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

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Key words: hypoxia-inducible factor, oxidative stress, neuronal migration, metastasis, hydroxylation, signal transduction, factor inhibiting HIF-1α

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)

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 domains6 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

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 H2O2 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 H2O2; (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.
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$_2$O$_2$ treatment, i.e., 13.2 or 22 μM. The 13.2 μM of H$_2$O$_2$ dose was below the level associated with increased AAH or HIF-1α expression, whereas 22 μM H$_2$O$_2$ was within the optimum concentration range for stimulating these proteins. Using the ATP Luminescence Motility and Invasion (ALMI) assay, we observed that treatment with H$_2$O$_2$ significantly altered cell motility (F = 10.9, 3 df, p = 0.004), and that cells treated with 22 μM H$_2$O$_2$ had significantly higher mean directional motility indices relative to cells that had been treated with vehicle (p < 0.01) or 13.2 μM of H$_2$O$_2$ (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. The same experiments demonstrated that treatment with 22 μM H$_2$O$_2$ 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) 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 with increased AAH or HIF-1α expression, whereas 22 μM H$_2$O$_2$ was within the optimum concentration range for stimulating these proteins. Using the ATP Luminescence Motility and Invasion (ALMI) assay, we observed that treatment with H$_2$O$_2$ significantly altered cell motility (F = 10.9, 3 df, p = 0.004), and that cells treated with 22 μM H$_2$O$_2$ had significantly higher mean directional motility indices relative to cells that had been treated with vehicle (p < 0.01) or 13.2 μM of H$_2$O$_2$ (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. The same experiments demonstrated that treatment with 22 μM H$_2$O$_2$ 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).

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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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ dose. Inter-group comparisons were made using ANOVA and post hoc Tukey Kramer tests. Significant p-values are shown above 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 H₂O₂ 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 H₂O₂-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 mRNA 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 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.
Previous studies demonstrated that AAH mediates its effects on cell motility by interacting with and hydroxylating Notch and Jagged, and that a downstream target of Notch signaling is HES-1. Since Notch-1 stimulates HES-1 transcription, 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. In addition, AAH overexpression stimulates Notch’s translocation to the nucleus where it regulates gene expression. 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

GSK-3β phosphorylates and destabilizes both AAH and HIF-1α. 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 H₂O₂) 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, and that a downstream target of Notch signaling is HES-1. Since Notch-1 stimulates HES-1 transcription, 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. In addition, AAH overexpression stimulates Notch’s translocation to the nucleus where it regulates gene expression. 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
mediated by post-translational mechanisms. Downstream Notch-regulated target genes that mediate cell motility include E-Cadherin,\textsuperscript{30} tenascin C,\textsuperscript{40} and other genes that regulate cell adhesion.\textsuperscript{31}

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.\textsuperscript{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 to interact with the intracellular domain of Notch-1,\textsuperscript{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.\textsuperscript{30} Increased Notch signaling through HES enhances expression of hypoxia responsive elements and hypoxia-inducible genes. However, attendant increased expression 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.\textsuperscript{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 were maintained as previously described.\textsuperscript{3,5} We examined the effects of oxidative stress on HIF-1α, AAH and FIH expression by treating 96-well microcultures with 0–45 μM H$_2$O$_2$ for 20 h and measuring immunoreactivity by a cellular enzyme-linked immunosorbent assay (ELISA).\textsuperscript{45} The protocol for the H$_2$O$_2$ 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$_2$O$_2$.\textsuperscript{46,47} Applying a more limited dose range of H$_2$O$_2$ (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_017667) 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.\textsuperscript{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.\textsuperscript{11,15,48} 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).\textsuperscript{49} 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.

Enzyme linked immunosorbent assay (ELISA). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors.\textsuperscript{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
Fluorescence was measured (Ex 530/Em 590) in a SpectraMax M5 microplate reader. Immunoreactivity was measured by indirect binding ELISA (see Methods; N = 8/group) with HRP-conjugated secondary antibodies and AmpliPlex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a SpectraMax S (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 1.** Primer pairs for quantitative RT-PCR<sup>a</sup>

| 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 ACT 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           |                   |
| FII HIF              | Forward        | CTT ACC TCT AAC CGT CTG CTC ATT G | 619           | 196               |
| FII 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 GGG GCA CAA CTG C | 6151          |                   |
| Jagged-1             | Forward        | TGT CTG TCC CAC TGG TTT TCT | 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 TT TCA | 408           |                   |

<sup>a</sup>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         | 4870.0 ± 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 AmpliPlex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a SpectraMax S (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 AmpliPlex Red soluble fluorophor. Fluorescence light units (FLU) were measured in a SpectraMax S (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.

Microtiter immunocytocchemical 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).<sup>45</sup> The main modification of the original protocol was that immunoreactivity was measured with the AmpliPlex Red fluorophore (Ex 579/Em 595 nm) (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. Briefly, culture medium containing 2% FCS was placed...
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 CO2 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 nant 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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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.
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