HIFs Enhance the Transcriptional Activation and Splicing of Adrenomedullin

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Abstract

Adrenomedullin (ADM) is important for tumor angiogenesis, tumor cell growth, and survival. Under normoxic conditions, the ADM gene was found to produce two alternative transcripts, a fully spliced transcript that produces AM and PAMP peptides and an intron-3-retaining transcript that produces a less functionally significant PAMP peptide only. ADM is a well-established hypoxia inducible gene; however, it is not clear which ADM isoform is induced by hypoxia. In this study, it was determined that various cancer and normal cells express two predominant types of ADM transcripts, a AM/PAMP peptide producing full-length transcript in which all introns are removed, and a nonprotein producing I1-3 transcript in which all introns are retained. Interestingly, hypoxia preferentially induced the full-length isoform. Moreover, hypoxia-inducible factors (HIF), but not hypoxia per se, are necessary and sufficient to increase splicing of ADM pre-mRNA. ADM splicing reporters confirmed that transcriptional activation by HIF or other transcription factors is sufficient to enhance splicing. However, HIFs are more potent in enhancing ADM pre-mRNA splicing than other transcriptional activators. Thus, ADM intron retention is not a consequence of abnormal splicing, but is an important mechanism to regulate ADM expression. These results demonstrate a novel function of HIFs in regulating ADM expression by enhancing its pre-mRNA splicing. Importantly, using endogenous and cloned ADM gene, further evidence is provided for the coupling of transcription and RNA splicing.

Implications: Here, a novel function of HIFs in regulating ADM gene expression is identified by enhancing ADM pre-mRNA splicing. Mol Cancer Res; 1–14. ©2014 AACR.

Introduction

Hypoxia is a common characteristic of many solid tumors. The hypoxic intratumoral microenvironment stabilizes hypoxia-inducible transcription factor-α (HIF-1α) and -2α (HIF-2α) that are normally degraded by the 26S proteasome upon pVHL-mediated ubiquitination under normoxia. Stabilized HIF-1α and HIF-2α proteins translocate to the nucleus and heterodimerize with a constitutive nuclear protein, the aryl hydrocarbon receptor nuclear translocator (ARNT, also called HIF-1) to form HIF-1α/ARNT (HIF-1) and HIF-2α/ARNT (HIF-2) heterodimers. Then, HIF-1 and HIF-2 bind to HIF-binding sites (HBS) on HIF target gene promoters and/or enhancers and activate genes involved in neovascularization, glycolysis, cellular proliferation, and metastasis. Thus, the HIF-mediated hypoxic transcriptional response is critical for tumor progression by allowing cancer cells to adapt to a low oxygen environment (1–4).

Adrenomedullin (ADM) is a well-established hypoxia-induced gene. The ADM gene codes for a 185–amino acid proadrenomedullin peptide that is cleaved into a 52–amino acid AM peptide and a 20–amino acid peptide called "proadrenomedullin N-terminal 20 peptide" or PAMP (Fig. 1D). AM peptide plays important roles in tumorogenesis by inducing tumor angiogenesis, enhancing tumor cell proliferation, and reducing tumor cell apoptosis (5–13). However, PAMP seems to be less important in tumorogenesis because PAMP has no activity in tumor cell proliferation and survival, although PAMP is a stronger vasodilator and angiogenic factor than the AM peptide (14–16). Thus, hypoxia-induced ADM gene expression is an important component of the hypoxia response that is crucial for tumor progression and metastasis.

Interestingly, various cancer cells cultured under normoxia were found to produce two isoforms (17). One isoform is devoid of introns (full-length; FL) and produces both PAMP and AM peptides. A second isoform, in which the third intron is retained (I3), produces only the PAMP peptide due to a premature stop codon in intron 3 (17). Moreover, the relative ratio of I3/FL was increased by hypoxia, resulting in...
an increased PAMP/AM peptide ratio even though both isoforms were induced by hypoxia (17). These data suggested that hypoxia favors intron 3 retention and expression of PAMP peptide. The goal of our study is to clarify whether hypoxia favors PAMP generation and to determine how hypoxia regulates the ADM isoform ratio change.

Materials and Methods

Cell culture

Hep3B cells were cultured in MEM/EBSS (Hyclone) containing 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100,000 units/L penicillin/streptomycin, 1.5 g/L sodium bicarbonate, and 1X nonessential amino acids (NEAA). Hela cells were grown in high-glucose Dulbecco’s Modified Eagle Medium (DMEM; Hyclone) with 10% FBS, 2 mmol/L L-glutamine, 100,000 units/L penicillin/streptomycin, and 1X NEAA. HEK293T, RCC4, and RCC4T cells were grown in high-glucose DMEM (Hyclone) with 10% FBS, 2 mmol/L L-glutamine, 100,000 units/L penicillin/streptomycin, and 1X NEAA. HK2 cells were grown in keratinocyte serum-free medium (GIBCO) with 0.05 mg/mL bovine pituitary extract, 5 mg/mL recombinant EGF, 2 mmol/L L-glutamine, 100,000 units/L penicillin/streptomycin, and 1X NEAA. Human umbilical vein endothelial cell (HUVEC) cells were grown in F-12K medium (American Type Culture Collection; ATCC) containing 10% FBS with 0.1 mg/mL heparin, 0.04 mg/mL endothelial cell growth supplement (ECGS), 2 mmol/L L-glutamine, 100,000 units/L penicillin/streptomycin, and 1X 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, HK2, and HUVEC) and modified cell lines (RCC4T) were authenticated by DNA profiling or “fingerprinting” by the University of Colorado (Aurora, CO) DNA Sequencing & Analysis Core.

Figure 1. Hypoxia increases the levels of fully spliced ADM transcripts in cancer and normal cells. RT-PCR (left) and qRT-PCR (right) analysis of ADM transcripts in normoxic or hypoxic Hep3B (A), HK2 (B), and HUVEC (C) cells. FL indicates ADM transcripts in which all introns are removed. I1 refers to ADM RNA containing intron 1. I1-3 refers to ADM RNA in which all three introns are retained. In the qRT-PCR panel, the numbers next to the sample labels represent the expression ratio of ADM FL to ADM I1-3 ± SD. D, schematic diagram of the ADM gene including 4 exons (boxes) and three introns (lines). The start and stop codons for the properADM protein were indicated. The exon regions that encode the PAMP and the AM peptides are indicated by black boxes and striped boxes, respectively. E, Western blot analysis of HIF-1α, HIF-2α, β-actin, and ADM proteins in normoxic and hypoxic Hep3B cell.

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Knockdown of endogenous mRNA using siRNAs
Control (Qiagen, 1027281) or siRNAs specific for human ARNT (Qiagen, equal mix of SI00304220, SI00304234, and SI03020913), HIF1α (Qiagen, SI02664053), or HIF2α (Qiagen, SI00380212) mRNAs were transfected into Hep3B cells at 50% confluence using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol. Thirty-two hours posttransfection, cells were cultured at 21% or 1.5% O2 for 12 to 16 hours and then collected to prepare mRNA or protein for analysis.

Plasmid constructs and viral transduction
The CA9/ADM, PA11/P/ADM, and 2HRE/ADM splicing reporters were in the pcDNA3.1 (+) vector. The CA9 and PA11 promoters were cloned from CA9/Luc and PA11/P/Luc (18, 19) using Advantage GC cDNA polymerase (Clontech) and then inserted into the pcDNA3.1 plasmid, replacing the cytomegalovirus (CMV) promoter. Two copies of hypoxia-responsive element (HRE/HBSs) from the PA11 promoter were added upstream of SV40 minimal promoter in the pGL3/Luc vector to produce the 2HRE/Luc construct, from which the 2HRE/SV40 was PCR amplified and replaced the CMV promoter in the pcDNA3.1 vector. Next, the ADM gene (full-length gene) from exon 1 to exon 4 was amplified from human genomic DNA and inserted downstream of the CA9, PA11, and 2HRE promoters to yield the CA9/ADM, PA11/ADM, or 2HRE/ADM splicing reporter constructs. The CA9Pm2HRE/ADM, PA11PmHRE/ADM, and ΔHRE/ADM constructs were synthesized from the CA9P/ADM, PA11P/ADM, and the 2HRE/ADM constructs using Pfu Ultra II polymerase (Invitrogen)-mediated mutagenesis PCR, in which the HREs were mutated or deleted from the constructs. The G5/ADM reporter was generated by replacing the CA9 promoter in CA9P/ADM with the five copies of Gal4 DNA-binding elements from G5/Luc. The HIF-1α/TM-Flag, HIF-2α/TM-Flag, HIF-1α/TDBD-Flag, HIF-1α/TDBD/VP16TAD-Flag, and USF expression plasmids were described previously (18, 20). The HIF-1α/TDBD/E2FITAD-Flag construct was synthesized by adding the E2F transcription factor 1 transactivation domain from the PHKgalE2F 380-437 plasmid (gift from David Bentley, Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine; ref. 21) to the HIF-1α/TDBD construct using PCR. The Gal4DBD fusion constructs were synthesized by replacing the HIF-1α/TDBD with the Gal4 DNA-binding domain (DBD).

The HIF-1α and HIF-2α lentiviral constructs were synthesized by PCR cloning the HIF-1α TM (triple mutation) and HIF-2α TM CDS, from the plasmids described above, into the pLEX-MCS vector containing a C-terminal 2X Flag tag (Thermo Scientific). The GFP lentiviral construct was synthesized by cloning GFP cDNA (CDS) into the pLEX-MCS vector containing a C-terminal 2X Flag tag. The GFP, HIF-1α/TM, or HIF-2α/TM plasmid were cotransfected with a pSIX2 packaging vector (Addgene) and a pmD2.G envelope vector (Addgene) into HEK293T cells, grown in 10 cm dishes (40% confluent), using TransIT LT-1 transfection reagent (Mirus). Twenty-four hours after transfection, transfection media were replaced with complete Hep3B media. The following day, viral media was filtered and viral particles were concentrated using high-speed ultracentrifugation at 26,000 × g. The viral pellet was resuspended in 1 mL of PBS and added to Hep3B cells (30% confluency) for 6 hours. The following day, a second round of viral PBS was added to Hep3B cells. Twenty-four hours after the second viral transduction, Hep3B cells were harvested for RNA and protein preparation.

RNA stability assay
Hep3B cells were grown to approximately 50% confluence in 6-well plates and placed under hypoxia for 16 hours. After which, the cells were treated with 2 μg/mL of actinomycin D to inhibit de novo transcription and placed back under normoxia or hypoxia for 2, 4, or 8 hours. Following treatment, cells were collected for RNA isolation using the SurePrep Nuclear or Cytoplasmic RNA Purification Kit (Fisher BioReagents, BP2805-25). cDNA synthesis was performed and ADM FL and ADM I1-3 mRNA stability was measured using quantitative reverse transcription PCR (qRT-PCR).

ADM splicing reporter assay
ADM splicing reporter assays were performed in Hep3B cells using Lipofectamine Reagent (Invitrogen 18324-012) and PLUS Reagent (invitrogen 11514-015) to transfact plasmid DNA. Typically, 2 × 10⁵ cells per well in 6-well plates were cotransfected with 0.2 μg of splicing report construct and 1.8 μg of transcription activator. Forty-eight hours after transfection, cells were collected for mRNA and protein analysis.

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-ADM (C20) pAb (SC-16496; Santa Cruz Biotechnology), anti-Flag mAb (F3165, Sigma), anti-HIF-1α mAb (610959; BD Bioscience), anti-HIF-2α mAb (D9E3, Cell Signaling Technology), anti-ARNT mAb (NB 100-124; Novus Biological), and anti-Actin pAb (SC-1616; Santa Cruz Biotechnology), and anti-Gal4DBD pAb (SC-577; Santa Cruz Biotechnology).

RNA preparation and reverse transcription PCR or quantitative PCR
RNA was isolated from cells using the RNaseasy Plus Mini Kit (Qiagen) which removes DNA, then was reverse transcribed using the iSCRIPT Advanced cDNA Synthesis Kit (Bio-Rad) containing oligo-dT and random hexamer. mRNA levels were semiquantified by RT-PCR or quantified by qRT-PCR using IQ Sybr Green supermix (Bio-Rad) in triplicate on the CFX384 Real-Time System (Bio-Rad). 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 ΔΔCt method using

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18s rRNA and β-actin as reference genes and untreated normoxia samples, GFP lentivirus, or empty vectors (His) as controls. At least three independent experiments were performed to generate the results presented in the figures.

**Statistical analysis**

One-way ANOVA was performed unless otherwise stated. Error bars in figures indicate SD. 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**

**Hypoxia preferentially increases fully spliced ADM transcript levels in various cell lines**

The ADM gene was reported to produce two isoforms, one isoform devoid of introns (ADM FL) and a second isoform in which the third intron is retained (ADM I3; ref. 17). To determine whether hypoxia differentially regulates the levels of these ADM transcripts, RNA prepared from normoxic and hypoxic Hep3B cells was used for RT-PCR. ADM transcripts were detected using forward and reverse primers of these ADM transcripts, respectively, of the ADM gene. Interestingly, normoxic Hep3B cells, a hepatocellular carcinoma cell line, expressed multiple ADM transcripts (Fig. 1A), a fully spliced transcript in which all introns were removed (Fig. 1A, full-length), a transcript in which all three introns were included (Fig. 1A, I1-3), a transcript in which intron 1 was included (Fig. 1A, I1), and two minor isoforms in which intron 2 was included (Fig. 1A, I2) and a previously reported intron 3-containing isoform (Fig. 1A, I3; ref. 17). Interestingly, hypoxia induced the levels of ADM FL but not the other intron-containing isoforms (Fig. 1A). To better quantify the ADM isoforms, qRT-PCR was used to measure the relative ratios of the ADM FL and I1-3 transcripts because they were the dominantly expressed transcripts in Hep3B cells. Using transcript-specific qPCR primers for ADM FL (E1-E2/E3-E2, detects both full-length and I3) and ADM I1-3 (I1-E2/I2-E2, detects I1-3 only), it was found that Hep3B cells exhibited an ADM FL/I1-3 ratio of 3.28 and 15.95 under normoxia and hypoxia (Fig. 1A, bar graph), suggesting that hypoxia favors ADM FL expression even though both isoforms are induced. We also found that normoxic MDA-MB-231, Hela, and HEK293 cells expressed ADM FL, I1-3, and I1 isoforms and that hypoxia preferentially induced the levels of ADM FL in these cells (data not shown).

To assess ADM expression in relatively normal cells, HK2 cells, an HPV-16 transformed kidney cell line was analyzed. Using RT-PCR, HK2 cells were found to express the full-length, I1, and I1-3 transcripts with full-length and I1-3 being the primarily expressed transcripts under normoxia. Interestingly, hypoxia increased the ADM FL but not the I1-3 transcript levels (Fig. 1B). According to qRT-PCR, hypoxia increased the levels of the ADM FL and I1-3 transcript by 5.98- and 1.16-fold (Fig. 1B, bar graph). As a result, the ADM FL/I1-3 ratio was increased from 11.7 under normoxia to 62.16 under hypoxia.

Similarly, HUVEC cells, a primary umbilical vein vascular endothelial cell line, also exhibited splicing patterns similar to those observed in Hep3B and HK2 cells under normoxia (Fig. 1C). Furthermore, hypoxia induced the levels of ADM FL, but not I1-3 transcripts (Fig. 1C, right). Again, qRT-PCR confirmed that the levels of ADM FL transcript were increased by 6.52-fold but the levels of ADM I1-3 transcript was not induced (0.91-fold; Fig. 1B, bar graph), thus hypoxia enhanced the FL/I1-3 ratio from 5.17 under normoxia to 37.62 under hypoxia. Because ADM FL and I1-3 transcripts are the dominantly expressed transcripts in all of the cell lines tested here, the full-length and I1-3 transcripts are the focus for the remainder of this study.

The ADM FL transcript codes for a 182-amino acid pre-adrenomedullin precursor protein that is cleaved to produce a 52–amino acid AM peptide and a 20–amino acid PAMP peptide (Fig. 1D). In contrast, the ADM I3 transcript only codes for the PAMP peptide due to a premature stop codon in intron 3, whereas the ADM I1, I2, and I1-3 isoforms do not code for either the AM or PAMP peptides due to a premature stop codon in introns 1 and 2 (Fig. 1D). After establishing that hypoxia increased the expression of the ADM FL transcript, Western blot analyses for the AM peptide were performed. As expected, high levels of HIF-1α and HIF-2α proteins were detected in hypoxic but not in normoxic Hep3B cells (Fig. 1E). Importantly, the ADM precursor (pro, 22 kDa) and the AM peptide (6 kDa, doublet likely due to posttranslational modifications) derived from the ADM FL transcript were detected under normoxia. Interestingly, hypoxia significantly increased the levels of AM peptide (Fig. 1E, anti-AM). However, the levels of the ADM precursor protein were not significantly different between normoxic and hypoxic Hep3B cells (Fig. 1E, pro), likely due to its cleavage which generates the AM and PAMP peptides. These results indicated that hypoxia increases the levels of ADM FL transcript in cancer and normal cell lines.

The increased ADM FL/I1-3 ratio is due to RNA splicing but not due to differential RNA stability

Intron-retaining transcripts frequently contain premature termination codons and are targeted for nonsense-mediated decay (NMD; refs. 22, 23). As stated above, ADM I1-3, I1, I2, and I3 transcripts contain premature stop codons in introns 1, 2, and 3. These intron-retaining ADM transcripts are predicted to undergo NMD when they are exported to the cytoplasm, which might explain why they exhibit lower induction under hypoxia. To test this, Hep3B cells were placed under hypoxia for 16 hours to increase the levels of both ADM FL and I1-3, 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 and harvested for RNA isolation and cDNA synthesis. Using qRT-PCR, both ADM FL and I1-3 transcripts were found to be very unstable because both transcripts were reduced by 82% to 97% 2 hours post-actinomycin D treatment under normoxia or hypoxia (Fig. 2A). In addition, the ADM FL and I1-3 mRNA levels did not change significantly from 2 to 8 hours following actinomycin D treatment (Fig. 2A).
Interestingly, we found that the mRNA stability of ADM FL did not significantly differ between normoxia and hypoxia at 2, 4, and 8 hrs (Fig. 2A). However, hypoxia reduced the stability of ADM I1-3. For instance, ADM I1-3 was reduced by 82%, 83%, and 84% at 2, 4, and 8 hours, respectively, under normoxia but was reduced by 91% to 93% under hypoxia at 2, 4, and 8 hours (Fig. 2A). It is not clear why ADM transcripts degrade rapidly although these results are consistent with a previous report, which demonstrates similar trends in ADM mRNA stability (24). Overall, ADM I1-3 was more stable than ADM FL both under normoxia and hypoxia; therefore, these data suggest that the hypoxia-induced increase of the ADM FL/I1-3 ratio is not due to preferential destabilization of ADM I1-3 under hypoxia.

ADM I1-3 transcripts are primarily located in the nucleus

Intron-retaining transcripts frequently contain premature termination codons and are either targeted for NMD or their presence is restricted to the nucleus (22, 23). Next, the subcellular localization of ADM FL and I1-3 transcripts was determined in normoxic or hypoxic Hep3B cells by examining the levels of ADM FL and I1-3 transcripts in the nucleus or cytoplasm. First, we measured the expression of β-actin RNA (B-ACT) as a positive control for mRNA splicing and nuclear export, and as a negative control for hypoxia induction. As expected, B-ACT expression was not induced by hypoxia and a majority of the RNA was localized in the cytoplasm, with cytoplasmic to nuclear ratios of 3.05 and 3.87 under normoxia and hypoxia, respectively (Fig. 2B). As expected of a mature transcript, ADM FL was found both in the nucleus and in the cytoplasmic fractions (Fig. 2C). However, more ADM FL transcript was found in the cytoplasmic fraction, with the cytoplasmic to nuclear ratios of ADM FL being 1.75 and 2.7 under normoxia and hypoxia, respectively. In contrast, a majority of the ADM I1-3 transcript accumulated in the nucleus compared with the cytoplasm, with the cytoplasmic to nuclear ratios being 0.029 and 0.15 under normoxia and hypoxia, respectively. These data suggested that ADM I1-3 is an unspliced ADM transcript that is restricted primarily to the nucleus (Fig. 2D). In addition, we measured the total levels of ADM
transcript (using primers located in exon 4) and found that more ADM transcripts were localized to the cytoplasm, with the cytoplasmic to nuclear ratio of total ADM being 1.33 under normoxia and 1.9 under hypoxia (Fig. 2E). In addition, we did not detect a signal by qRT-PCR in our nuclear or cytoplasmic fractions in which we excluded reverse transcriptase from our cDNA synthesis (Fig. 2B–E, no reverse transcriptase), suggesting that all of our transcripts including ADM 1-3 are in fact mRNAs and not DNA contamination.

**HIF activity is required for increased splicing of ADM pre-mRNA**

*ADM* is a HIF-regulated gene; therefore, we wanted to determine whether HIF activity was necessary for the hypoxia-induced ADM FL/I1-3 ratio increase. To test this, the levels of ARNT, HIF-1α, or HIF-2α mRNAs in Hep3B cells were reduced by 75% to 87% using siRNAs (Fig. 3A). ARNT knockdown inhibited hypoxic induction of HIF-1α target genes, *LDHA* and *PGK1*, and HIF-2α target genes, *EPO* and *PAI1* (Fig. 3B). On the other hand, HIF-1α knockdown only inhibited hypoxic induction of HIF-1α target genes, whereas HIF-2α knockdown only inhibited hypoxic induction of HIF-2α target genes (Fig. 3B). Hypoxia preferentially increased the levels of the ADM FL transcript in Hep3B/control and HIF-2α siRNA cells (Fig. 3C), whereas knockdown of ARNT and HIF-1α dramatically inhibited the hypoxic induction of the ADM FL mRNA (Fig. 3C). Using qRT-PCR, we found that hypoxia induced the expression of both the ADM FL and I1-3 transcripts but favored the induction of the ADM FL transcript in Hep3B/control siRNA cells (Fig. 3D). Interestingly, ARNT knockdown reduced the hypoxic induction of both the ADM FL and I1-3 transcripts and also reduced the ADM FL/I1-3 ratio to 7.4 versus 11.4 observed in hypoxic Hep3B/control siRNA cells. In addition, HIF-1α and HIF-2α knockdown also decreased the hypoxia induction of ADM FL and I1-3 transcripts; however, individual knockdown of either HIF-1 or HIF2 did not significantly

![Figure 3. HIF activity is required for hypoxia-induced splicing of ADM pre-mRNA. A, qRT-PCR analysis of ARNT, HIF-1α, and HIF-2α mRNA levels in normoxic and hypoxic Hep3B cells targeted with control, ARNT, HIF-1α, or HIF-2α siRNAs. B, qRT-PCR analysis of the levels of HIF-1 target genes, LDHA and PGK1, and HIF2 target genes, EPO and PAI1, in normoxic and hypoxic Hep3B cells targeted with control, ARNT, HIF-1α, or HIF-2α siRNAs. C, RT-PCR analysis of ADM transcripts in normoxic and hypoxic Hep3B cells targeted with control, ARNT, HIF-1α, or HIF-2α siRNAs. D, qRT-PCR analysis of ADM FL, I1-3, and total transcripts in normoxic and hypoxic Hep3B cells targeted with control, ARNT, HIF-1α, or HIF-2α siRNAs.](image-url)
alter the ADM FL/I1-3 ratio. For instance, HIF-1 knockdown reduced the ADM FL/I1-3 ratio to 9.9, whereas HIF2 knockdown increased the ADM FL/I1-3 ratio to 12.9. This is likely because ADM is a common HIF-1 and HIF-2 target gene. These data suggested that HIF activity, but not hypoxia per se, is necessary for hypoxia-induced pre-mRNA splicing of ADM.

**HIF activity is sufficient for increased splicing of ADM pre-mRNA**

Next, we determined whether HIF activity is sufficient for increased splicing of ADM pre-mRNA. To test this, Hep3B cells were transduced with lentiviruses expressing normoxia active, Flag-tagged HIF-1α, HIF-2α, or GFP. Western blot analysis using an anti-Flag antibody detected expression of HIF-1α, HIF-2α, and GFP proteins in virus-infected Hep3B cells under normoxia (Fig. 4A). In addition, HIF-1α and HIF-2α proteins were functional under normoxia because HIF-1α target genes, LDHA and PGK1, and HIF-2α target genes, EPO and PAI1, were induced in HIF-1α– or HIF-2α–transduced cells compared with the GFP-transduced cells (Fig. 4B). Importantly, both HIF-1α and HIF-2α increased the levels of ADM FL, but not I1-3, as determined by RT-PCR (Fig. 4C). qRT-PCR also determined that both HIF-1α and HIF-2α induced the expression of ADM FL 4.8- and 6.2-fold, respectively, and also induced the expression of ADM I1-3 2.3- and 2.35-fold, respectively (Fig. 4C, left). Thus, HIF-1α or HIF-2α transduction increased the ADM FL/I1-3 ratio to 19.36 or 25.19 versus 4.19 for the GFP-transduced cells.

To further validate that HIFs are sufficient to promote splicing of ADM pre-mRNA, we used RCC4 cells, a renal cell carcinoma cell line that expresses constitutively active HIF-1α and HIF-2α protein even under normoxia due to mutation of the VHL gene. In addition, we used RCC4T cells in which functional pVHL is reintroduced into RCC4 cells and therefore HIF proteins are only active under hypoxia (25). RCC4T cells expressed both ADM FL and I1-3 transcripts (Fig. 4D); moreover, hypoxia preferentially induced the levels of the ADM FL transcript (Fig. 4D).

**Figure 4.** HIF activity is sufficient to promote splicing of ADM pre-mRNA. A, Western blot analysis of Flag-tagged HIF-1α, HIF-2α, and GFP proteins in Hep3B cells transduced with lentivirus expressing normoxia active Flag-tagged HIF-1αTM, or normoxia active HIF-2αTM or GFP proteins under normoxia. B, qRT-PCR analysis of HIF-1 target genes, LDHA and PGK1, and HIF2 target genes, EPO and PAI1 in the above described cells. C, RT-PCR (left) and qRT-PCR (right) analysis of ADM FL and I1-3 transcripts in the above described cells. The ADM FL/I1-3 ratio is indicated next to the graph legend. D and E, RT-PCR (D) and qRT-PCR (E) analysis of ADM transcripts in normoxic and hypoxic RCC4 or RCC4T cells with ADM FL/I1-3 ratio indicated next to the graph legend.
However, RCC4 cells mainly expressed the ADM FL transcript under both normoxia and hypoxia (Fig. 4D). Using qRT-PCR, hypoxia was found to induce the expression of ADM FL and I1-3, 10- and 1.4-fold above normoxic RCC4 cells, respectively, and also increased the ADM FL/I1-3 ratio to 54.16 versus 8.72 in normoxic RCC4 cells (Fig. 4E). However, hypoxia only weakly increased the ADM FL/I1-3 ratio in RCC4 cells because ADM FL was already favored even under normoxia. For instance, the ADM FL/I1-3 ratio was 53.41 under normoxia and 67.11 under hypoxia. These findings supported the idea that HIFs are sufficient to increase splicing of ADM pre-mRNA.

ADM splicing reporters recapitulate splicing changes observed for the endogenous ADM gene

After determining the relationship between ADM gene activation and increased intron removal using the endogenous ADM gene, we wanted to see whether HIF-mediated transcription activation of an ADM splicing reporter would result in increased ADM splicing. To test this idea, the full-length ADM gene (exons 1–4 including introns) was cloned and placed downstream of the CA9 promoter, a HIF-1 target gene promoter, or the PAI1 promoter, a HIF-2 target gene promoter, or an artificial hypoxia/HIF-induced promoter containing two HREs (2HRE) and the SV40 minimal promoter (Fig. 5A). In addition, splicing reporters in which the HREs were mutated (mHRE) or deleted (ΔHRE) were generated. These ADM splicing reporters were then cotransfected into Hep3B cells with empty vector expressing a His tag (His) or with normoxia active HIF-1α or HIF-2α expression plasmids. All of the splicing reporters expressed two major transcripts, a transcript in which introns 1–3 were removed (intron skipping or IS, to distinguish from the endogenous full-length transcript) and a second transcript in which introns 1–3 were retained (intron retaining, IR; Fig. 5B and C). The ADM IS and IR transcripts corresponded to endogenous ADM FL and ADM I1-3, respectively, demonstrating that the splicing reporters recapitulated the expression patterns of the endogenous ADM gene.

Figure 5. HIFs regulate splicing of ADM splicing reporters. A, diagram of ADM splicing reporters. Arrows represent primers used for RT-PCR and qRT-PCR. Forward primer in the promoter region (CA9P, PAI1P, or 2HRE) and ADM RT-PCR primer (in E4) were used in RT-PCR. CA9P, PAI1P, or 2HRE primers in conjunction with ADMIS, ADMIR, or ADM total were used in qRT-PCR. B, RT-PCR (top) and qRT-PCR (bottom) detection of ADM transcripts in normoxic Hep3B cells transfected with CA9P/ADM or CA9Pm2HRE/ADM and with His tagged empty vector control (His). HIF-1α or HIF-2α expression plasmids. C, RT-PCR (top) and qRT-PCR (middle and bottom) analysis of ADM transcripts in normoxic Hep3B cells transfected with PAI1P/ADM, PAI1PmHRE/ADM, 2HRE/ADM, or ΔHRE/ADM splicing reporters and empty vector (His), HIF-1α or HIF-2α expression plasmids.
Using reporter-specific primers and RT-PCR, we found that cotransfected HIF-1α activated the CA9P/ADM reporter and increased the levels of the ADM IS transcript but not the ADM IR transcript (Fig. 5B). Cotransfected HIF-2α also increased the levels of the ADM IS transcript to a lesser extent than HIF-1 (Fig. 5B). In contrast, the CA9Pm2HRE/ADM reporter was not activated by HIF-1 or HIF-2 nor were the ADM IS and IR transcripts induced (Fig. 5B). qRT-PCR confirmed that HIF-1α induced the expression of ADM IS and IR by 16- and 2.6-fold, respectively. Similarly, HIF-2α induced the expression of the ADM IS and IR transcripts by 4- and 1.7-fold, respectively. Moreover, HIF-1α or HIF-2α increased the ADM IS/IR ratio to 4.22 or 2.23, respectively, versus 0.93 for the empty vector (His; Fig. 5B, bar graph). In contrast, the CA9Pm2HRE/ADM reporter was not induced by HIF-1α or HIF-2α, nor did HIF-1α or HIF-2α alter the ADM IS/IR ratio.

Next, similar experiments were performed on ADM splicing reporters driven by the PAI1 promoter (PAI1P/ADM and PAI1PmHRE/ADM). Using reporter-specific primers and RT-PCR, we found that HIF-1α activated the PAI1P/ADM reporter and increased the levels of the ADM IS, but not the IR transcript (Fig. 5C, top). Similarly, HIF-2α also increased the levels of ADM IS but not IR (Fig. 5C, top). In contrast, the PAI1PmHRE/ADM reporter was not activated by HIF-1α or HIF-2α and nor did HIF change the levels of ADM IS and IR transcripts (Fig. 5C, top). Using qRT-PCR, we found that HIF-1α induced the levels of ADM IS and IR by 2.8- and 1.3-fold, respectively (Fig. 5C, middle), whereas HIF-2α increased the levels of the ADM IS and IR transcripts by 18- and 3.3-fold, respectively. As a result, HIF-1α or HIF-2α increased the ADM IS/IR ratio to 9.85 or 19.73, respectively versus 3.31 for the His vector (Fig. 5C, middle). In contrast, the PAI1PmHRE/ADM reporter was not activated by HIF-1α or HIF-2α and nor did HIF-1α or HIF-2α significantly alter the ADM IS/IR expression ratio (Fig. 5C, middle left).

Finally, we utilized ADM splicing reporters driven by the SV40 minimal promoter in which two HREs from the PAI1 promoter were added to produce an artificial HIF/hypoxia-responsive promoter (2HRE/ADM and ΔHRE/ADM). Using reporter-specific primers and RT-PCR, we found that both HIF-1α and HIF-2α activated the 2HRE/ADM reporter and increased the expression of the ADM IS transcript (Fig. 5C, top left). In contrast, the ΔHRE/ADM reporter was not activated by HIF-1α or HIF-2α and therefore the ADM IS and IR transcripts were not induced (Fig. 5C, top left). Again, qRT-PCR confirmed that HIF-1α induced the expression of ADM IS 4-fold but reduced the expression of ADM IR 1.4-fold (Fig. 5C, bottom right). Moreover, HIF-2α induced the expression of the ADM IS and IR transcripts 17- and 1.2-fold, respectively (Fig. 5C, bottom right). HIF-1α and HIF-2α increased the ADM IS/IR ratio to 4.55 and 11.28, respectively versus 0.5 for the His vector (Fig. 5C, bottom right). In contrast, the ΔHRE/ADM reporter was not activated by HIF-1α or HIF-2α, nor did HIF-1α or HIF-2α significantly alter the ADM IS/IR ratio (Fig. 5C, bottom left). Taken together, these data showed that splicing of the ADM splicing reporters can recapitulate the HIF-dependent splicing changes observed for the endogenous ADM gene. These data further supported our conclusion that HIF activity is sufficient to promote splicing of ADM pre-mRNA. In addition, these data suggested that transcription activation of ADM is necessary to promote splicing of ADM pre-mRNA because mutation or deletion of HREs in the reporters prevented transcription activation and also the increase in the ADM IS/IR ratio.

**The transactivation domain of HIF-α protein is not required for increased RNA splicing of the ADM splicing reporter**

Some transcription factors have dual roles in RNA splicing and gene transcription (26–28). To determine whether the activation domain of HIF-α protein is important for ADM pre-mRNA splicing, we constructed hybrid proteins that contained the HIF-1α DBD and ARNT dimerization domain, fused to the transactivation domains from the E2F1 or VP16 transcription factors (Fig. 6A). To facilitate the detection of these hybrid proteins, 2X Flag tag was added at the C-terminuses of these constructs (Fig. 6A). As determined by anti-Flag Western blot analysis, the HIF-1αDBD, HIF-1αDBD/E2F1TAD, and HIF-1αDBD/VP16TAD expression plasmids expressed higher levels of proteins than the normoxia active HIF-1α and HIF-2α expression plasmids even though the same amount of plasmid DNAs were transfected (Fig. 6B). However, the CA9P/ADM reporter was activated similarly by HIF-1α (2.2-fold) and HIF-1αDBD/E2F1 (2.1-fold), whereas the HIF-2α and HIF-1αDBD/VP16 weakly activated the CA9P/ADM reporter (Fig. 6C, ADM total). Interestingly, HIF-1α was more potent in increasing the ADM IS/IR ratio than HIF-1αDBD/E2F1, increasing the ADM IS/IR ratio to 4.37, whereas HIF-1αDBD/E2F1 increased the ADM IS/IR ratio to 2.47 versus 0.94 for the His control, although the constructs were able to activate the reporter similarly (Fig. 6C). In addition, HIF-2α and HIF-1αDBD/VP16 only increased the ADM IS/IR ratio slightly (Fig. 6C).

In addition, the above described fusion constructs were able to activate the PAI1P/ADM reporter in Hep3B cells (Fig. 6D, ADM total). HIF-1α activated the reporter 1.96-fold, whereas HIF-2α activated the reporter more than any other expression plasmid, 8.84-fold. Interestingly, even the HIF-1αDBD slightly activated this reporter, 1.67-fold. In addition, the HIF-1αDBD/E2F1 and HIF-1αDBD/VP16 activated the reporter 2.17- and 2.35-fold, respectively (Fig. 6D, ADM total). In addition, HIF-1α and HIF-2α were able to increase the ADM IS/IR ratio to 15.09 and 23.4 versus 7.5 for the His control (Fig. 6D). Although HIF-1αDBD activated the reporter slightly, it did not significantly change the ADM IS/IR splicing ratio (7.7) compared with the His vector (7.5). On the other hand, HIF-1αDBD/E2F1 and HIF-1αDBD/VP16 increased the ADM IS/IR splicing ratio to 13.3 and 9.85, respectively, versus 7.5 for the His control (Fig. 6D). Furthermore, the fusion constructs were also able to activate and promote splicing of endogenous ADM even under normoxia (data not shown). In summary, these data...
suggested that other transcription factors are also able to promote splicing of the ADM pre-mRNA and the transactivation domain of the HIF-α protein is replaceable for increased RNA splicing of the ADM splicing reporter.

**Activation of endogenous HIF target genes is not absolutely required for increased splicing of the ADM splicing reporter**

As stated above, transcription activation by HIF-1 or HIFDBD hybrid constructs is sufficient to promote splicing of the ADM pre-mRNA. However, both HIF and the fusion constructs can activate endogenous HIF target genes; therefore it is possible that HIF target genes may be responsible for promoting splicing of ADM pre-mRNA. To rule out or confirm this possibility, we utilized an ADM splicing reporter driven by the PAI1 promoter (PAI1P/ADM), which contains an HRE and also binding sites for the upstream stimulatory factor 2 (USF2; ref. 18). In contrast with HIFs and the HIF fusion constructs, USF2 does not activate endogenous HIF target genes. To test this, normoxic Hep3B cells were cotransfected with the PAI1P/ADM splicing reporter and either HIF-2α or USF2 expression plasmid. As determined by qRT-PCR using primers that detect endogenous and transfected HIF-2α or USF2 RNA, we found that HIF-2α transfection increased HIF-2α mRNAs levels 18.31-fold and USF2 transfection increased USF2 levels 105.94-fold above the His vector (Fig. 6E, top). As determined by qRT-PCR using reporter-specific primers that detect total reporter-specific ADM transcripts, we found that HIF-2α activated the PAI1P/ADM reporter 3-fold, whereas USF2 activated the reporter 2-fold compared with the His vector (Fig. 6E, bottom). Also, HIF-2α induced the expression of ADM IS and IR 11.4- and 1.7-fold, respectively, whereas USF2 induced the expression of ADM IS and IR 5.2- and 1.9-fold, respectively. Thus, HIF-2α or USF2 increased the ADM IS/IR ratio to 9.13 or 3.71 versus 1.42 for the His control (Fig. 6E, bottom).
To further validate that transcription activation of ADM was sufficient to promote splicing of ADM pre-mRNA, the ADM splicing reporter was placed under the control of a promoter containing five copies of the Gal4 DNA-binding element (Fig. 7A). In addition, fusion constructs containing the Gal4 DBD fused to the transactivation domains of normoxia active HIF-1α, normoxia active HIF-2α, VP16, or E2F1 were generated (Fig. 7A). Therefore, the Gal4 DBD fusion constructs are expected to activate the G5P/ADM splicing reporter but not endogenous ADM target genes. Next, we cotransfected Hep3B cells with the G5P/ADM splicing reporter and the Gal4 DBD fusion constructs. An anti-gal4 Western blot analysis indicated that the fusion constructs expressed proteins as expected (Fig. 7B). Using qRT-PCR to assess activation of the G5P/ADM reporter, we found that the Gal4 DBD did not significantly activate the reporter above the His control (Fig. 7C, ADM total). In contrast, Gal4/HIF-1αTAD, HIF-2αTAD, VP16TAD, and E2F1TAD were able to activate the reporter 16.4-, 4.05-, 71.4-, and 66.75-fold, respectively (Fig. 7C, ADM total). Moreover, the Gal4 DBD expression plasmid did not alter the expression of ADM IS and IR transcripts and did not change the ADM IS/IR ratio (Fig. 7C). However, the Gal4/HIF-1αTAD expression plasmid increased the expression of ADM IS and IR by 80.95- and 3-fold and also increased the ADM IS/IR ratio to 39.4 (Fig. 7C). Moreover, the Gal4/E2F1TAD expression plasmid increased the expression of ADM IS and IR by 255.13- and 8.03-fold and increased the ADM IS/IR ratio to 52.12 (Fig. 7C). Taken together, these data further confirmed that transcriptional activation of ADM is sufficient to enhance intron removal in the absence of endogenous HIF target gene activation.

Discussion

Martinez and colleagues reported that the ADM gene expresses full-length and I3-containing isoforms in normoxic cell lines (17). By analyzing ADM RNA transcripts using RT-PCR, we found that normoxic cancer cells express fully spliced (full-length) and several intron-retaining RNA transcripts including I1, I2, I3, and I1-3 isoforms (Fig. 1). Furthermore, the full-length and I1-3 isoforms, but not the I3 isoform, are the predominate isoforms expressed in normoxic cells (Figs. 1 and 4). To our knowledge, this study is the first to report that the ADM gene expresses I1-3, I1, and I2 isoforms in normoxic cells. Importantly, in addition to cancer cells, we found that intron-retaining ADM isoforms are expressed in normal human tissues (data not shown) and normal HUVEC cells, suggesting that expression of intron-retaining isoforms is not a consequence of abnormal splicing, but a normal control mechanism of gene expression.

ADM is a well-established HIF target gene (25, 29). However, it was reported that the I3 but not the full-length isoform was preferentially induced by hypoxia (17). Because the ADM FL RNA codes for a more functionally important AM peptide than the ADM I3 RNA, it is puzzling that I3 is preferentially induced by hypoxia. Thus, we investigated ADM isoform expression in hypoxic cells. Interestingly, we found that hypoxia preferentially induces the levels of ADM FL over intron-containing ADM transcripts. It is not clear whether this discrepancy is due to different cell lines used or other differences.
To address the molecular mechanism concerning preferential induction of ADM FL RNA under hypoxia, we first tested the hypothesis that ADM I1-3 transcripts are selectively degraded during hypoxia. However, we found that ADM FL is even less stable than the ADM I1-3 isoform, suggesting that differential transcript stability does not account for the hypoxia-induced ADM FL/I1-3 ratio increase. Furthermore, we found that the both ADM I1-3 and full-length transcripts are very unstable both under normoxia and hypoxia (Fig. 2A), a result consistent with a previous report that demonstrates similar patterns of ADM mRNA degradation (24). Because there are significant amounts of ADM FL and I1-3 in the nucleus, one possibility is that the ADM FL and ADM I1-3 transcripts undergo Traf4/Trf5-Air1/Air2-Mtr4 (TRAMP)-mediated polyadenylation and RNA degradation by the nuclear exosome complex (30–32). In the future, it will be interesting to address why ADM transcripts are so unstable.

After establishing that increased splicing of ADM pre-mRNA is responsible for the increased ADM FL/I1-3 ratio under hypoxia, using the endogenous ADM gene (Figs. 3 and 4) as well as ADM splicing reporters (Fig. 5), we determined that HIF activity, but not hypoxia per se, is necessary and sufficient to regulate hypoxia-induced splicing of ADM pre-mRNA. Furthermore, we determined that transcription activation of ADM is necessary and sufficient to regulate splicing of ADM pre-mRNA independent of endogenous HIF target gene activation (Figs. 6E and 7). Thus, our data demonstrated that ADM gene transcription and pre-mRNA splicing are coupled. Although it is well established that transcription and splicing are functionally coupled (33–35), these conclusions are mainly derived from studies using chimeric splicing reporters in which an alternatively spliced exon is placed between two constitutive exons of a different gene. To our knowledge, this is the first report that utilizes endogenous and cloned genes in the same study as models to demonstrate functional coupling of transcription and RNA splicing.

In addition, our data indicated that transcription activation strength contributes to splicing of ADM pre-mRNA. For instance, HUVEC cells exhibit an ADM FL/I1-3 splicing ratio of 5.17 (Fig. 1C) under normoxia. In contrast, Hep3B cells exhibit an ADM FL/I1-3 ratio of 3.28 under normoxia (Fig. 1A). Interestingly, basal level expression of the ADM FL and the ADM I1-3 isoforms are 2.62- and 2.89-fold higher in HUVECs than in Hep3B cells (data not shown). In addition, the HIF-2α virus is a stronger inducer of ADM gene transcription and increases the ADM FL/I1-3 ratio more than the HIF-1α virus (Fig. 4C). Further support is garnered from our ADM splicing reporter studies. For instance the ADM IS/IR ratio is 0.5 under basal conditions (His) for the 2HRE/ADM reporter, whereas the ADM IS/IR ratio is 3.31 for the PAI1P/ADM reporter under basal conditions (His; Fig. 5C). Interestingly, the basal expression of PAI1P/ADM reporter is 2.8-fold higher than the basal expression of the 2HRE/ADM reporter (data not shown). Moreover, higher ADM IS/IR expression ratios correlate with higher levels of reporter activation. For instance, if HIF-1α activates the reporter more than HIF-2α, HIF-1α also increases the ADM IS/IR ratio more than HIF-2α (Fig. 5B) or if HIF-2α activates the reporter more than HIF-1α, HIF-2α also increases the ADM IS/IR ratio more than HIF-1α (Fig. 5C). In summary, these findings indicate that transcription activation strength regulates pre-mRNA splicing efficiency of ADM independent of promoter differences. These findings are novel and in contrast with what have been observed for alternatively spliced genes. For instance, previous studies suggest that differences in promoter structure lead to differences in alternative splicing of particular transcripts; however, transcription activation strength is not responsible for this effect (35–37). It is not clear why our results differ from previous studies. We speculate that these differences may be due to the fact that we studied intron removal, whereas previous studies assessed alternative exon inclusion. Moreover, although previous studies compared different transcription factors in regulating alternative splicing, we compared basal level expression of ADM transcripts in different cell lines. In fact, if we compared HIF with other transcription factors in regulating ADM splicing (Figs. 6C and D and Fig. 7C), our data are in agreement with previous studies and suggest that activation strength does not determine the efficiency of ADM splicing (see below).

Although transcription activation and activation strength influence ADM pre-mRNA splicing, HIFs are more potent than other transcriptional activators in promoting intron removal under condition in which both endogenous HIF target genes are activated or not activated. For example, HIF-1α is more potent than HIF-1αDBD/E2F1 in inducing ADM IS from the CA9P/ADM splicing reporter despite similar activation of the reporter by the two activators (Fig. 6C). Furthermore, Gal4/HIF-1αTAD is also a stronger inducer of ADM IS than Gal4/VP16TAD or Gal4/E2F1TAD although Gal4/VP16TAD or E2F1TAD activates the G5/ADM reporter more than Gal4/HIF-1αTAD (Fig. 7C). It is not clear why HIFs are more efficient than other transcription factors in increasing ADM pre-mRNA splicing. On the basis of the recruitment model of RNA splicing (38–40), we speculate that HIF may have stronger activity than other transcription factors in recruiting splicing factors to HIF target gene promoters. So far, HIFs have not been reported to interact with splicing factors such as serine-arginine rich proteins or heterogeneous nuclear ribonucleoproteins or with components of the spliceosome. However, HIFs have been shown to recruit cotranscription factors that can promote splicing. For example, studies indicated that BRAHMA (BRM), a component of the SWI/SNF chromatin remodeling complex, can act as a coactivator for HIF target genes (41). Moreover, a separate study showed that BRM could regulate alternative splicing by slowing the rate of RNA Pol II elongation (42). Therefore, it is possible that HIFs are more efficient than other transcription factors in recruiting BRM to the ADM promoter and regulate splicing by altering the elongation rate of RNA Pol II (38–40, 43).

In summary, we demonstrate for the first time that HIFs enhance pre-mRNA splicing of ADM in addition to regulating ADM transcription. In the future, it will be interesting...
to assess whether other HIF target genes also express intron-retaining or exon-skipping isoforms under normoxia and whether HIF promotes RNA splicing of additional HIF target genes. In addition, it will be interesting to know whether activation of other HIF target genes will lead to increased RNA splicing independent of endogenous HIF target gene activation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.A. Sena, M.R. Pawlus, C.-J. Hu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.A. Sena, L. Wang, C.-J. Hu

References


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, 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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