Hypoxia Regulates Alternative Splicing of HIF and non-HIF Target Genes

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Abstract

Hypoxia is a common characteristic of many solid tumors. The hypoxic microenvironment stabilizes hypoxia-inducible transcription factor 1α (HIF1α) and 2α (HIF2α) that are normally degraded under normoxia. The stabilized HIF1α and HIF2α proteins translocate to the nucleus, where they dimerize with aryl hydrocarbon receptor nuclear translocator (ARNT) to form HIF1α–ARNT (HIF1) and HIF2α–ARNT (HIF2) complexes. HIF1 and HIF2 activate gene transcription to promote tumor progression and metastasis (1–4). Thus, HIF-mediated transcriptional response is very important in cancer biology.

Recently, it was found that 95% of human genes are alternatively spliced, producing RNA isoforms that code for functionally distinct proteins. Thus, an effective hypoxia response requires increased HIF target gene expression as well as proper RNA splicing of these HIF-dependent transcripts. However, it is unclear if and how hypoxia regulates RNA splicing of HIF targets. This study determined the effects of hypoxia on alternative splicing (AS) of HIF and non-HIF target genes in hepatocellular carcinoma cells and characterized the role of HIF in regulating AS of HIF-induced genes. The results indicate that hypoxia generally promotes exon inclusion for hypoxia-induced, but reduces exon inclusion for hypoxia-reduced genes. Mechanistically, HIF activity, but not hypoxia per se is found to be necessary and sufficient to increase exon inclusion of several HIF targets, including pyruvate dehydrogenase kinase 1 (PDK1). PDK1 splicing reporters confirm that transcriptional activation by HIF is sufficient to increase exon inclusion of PDK1 splicing reporter. In contrast, transcriptional activation of a PDK1 minigene by other transcription factors in the absence of endogenous HIF target gene activation fails to alter PDK1 RNA splicing.

Implications: This study demonstrates a novel function of HIF in regulating RNA splicing of HIF target genes. Mol Cancer Res; 1–11. ©2014 AACR.

Introduction

Hypoxia is a common characteristic of many solid tumors. The hypoxic microenvironment stabilizes hypoxia-inducible transcription factor 1α (HIF1α) and 2α (HIF2α) that are normally degraded under normoxia. The stabilized HIF1α and HIF2α proteins translocate to the nucleus, where they dimerize with aryl hydrocarbon receptor nuclear translocator (ARNT) to form HIF1α–ARNT (HIF1) and HIF2α–ARNT (HIF2) complexes. HIF1 and HIF2 activate gene transcription to promote tumor progression and metastasis (1–4). Thus, HIF-mediated transcriptional response is very important in cancer biology.

Recently, it was found that 95% of human genes are alternatively spliced and RNA isoforms produced from a single gene code proteins with distinct or opposing functions (5). Thus, it becomes increasingly clear that assessing isoforms expression will be more informative than quantifying total transcripts to appreciate the functional consequence of gene regulation.

Exon arrays contain probe sets interrogating every exon of the transcriptome, thereby permitting the distinguishing of different isoforms of a gene as well as measuring gene expression levels. So far, two studies have examined the effect of hypoxia in regulating RNA splicing using exon arrays (6, 7). However, both studies used endothelia cells, a normal cell type. So far, hypoxia-mediated RNA splicing changes have not been measured in cancer cells. In addition, these previous studies in endothelial cells focused on non-HIF target genes and reported that hypoxia, like other stresses, inhibits RNA splicing of non-HIF target genes by promoting exon skipping and/or intron inclusion (6, 7). Thus, role of hypoxia in regulating RNA splicing of HIF-induced genes, the most important genes in hypoxia response is still unknown. Furthermore, none of these studies have addressed the molecular mechanisms about how hypoxia regulates alternative splicing (AS) of HIF target genes. In this study, we used exon arrays to assess global effects of hypoxia on RNA splicing of HIF and non-HIF target genes in Hep3B cells, a cancer cell line. In addition, we studied if HIF or hypoxia per se is important for RNA splicing of several HIF target genes. These findings have important implications for our understanding of how HIFs promote tumorigenesis in response to hypoxia.
Materials and Methods

Cell culture
Hep3B cells were cultured in Minimum Essential Medium with Earle’s Balanced Salt Solution (HyClone) containing 10% FBS, 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 100,000 U/L penicillin/streptomycin, 1.5 g/L sodium bicarbonate, and 1× nonessential amino acids (NEAA). HEK293T, RCC4, RCC4T, and UM-SCC-22B cells were grown in high-glucose Dulbecco’s Modified Eagle Medium (DMEM; HyClone) with 10% FBS, 2 mmol/L l-glutamine, 100,000 U/L penicillin/streptomycin, and 1× NEAA. SK-N-MC cells were grown in RPMI-1640 (HyClone) with 10% FBS, 2 mmol/L l-glutamine, 100,000 U/L penicillin/streptomycin, and 1× NEAA. Before hypoxia treatment, 25 mmol/L HEPES was added to growth media and cells were incubated under normoxia (21% O2) or hypoxia (1.5% O2) for 12 to 16 hours. All parental cell lines were purchased from ATCC. After completing the experiments, the parental (Hep3B, HEK293T, RCC4, UM-SCC-22B, and SKNMC) and modified cell lines (RCC4T) were authenticated by DNA profiling or "fingerprinting" by the University of Colorado DNA Sequencing & Analysis Core.

Exon array analysis of AS
The genome-wide effect of hypoxia on AS was determined in Hep3B cells using the Affymetrix GeneChip Human Exon 1.0 ST array. Hep3B cells were cultured, as described above, under normoxic (21%) or hypoxic (1.2%) conditions for 12 hours. RNA was prepared using a Qiagen RNeasy Plus RNA extraction kit. RNA was reverse transcribed and assessed for hypoxic induction of HIF target genes using qPCR. RNAs that passed quality control were submitted to the Gene Expression Core at the University of Colorado Comprehensive Cancer Center for exon array analysis. Three independent replicate samples were analyzed for each treatment. The Core performed target labeling, hybridization, and chip scanning. To assess AS events, raw CEL files were analyzed using the freely available AltAnalyze software (http://www.altanalyze.org; ref. 8) with the following settings: species, human; gene and exon set, both core; and gene and exon level normalization FIRMA. The array data can be accessed at GEO (GSE57613). Raw CEL files were alternatively analyzed using easyExon (http://microarray.ym.edu.tw:8080/tools/module/easyexon/index.jsp?mode=home), with default settings and using RNA filtering (9). In addition, AltAnalyze software was used for gene expression analysis. Genes predicted to undergo AS by AltAnalyze and easyExon were then compared with genes that were induced by hypoxia (AltAnalyze) using Venny (http://bioinfo.cnb.csic.es/tools/venny/) to identify genes that were predicted to undergo AS and hypoxia induction.

Functional clustering of hypoxia-inducible genes and alternatively spliced genes using DAVID bioinformatics resources
DAVID Bioinformatics (10) was used to create functionally annotated clusters of (i) genes that were hypoxia induced, (ii) genes that were predicted to undergo hypoxia-induced AS, and (iii) genes that were hypoxia induced and exhibiting AS.

Knockdown of endogenous mRNA using small interfering RNAs
Control (Qiagen; 1027281) or siRNAs specific for human ARNT (Qiagen; equal mix of S100304220, S100304234, and S103020913), HIF1α (Qiagen; S102664053), and HIF2α (Qiagen; S100380212) mRNAs were transfected into Hep3B cells at 40% confluency using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol. Thirty-two hours after transfection, cells were cultured at 21% or 1.5% O2 for 12 to 16 hours and then collected for mRNA or protein purification.

Viral transduction of Hep3B cells
The GFP-Flag, HIF1αTM-Flag (triple mutation to make HIFα protein active under normoxia, with Flag tag), and HIF2αTM-Flag lentiviral constructs were described (11). The GFP-Flag, HIF1αTM-Flag, or HIF2αTM-Flag plasmid was cotransfected with psPAX2 packaging vector (Addgene), and pMD2.G envelope vector (Addgene) into HEK293T cells. Twenty-four hours after transfection, transfection media were replaced with complete Hep3B media. The following day, viral media were filtered to remove possible 293T cells and viral particles were concentrated using high-speed ultracentrifugation at 26,000 × g for 3 hours. The viral pellet was resuspended in 1 mL of PBS and added to 6 cm Hep3B cells (30% confluency) for 6 hours. The following day, a second round of concentrated virus was added to the same Hep3B cells. Twenty-four hours after the second viral transduction, Hep3B cells were harvested for RNA and protein analysis.

Plasmid constructs
The CA9P/ADM, PAI1P/ADM, and PAI1PmHRE/ADM constructs were described previously (11). The CA9P/PDK1, PAI1P/PDK1, and PA11PmHRE/PDK1 constructs were generated by replacing the ADM gene with PDK1 minigene that was PCR amplified from human genomic DNA and contained exon 3 to exon 5, including introns 3 and 4. The HIF1αTM-Flag, HIF2αTM-Flag, HIF1αDBD-Flag, HIF1αDBD/VP16-Flag, HIF1αDBD/E2F-Flag, and upstream stimulatory factor 2 (USF2) expression plasmids were described previously (11–13). The G5/PDK1 minigene was generated by replacing the ADM gene with PDK1 minigene in the G5/ADM construct that was described previously (11). The Gal4 DNA binding domain (Gal4), Gal4/HIF1αTAD, Gal4/VP16, and Gal4/E2F1 have been described in ref. (11).

PDK1 splicing reporter assay
Typically, HEK293T cells were grown to approximately 30% confluency in 6-well plates and cotransfected with 200 ng of splicing reporters (CA9/PDK1, PAI1/PDK1, PAI1mHRE/PDK1, or G5/PDK1) and 1.8 μg of His (empty vector control), HIF1αTM, HIF2αTM, USF2,
HIF1αDBD, HIF1αDBD/E2F1, or HIF1DBD/VP16 expression constructs or Gal4 expression construct for G5/PDK1. Forty-eight hours after transfection, cells were collected for RNA or protein preparation. Results were the average of at least three experiments.

**Protein analysis**
Whole-cell lysates were prepared and quantified for protein concentration. Western blot analysis was performed using standard protocols with the following primary antibodies: anti-CA9 (H-120) pAb (SC-25599; Santa Cruz Biotechnology), anti-ANGPTL4 (H-200) pAb (SC-66806; Santa Cruz Biotechnology), anti-Flag mAb (F3165; Sigma), anti-Gal4DBD pAb (SC-577; Santa Cruz Biotechnology), or anti-beta Actin pAb (SC-1616; Santa Cruz Biotechnology).

**RNA preparation and reverse transcription PCR or quantitative PCR**
RNA was isolated from cells using the RNeasy Plus mini kit (Qiagen), which removes DNA, then was reverse transcribed using the iSCRIPT Advanced cDNA synthesis kit (Bio-Rad) containing both oligo-dT and random hexamer as primers because using both random hexamers and oligo-dT allows efficient reverse transcription of the whole mRNA transcript as well as 18 rRNA. Alternative splicing analysis was then validated by RT-PCR using forward and reverse primers flanking AS events predicted by both AltAnalyze and easyExon software. Genes that expressed multiple transcripts were Topo TA cloned and submitted to the Gene Expression Core at the University of Colorado Cancer Center for sequencing. qRT-PCR using iQ SYBR Green Supermix (Bio-Rad) was used to quantify the levels of RNA isoforms. qRT-PCR using iQ SYBR Green Supermix in triplicate on the CFX384 Real-Time System (Bio-Rad) was used to quantify the levels of RNA isoforms. All primer sets for qRT-PCR designed to measure mRNA levels were validated for their specificity and amplification efficiency (85%-110%) using melt curve analysis, qRT-PCR product sequencing, and standard dilution analysis. qRT-PCR results were normalized using the PfafflΔΔCt method using18s rRNA and β-actin as reference genes because their expression are not affected by hypoxia and untreated normoxia samples, GFP lentivirus, or empty vectors (His) as controls. The RNA ratio of splice variants was calculated by dividing the expression level of the full-length (FL) isoform by the expression level of the ΔE isoform. The expression level for each isoform was determined by the following equation: Expression level = efficiencytargetΔΔCt(target − control)sample/referenceΔΔCtreference(control − sample), where the efficiency = 10−1/slope (slope generated by standard curve analysis). At least three independent experiments were performed to generate the results presented in the Figures. All the primers used for RT-PCR and qRT-PCR were included in Supplementary File S3.

**Statistical analysis**
One-way analysis of variance was performed unless otherwise stated. Error bars in figures indicate standard deviation. Asterisks indicate statistical significance as follows: *, P < 0.05; **, P < 0.01. Controls for statistical analysis are specified in each figure. All experiments were performed at least three separate times.

**Results**
Genome-wide exon array analysis determines that hypoxia alters RNA splicing of HIF and non-HIF target genes in Hep3B cells
To assess the effects of hypoxia on RNA splicing, Affymetrix GeneChip Human Exon 1.0 ST arrays were used to probe RNAs isolated from normoxic or hypoxic Hep3B cells. Hep3B cells were chosen because of their high expression levels of HIFα proteins and high hypoxic induction of HIF target genes (12–14). AltAnalyze (FIRMA) and easyExon software predicted that hypoxia regulated AS of about 2,005 (Fig. 1A, yellow and gray) and 3,919 genes, respectively (Supplementary File S1; AS by Firma or AS by easyExon). Venn diagrams using Venny indicated that 1,012 genes were predicated to undergo AS by both programs, whereas an additional 957 and 2,634 genes were predicated to undergo AS by FIRMA and easyExon, respectively. FIRMA was used to further characterize the hypoxia-mediated splicing events, as FIRMA predictions of AS are more stringent than easyExon. FIRMA analysis indicated that hypoxia produced 3,059 AS events in 2,005 genes, averaging 1.53 splicing events per gene (Fig. 1A, all genes). As expected, alternative cassette-exons were the most abundant type of splicing events, making up 51% of all splicing events. Other AS events included alternative 5′ splice-sites (16%), alternative 3′ splice-sites (14%), intron retention (11%), mutually exclusive exon (4%), and others (4%).

In addition, AltAnalyze determined that hypoxia induced 2,439 genes, for at least 1.4 fold, when compared with normoxic Hep3B controls (Fig. 1A, blue and gray; also see Supplementary File S1, gene expression). Venny analysis of hypoxia-inducible genes and AS genes indicated that 134 genes were hypoxia induced and alternatively spliced and these 134 genes were called Hx AS genes (Fig. 1A, in gray). Interestingly, these 134 Hx AS genes had 238 splicing events, averaging 1.78 AS events per gene (Fig. 1A, Hx-induced genes), suggesting that AS is enriched in hypoxia-induced genes, compared with the total population of genes that underwent AS (1.53 AS events per gene). Similar to the total population of AS genes, alternative cassette-exons were the most abundant type of splicing events (62%) in Hx AS genes. Other AS events for these Hx AS genes included alternative 5′ splice sites (16%), alternative 3′ splice sites (13%), intron retention (9%), and mutually exclusive exons (0%). However, Hx AS genes exhibited 1.2-fold enrichment in alternative cassette-exon inclusion and a 1.22-fold reduction in intron retention in comparison with the total population of AS genes.

Because cassette-exon AS is the most abundant splicing event, hypoxia-mediated cassette-exon regulation was further analyzed. In the total population of cassette-exon genes (all cassette, Fig. 1B), 44% of cassette-exon genes exhibited exon inclusion (454 out of 1,022 genes), whereas 56% of the
Functional clustering of hypoxia-mediated AS genes using DAVID reveals new pathways regulated by hypoxia

To determine whether hypoxia-induced AS was enriched in specific cellular pathways, DAVID (10) was used to create functional annotation clusters of genes that were hypoxia induced (Fig. 1A, left column), the entire population of AS genes (Fig. 1A, right column), and the Hx AS genes (Fig. 1A, top; see Supplementary File S2 for the gene lists). As expected, hypoxia induced the expression of genes in pathways allowing cancer cells to adapt to a hypoxic microenvironment such as vascular development, glycolysis, oxidoreductase activity, and cell adhesion/cadherins (Fig. 1A, left column). On the other hand, when analyzing all AS genes, pathways involved in oxidoreductase activity, glycolysis, and positive regulation of glucose transport that were enriched in gene expression were also enriched in AS (Fig. 1A, right column and Supplementary File S2). However, for the same pathway, the genes involved in AS could be different from the genes enriched in expression analysis, demonstrating the importance of these pathways in the hypoxia response. Importantly, several novel cellular pathways, including ATP-binding/protein kinase activity, pleckstrin homology...
Hypoxia Regulates Alternative Splicing

Expression of multiple isoforms and hypoxia-regulating RNA splicing of additional HIF target genes, pyruvate dehydrogenase kinase 1 (PDK1, FL, and ΔE4), WNK lysine deficient protein kinase 1 (WNK1, FL, and ΔE11–12), and prolyl 4-hydroxylase alpha polypeptide II (P4HA2, FL, and ΔE2), were also confirmed by RT-PCR and qRT-PCR (Supplementary Fig. S2A–C).

CA9, ANGPTL4, PDK1, WNK1, and P4HA2 exhibited preferential induction of the FL isoform. However, AltAalyze (FIRMA) predicts that hypoxia promotes exon skipping of some HIF target genes, including procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2, FL, and ΔE14) and enolase 2 (ENO2, FL, and ΔE8), which are also confirmed (Supplementary Fig. S2D and S2E). These data demonstrated that hypoxia promotes exon inclusion for most HIF target genes; however, hypoxia promotes exon skipping for some HIF target genes such as PLOD2 and ENO2.

Hypoxia changes isoform ratios of HIF target genes at the transcriptional level

After confirming AS of several HIF target genes, we addressed the molecular mechanism on how hypoxia regulates AS of HIF target genes. Although AS was proposed to explain the observed FL to skipping isoform ratio changes in the above sections, the hypoxia-induced isoform ratio variations could be caused by RNA splicing or other mechanisms. For instance, preferential degradation of exon skipping isoforms under hypoxia might explain the hypoxia-induced isoform ratio changes as exon skipping RNA isoforms may incur premature termination codons and such transcripts are often targeted for nonsense-mediated decay (NMD; ref. 18). In the seven genes analyzed, only RNA of CA9 ΔE89 (E8–9, 172 bp), but not ANGPTL4 (E4, 114 bp), PDK1 (E4, 138 bp), WNK1 (E11–12, 609 bp), P4HA2 (E2, 136 bp in 5′-UTR), PLOD2 (E13, 114 bp), and ENO2 (E8, 198 bp) had premature stop codon. To test the possibility that preferential instability of CA9 ΔE89 contributes to the increased CA9 FL/ΔE89 ratio under hypoxia, Hep3B cells were placed under hypoxia for 16 hours to increase the levels of both CA9 FL and ΔE89 transcripts, followed by treatment with actinomycin D to inhibit de novo transcription. Cells were then placed back under normoxia or hypoxia for 0, 2, 4, or 8 hours to allow RNA decay. Using qRT-PCR, both CA9 FL and ΔE89 transcripts were found to be very stable, as 90% of the FL and ΔE89 transcripts were detected even after 8 hours. Moreover, CA9 FL and ΔE89 transcripts exhibited similar stability under normoxia and hypoxia at every time point (data not shown). ANGPTL4 FL and ΔE4 transcripts were far less stable than CA9 transcripts as only 40%, 28%, and 20% of the transcripts remained after 2, 4, and 8 hours. However, ANGPTL4 FL and ΔE4 transcripts also exhibited similar stability under normoxia and hypoxia (data not shown). Furthermore, actinomycin D treatment in Hep3B cells blocked hypoxic induction of HIF target genes and blocked splicing change of HIF target genes, indicating that the hypoxia-mediated isoform shift required active transcription. These data supported the idea that transcription...
regulation, not posttranscriptional regulation is responsible for the hypoxia-induced increased CA9 and ANGPTL4 FL/exon-skipping ratio.

**HIF activity, not hypoxia per se, is necessary to change AS of HIF target genes**

To test whether hypoxic stress or HIF activity is responsible for the splicing changes of HIF target genes, ARNT, HIF1α, or HIF2α mRNA levels were reduced by 80% using siRNAs in normoxic or hypoxic Hep3B cells (data not shown). ARNT and HIF1α, but not HIF2α knockdown dramatically reduced the hypoxic induction of CA9, ANGPTL4, and PDK1, consistent with the idea that CA9, ANGPTL4, and PDK1 are primarily regulated by HIF1α in Hep3B cells (Fig. 3A). qRT-PCR confirmed that ARNT and HIF1α knockdown significantly reduced the levels of both FL and exon skipping isoforms of CA9, ANGPTL4, and PDK1 and prevented the splicing changes of these genes (Fig. 3B–D). In contrast, HIF2α knockdown only mildly reduced hypoxic induction of CA9FL (1.44 fold) and PDK1ΔE4 (1.6 fold), similarly reduced hypoxic induction of ANGPTL4FL and ΔE4. Thus, HIF2α knockdown only reduced the CA9FL/ΔE89 ratio by 1.33 fold (Fig. 3B), maintaining the ANGPTL4FL/ΔE4 ratio (Fig. 3C), but enhanced the PDK1FL/ΔE4 ratio 1.5 fold (Fig. 3D). Knockdown of ARNT and HIF1α also inhibited hypoxic induction of the FL and exon skipping isoforms of WNK1, PLOD2, ENO2, and P4HA2 in Hep3B cells and prevented splicing ratio changes for these genes (data not shown). These data suggested that HIF activity, but not hypoxia per se is necessary for increased gene expression as well as hypoxia-mediated splicing changes of these HIF target genes.
HIF activity is sufficient to regulate AS of HIF target genes

Next, we wanted to determine whether HIF activity is sufficient for hypoxia-regulated AS of HIF target genes. To test this, normoxic Hep3B cells were transduced with lentiviruses expressing normoxia active, flag-tagged, HIF1α, or HIF2α, or GFP as a negative control (Fig. 4A). HIF1α and HIF2α transduction induced the expression of CA9, ANGPTL4, and PDK1 as determined by RT-PCR (Fig. 4B). More importantly, qRT-PCR determined that HIF1α and HIF2α increased both FL and exon-skipping isoforms of CA9 (Fig. 4C), ANGPTL4 (Fig. 4D), and PDK1 (Fig. 4E). However, FL transcripts of CA9, ANGPTL4, and PDK1 were preferentially induced (Fig. 4C–E). Thus, HIF1 or HIF2 increased the FL/exon skipping ratio for CA9 by 14.5 or 6.0 fold (Fig. 4C), ANGPTL4 by 3.41 or 5.7 fold (Fig. 4D), and PDK1 by 1.43 or −1.2 fold (Fig. 4E).

To further validate these results, the RNA splicing of CA9, ANGPTL4, and PDK1 were examined in RCC4 cells, a renal cell carcinoma cell line that expresses constitutively active HIF1α and HIF2α proteins due to loss of functional pVHL (15, 19). In addition, the RNA splicing of the above three genes were also assessed in RCC4T cells in which functional pVHL is reintroduced into RCC4 cells and, therefore, HIF proteins are only active under hypoxia (15, 19). RT-PCR and qRT-PCR analysis determined that hypoxia increased the levels of CA9FL and ΔE89 by 41.8 and 7 fold, respectively, in RCC4T cells, increasing the FL/ΔE89 ratio by 6.2 fold (Supplementary Fig. S3B). In contrast, hypoxia did not induce the levels of CA9FL and ΔE89 isoforms nor did hypoxia enhance the FL/ΔE89 ratio in RCC4 cells (Supplementary Fig. S3B). However, the CA9 FL/ΔE89 ratio in RCC4 cells was already high even under normoxia (Supplementary Fig. S3B). Similar findings were observed for AS of ANGPTL4; however, hypoxia was still able to enhance the ANGPTL4 FL/ΔE4 ratio in RCC4 (Supplementary Fig. S3C). Although hypoxia was able to increase the expression of the PDK1FL and ΔE4 isoforms and to enhance the PDK1FL/ΔE4 ratio in RCC4T cells, PDK1 FL/ΔE4 ratio was not elevated in normoxic or hypoxic RCC4 cells (Supplementary Fig. S3D).

Taken together, these data suggested that HIF activity is necessary and sufficient to regulate AS of a subset of HIF target genes independent of hypoxia.

PDK1 splicing reporters recapitulate splicing changes observed for the endogenous PDK1 gene when activated by HIF under normoxia

Next, we wanted to see if similar splicing changes could be observed in splicing reporter gene. We selected the PDK1 gene for splicing reporter model as both the FL and ΔE4 isoforms were highly expressed in 293T cells (data not shown). Exons 3 to 5, including introns 3 and 4 of the PDK1 gene, were PCR amplified from human genomic DNA and placed downstream of the CA9 promoter, a HIF1 target gene promoter, or the PAI1 promoter, a HIF2 target gene promoter (Fig. 5A). RT-PCR using minigene-specific primers determined that the PDK1 minigene expressed both FL and ΔE4 isoforms in 293T and Hep3B cells (Fig. 5B). In addition, the PDK1ΔE4 isoform was the dominantly expressed isoform in both 293T and Hep3B cells (Fig. 5B). Furthermore, PDK FL isoforms were also highly expressed in 293T cells (Fig. 5B). These data demonstrated that the PDK1 splicing reporter recapitulated the expression
patterns of the endogenous PDK1 gene in 293T and Hep3B cells (Supplementary Fig. S2A and data not shown).

To assess AS of the PDK splicing reporter in response to HIF activation, 293T cells were transfected with the PDK1 minigene and normoxia active HIF1α expression plasmids. HIF1α induced the levels of total PDK1 minigene transcripts, FL, and ΔE4 isoform by 3.65, 24.9, and 10.6 fold, respectively, and enhanced the FL/ΔE4 ratio by 2.34 fold (Fig. 5D). Next, similar experiments were performed using the PAI1P/PDK1 splicing reporter (Fig. 5A). Normoxia induced the expression of PDK1FL and PDK1ΔE4 ratio by reducing the levels of the CA9 and ANGPTL4 (D), and PDK1 (E) isoforms by 3.65, 24.9, and 10.6 fold, respectively, and enhanced the FL/ΔE4 ratio by 2.34 fold (Fig. 5D). Similarly, HIF1α or HIF2α proteins were used to activate CA9, ANGPTL4, and PDK1 transcripts in normoxic Hep3B cells transduced with GFP, or normoxia-active HIF1α, or HIF2α proteins. C to E, qRT-PCR analysis of FL and exon-skipping RNA isoforms of CA9 (C), ANGPTL4 (D), and PDK1 (E) in the above described cells.

These data indicated that HIF transactivation domain is not required for AS of the PDK1 minigene.

In summary, these data indicated that HIF1 or HIF2–mediated activation of the PDK1 minigene is sufficient to increase PDK1 FL/ΔE4 ratio.

Activation of endogenous HIF target genes contributes to increased FL/ΔE4 ratio of PDK1 splicing reporter

As stated above, transcription activation of PDK1 by HIF or HIFDBD hybrid constructs is sufficient to increase the PDK1 minigene FL/ΔE4 ratio. However, HIF and the fusion constructs can activate endogenous HIF target genes; therefore, it is possible that activation of endogenous HIF target genes may increase FL/ΔE4 ratio. To rule out or confirm this possibility, we used USF2 and the PAI1P/PDK1 minigene as USF2 can activate the PAI1 promoter (13), but not endogenous HIF target genes. USF2 induced the expression of the PDK1FL and ΔE4 isoforms by 2.95 and 2.96 fold, respectively, but was not able to enhance the FL/ΔE4 ratio even though USF2 was able to activate the minigene to a similar extent as HIF2α (USF2 = 1.97 and HIF2α = 1.52-fold induction of total transcripts; Fig. 5E). To test if activation of endogenous HIF target gene could regulate AS of the PDK1 minigene, HIF-binding site on the PAI1 promoter was mutated to produce the PAI1PmHRE/PDK1 minigene (Fig. 5F). Interestingly, although HIF2α was not able to activate the expression of PAI1PmHRE/PDK1 as assessed by total as well as FL transcripts, HIF2α increased the FL/ΔE4 ratio by reducing the levels of the PDK1 ΔE4 transcript (Fig. 5F).
To further validate that transcription activation of endogenous HIF target genes increases the PDK1 FL/ΔE4 ratio, the PDK1 splicing reporter was placed under the control of a promoter containing five copies of the Gal4 DNA binding element (Supplementary Fig. S4A, 5xUAS). Fusion constructs containing the Gal4 DNA binding domain fused to the transactivation domains of normoxia active HIF1α, VP16, or E2F1 were used to activate the G5P/PDK1 minigene in the presence or absence of normoxia-active HIF1α and HIF2α that activate endogenous HIF target genes (Supplementary Fig. S4B). Interestingly, although Gal4/HIF1αTAD, Gal4/VP16TAD, and Gal4/E2F1TAD proteins in 293 T cells for experiments whose results are presented in Fig. 5D, HIF1αDBD/E2F1TAD construct produces two proteins. D, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with CA9P/PDK1 minigene and HIF1α or HIF1αDBD expression constructs. E, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with PAI1P/PDK1 minigene and HIF2α or USF2 expression constructs. F, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with PAI1PmHRE/PDK1 minigene and HIF2α or USF2 expression constructs.

Figure 5. Transcription activation is not sufficient to regulate splicing of a PDK1 minigene. A, schematic of a PDK1 splicing reporter driven by CA9 or PAI1 promoter. Arrows, primers used for RT-PCR (E3/vector R) and qRT-PCR (E3/5, E4/5, or total with Vector R for ΔE4, FL, or total transcript respectively). B, RT-PCR analysis of PDK1 transcripts expressed from CA9P/PDK1 in 293T and Hep3B cells. C, Western blot analysis of Flag-tagged HIF1α, HIF1αDBD/VP16, and HIF1αDBD/E2F1TAD proteins in 293 T cells for experiments whose results are presented in Fig. 5D. HIF1αDBD/E2F1TAD construct produces two proteins. D, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with CA9P/PDK1 minigene and HIF1α or HIF1αDBD expression constructs. E, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with PAI1P/PDK1 minigene and HIF2α or USF2 expression constructs. F, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with PAI1PmHRE/PDK1 minigene and HIF2α or USF2 expression constructs.

To further validate that transcription activation of endogenous HIF target genes increases the PDK1 FL/ΔE4 ratio, the PDK1 splicing reporter was placed under the control of a promoter containing five copies of the Gal4 DNA binding element (Supplementary Fig. S4A, 5xUAS). Fusion constructs containing the Gal4 DNA binding domain fused to the transactivation domains of normoxia active HIF1α, VP16, or E2F1 were used to activate the G5P/PDK1 minigene in the presence or absence of normoxia-active HIF1α and HIF2α that activate endogenous HIF target genes (Supplementary Fig. S4B). Interestingly, although Gal4/HIF1αTAD, Gal4/VP16TAD, and Gal4/E2F1TAD proteins in 293 T cells for experiments whose results are presented in Fig. 5D, HIF1αDBD/E2F1TAD construct produces two proteins. D, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with CA9P/PDK1 minigene and HIF1α or HIF1αDBD expression constructs. E, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with PAI1P/PDK1 minigene and HIF2α or USF2 expression constructs. F, qRT-PCR analysis of minigene-specific PDK1 isoforms in 293 T cells cotransfected with PAI1PmHRE/PDK1 minigene and HIF2α or USF2 expression constructs.

Discussion

Most analyses of hypoxia-mediated gene expression changes are conducted at the gene level using traditional microarrays (15, 23–26). However, due to significant functional difference of the proteins encoded by RNA isoforms and the fact that a majority of genes express multiple RNA isoforms (5), gene expression analysis by exon array or RNA
deep sequencing is necessary to fully understand gene expression programs. Our exon array analysis in normoxic and hypoxic Hep3B cells not only confirms hypoxic induction of previously identified hypoxia-inducible genes, but also reveals significant roles of hypoxia in regulating RNA splicing of genes whose transcription are induced by hypoxia or whose transcription are not changed or reduced by hypoxia.

Here, we first reported novel hypoxia-regulated genes/pathways that cannot be identified using traditional microarrays as expression levels of these genes are typically not changed by hypoxia or reduced by hypoxia. In addition, we found that hypoxia primarily promotes exon skipping of these non-HIF target genes in Hep3B cells, a result consistent with what is reported in endothelial cells (6, 7). We proposed that increased exon skipping of these non-HIF target genes also have functional consequences. For example, genes involved in ATP binding and protein kinase activity are not hypoxia induced, but exhibit AS. Increased exon skipping of these genes in hypoxic Hep3B cells, presumably acts to reduce ATP usage to maintain cellular ATP levels. AS of some of these non-hypoxia-induced genes are not associated with enriched pathways identified by DAVID Bioinformatics; however, their splicing are also altered and expected to be functionally important. For example, the KRAS 4B isoform (exon 4a is skipped in KRAS 4B, but included in KRAS 4A) is induced in hypoxic Hep3B, thus reducing the KRAS 4A/4B ratio. Interestingly, a decreased KRAS 4A/4B ratio is often observed in human colorectal cancer, but not in normal colon (27, 28). Furthermore, decreased KRAS 4A/4B ratios through reduction of KARS 4A expression or increased KRAS 4B expression promotes DMH-induced colonic tumorigenesis in in vivo mouse models independent of KRAS mutations (29), demonstrating the functional consequence of reduced KRAS 4A/4B ratio in tumor biology.

More importantly, we reported here, for the first time the role of hypoxia in regulating RNA splicing of hypoxia-inducible genes. We found that hypoxia promotes exon inclusion for 75% of Hx AS genes. Although no functional differences have been examined for the isoforms of most HIF target genes, FL CA9 protein was reported to be a membrane-associated, tumor-promoting protein by regulating intercellular pH via transporting protons out of the cells during hypoxia. In contrast, the CA9ΔE89 protein is a soluble protein and is not able to transport protons out of the cells (17). Thus, it makes sense that CA9 FL is preferentially induced by hypoxia. In addition, preferential induction of nerve growth factor receptor TrkA AE679 isoform, but not FL leads to nerve growth factor–independent receptor activity and neuroblastoma tumor-promoting activity (30). Again, specific promotion of Cysteine-Rich protein 61 FL, but not intron-3 retaining isoform (producing no functional protein) promotes tumor angiogenesis (31).

While isoform ratio changes of several HIF target genes have been reported under hypoxia (17, 30–34), none of these studies have addressed the molecular mechanisms about how hypoxia regulates isoform ratio changes of HIF target genes. We demonstrated in this study that the increased FL/exon-skipping ratio of several HIF target genes, including CA9 and ANGPTL4, is the result of AS, not preferential degradation of exon-skipping isoforms. Moreover, we determined that HIF activity, but not hypoxic stress per se is necessary and sufficient to regulate AS of CA9, PDK1, and ANGPTL4 pre-mRNAs. Furthermore, we found that activation of endogenous HIF target genes contributes to AS of PDK1 minigenes.

Our studies also indicated that there is an opposing effect on HIF target genes versus non-HIF target genes for RNA splicing in which hypoxia primarily promotes exon inclusion of HIF target genes, but inhibits exon inclusion of genes repressed under hypoxia. This opposing effect is similarly observed for gene transcription and protein translation in which, transcription and protein translation of non-HIF target genes are generally reduced, whereas HIF target genes are transcriptionally induced and protein translation of genes involved in hypoxia response such as HIF1A, HIF2A, and VEGF are maintained due to internal ribosome entry sites (IRES). Our data indicated that exon inclusion of HIF target genes is due to HIF activity, but not hypoxic stress. However, we do not know if exon skipping of hypoxia-repressed genes is dependent on HIF activity or hypoxic stress. This is a very interesting question that should be assessed in future studies.

In summary, this study suggests that hypoxia regulates AS of hypoxia-induced genes, hypoxia-reduced genes, and genes whose transcription is not changed by hypoxia. In addition, HIF activity, not hypoxic stress is found to be necessary and sufficient to regulate AS of a subset of hypoxia-inducible genes. Moreover, activation of endogenous HIF target genes contributes to AS of some HIF target genes. These findings significantly increase our understanding of how cells regulate gene transcription as well as RNA splicing to adapt to a hypoxic microenvironment. In the future, it will be interesting to examine the role of hypoxia in regulating RNA splicing in primary tumors.

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

Authors’ Contributions
Conception and design: J.A. Sena, L. Wang, C.-J. Hu
Development of methodology: J.A. Sena, L. Wang, C.-J. Hu
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.A. Sena, L. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.A. Sena, L. Wang, C.-J. Hu
Writing, review, and/or revision of the manuscript: J.A. Sena, L.E. Healey, C.-J. Hu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.A. Sena, C.-J. Hu
Study supervision: C.-J. Hu

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