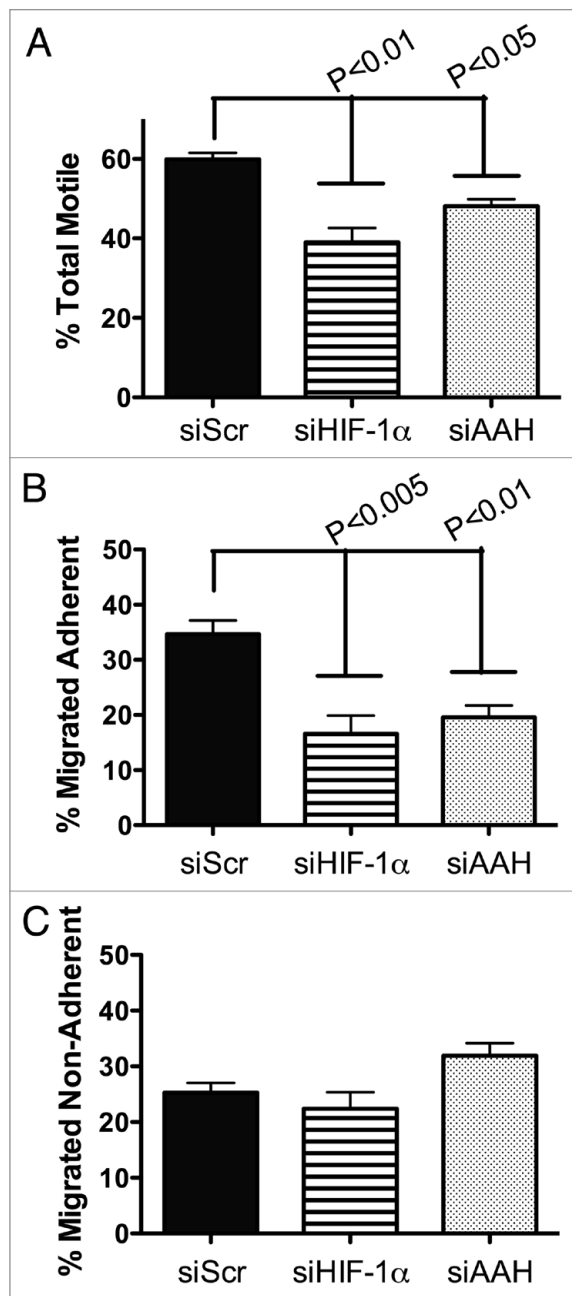
Figure 2. Inhibition of HIF-1α or AAH impairs directional motility. PNET2 cells were transfected with siRNA targeting no specific sequences (siScr), HIF-1α (siHIF-1α) or AAH (siAAH) using the Amaxa electroporation system (see Methods). 24 hours later, directional motility was measured using the ALMI assay, which enables one to quantify the percentages of non-motile, motile-adherent and motile non-adherent cells. The total percentage of motile cells was calculated from the sum percentages of motile adherent plus motile non-adherent cells. The graphs depict the mean ± S.E.M percentages of (A) total motile, (B) motile adherent and (C) motile non-adherent cells after 30 min incubation in blind-well Boyen chambers. 2% fetal bovine serum was supplied in the lower chamber as a trophic factor. Inter-group statistical comparisons were made using one-way ANOVA with the post hoc Tukey-Kramer significance test. Significant p-values are indicated over the bars.

with recent findings demonstrating that hypoxia and HIF-1α can potentiate Notch signaling.[30,31]

Discussion

This study was designed to investigate the role of cross-talk between AAH and HIF-1α as a means of regulating cell motility. First, we demonstrated that AAH and HIF-1α expression and directional motility in PNET2 CNS-derived neuronal cells were stimulated by mild oxidative stress induced by low dose H_2O_2 treatments. Then, we showed that si-RNA inhibition of either AAH or HIF-1α significantly impaired directional motility, particularly with regard to adherent cells. These results indicate that oxidative stress regulates expression of both AAH and HIF-1α, and that both molecules play key roles in regulating cell motility. Since both AAH and HIF-1α are also regulated by insulin/IGF stimulation,[4,18,32] dual signaling pathways and mechanisms modulate cell motility. It would seem that while insulin/IGF regulatory mechanisms are important for effectuating long-term changes in cellular responses, including at the level of transcription,[33] the role of redox regulation and signaling may be to modulate short-term responses to environmental cues such as those produced by acute injury. Whether AAH and/or Notch have protective roles in the context of oxidative stress, as previously demonstrated for DJ-1, which is induced in astrocytes in response to ischemic injury,[34] or H_2O_2-removing enzymes, such as catalase, which is induced in hypercoagulable states that cause ischemic injury,[35] cannot be determined from the data at hand. However, since si-RNA inhibition of AAH and HIF-1α impaired motility and not cell viability, it is unlikely that either molecule mediates anti-stress responses at the low levels of oxidative stress produced in our experiments.

Given the overlapping mechanisms of gene regulation, their roles in cell motility, and the fact that AAH is a hydroxylase enzyme while HIF-1α is regulated by FIH, which is also a hydroxylase enzyme, it was of interest to explore potential functional connections between AAH and HIF-1α. The main approach used was to inhibit gene expression through transient transfection of PNET2 cells with siRNA duplexes and examine the effects on AAH, HIF-1α and related signaling molecules by qRT-PCR analysis and ELISA. The findings that both si-AAH and si-HIF-1α inhibited AAH mRNA, while si-HIF-1α inhibited HIF-1α and si-AAH did not, place HIF-1α upstream of AAH in terms of gene regulation and functionally connect these genes at the level of transcription. At the protein level however, the effects were mixed in that si-RNA suppression of AAH caused parallel shifts in expression of Humbug, a truncated AAH-related protein,[7,8] but had either no effect, or it significantly increased HIF-1α and/or HIF-1β expression. Transfection with si-HIF-1α did not suppress AAH or Humbug protein expression. While the explanation for this discrepancy is not clear, conceivably other interconnecting pathways may permit AAH protein stabilization under normoxic conditions, including trophic factor stimulation (insulin/IGF in medium). In this regard, it is noteworthy that trophic factors stimulate Akt and inhibit glycogen synthase kinase 3β (GSK-3β). Since
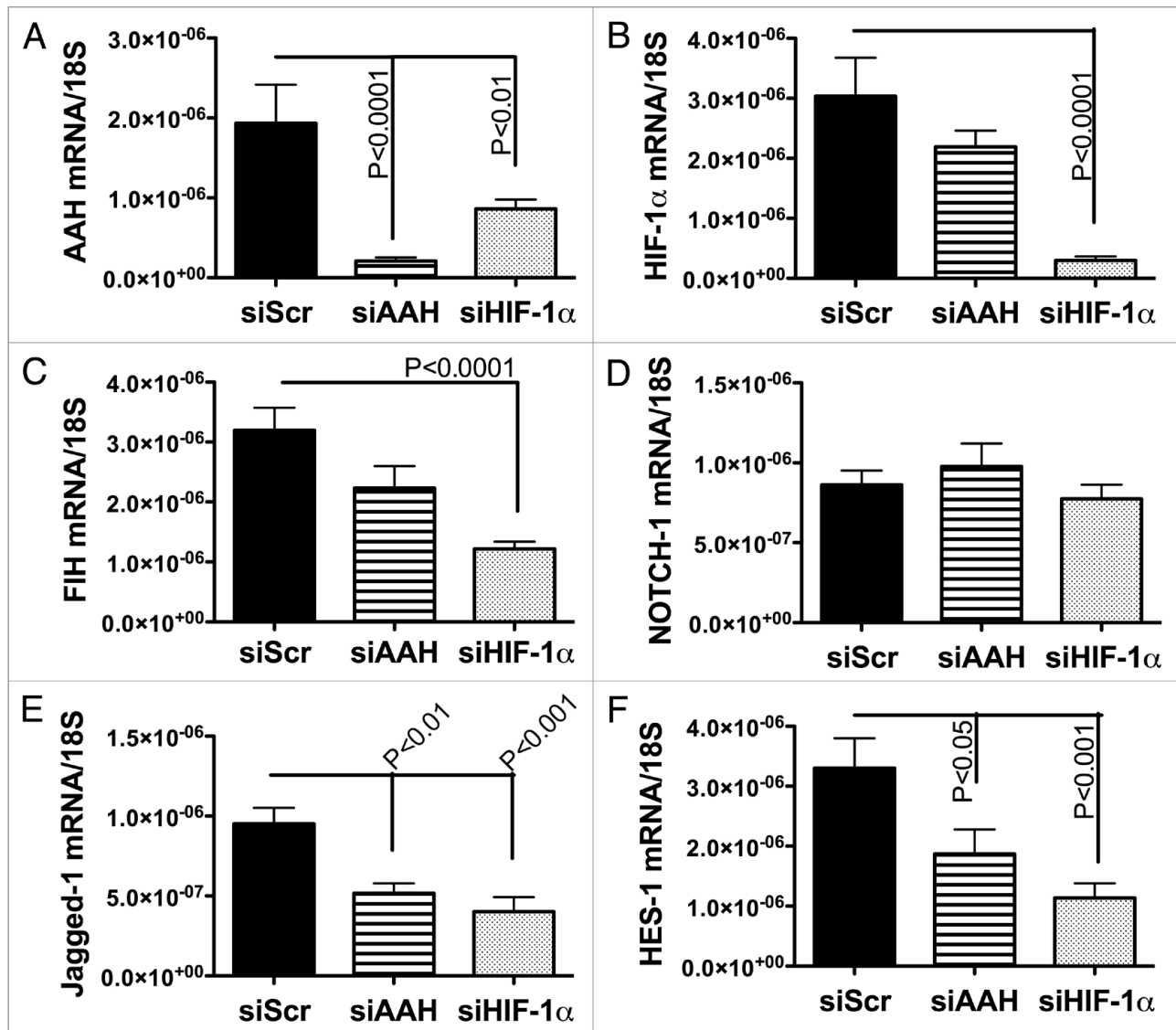
Figure 3. Effects of siAAH and HIF-1α transfection on PNET cell mRNA expression of target genes. PNET cells were transfected with siAAH, siHIF-1α or negative control siSCR. Total RNA was isolated from cells and reverse transcribed. The resulting cDNA templates were used in qPCR amplification assays. The mRNA levels were normalized to 18s rRNA measured in parallel reactions. Graphs depict the mean relative mRNA ± S.E.M levels of (A) AAH, (B) HIF-1α, (C) FIH, (D) Notch-1, (E) Jagged-1 and (F) HES-1. Inter-group comparisons were made using one-way ANOVAs with the post hoc Tukey-Kramer significance test. Significant p-values are indicated over the bars.

GSK-3β phosphorylates and destabilizes both AAH[3,36] and HIF-1α,[37] si-RNA inhibition of HIF-1α may not necessarily inhibit AAH protein in the context of trophic factor inhibition of GSK-3β.

Under normoxic conditions, FIH hydroxylates HIF-1α, signaling it to undergo degradation. Under hypoxic conditions, FIH's hydroxylase is inactivated, permitting HIF-1α to enter the nucleus where it serves as a transcription factor. Our results demonstrate that, in addition to hypoxia, mild oxidative stress (induced with H_2O_2) preserves HIF-1α protein. Thus, we propose that mild oxidative stress stimulates cell motility and regulates AAH protein expression by inhibiting FIH hydroxylation of HIF-1α, allowing HIF-1α to enter the nucleus and serve as a transcription factor for AAH.

Previous studies demonstrated that AAH mediates its effects on cell motility by interacting with and hydroxylating Notch and Jagged,[8] and that a downstream target of Notch signaling is HES-1.[38] Since Notch-1 stimulates HES-1 transcription,[39] the reductions in HES-1 mRNA associated with si-RNA inhibition of AAH and HIF-1α suggest that Notch transcriptional activity is regulated by both AAH and HIF-1α. As demonstrated herein and in previous reports, overexpression of AAH increases Notch-1 protein levels.[11] In addition, AAH overexpression stimulates Notch's translocation to the nucleus where it regulates gene expression.[11] Once in the nucleus, Notch-1 serves as a transcription factor for other genes involved in motility. However, since si-AAH had no significant effect on Notch's mRNA levels, AAH's regulation of Notch is most likely
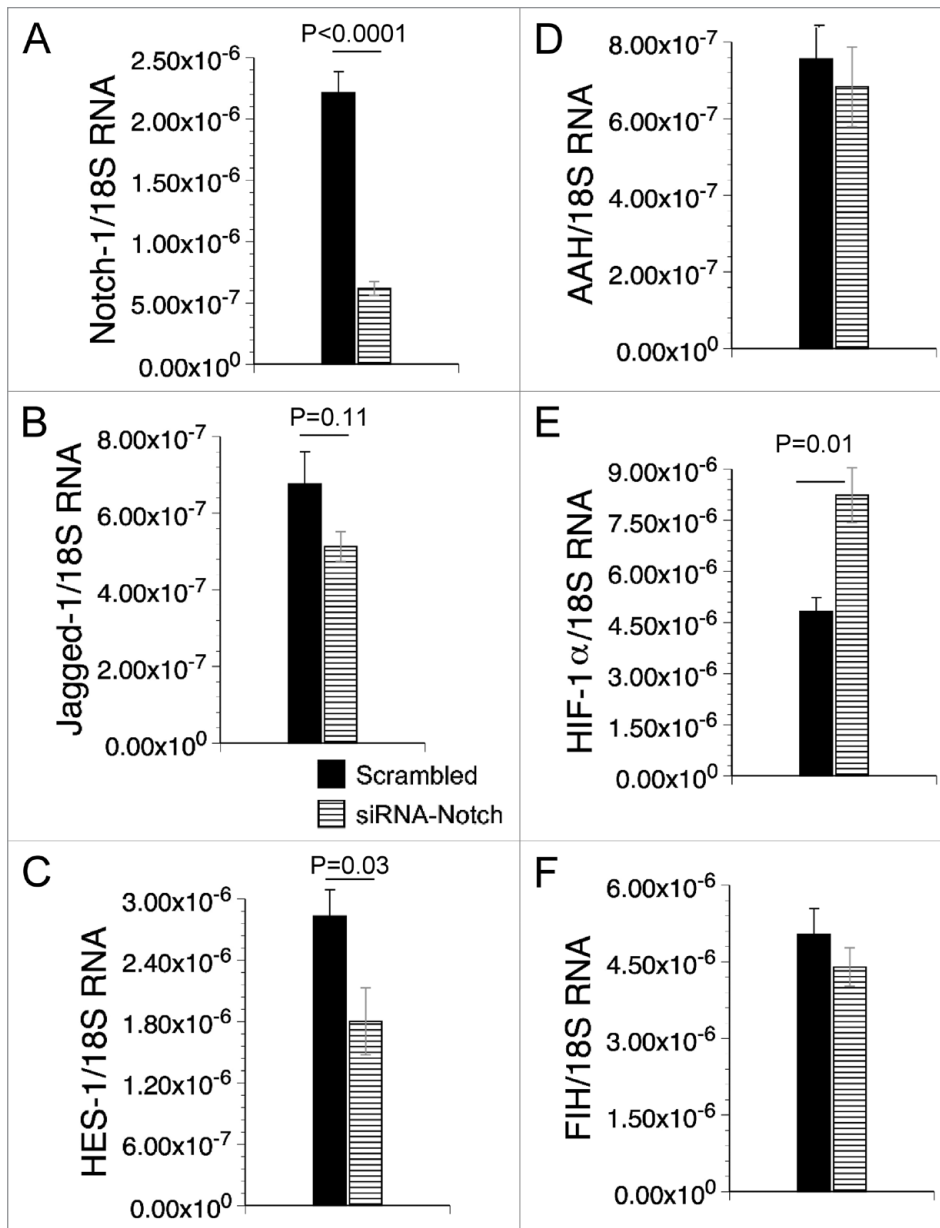
Figure 4. Effects of siNotch-1 transfection on PNET cell mRNA expression of target genes. PNET cells were transfected with either siNotch-1 or negative control, siSCR. Total RNA was isolated from cells and reverse transcribed. The resulting cDNA templates were used in qPCR amplification assays. The mRNA levels were normalized to 18S rRNA measured in parallel reactions. Graphs depict the mean relative mRNA ± S.E.M levels of (A) Notch-1, (B) Jagged-1, (C) HES-1, (D) AAH, (E) HIF-1α and (F) FIH. Inter-group comparisons were made using Student t-tests. Significant p-values are shown over the bars.

to interact with the intracellular domain of Notch-1,[25] or regulate AAH mRNA expression. The reduced expression of FIH mRNA effectuated by si-AAH or si-HIF-1α transfection could represent a feedback mechanism to negatively regulate HIF-1α and eventually AAH expression, thereby halting cell motility.

We summarize our proposed scheme for extrinsic regulation and cross-talk between the AAH-Notch-Jagged-HES and FIH-HIF-1α hydroxylase signaling pathways in **Figure 5**. In brief, insulin and IGF regulate AAH and HIF-1α protein expression and function through post-translational mechanisms including phosphorylation and attendant inhibition of GSK-3β activity. In addition, insulin and IGF stimulate AAH and HIF-1α gene expression, increasing their mRNA levels. Oxidative stress and hypoxia activate HIF-1α signaling by inhibiting FIH. This results in HIF-1α-mediated increases in AAH mRNA. Attendant increases in AAH protein expression lead to increased interactions between AAH and Notch/Jagged. AAH and HIF-1α both increase Notch signaling and cell motility. AAH functions by interacting with and hydroxylating Notch and Jagged. The cleaved N-terminal fragment of Notch translocates to the nucleus where it functions as a transcription factor and regulates target genes such as HES-1. HIF-1α potentiates Notch signaling via the mastermind-like protein 1 (MAML1) co-activator, with attendant stimulation of Notch target genes including HES-1 and HEY-1.[30] Increased Notch signaling through HES enhances expression of hypoxia responsive elements and hypoxia-inducible genes. However, attendant increased expres-

mediated by post-translational mechanisms. Downstream Notch-regulated target genes that mediate cell motility include E-Cadherin,[30] tenascin C,[40] and other genes that regulate cell adhesion.[41]

Jagged is a ligand for Notch, and its binding to Notch is needed for Notch cleavage and its release from the membrane for translocation to the nucleus.[42,43] The finding that both si-AAH and si-HIF decreased Jagged-1 expression suggests an additional mechanism by which AAH and HIF-1α regulate Notch signaling. The si-HIF-1α inhibition of HES-1 mRNA could be explained by HIF-1α's ability

sion of FIH could serve as a negative feed-back mechanism for HIF-1α-AAH-Notch signaling. Notch activated signaling increases cell motility in part by altering expression of cell adhesion molecules.[30,40,41] We hypothesize that AAH and HIF-1α cross-talk within a hydroxylation-regulated signaling pathway that is transiently driven by fluctuations in oxidative stress, while more sustained stimulation of motility is mediated by signaling through insulin/IGF cascades. Therefore, therapeutic measures to prevent or limit invasion and metastatic spread of neuroblastic tumor cells will likely require inhibition of both redox- and

growth factor-mediated mechanisms. Prevention of neuroblastic tumor metastasis will likely require inhibition of growth factor and redox-mediated mechanisms.

Methods

Cell culture. Human CNS-derived Primitive Neuroectodermal Tumor 2 (PNET2) cells[44] were maintained as previously described.[3,32] We examined the effects of oxidative stress on HIF-1α, AAH and FIH expression by treating 96-well microcultures with 0–45 μM H_2O_2 for 20 h and measuring immunoreactivity by a cellular enzyme-linked immunosorbent assay (ELISA).[45] The protocol for the H_2O_2 exposures was based on previous studies demonstrating that CNS neuronal cells exhibit oxidative injury, but remain viable with altered gene expression, 24 to 48 hours after treatment with up to 45 μM H_2O_2.[46,47] Applying a more limited dose range of H_2O_2 (0, 13.2 and 22 μM), we examined the effects of mild oxidative stress on directional motility. We assessed cross-talk among the AAH, FIH, HIF-1α and Notch signaling pathways in cells transfected with commercially prepared (Dharmacon, Inc., Chicago, IL) small interfering RNA duplexes (si-RNA) that targeted the AAH (ASPH NM_004318), HIF-1α (NM_001530, NM_181054) or Notch-1 (NM_0176617) genes. Finally, we examined the consequences of AAH overexpression in cells transfected with recombinant plasmid carrying the full-length AAH cDNA (pAAH) in which gene expression was under the control of a CMV promoter.[1] Control cells were transfected with recombinant plasmid carrying the green fluorescent protein gene (pGFP). Cells were transfected in suspension using the Amaxa "v" nucleofector cell line reagents and the Amaxa nucleofector apparatus (Amaxa, Inc., Gaithersburg, MD) according to the manufacturer's protocol. With this approach, we consistently achieved 75–90% transfection efficiencies as determined by GFP labeling of co-transfected cells. 16–24 hours later, cells were used to examine protein and mRNA expression.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. We used qRT-PCR to measure mRNA expression.[11,48,49] In brief, cells were lysed in Qiazol reagent (Qiagen Inc., Valencia, CA) and total RNA was isolated using the EZ1 RNA universal tissue kit and the BIO Robot EZ1 (Qiagen, Inc.). RNA was reverse transcribed using random oligodeoxynucleotide primers and the AMV First Strand cDNA synthesis kit (Roche Diagnostics Corporation, Indianapolis, IN). The resulting cDNA templates were used in qPCR amplification reactions with gene specific primer pairs (**Table 1**).[48] Primers were designed using MacVector 10 software (MacVector, Inc., Cary, NC) and their target specificity was verified using NCBI-BLAST (Basic Local Alignment Search Tool). The amplified signals from triplicate reactions were detected and analyzed using the Mastercycler ep realplex instrument and software (Eppendorf AG, Hamburg, Germany). Relative mRNA abundance was calculated from the ng ratios of specific mRNA to 18S rRNA measured in the same samples. Inter-group statistical comparisons were made using the calculated mRNA/18S ratios.
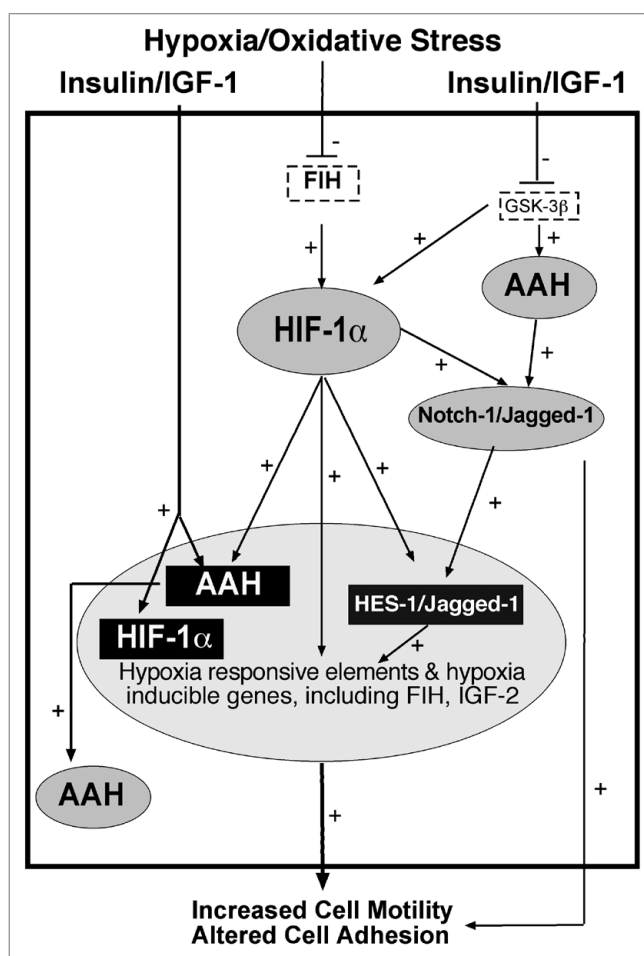

Figure 5. Proposed scheme for extrinsic regulation and cross-talk between the AAH-Notch-Jagged-HES and FIH-HIF-1α hydroxylase signaling pathways. Large shaded oval toward the bottom of the diagram represents the nucleus, large rectangle represents the cytoplasm and outside the rectangle, extrinsic stimuli and cellular responses are indicated. Small shaded ovals represent proteins, black shaded rectangles represent genes and dash-lined rectangles depict post-transcriptional regulators of AAH and HIF-1α signaling. Pluses correspond to positive stimulatory effects and minus signs represent inhibitory effects. Insulin and IGF regulate AAH and HIF-1α through inhibition of GSK-3β and by increasing their mRNA levels. Oxidative stress and hypoxia activate HIF-1α via inhibition of FIH, resulting in increased AAH gene expression. Both AAH and HIF-1α increase Notch signaling. AAH interacts with and hydroxylates Notch and Jagged, resulting in nuclear translocation of Notch and increased expression of Notch-regulated genes, e.g., HES-1. HIF-1α potentiates Notch signaling and stimulation of Notch target genes.[30] Consequences include increased expression of hypoxia responsive elements and hypoxia-inducible genes that promote cell motility and alter cell adhesion.

Enzyme linked immunosorbent assay (ELISA). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors.[49,50] Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). We performed direct binding ELISAs to measure AAH, HIF-1α, HIF-1β, FIH, Notch-1, Jagged-1 and β-Actin immunoreactivity. Samples containing 50 ng protein diluted in Tris-buffered saline, pH

Table 1. Primer pairs for quantitative RT-PCR[*]

Gene-specific primer	Forward/Reverse	Sequence 5'-3'	Position (mRNA)	Amplicon size (bp)
18S rRNA	Forward	GGA CAC GGA CAG GAT TGA GCA	1274	50
18S rRNA	Reverse	ACC CAC GGA ATC GAG AAA GA	1323	
AAH	Forward	GGG AGA TTT TAT TTC CAC CTG GG	1650	257
AAH	Reverse	CCT TTG GCT TTA TCC ATC ACT GC	1906	
HIF-1α	Forward	GCC TTG GAT GGT TTT GTT ATG G	567	385
HIF-1α	Reverse	TCA GCA CCA AGC AGG TCA TAG G	951	
FIH HIF	Forward	CTT ACC TCT AAC CTG CTG CTC ATT G	619	196
FIH HIF	Reverse	GAT TGT CAA AGT CCA CCT GGC T	814	
Notch-1	Forward	AGG ACC TCA TCA ACT CAC ACG C	6035	117
Notch-1	Reverse	CGT TCT TCA GGA GCA CAA CTG C	6151	
Jagged-1	Forward	TGT CTG TCC CAC TGG TTT CTC	1950	142
Jagged-1	Reverse	AGT TCT TGC CCT CAT AGT CCT CG	2091	
HES-1	Forward	CCA AAG ACA GCA TCT GAG CA	318	91
HES-1	Reverse	TCA GCT GGC TCA GAC TTT CA	408	

[*]Nucleic acid sequences of gene specific forward and reverse oligodeoxynucleotide primers used for quantitative RT-PCR analysis. Position refers to 5' binding site on cDNA. Amplicon is the length of the PCR amplified product in base pairs (bp).

Table 2. Effects of siAAH on AAH, NOTCH and HIF-1α signaling molecules

Protein	siScr	siAAH	p-value
HIF-1α	3983.1 ± 164.9	4562.7 ± 135.8	0.01
HIF-1β	2794.2 ± 264.2	4248.9 ± 280.3	0.001
FIH	487.0 ± 76.6	324.8 ± 31.6	0.04
β-Actin	1586.0 ± 42.4	967.5 ± 99.1	<0.0001
Notch-1	140.0 ± 3.1	153.7 ± 20.5	
Jagged-1	141.0 ± 5.0	211.6 ± 27.2	0.01
AAH	722.5 ± 17.9	610.9 ± 4.8	<0.0001
Humbug	682.0 ± 16.8	619.7 ± 5.6	0.0005

Cultured PNET cells were transiently transfected with siAAH or siScr (negative control) RNA duplexes. Immunoreactivity was measured by direct binding ELISA (see Methods; N = 8/group) with HRP-conjugated secondary antibodies and Amplex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a Spectramax 5 (Ex 579 nm/Em 595 nm). Data represent mean ± S.E.M. FLU corrected for protein loading. Between group comparisons were made using Student t-tests. Computer-generated significant p-values are listed in Column 4.

Table 3. Effects of pAAH on NOTCH and HIF-1α signaling molecules

Protein	GFP-cDNA	AAH-cDNA	p-value
HIF-1α	4666.1 ± 154.6	4109.5 ± 281.9	
HIF-1β	3723.4 ± 264.2	3125.7 ± 280.3	0.02
FIH	204.3 ± 23.8	193.6 ± 16.6	
β-Actin	821.1 ± 39.1	784.8 ± 43.3	
Notch-1	166.7 ± 9.9	234.8 ± 9.6	0.0003
Jagged-1	366.2 ± 68.4	385.5 ± 28.1	

Cultured PNET cells were transfected with recombinant plasmid DNA carrying full-length AAH or green fluorescent protein (GFP; negative control) cDNA, and 24 hours later, immunoreactivity was measured by direct binding ELISA (see Methods; N = 8/group) with HRP-conjugated secondary antibodies and Amplex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a Spectramax 5 (Ex 579 nm/Em 595 nm). Data represent mean ± S.E.M. FLU corrected for protein loading. Between group comparisons were made using Student t-tests. Computer-generated significant p-values are listed in Column 4.

7.4 (TBS) were adsorbed to the bottom flat surfaces of 96-well polystyrene plates (Nunc, Rochester, NY) overnight at 4°C.[36] Non-specific binding sites were blocked by a 3-hour room temperature incubation with 300 μl/well of TBS + 0.05% Tween 20 + 3% BSA. Samples were then incubated with 0.1–0.5 μg/ml primary antibody for 1 h at 37°C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody and Amplex Red soluble fluorophore (Molecular Probes, Eugene, OR).[36,50] Fluorescence was measured (Ex 530/Em 590) in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Parallel negative control assays had primary, secondary or both antibodies omitted. Between steps, reactions were rinsed 3 times with TBS + 0.05% Tween 20 using a Nunc ELISA plate washer.

Microtiter immunocytochemical ELISA (MICE) assay. The MICE assay is a cellular ELISA that was used to measure the effects of oxidative stress on AAH, HIF-1α, FIH and β-Actin immunoreactivity directly in fixed cultured cells (96-well plates).[45] The main modification of the original protocol was that immunoreactivity was measured with the Amplex Red fluorophore (Ex 579/Em 595) (Molecular Probes, Eugene, OR) instead of a colorimetric reagent. Cell density was assessed by subsequently staining the cells with Hoechst H33342 (Molecular Probes, Eugene, OR) and measuring fluorescence (Ex360 nm/Em460 nm) in a Spectramax M5 microplate reader (Molecular Dynamics, Inc., Sunnyvale, CA). The calculated ratios of fluorescence immunoreactivity to H33342 were used for inter-group comparisons. At least eight replicate cultures were analyzed in each experiment.

Directional motility assay. Directional motility was measured using the ATP Luminescence-Based Motility-Invasion (ALMI) assay.[28] Briefly, culture medium containing 2% FCS was placed

Table 4. Effects of siHIF-1α on AAH, NOTCH and HIF-1α signaling

Protein	siScr	siHIF-1α	p-value
HIF-1α	6333.3 ± 249.1	4374.2 ± 229.3	<0.0001
HIF-1β	3546.4 ± 286.2	5551.5 ± 507.7	0.001
FIH	4748.8 ± 318.5	9337.0 ± 425.4	<0.0001
β-Actin	16168 ± 1163	21068 ± 1703	0.013
Notch-1	2668.9 ± 241.9	4563.1 ± 481.3	0.0009
Jagged-1	4766.6 ± 740.8	11715 ± 1493	0.0001
AAH	7754.1 ± 844.5	12549 ± 1127	0.001
Humbug	7016.7 ± 1161.9	9505.3 ± 1017.4	

Cultured PNET cells were transfected with siScr (negative control) or siHIF-1α RNA duplexes, and 48 h later, cells were harvested to measure immunoreactivity by direct binding ELISA (see Methods; N = 8/group) using HRP-conjugated secondary antibodies and Amplex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a Spectramax 5 (Ex 579 nm/Em 595 nm). Data represent mean ± S.E.M. FLU corrected for protein loading. Between group comparisons were made using Student t-tests. Computer-generated significant p-values are listed in Column 4.

Table 5. Effects of siNOTCH on AAH, NOTCH and HIF-1α signaling

Protein	siScr	siNotch-1	p-value
HIF-1α	497.3 ± 4.2	529.5 ± 8.7	0.0008
HIF-1β	478.3 ± 4.2	539.3 ± 8.0	<0.0001
FIH	495.8 ± 3.6	485.6 ± 8.5	
β-actin	225.9 ± 27.6	158.8 ± 34.8	
Notch-1	344.1 ± 52.7	108.7 ± 24.9	0.001
Jagged-1	201.7 ± 8.1	163.5 ± 14.9	0.024
AAH	722.5 ± 17.9	610.9 ± 4.8	<0.0001
Humbug	682.0 ± 16.8	619.7 ± 5.6	0.0005

Cultured PNET cells were transfected with siScr (negative control) or siNotch-1 RNA duplexes, and 48 h later, cells were harvested to measure immunoreactivity by direct binding ELISA (see Methods; N = 8/group) using HRP-conjugated secondary antibodies and Amplex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a Spectramax 5 (Ex 579 nm/Em 595 nm). Data represent mean ± S.E.M. FLU corrected for protein loading. Between group comparisons were made using Student t-tests. Computer-generated significant p-values are listed in Column 4.

in the lower chambers (Neuro Probe, Gaithersburg, MD) and 8-micrometer pore diameter polycarbonate filters divided the upper and lower chambers. 100,000 viable (Trypan Blue exclusion) PNET2 cells were seeded into the upper chambers and cell migration was allowed to proceed for 30 minutes at 37°C in a CO_2 incubator. Cells collected from the upper chambers (non-motile), under surfaces of the filters (motile adherent) and bottoms of the wells (motile non-adherent) were quantified using ATPLite reagent (Perkin-Elmer, Waltham, MA) because ATP luminescence is linearly correlated with cell density.[28] Luminescence was measured in a TopCount Machine (Perkin-Elmer). The percentages of non-motile, motile adherent, motile non-adherent cells in 8 replicate assays were calculated and used for statistical analysis. Because this assay separately quantifies motile-adherent and motile-non-adherent sub-populations, it provides information about cell motility and adhesion.

Sources of reagents. QuantiTect SYBR Green PCR Mix was obtained from (Qiagen Inc.). Monoclonal antibodies to HIF-1α, HIF-1β, FIH and Notch-1 and Jagged-1, β-Actin and were purchased from Abcam Inc. (Cambridge, MA), Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or Chemicon International (Tecumsula, CA). The 85G6 and 85E6 AAH and Humbug monoclonal antibodies were generated to human recombinant protein and purified over Protein G columns (Healthcare, Piscataway, NJ).[36]

Statistical analysis. Data depicted in the graphs represent the means ± S.E.M.'s for each group. Inter-group comparisons were made using Student t-tests or analysis of variance (ANOVA) with the Tukey post-hoc test. Statistical analyses were performed using the GraphPad Prism 5 software (San Diego, CA) and significant p-values (<0.05) are indicated over the graphs.

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