Cancer Biology and Signal Transduction

Nuclear Translocation of Hand-1 Acts as a Molecular Switch to Regulate Vascular Radiosensitivity in Medulloblastoma Tumors: The Protein uPAR Is a Cytoplasmic Sequestration Factor for Hand-1

Swapna Asuthkar1, Venkateswara Rao Gogineni1, Jasti S. Rao1,2, and Kiran Kumar Velpula1

Abstract

Urokinase-type plasminogen activator receptor (uPAR) is overexpressed in the tumor–stromal invasive microenvironment in many human cancers, including medulloblastoma. The role of uPAR in tumor progression and angiogenesis has been well characterized. Previously, in medulloblastoma cells, we showed that ionizing radiation (IR)–induced uPAR is a potent activator of cancer stem cell (CSC)–like properties and is associated with various transcription factors that are involved during embryonic development and cancer. In the present study, we show that uPAR protein acts as a cytoplasmic sequestration factor for a novel basic helix-loop-helix transcription factor, Hand-1. The Hand-1 protein plays an essential role in the differentiation of trophoblast giant cells and cardiac morphogenesis, and yet its precise cellular function and its contribution to cancer remain mostly unknown. We also observed that the Hand-1 protein is upregulated in uPAR short hairpin RNA–treated medulloblastoma cells and accompanies sustained cell growth and angiogenesis. Furthermore, IR-induced uPAR overexpression negatively regulates Hand-1 activity and results in the stabilization of angiogenesis-promoting molecules, including hypoxia-inducible factor-1α. Finally, uPAR overexpression and its association with Hand-1 after IR treatment indicate that uPAR is capable of regulating Hand-1 and that uPAR has a role in the process of IR-induced tumor angiogenesis. Mol Cancer Ther; 1–14. ©2014 AACR.

Introduction

Medulloblastoma is the most frequent malignant brain tumor in children and adolescents (1). Following surgery and treatment with chemotherapy and radiation, medulloblastomas often recur as a consequence of a rare subpopulation of cells that exhibit stem cell properties such as self-renewal capacity and multilineage differentiation (2). Interestingly, these cancer stem cells (CSC) exist closer to tumor vasculature and interact physically with vascular endothelial cells (3). This highlights the fact that, to target the putative CSC vascular niche, it is first necessary to understand the mechanism by which CSCs are protected by tumor vasculature.

Urokinase-type plasminogen activator receptor (uPAR) is part of a cell surface system that also consists of the serine protease uPA and several specific inhibitors (plasminogen activator inhibitors 1 and 2; refs. 4, 5). This system has classically been thought to drive tumor progression by mediating directed extracellular proteolysis on the surface of invading cancer cells (6). In addition to mediating proteolysis, several mechanistic studies demonstrated that uPAR also had a role in cancer cell survival, invasion, migration, and angiogenesis in vitro (7–12). Recent data provided new insights into the role of uPAR to facilitate epithelial–mesenchymal transition and to induce CSC-like properties in medulloblastoma and breast cancer cells (13, 14). Downregulation of uPAR abolishes Notch 1 and WNT–β-catenin–pertinent gene expression and signaling events in cancer cells (13, 15). Although whether or not nonproteolytic function of uPAR requires uPA–uPAR interaction remains to be answered, it is clear that uPAR can promote tumor progression independent of its proteolytic function (13, 16). The fact that uPAR lacks intracellular domain suggests that its signaling must be mediated through its coreceptors. Indeed, uPAR influences a range of biologic functions due to its ability to engage in multiple protein–protein interactions (13, 17–19), which emphasizes the possibility of other unidentified proteins that may interact with uPAR.

In this study, we showed that uPAR associates with the basic helix-loop-helix (bHLH) transcription factor (TF),
Hand-1. Hand-1 induction in mouse embryonic stem cells promotes cellular differentiation (20), whereas uPAR is not required for mouse embryonic development, growth to adulthood, or reproduction (21), indicating that uPAR may have an acquired role in CSC maintenance. Our data suggest that the antagonistic activities of uPAR and Hand-1 in cancer cells must be closely regulated.

Hand-1 protein can form homo- and heterodimer combinations with multiple bHLH partners (22, 23), which reflects the mechanisms of Hand-1-mediated transcriptional activation in the nucleus. Furthermore, Hand-1 plays a functional role in vasculogenesis and embryonic survival by both transcriptional activation and suppression of thymosin β4 protein, which has been implicated in cell migration and angiogenesis (24). Here, we present evidence for a novel differential regulation of Hand-1 via the proposed interaction with a non-bHLH protein and uPAR, and we determined that aberrant uPAR expression in cancer cells can function as a repressor of Hand-1 transcriptional activity. In colon cancer, the hand-1 gene was shown to be epigenetically silenced and ectopic expression of Hand-1 caused significant repression of cancer cell growth when compared with normal colon cells (25, 26). Although little is known about the Hand-1 protein’s role in cancer, identification of Hand-1 and the functional significance of its interactions are of interest in cancer cells. Our studies explored the functional role of uPAR in the negative regulation of Hand-1 activity in medulloblastoma tumor growth and angiogenesis.

Materials and Methods

Cell lines, transfection, and ionizing radiation

The medulloblastoma cell line D283 Med (HTB-185) was obtained from American Type Culture Collection (ATCC). D425, D458, H2402, and H2405 cells were kindly provided by Dr. Darell D. Bigner (Duke University Medical Center, Durham, NC); UW228 cells were kindly provided by Dr. Ali-Osman (Duke University Medical Center, Durham, NC); D425, D458, H2402, and H2405 cells were kindly provided by Dr. Ali-Osman (Duke University Medical Center, Durham, NC); UW228 cells were kindly provided by Dr. Ali-Osman (Duke University Medical Center, Durham, NC); D425, D458, H2402, and H2405 cells were kindly provided by Dr. Ali-Osman (Duke University Medical Center, Durham, NC); UW228 cells were kindly provided by Dr. Ali-Osman (Duke University Medical Center, Durham, NC). These cells were authenticated on the basis of c-Myc amplification and chromosomal aberrations by the provider (27, 28). These cells were cultured and transfected following standard protocols (29, 30). Human microvascular endothelial cells (HMEC) were purchased from ATCC and cultured according to standard protocols (31). For combination treatments, an ionizing radiation (IR) dose of 6 Gy was given after 48 hours of transfection using the RS 2000 Biological Irradiator X-ray unit (Rad Source Technologies Inc.). Irradiated cells were further incubated for 24 hours.

Plasmids, short hairpin RNA construct, antibodies, inhibitor, and reagents

We used uPAR human cDNA cloned in pCMV6-AC vector (Origene) for full-length uPAR (FL-uPAR). We used Hand-1 human cDNA cloned in pDsRed Express-C1 vector (Clontech) for full-length Hand-1 (FL-Hand-1) overexpression. The cells overexpressing uPAR and Hand-1 in our study will be referred to as uPAROE and Hand-1OE cells, respectively. A monocistronic-pU construct designed to knock down uPAR was generated in our laboratory (32). A pCDNA3-scrambled vector with an imperfect sequence (pSV) was used as a control. An insertion mutation was introduced within the predicted monopartite nuclear localization signal (NLS) fragment of FL-Hand-1 plasmid using a QuikChange XL site-directed mutagenesis kit (Stratagene) and specific primers (Supplementary Table S1) following the manufacturer’s instructions. This mutant plasmid was termed Hand-1 (ΔNLS). Antibodies such as anti-uPAR, Hand-1, DsRed, factor inhibiting HIF-1 (FIH-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin, and lamin B were purchased from Santa Cruz Biotechnology; Myc-tag was purchased from Cell Signaling Technology; and hypoxia-inducible factor-1α (HIF-1α) was purchased from Novus Biologicals. Recombinant human Hand-1 partial recombinant protein was purchased from Novus Biologicals. N-(methoxyoxoacetyl)-glycine methyl ester (DMOG) was purchased from Cayman Chemicals.

Establishment of stable cells overexpressing uPAR and Hand-1 (uPAROE and Hand-1OE)

Cells were transfected at 80% confluence in 6-well culture plates with FL-uPAR (with Myc-tagged or without Myc-tagged) and FL-Hand-1 (DsRed-tagged) constructs. The medium was replaced with normal growth medium and incubated for 48 hours. On day 3, cells were grown in 10 μg/mL neomycin (Cayman Chemicals) containing medium and the clones with stable expression of uPAR and Hand-1 were selected according to previously established protocols (13).

Subcellular protein fractionation, immunoblotting, and immunoprecipitation

Nuclear extracts and Triton X-114–mediated phase partitioning was performed according to standard protocol (33). Immunoprecipitation (IP) assays were carried out by incubating 800 μg of nuclear/cytoplasmic extracts with specific primary antibody (2 μg) overnight at 4°C on an end-to-end rotator following a previously established protocol (33). Immunoprecipitated proteins were immunoblotted using specific primary antibodies.

Ubiquitination assay

For the ubiquitination assay, whole cell lysates (500 μg protein/sample) were incubated with UbiCapture-Q Matrix (VWR International) by gentle agitation at 4°C overnight to pull down all ubiquitinated proteins according to the manufacturer’s instructions. After washing three times, captured proteins were eluted with 2× SDS-PAGE loading buffer and analyzed by Western blotting using anti–HIF-1A antibody.

Immunocytochemistry and immunohistochemistry

For immunocytochemical analysis, treated cells were seeded onto 4-well chamber slides, fixed, permeabilized with ice-cold methanol, and blocked for 1 hour using 3% bovine serum albumin in PBS. Cells were incubated with...
primary antibody and Alexa Fluor–conjugated secondary antibody for 60 minutes at room temperature. Before mounting, the slides were washed with PBS and incubated for 5 minutes with a 1:100 dilution of 4′,6-diamidino-2-phenylindole for nuclear staining and analyzed using confocal microscopy (Olympus BX61 Fluoview) at 40× magnification. For immunohistochemical analysis, tissue sections (3–6 μm thick) were deparaffinized in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was carried out with 10 mmol/L citrate buffer (pH 6) at boiling temperature for 60 minutes and permeabilization was carried out with 10 mmol/L citrate buffer (pH 6) at 37°C. The formation of capillary-like structures was captured. The degree of angiogenesis was quantified by the number of vessel length in a frame by the area of the frame (35).

**In vitro angiogenic assay**

Angiogenic assay was performed as previously described (34) with minor modifications. HMECs (2 × 10^4 cells/well) were grown in the presence of cancer cell culture filtrates in 96-well plates coated with growth factor–reduced Matrigel and incubated for 16 hours at 37°C. The formation of capillary-like structures was captured. The degree of angiogenesis was quantified by the number of branch points per view and cumulative tube length.

**Dorsal skin-fold chamber model**

Athymic nude female mice (6903 F) obtained from Harlan Laboratories were maintained within a specific-pathogen, germ-free environment. The implantation technique of the dorsal skin-fold chamber model has been described previously (34). Briefly, diffusion chambers with control-, Hand-1OE–, or FL-uPAR–transfected stable cells (2 × 10^6) were placed subcutaneously in the skin through the superficial incision made horizontally along the edge of the dorsal air sac. After 10 days, the animals were anesthetized with ketamine/xylazine and sacrificed by intracardial perfusion with saline (10 mL) followed by 10 mL of 10% formalin/0.1 mol/L phosphate solution. The mice were carefully skinned around the implanted chambers, and the skin fold covering the chambers was photographed under a visible light microscope. The vascular length density was calculated by dividing the total vessel length in a frame by the area of the frame (35).

**Surgical orthotopic implantation**

D283 control and Hand-1OE stable cells (1 × 10^6) with or without IR treatment were injected intracerebrally into nude mice following previously established protocols (13). The Institutional Animal Care and Use Committee of the University of Illinois College of Medicine, Peoria, IL, approved all surgical interventions and postoperative animal care (protocol #857, dated May 27, 2009 and renewed on June 30, 2012).

**Chromatin immunoprecipitation, cloning, DNA sequencing, TF-binding site prediction, and TF interaction array**

ChIP assay was performed using the ChIP-IT Express Kit from Active Motif, and cloning, DNA sequencing, TF-binding site prediction analysis were done following previously standardized protocol (13). TF protein interaction array versions I (MA3501) and II (MA3502) from Panomics were used to study protein–protein interactions between human recombinant uPAR (ruPAR; R&D Systems, Inc.) protein (bait) with Hand-1 and Hand-2 TF immobilized on membranes following the established protocol (13).

**Reverse transcription PCR**

Reverse transcription (RT)-PCR reactions were performed according to standard protocol using specific primers listed in Supplementary Table S1.

**Electrophoretic mobility shift assays**

Electrophoretic mobility shift assays (EMSA) was performed using 2 μg of nuclear extract and specific biotinylated probes (Supplementary Table S2) following the manufacturer’s instructions (Panomics).

**Statistical analysis**

All the data presented (immunoblots, RT-PCR, and immunocytochemistry and immunohistochemistry) are representative of three independent experiments. All data are presented as means ± SE of at least three independent experiments. One-way ANOVA combined with a Tukey post hoc test of means was used for multiple comparisons. Statistical differences are presented at P < 0.05, P < 0.01, and P < 0.001.

**Results**

**IR-induced uPAR is inversely associated with Hand-1 protein**

uPAR is well known to establish multiple interactions with various proteins (36). Previously, from the TranSignal TF protein array, we found a number of factors that interacted with uPAR involved in neurogenesis during embryonic development and cancer (13). Here, we revealed one such most significant interacting factor, Hand-1 with recombinant uPAR (ruPAR) protein. However, we observed that ruPAR binds to the Hand-1 but not the Hand-2 protein (Fig. 1A). We then screened six different (UW228, D425, D283, D458, H2402, and H2405) medulloblastoma cell lines and found that while some (UW228, D425, D283, and H2402) expressed higher levels of uPAR and lower levels of Hand-1, whereas H2405 cells expressed higher levels of uPAR and Hand-1 and D458 cells expressed lower levels of uPAR and Hand-1 (Fig. 1B). Most of the medulloblastoma cell lines showed higher uPAR levels when compared with Hand-1, suggesting mutually negative regulation of their expression. Because the IR treatment induces overexpression of uPAR in medulloblastoma cells (13), we next asked whether
Hand-1 expression is affected in IR-treated medulloblastoma cells. Consistent with our earlier reports, uPAR was found to be upregulated by IR treatment in a dose-dependent (up to 8 Gy) manner. Interestingly, we also observed a concurrent IR dose-dependent decrease in Hand-1 expression (Fig. 1C). Our previous studies reported that IR treatment induces the expression of uPAR and other CSC markers, thereby promoting cancer stemness (13). Here, we further observed that the addition of increasing concentrations of human recombinant stem cell factor increased uPAR levels while suppressing Hand-1 expression in UW228 cells (Supplementary Fig. S1A and S1B). These findings are suggestive of the negative regulation of Hand-1 in cancer stemness.

Next, we compared the specific localization of Hand-1 with that of uPAR in control- and IR-treated UW228 and D283 cells. Consistent with previously published data, we found higher levels of Hand-1 in the nucleus, especially in the control cells, which is believed to lead to increased transcriptional activation (37). In contrast with control, we localized Hand-1 to the cytoplasmic fraction in IR-treated cells. In addition, Hand-1 and uPAR proteins were found to colocalize in the cytoplasm but were excluded from the nuclei in the IR-treated cells (Fig. 1D). This colocalization was confirmed by the co-IP experiments. Myc-tagged uPAR or DsRed-tagged Hand-1 was over-expressed (uPAROE/Hand-1OE) in both control- and IR-treated cells. Consistent with immunocytochemistry results, the co-IP of cytoplasmic extract proteins using anti-uPAR antibody and the reverse pull down co-IP using anti-DsRed antibody confirmed the association between uPAR and Hand-1 in both control- and IR-treated cells. These experiments confirmed the physical interaction between uPAR and Hand-1 in the cytoplasmic extract, which was more...
prominent in the IR-treated cells. Unlike in the cytoplasmic extract, we could not detect any physical interaction in the nuclear extracts (data not shown).

Nuclear localization of Hand-1 correlates inversely with uPAR overexpression and IR treatment

Hand-1 is predominantly a nuclear protein and, upon closer inspection, showed a putative bipartite nuclear localization signal (NLS) at the C-terminus, RESKKRR [EL-VE] KRIKGR (Fig. 2A). To test the function of this putative NLS, we generated constructs for the expression of a series of DsRed fusion proteins (Fig. 2B). We transfected UW228 and D283 cells with these constructs and analyzed the subcellular localization of the DsRed fusion proteins by immunoblot (Fig. 2C) and immunocytochemistry (Fig. 2D) analysis. Consistent with the localization of endogenous Hand-1 in medulloblastoma cells, we found that DsRed-Hand-1 localized mainly to the nucleus. However, in IR-treated cells, DsRed-Hand-1 predominantly localized to the cytoplasm, recapitulating the cytoplasmic localization of endogenous Hand-1 observed in IR-treated cells. When we exogenously expressed the DsRed-Hand-1 (ANLS) truncation, we observed increased signal in the cytoplasm of control cells (Non-IR). Indeed, the mutant DsRed-Hand-1 (ANLS) protein showed cellular localization similar to that of IR-treated cells (Fig. 1C), suggesting that the NLS element is important for nuclear and nuclear localization of Hand-1. To further determine whether the above predicted sequence represented functional NLS, we examined the localization of the DsRed-NLS (Fig. 2D). When exogenously expressed in medulloblastoma cells, DsRed-Hand-1 localized nearly entirely throughout the nucleus including the nucleoli. This was in stark contrast to the control DsRed construct that was broadly distributed throughout the cell (Fig. 2D). Together, these results confirmed that Hand-1-NLS amino acid sequence represented a functional NLS.

Moreover, the anti-Hand-1 antibody–precipitated chromatin immunoprecipitation (ChIP) DNA was isolated from UW228 and D283 cells, which were then cloned, sequenced, and analyzed. The ChIP analyses identified a number of short individual sequences that revealed E12/E47/MyoD, GATA-1, and c-Myb TF binding consensus sequences (Fig. 2E). Following this, to examine the role of Hand-1 in the aforementioned TF-mediated transactivation activity, we performed EMSA using double-stranded biotinylated oligos identified by ChIP. We observed that although IR treatment downregulated E12/E47/MyoD and GATA-1 activity, c-Myb activity was increased when compared with control cells. The incubation of nuclear extracts obtained from Hand-1 overexpressing (Hand-OE) cells and Hand-1–treated cells with anti-Hand-1 antibody induced a supershift in E12/E47/MyoD complex migration. These results are indicative of heterodimerization of Hand-1 with transcription factor E12/E47/MyoD, although not all the E12/E47/MyoD was bound to Hand-1. These experiments recapitulated previously published results demonstrating that both Hand-1 and Hand-2 proteins directly compete for dimerization with MyoD/E12 during embryogenesis (38). Indeed, Hand-OE in medulloblastoma cells upregulated all of the TF-mediated transcriptional activity (Fig. 2E).

Hand-1 antagonizes the uPAR-induced angiogenesis and tumor growth

Both Hand-1 and uPAR proteins are implicated in angiogenesis. Hand-1 is required during normal angiogenesis for vascular smooth muscle recruitment to the endothelial tissue in the yolk sac (39). The abundance of uPAR on aggressive tumor cells relative to cells in otherwise normal states (40, 41) is considered to be a key angiogenic mediator contributing to pathologic angiogenesis. Using protein array analysis, we found increased expression of several proangiogenic factors in uPAROE cells relative to Hand-OE cells. Interestingly, we observed a slight upregulation in uPA and FGF-2 levels in Hand-OE cells when compared with uPAROE cells (Fig. 3A), suggesting their role in early embryogenesis and development (42–45). Earlier, we showed that increased expression of uPAR with IR treatment induces the angiogenic ability of medulloblastoma cells (7, 44). In addition, in the present study, we sought to confirm and expand on the findings of our previous work demonstrating similar increases in the angiogenesis network formation with IR treatment and uPAROE. We also observed that both short hairpin RNA (shRNA)-mediated uPAR knockdown (pU) and Hand-OE in UW228 and D283 were associated with marked impairment of tumor angiogenesis (Fig. 3B). Similarly, the orthotopic implantation of D283-Hand-OE cells in nude mice showed regression of tumor angiogenesis and growth as determined by dorsal air sac mouse model (Fig. 3C) and bioluminescence imaging (Fig. 3D). Furthermore, the intravenous delivery of uPAROE plasmid alone and in combination with IR treatment aggravates vascular network formation and also increases tumor growth (Fig. 3C and D). We further show that Hand-OE suppresses IR-induced tumor angiogenesis in both in vitro and in vivo experiments (Fig. 3B and C). Overall, our results indicate the antiangiogenic effect of Hand-OE in medulloblastoma cells.

Nuclear Hand-1 regulates tumor angiogenesis through a FIH-1–dependent mechanism

Next, to address the functional role of Hand-1 in uPAR-mediated angiogenesis, we performed a series of experiments using shRNA specific for uPAR (pU) and Hand-OE plasmids in a panel of four human medulloblastoma cell lines. The pU, Hand-OE, and Hand-OE (ANLS) treatments showed increased Hand-1 mRNA expression by quantitative RT-PCR (qRT-PCR). Interestingly, Hand-1 overexpression, in all treatments, led to a marked decrease in the mRNA levels of Hand-2. Furthermore, both shRNA-mediated depletion of uPAR and overexpression of Hand-1 in medulloblastoma cells led to decreased expression of HIF-1A, whereas levels of FIH-1 and tumor suppressor von Hippel–Lindau (VHL) were elevated.
Figure 2. Hand-1 is differentially localized within the control and IR-treated cells. A, schematic representation of NLS sequence in the human Hand-1 (hHand-1) protein identified using cNLS mapper. B, the predicted monopartite NLS sequence in the Hand-1 protein was cloned and the two lysines (K) at position 176 and 178 replaced by asparagine (N) residues are shown in the box. C, immunoblot analysis of cytoplasmic extract (CE) and nuclear extract (NE) proteins obtained from Hand-1 and Hand-1 (ΔNLS) overexpressing control and IR-treated (6 Gy) cells. The immunoblots were developed using antibodies specific for DsRed, tubulin, and lamin B were used as loading controls for CE and NE, respectively. D, immunolocalization of DsRed in Hand-1, Hand-1 (ΔNLS), and Hand-1NLS expressing UW228 and D283 cell (C, cytoplasmic; N, nuclear). E, the Hand-1 ChIP was performed in Hand-1OE UW228 and D283 cells using a ChIP-IT express kit (Active Motif). (Continued on the following page).
Hand-1 is an important regulatory factor in the initiation of angiogenesis and in radiotherapy response (46, 47). Hypoxia in tumor cells is known to regulate angiogenesis via activation of HIF-1α-mediated transcriptional upregulation of uPAR (48). Under normoxic conditions, HIF-1α is targeted for proteasomal degradation involving the E3 ubiquitin ligase complex containing VHL (49–51). Furthermore, FIH-1 inhibits the transactivation domain activity of HIF-1 by preventing HIF-1α binding to p300/CREB and also by directly interacting with HIF-1α (52–54). Interestingly, elevated levels of FIH-1 were detected with Hand-1 overexpression in all four medulloblastoma cell lines by using qRT-PCR and Western blot analysis. In contrast, overexpression of Hand-1 (∆NLS) suppressed FIH-1 and resulted in upregulation of HIF-1α mRNA expression (Fig. 4A–D). These results indicate the role of nuclear Hand-1 in regulating FIH-1 expression.

Most interestingly, we observed around 8 putative bHLH-binding sites in the promoter region of the human FIH-1 gene. To investigate whether Hand-1 binding to FIH-1 promoter was localized to these sites, we used UW228 and D283 ChIP DNA immunoprecipitated by anti-Hand-1 and anti-immunoglobulin G (IgG) antibodies. ChIP was carried out by scanning the bHLH binding in the first 1463 bp 5′-flanking region of the human FIH-1 gene (EMBL accession number X166135) using semiquantitative qRT-PCR with primers for regions (R) named 1–8 (Fig. 4E). Hand-1 enrichment at R2 and R8 (P < 0.01), which includes three and one putative bHLH-binding sites respectively, was greater than at other regions containing bHLH- (R1, R3, and R6) or no bHLH- (R3, R4, and R7) binding sites (Fig. 4F). The coefficient of Hand-1 interaction to different bHLH-binding regions in the FIH-1 promoter indicated that Hand-1 may bind to FIH-1 promoter in a region detected by the R2 and R8 primers (Fig. 4F).

**Hand-1 inhibits radiation-mediated HIF-1α activation and angiogenesis in vitro**

Both VHL and FIH-1 specifically interact with HIF-1α, and overexpression of either protein is thought to inhibit angiogenesis as a result of decreased HIF-1α-mediated VEGF expression (52). Therefore, we next examined the role of Hand-1–induced FIH-1 in the regulation of angiogenesis and HIF-1α–mediated transcriptional activation. To investigate this, we added DMOG, a potent inhibitor of FIH-1 asparaginyl hydroxylase activity (55), to Hand-1 and Hand-1 (∆NLS) overexpressing cells. The addition of DMOG (200 μmol/L) stimulated angiogenesis network formation in Hand-1OE cells, whereas there was no significant change in Hand-1OE (∆NLS) cells (Fig. 5A and B and Supplementary Fig. S1C).

Experiments have also shown that DMOG induces stabilization of HIF-1α and downstream gene expression under hypoxic conditions (56).

Local recurrence after radiation therapy is mediated, at least in part, by a hypoxic tumor microenvironment (57). Moreover, HIF-1 was identified to play a pivotal role in hypoxia-mediated radioresistance and provided a rational basis for targeting HIF-1 after radiation therapy (58, 59). Previous studies have shown that radiation treatment enhances HIF-1α expression in cancer cells (46, 60). We therefore first investigated whether IR treatment upregulates HIF-1α activity in medulloblastoma cells. We used the TransAM transcription factor assay Kit to determine the levels of activated HIF-1α in nuclear extracts of UW228 and D283 cells. We observed that with IR treatment, HIF-1α protein binds to an immobilized oligonucleotide probe containing the hypoxia responsive element, and its binding efficiency to this probe decreases with Hand-1OE and shRNA-mediated knockdown of uPAR (pU; Fig. 5C). These results demonstrate negative regulation of HIF-1α activation with Hand-1 overexpression. Quantification of HIF-1α reporter fluorescence intensity over time revealed that IR treatment caused a statistically significant increase in HIF-1α activity when compared with non–IR-treated controls. Again, the Hand-1OE (∆NLS) cells demonstrated no changes in HIF-1α activation following IR treatment (Fig. 5C). To determine whether the observed activity data were reflective of actual changes in HIF-1α signaling, we studied the effect of IR on protein levels for both HIF-1α and cytokines expressed under its control. IR treatment resulted in a slight increase in the nuclear levels of HIF-1α protein, whereas it did lead to a marked increase in the secretion of HIF-1α–regulated cytokine such as VEGF-D in medulloblastoma cells (Fig. 5D).

In normoxia, HIF-1α is rapidly degraded by the ubiquitin–proteasome system; under hypoxic conditions, however, this degradation is blocked. We examined HIF-1α stabilization in response to IR treatment alone and in combination with Hand-1OE and Hand-1OE (∆NLS). To test for the possible ubiquitination of HIF-1α, we used the Ubiquitination Kit, which showed increased capture of HIF-1α protein with antiubiquitin antibody in control cells. IR treatment further increased this degradation when compared with IR-treated D283 cells. These results indicate increased degradation of HIF-1α in control cells. Moreover, IR in combination with Hand-1OE also showed increased HIF-1α degradation when compared with IR-treated cells. In contrast, IR in combination with Hand-1OE (∆NLS) showed no significant increase in HIF-1α degradation (Fig. 5E).

All of these data suggest that Hand-1 has a functional role in the stabilization of HIF-1α.
Hand-1 overexpression suppresses radiation-induced HIF-1A expression in mouse brain tumors

We next sought to determine to what degree Hand-1OE regulates tumor radiosensitization. As shown above, Hand-1 upregulates the expression of an important protein called FH1-1 that targets HIF-1A. It would be informative to know whether Hand-1–induced FH1-1 expression regulates HIF-1A stabilization in mouse brain tumors. D283 cells were stably transfected with Hand-1OE plasmids followed by IR treatment, after which the cell suspension was implanted in the cerebellum of nude mice. Tumors were allowed to grow for 45 days and confirmed by hematoxylin and eosin staining (Fig. 6A). The IR-treated D283–Hand-1OE stable cells showed lower tumor growth and migration when compared with IR-treated control cells. Alternatively,
the intravenous administration of full-length uPAROE plasmid inhibited the tumor suppressor property of Hand-1OE+IR–treated cells (Fig. 6A).

We further performed DAB staining, which confirmed increased HIF-1A expression in IR and uPAROE treatments. Through DAB staining, we were able to observe several areas of FIH-1 staining within the nucleus which were more prominent in control and Hand-1OE tumors. The IR-treated tumors demonstrated lower levels of FIH-1 as well as its nuclear localization (Fig. 6B). In addition, immunofluorescence studies using Alexa Fluor–conjugated secondary antibody against Hand-1 (red) and uPAR (green) confirmed their association (yellow) in IR and uPAR overexpressing in vivo tumors (Fig. 6C). In control tumors, we observed increased Hand-1 and FIH-1 levels that were spread throughout the tumor region when compared with in vitro medulloblastoma cells (Supplementary Fig. S2). These results argue that radiation causes prominent HIF-1A upregulation in vivo when compared with in vitro, suggesting that an intact tumor–host interface may be required for this radioprotective response to occur.

Figure 4. Hand-1 regulates FIH-1 biogenesis through a uPAR-dependent mechanism. RT-PCR and immunoblot analysis of shRNA specific for uPAR–(pU), Hand-1OE–, and Hand-1–(ΔNLS) transfected UW228 (A), D283 (B), D458 (C), and H2402 (D) medulloblastoma cells. The fold change to control cells was calculated using 2−ΔΔCT (in which ΔΔCT = ΔCT of treatment − ΔCT of control) from three independent experiments and is represented graphically (mean ± SE, n = 3; *, P < 0.05; **, P < 0.01; ′′′, P < 0.001; ′′′′, the significant increase when compared with control cells). E and F, analysis of 1463 bp 5′-flanking region of the human FIH-1 gene (EMBL accession number X166135) identified 8 putative bHLH-binding sites. qPCR amplicons covering R1, R2, R3, R4, R5, R6, R7, and R8 are indicated by black lines below. E, to investigate whether Hand-1 binding to the FIH-1 promoter is localized around these bHLH-binding sites, we used UW228 and D283 ChIP DNA immunoprecipitated by anti-Hand-1 antibody. Immunoprecipitated DNA was analyzed by semiquantitative RT-PCR using ChIP-specific primers covering FIH-1 promoter regions R1–R8 (primers are listed in Supplementary Table S3). F, the fold enrichment of Hand-1 interaction with different regions (R1–R8) in the FIH-1 promoter was quantified and represented graphically. The negative control anti-immunoglobulin G (IgG) ChIP DNA did not show any amplification with R1–R8 primers.
Figure 5. Hand-1 suppresses medulloblastoma cell angiogenesis in vitro. A, quantitative analysis of tube-forming ability of HMEC cells upon treatment with culture filtrates of Hand-1OE and Hand-1 (JNLS) cells treated with or without DMOG inhibitor. B, the percentage of branch points times the branches was calculated and represented graphically. C, monitoring HIF-1A activation as assessed by HIF-1 DNA binding in nuclear extracts (5 μg) using a TransAM assay kit (TransAM, HIF-1 Transcription Factor Assay Kit; Active Motif) following the manufacturer’s protocol. Nuclear extract of CoCl2-treated HeLa cells (provided by the manufacturer) served as a positive control. D, to determine the activation of HIF-1A, immunoblot analysis of nuclear extract proteins (80 μg) was done using antibodies specific for HIF-1A; lamin B was used as a loading control. The VEGF-D protein was analyzed in culture filtrates (CF) by immunoblot. E, the control and Hand-1OE with or without IR-treated cells were lysed for ubiquitination assays to detect endogenous ubiquitinated HIF-1A protein. F, diagramatic representation of antagonistic activities of uPAR and Hand-1 signaling. The IR treatment induces uPAR expression and activates uPAR-dependent cell signaling. We showed that aberrant expression and stabilization of uPAR in IR-treated cells is associated with increased cytoplasmic sequestration of Hand-1 protein. This uPAR-mediated cytoplasmic anchoring of Hand-1 suppresses Hand-1 heterodimer-induced transcription of FIH-1 in the nucleus of IR-treated cells when compared with the nonirradiated control cells. In summary, we showed the negative regulation of Hand-1 by uPAR in IR-treated cells and its association with increased angiogenesis. In view of these findings, downregulation of uPAR or upregulation of Hand-1 accompanied by IR has significant therapeutic implications through the regulation of tumor growth and angiogenesis.
Discussion

The Hand-1 protein falls into a large phylogenetic group of transcriptional regulators referred to as the "bHLH family." bHLH proteins exhibit the unique and important ability to regulate a variety of different transcriptional programs via the formation of homodimers or heterodimerization with several different bHLH partners (61, 62). This dimerization is a prerequisite for DNA binding to E-box (CANNTG). As a member of the bHLH class B (tissue-restricted) TFs, Hand-1 is capable of forming heterodimers with bHLH class A (near-ubiquitous) TFs (63) and may also form homodimers with other tissue-restricted bHLH proteins such as HEY and most closely related factor Hand-2 (38). Aside from its ability to dimerize with other nuclear proteins, the mechanism by which Hand-1 regulates transcription is complex and may require tertiary Hand-1 protein interactions with non-bHLH factors (64). However, little is known about the function of these dimers or, more importantly, how they are regulated in cancer cells.

Here, we describe the negative regulation of Hand-1 by a non-bHLH protein uPAR, a known activator of tumor progression and angiogenesis. Earlier, we correlated increased uPAR signaling with IR treatment, and found that its suppression caused reduced invasion and migration in medulloblastoma cells (7). We also showed that the overexpression of uPAR was associated with activation of WNT/β-catenin signaling and cancer stemness, which was enhanced upon radiation treatment (13). Furthermore, the role of uPAR in angiogenesis, inflammation, wound repair, and tumor progression is due to its ability to engage in multiple protein–protein interactions (19, 65–67), which indicates that uPAR is capable of regulating other molecules to a large extent. Numerous studies confirmed the stable localization of uPAR on the cell surface and cytoplasm of the malignant cells (68, 69). Very importantly, we identified HSP90β as the prominent interacting molecule in uPAR stabilization after IR treatment (13). In continuation of our previous study, we demonstrate that aberrant expression and stabilization of uPAR in IR-treated cells is associated with increased cytoplasmic sequestration of Hand-1 protein. As a result, the uPAR-mediated cytoplasmic anchoring of Hand-1 protein represses Hand-1 heterodimer-induced transcription in the nucleus and
alters the regulation of angiogenesis-related processes (Fig. 5F). Previous studies of differential expression of vascular genes downstream of Hand-1 have indicated that Hand-1 is required for normal angiogenesis and extraembryonic vasculature development (39). However, there have been no conjectures into how Hand-1 might function at the level of direct downstream regulation of targets of angiogenesis.

We observed a synergistic increase in the expression of a major angiogenesis regulator, HIF-1A, in response to IR treatment. The literature has shown that HIF-1A activation promotes transcriptional activation of uPAR in cancer cells (48, 70, 71), thereby indicating that a mutual regulatory mechanism exists between HIF-1A and uPAR signaling mechanisms, which regulates the angiogenesis tube-induced ability and adaptation to radiation treatment of both cancer cells and CSCs (Fig. 5F). The role of HIF-1A activation (46) and uPAR overexpression (7, 13) in enhancing tumor- and endothelial cell radioresistance has stimulated an increased interest in research to block these molecules to improve treatment efficacy.

Interestingly, we also observed for the first time that overexpression of Hand-1 and its localization in the nucleus were associated with increased transcription of FIH-1. We identified that the promoter region of FIH-1 possessed 8 putative bHLH-binding regions and that the Hand-1 binding to FIH-1 promoter was localized to these sites. Furthermore, the Hand-1-ChIP and EMSA analyses indicated that nuclear Hand-1 associates with E12/E47/MyoD/GATA-1, or c-Myc proteins and their respective consensus sequences. The results also indicate that it may mediate FIH-1 gene transcription. Earlier studies have suggested that FIH-1 along with VHL protein functions cooperatively to suppress HIF-1A-mediated transactivation under nonhypoxic conditions (52). The FIH-1 loss-of-function may also contribute to increased HIF-1-mediated transactivation of downstream target genes such as VEGF in gliomas and other human cancers (72, 73).

It is evident that Hand-1 facilitates adaptive reprogramming of gene expression in adult and embryonic cells in response to physiologic, pathologic, and external cues (23, 74, 75). All of our data and the functional redundancy reported thus far suggested a wide range of upstream signals/mediators that can regulate the localization and functioning of intracellular Hand-1. The increased expression of angiogenesis-promoting molecules such as HIF-1A/VEGF-D/uPAR among IR-treated cells has led us to relate their regulatory role in decreased Hand-1–mediated FIH-1 transcription. Further, we showed that upregulation of uPAR during pathologic angiogenesis is highly associated with decreased Hand-1 expression in medulloblastoma cells. We have discovered a novel function of Hand-1 in regulating angiogenesis. Overexpression of Hand-1 in cancer cells leads to the upregulation of FIH-1, which inhibits angiogenesis in vivo and in vitro. Furthermore, this inhibition of angiogenesis is mediated through the uPAR–FIH-1 pathway. Taken together, our results demonstrate that Hand-1 expression and its nuclear translocation suppress the growth and angiogenic potential of medulloblastoma cells. This suppression was found to be mediated by downregulation of uPAR. Our data show that IR treatment upregulated uPAR stabilization, possibly via HSP90 association, decreased accumulation of nuclear Hand-1, and consequently activated the FIH-1A target gene transcription such as VEGF-D. In conclusion, our study has demonstrated the antagonistic regulation of Hand-1 by uPAR in IR-treated cells and its association with increased angiogenesis (Fig. 5F). In view of these findings, downregulation of uPAR or upregulation of Hand-1 accompanied by IR has significant therapeutic implications through the regulation of tumor growth and angiogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Asuthkar, V.R. Gogineni, J.S. Rao, K.K. Velpula
Development of methodology: S. Asuthkar, K.K. Velpula
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Asuthkar, K.K. Velpula
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): S. Asuthkar, J.S. Rao, K.K. Velpula
Writing, review, and/or revision of the manuscript: S. Asuthkar, V.R. Gogineni, J.S. Rao, K.K. Velpula
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.K. Velpula
Study supervision: J.S. Rao, K.K. Velpula

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