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Cell type-specific reciprocal regulation of *HIF1A* gene expression is dependent on 5′- and 3′-UTRs

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ABSTRACT

In the present study, we demonstrated the reciprocal regulation of hypoxia-inducible factor 1 alpha (*HIF1A*) gene expression via untranslated region-(UTR) dependent mechanisms. A 151 nucleotide sequence found in the *HIF1A* 5′-UTR is sufficient for significant translational up-regulation. On the other hand, the 3′-UTR of *HIF1A* has been implicated in mRNA degradation. In the non-metastatic breast cancer cell line MCF7, the 3′-UTR-dependent down-regulatory machinery predominates over the 5′-UTR-dependent up-regulation of *HIF1A*. However, 5′-UTR-dependent up-regulation is dominant among metastatic cell lines (MDA-MB453, U87MG). It is therefore likely that the predominance of 5′-UTR-dependent translational enhancement of *HIF1A* is critical for the malignant phenotype of cancer cells. PTBP-1, but not HuR, is a candidate RNA binding protein for the translational control of *HIF1A*.

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1. Introduction

Hypoxia-inducible factor 1 alpha (HIF-1α) is a key transcription factor responsible for the induction of genes that facilitate adaptation to local and general reduction of oxygen tension [1]. The HIF-1 heterodimer (HIF-1α and HIF-1β) recognizes a consensus element designated as hypoxia responsible element (HRE) within the enhancer regions of target genes that are implicated in many different cellular functions such as cell survival, proliferation, apoptosis, glucose metabolism and angiogenesis [2,3]. In the microenvironment of most cancers, conditions are hypoxic because the oxygen tension is not well maintained due to structurally or functionally inadequate vasculature. Therefore, HIF-1α expression-dependent angiogenesis plays a critical role for cancer growth; high expression of HIF-1α is found in malignant solid tumors, but not in normal tissues or slowly growing tumors [4,5].

In human tissues, mRNA expression of *HIF1A* is ubiquitous [6]; however, HIF-1α protein is not detectable under normoxic conditions because the protein has a very short half-life (less than 10 min) [7]. Under normoxic conditions, prolyl and asparagyl residues of HIF-1α are hydroxylated by a family of prolyl-hydroxylase domain-containing proteins (PHDs). Hydroxylated HIF-1α is a target of the Von Hippel–Lindau syndrome (VHL) protein, the recognition factor of a ubiquitin E3 ligase [8,9]. During hypoxia, hydroxylation is immediately inhibited and followed by robust stabilization of HIF-1α. It is widely accepted that silencing or mutation of the *VHL* gene activates HIF-1-dependent gene expression and some cancer cells harboring these defects exhibit aggressive phenotypes, such as increased cell motility [10].

HIF-1α expression is also regulated at the post-transcriptional level. Several studies suggested a possible contribution of the 5′-untranslated region (UTR) and the 3′-UTR of *HIF1A* for mRNA turnover and translation but those reports are controversial [11–14]. It seems likely that a candidate sequence for the internal ribosome entry site (IRES) in the 5′-UTR of *HIF1A* might represent an up-regulatory factor. On the other hand, AU-rich elements (ARE) that are found in the 3′-UTR of *HIF1A* mRNA might function as a destabilizer. It is paradoxical that a single transcribed mRNA contains both up- and down-regulatory cis-elements in the 5′- and 3′-UTRs. In the present study, we demonstrated that the 5′-UTR-dependent up-regulatory function is predominant in malignant cancer cells such as malignant glioma. We also found that the 3′-UTR-dependent down-regulatory function is significant in relatively benign cancer cell lines, such as MCF7 (breast cancer). It seems likely that the balance between 5′- and 3′-UTR dependent regulation of *HIF1A* could have implications for cancer cell phenotypes.

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2. Materials and methods

2.1. Cell culture and transfection

HeLa, MCF7, MDA-MB453, U87MG and H1299 cell lines were obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were seeded in 24-well plates and transfected with 0.8 μg of DNA per well using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and were harvested 24 h after transfection for analysis. To mimic hypoxic conditions, cells were exposed to DMEM containing 200 μM $CoCl_2$ for 16 h.

2.2. Plasmid construction and luciferase reporter assay

Full length *HIF1A* (the 294 bp of the 5′-UTR, the 2481 bp of the coding region and the 1195 bp of the 3′-UTR) was cloned into pcDNA3 (Invitrogen, Carlsbad, USA) and designated as pcDNA HIF 5C3. pcDNA HIF 5C lacked the 3′-UTR and pcDNA HIF C3 lacked the 5′-UTR of *HIF1A*. pcDNA HIF C contained only the coding region. pGL-HRE contained the pGL3 basic vector (Promega, Madison, USA) backbone and four HRE (GGGTACGTGCTGTACGTGCTGTACGTGCT GTACGTGCTG) sequences. The coding region of luciferase was cloned into pcDNA plasmid (pcDNA LUC). Several 5′-UTR sequences of *HIF1A* (294 bp, 257 bp and 151 bp) and the IRES from the foot-and-mouth disease virus (FMDV) were cloned upstream from the luciferase coding sequence of pcDNA LUC (pcDNA 5UTR LUC_294, pcDNA 5UTR LUC_257, pcDNA 5UTR LUC_151 and pcDNA FMDV LUC). Two independent 3′-UTR sequences (*c-MYC*: BC000141 and *HIF1A*: NM 001530) were cloned downstream from the luciferase sequence of the pmir GLO vector (Promega, Madison, USA) that contains both firefly luciferase and *Renilla* luciferase sequences. Coding sequence of *PTBP1* (GenBank accession no. NM 002819) and *HuR*/*ELAVL1* (GenBank accession no. NM 001419) were cloned into pcDNA3 (Invitrogen, Carlsbad, USA).

Luciferase constructs or HRE luciferase reporter plasmids, except pmir GLO constructs, were transfected with reference *Renilla* luciferase plasmids (pGL 4.72, Promega), and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega, Madison, USA) according to the manufacturer's instructions.

2.3. RT-PCR and Real Time PCR

Total RNA was extracted by CellAmp Direct RNA Prep Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. Then, cDNAs were synthesized using PrimeScript RT Reagent Kit (Takara, Otsu, Japan). Target sequences were amplified by use of the following four primer sets: (1) firefly luciferase, forward primer-TTC GAC CGG GAC AAA ACC AT and reverse primer-GGG ATG ATC TGG TTG CCG AA; (2) *Renilla*-luciferase, forward primer-GGG CGA GAA AAT

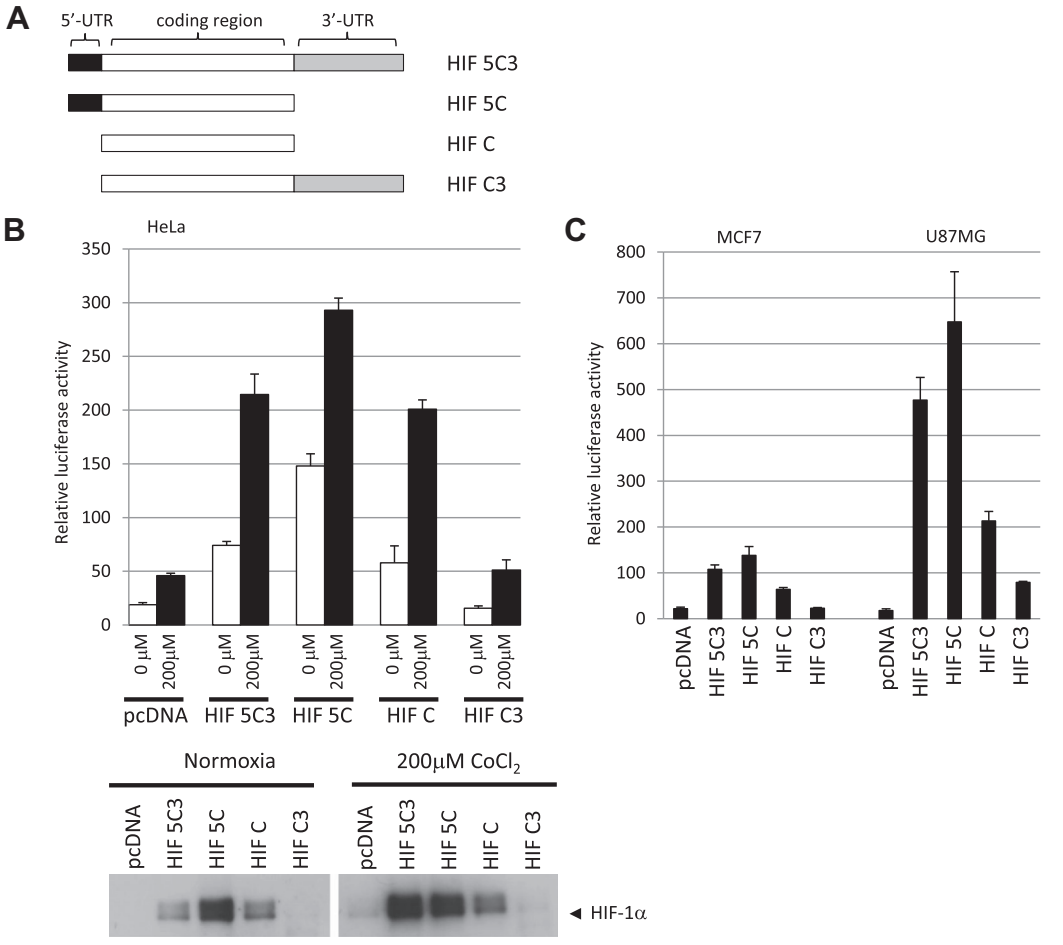

Fig. 1. Expression of *HIF1A* is regulated in both 5′- and 3′-UTR-dependent mechanisms. (A) Schematic illustration of *HIF1A* constructs. (B) UTR sequence-dependent control of *HIF1A* expression in HeLa cells. HeLa cells were transfected with pGL-HRE, pGL4.72 and the indicated *HIF1A* construct. Expression (relative light units) in HIF C3-transfected HeLa cells was only 25% of that for HIF C (coding region only construct) under both normoxic and hypoxia-mimicking conditions (200 μM $CoCl_2$ for 16 h). Cell lysates were made and luciferase activity was measured using a Dual-Luciferase reporter assay system. Error bars represent standard deviations for triplicate wells in a single experiment; data are representative of three independent experiments. Total cell lysates of transfected HeLa cells were blotted with anti-HIF-1α monoclonal antibody. (C) None-metastatic breast cancer MCF7 cells and malignant glioblastoma U87MG cells were transfected with pGL-HRE, pGL4.72 and the indicated *HIF1A* construct. MCF7 and U87MG cells demonstrated different UTR-dependent expression control. Error bars represent standard deviations for triplicate wells in a single experiment.

GGT GCT TG and reverse primer-GGA GAG GGT AGG CCG TCT AA; (3) beta-actin, forward primer-AAC ACC CCA GCC ATG TAC GT and reverse primer- AGT ACT TGC GCT CAG GAG GA); and (4) neomycin resistant gene, forward primer-ACA ATC GGC TGC TCT GAT and reverse primer-CAG TGA CAA CGT CGA GCA. Real Time PCR experiments were performed by KAPA SYBR FAST qPCR kit (KAPA BIOSYSTEMS, Boston, USA) according to the manufacturer's instructions.

2.4. Western blot analysis

Cells were lysed in 1× SDS PAGE sample buffer and subjected to Western blotting analysis. Anti-HIF-1α monoclonal antibody (BD Bioscience, Franklin Lakes, USA) and anti-luciferase monoclonal antibody (Sigma–Aldrich, St. Louis, USA) were used as primary antibodies. Immobilon Western blotting detection reagents (Millipore, Billerica, USA) were used for signal detection.

3. Results

Schematic illustrations of *HIF1A* constructs are shown in Fig. 1A. A construct designated as HIF 5C3 contained both the 5′-UTR (294 bp) and the 3′-UTR (1195 bp). HIF 5C contained the 5′-UTR plus the coding region, whereas HIFC3 contained the coding region and the 3′-UTR. HIF C contained only the coding region of *HIF1A*. Those *HIF1A* plasmids together with HRE luciferase reporter and *Renilla*-luciferase plasmid were introduced into HeLa cells. As indicated in Fig. 1B, HIF 5C3 or HIF 5C expressed a significantly larger amount of HIF-1α protein under normoxic and hypoxia-mimicking conditions. On the other hand, only modest expression of HIF-1α protein was observed in HIF C3 transfected cells. We next introduced the same plasmids into the malignant glioblastoma cell line U87MG and the non-metastatic breast carcinoma cell line MCF7. Only U87MG cells showed accelerated *HIF1A* expression via a 5′-UTR-dependent mechanism among the three cell lines (Fig. 1C). It should be noted that all constructs contained the same promoter sequence.

To investigate the nature of the 5′-UTR-dependent mechanism that regulated expression, we cloned several segments of the 5′-UTR in the 5′ region of the luciferase gene that was driven by the same CMV promoter (Fig. 2A). As a control for the typical IRES, the IRES sequence of bovine foot-and-mouth disease virus (FMDV) was also cloned (Fig. 2A). RT-PCR and real time PCR experiments (data not shown) indicated that comparable amounts of luciferase mRNAs were expressed in each type of transfected cell, suggesting that none of the 5′-UTR sequences possessed promoter/enhancer functions (Fig. 2B). It was surprising that a ten-fold greater expression of luciferase protein was observed with *HIF1A* 5′-UTR-containing luciferase plasmid transfectants than in FMDV IRES-containing luciferase transfected cells (Fig. 2B). Since there was no significant difference in the amount of transcribed mRNA, it is likely that the up-regulatory effect of *HIF1A* 5′-UTR was due to the translational machinery under our experimental conditions. It was also demonstrated that the 151 bp of *HIF1A* 5′-UTR was sufficient for translational up-regulation.

HIF1A contains a relatively long 3′-UTR sequence (1197 bp) that includes at least eight ARE motifs (AUUUA). To further investigate the 3′-UTR-dependent down-regulatory mechanism, the whole 3′-UTR sequence of *HIF1A* was cloned downstream from the luciferase gene that was driven by the human phosphoglycerate kinase (PGK) promoter. As a typical ARE sequence, the 3′-UTR of *MYC* was also cloned in the 3′ region (Fig. 3A). As demonstrated in Fig. 3B, a decrease in luciferase activity of more than 50% was observed both with the luciferase construct containing the *HIF1A* 3′-UTR (designated "pmir HIF-1") or the *MYC* 3′-UTR (designated "pmir *MYC*"). Real-time PCR experiments also demonstrated that the amounts of luciferase mRNAs were decreased in pmir HIF-1- and pmir *MYC*-transfected HeLa cells (Fig. 3C). It seemed likely that the

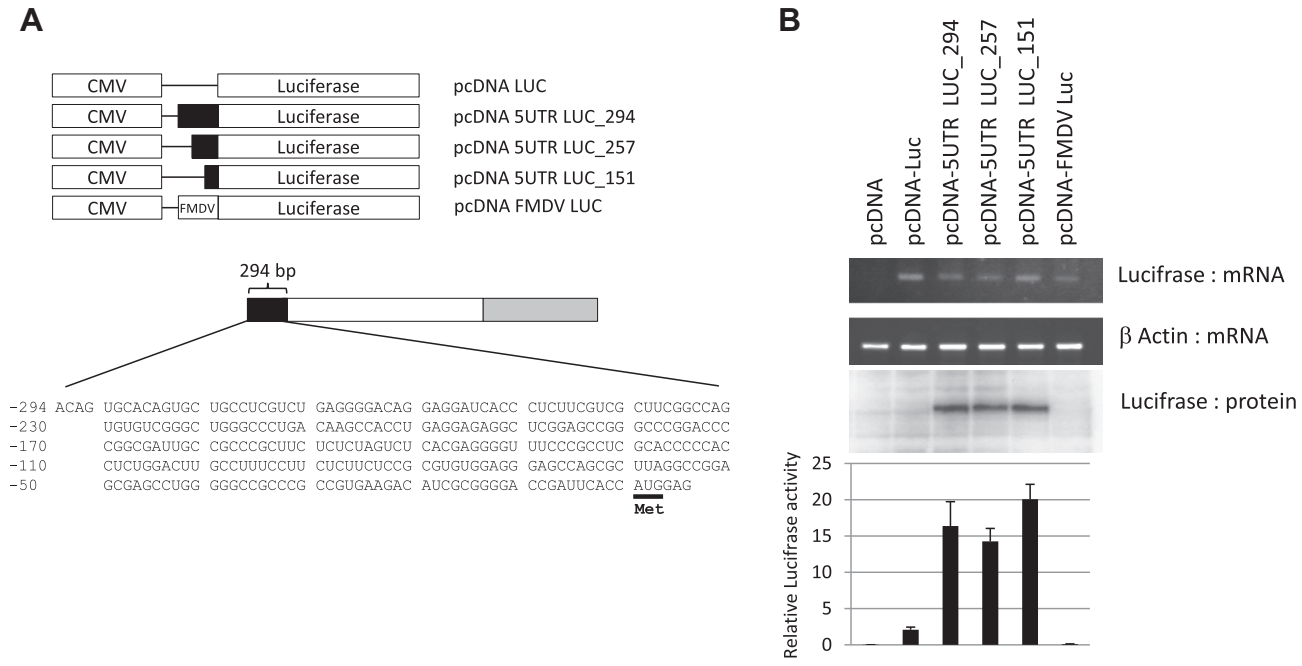

Fig. 2. Translational control via the 5′-UTR of *HIF1A* (A) Schematic illustration of 5′-UTR-tagged luciferase plasmids and 5′-UTR sequence of *HIF1A*. A start codon was underlined. (B) Discrepancies between transcription levels and translation of *HIF1A*. HeLa cells were transfected with indicated luciferase constructs or indicated constructs plus reference *Renilla* luciferase plasmid. Upper two rows show semi-quantitative PCR for indicated target. Total cell lysates of transfected HeLa cells were blotted with anti-luciferase monoclonal antibody or subjected to dual luciferase assays. Error bars represent standard deviations for triplicate wells in a single experiment; data are representative of three independent experiments.

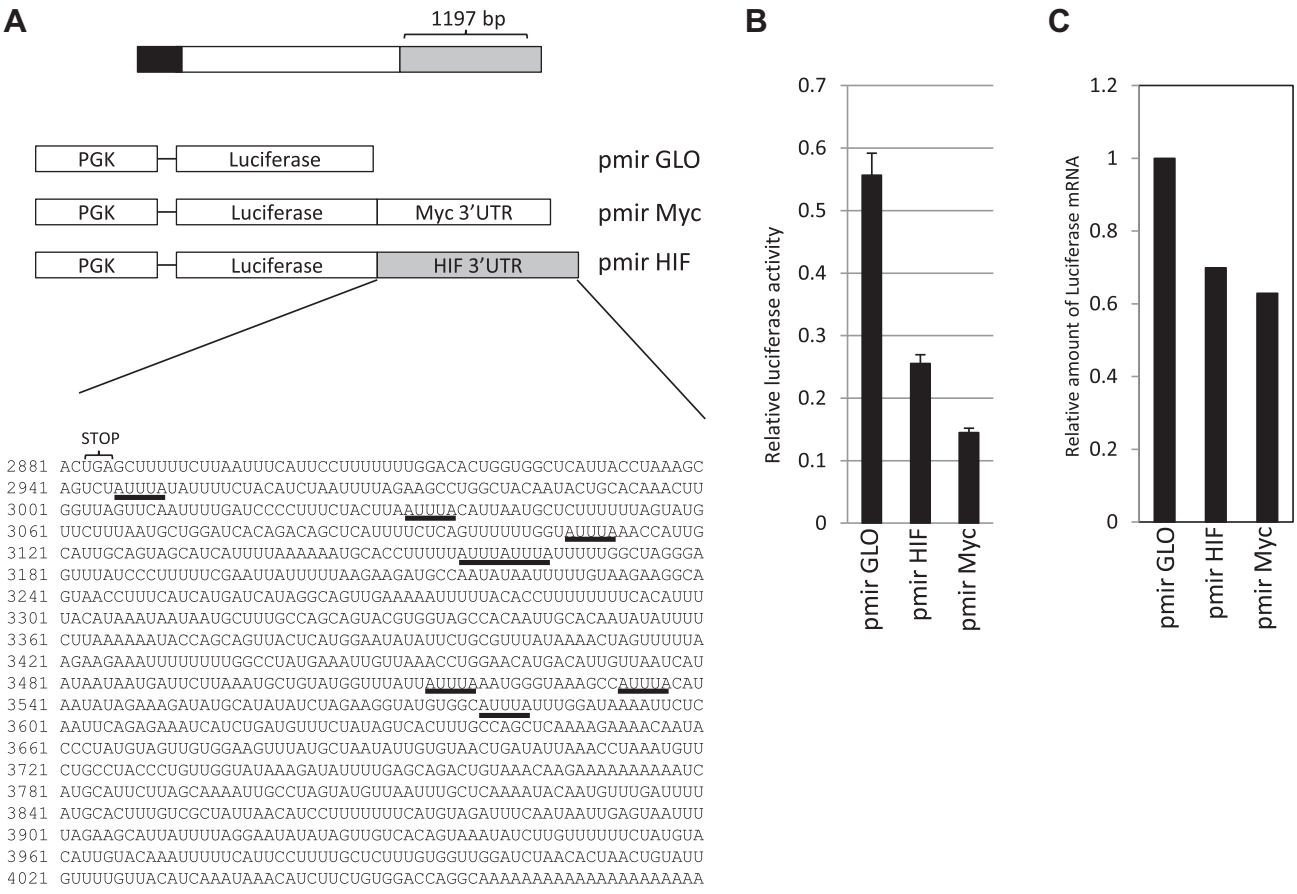

Fig. 3. *HIF1A* mRNA degradation via 3′-UTR dependent mechanism (A) Schematic illustration of 3′-UTR-tagged luciferase plasmids. 3′-UTRs of human *MYC* and *HIF1A* were cloned into the 3′ region of the firefly luciferase sequence of pmir GLO vector. Typical AU-rich sequences of *HIF1A* are underlined. (B) HeLa cells were transfected with indicated constructs and the lysates were subjected to dual luciferase assays. Error bars represent standard deviations for triplicate wells in a single experiment; data are representative of three independent experiments. (C) HeLa cells were transfected with the indicated constructs and total RNA was extracted for cDNA synthesis. The reference target for real time PCR was *Renilla* luciferase that was transcribed from pmir GLO back bone.

down-regulatory function of *HIF1A* 3′-UTR was involved in the degradation of mRNA.

These controversial findings, i.e., opposing functions of 5′- and 3′-UTRs of *HIF1A* mRNA, prompted us to investigate which function was predominant in malignant tumor cells. Several luciferase constructs, including pcDNA 5′-UTR LUC_294 and pmir HIF, were introduced into several cancer cell lines. As expected, 5′-UTR-dependent up-regulation was predominant in U87MG (Fig. 4A). On the other hand, MCF7 cells showed significant down-regulation with the 3′-UTR-tagged luciferase construct (Fig. 4B). It is intriguing that MDA-MB 453, a metastatic breast cancer-derived cell line, showed the same regulatory patterns as U87MG (Fig. 4A and B). A microarray analysis also demonstrated up-regulation of HRE-containing genes such as *VEGFA* in both MDA-MB 453 and U87MG compared with MCF7 (data not shown). It is plausible that the balance of 5′-UTR-dependent up-regulation and 3′-UTR-dependent down-regulation is relevant for *HIF1A* expression and might modulate the degree of malignancy. It is also clearly demonstrated that these UTR-dependent regulatory mechanisms are independent of pVHL-dependent degradation cascades of HIF-1α.

RNA binding proteins HuR and PTBP-1 are probably associated with the 5′- and 3′-UTRs of *HIF1A* mRNA [12]. To confirm the biological effect of those proteins on *HIF1A* expression, we introduced those plasmids together with *HIF1A* constructs and reporters into HeLa cells. It was surprising that PTBP-1, but not HuR, upregulated *HIF1A* expression (Fig. 4C). It is likely that the up-regulatory function of PTBP-1 is 5′-UTR-dependent because this effect was not observed when cells were transfected with HIF C or HIF C3 constructs (Fig. 4C). To further investigate whether over-expression of PTBP-1 could upregulate endogenous *HIF1A* expression, we introduced PTBP-1 or HuR with reporter plasmids into HeLa cells followed by the addition of the hypoxia-mimicking agent. As expected, only PTBP-1-transfected cells showed more than a two-fold increase in luciferase activity under hypoxia-mimicking conditions (Fig. 4D). It is likely that higher-PTBP-1 expressing cells might express larger amount of HIF-1α under hypoxic conditions compared with lower-PTBP-1 expressing cells.

4. Discussion

In the present study, we have demonstrated that *HIF1A* expression was regulated by dual post-transcriptional mechanisms that were independent of protein degradation cascades. These mechanisms are implicated in both translational enhancement (up-regulation) and mRNA decay (down-regulation) in a UTR cis-element-dependent manner. Most importantly, these two opposing mechanisms appeared to modulate tumor malignancy because HIF-1α has been implicated in aggressive tumor phenotypes. Besides angiogenesis, HIF-1α-dependent gene expression plays a major role in aggressive tumor behavior. Recent reports have demonstrated that TWIST, the key regulator for the epithelial–mesenchymal transition (EMT), contains HRE sequence in the proximal promoter [15–17]. It has also been reported that one of the cancer stem cell markers (CD44) is positively regulated by HIF-1α [18].

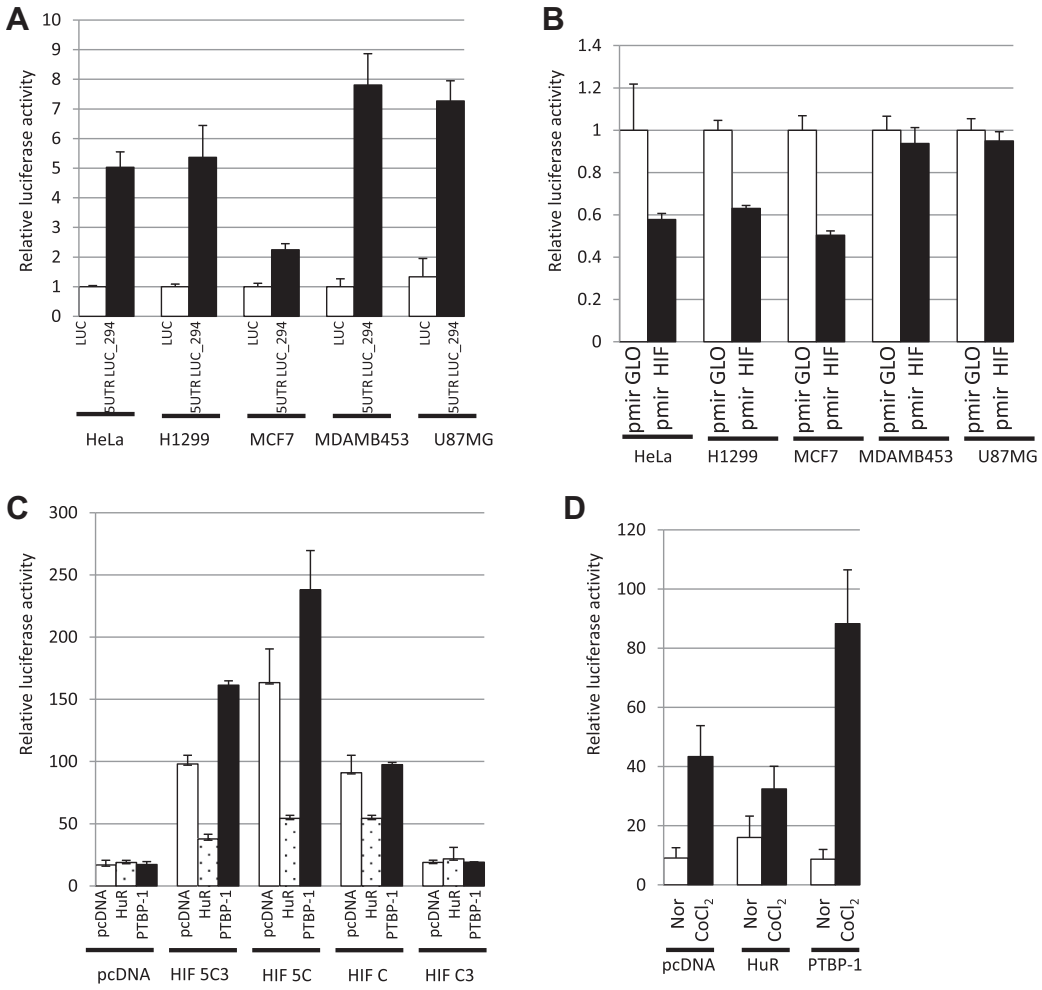

Fig. 4. Tumor malignancies and UTR-dependent regulation of *HIF1A* (A) Indicated cell lines were transfected with pcDNA LUC or pcDNA 5UTRLUC_294 together with pGL4.72. Cell lysates were analyzed by use of a dual luciferase system. (B) Cells were transfected with the indicated constructs and the cell lysates were subjected to dual luciferase assays. (C) HeLa cells were transfected with indicated plasmid together with HRE-reporter plasmid and reference *Renilla* luciferase plasmid. (D) HeLa cells were transfected with the indicated plasmid and reporter plasmids (pGL-HRE and pGL4.72). Five hour after transfection, cells were incubated with DMEM containing 200 μM $CoCl_2$ for 16 h followed by dual luciferase assay. A–D: Error bars represent standard deviations for triplicate wells in a single experiment. All data are representative of three independent experiments.

The *HIF1A* transcript (GenBank accession number: NM 001530) contains relatively long 5′- and 3′-UTRs, 404 and 1197 nucleotides, respectively. It has been documented that the 5′-UTR of the *HIF1A* transcript functions as an internal ribosome entry site (IRES) in a bicistronic reporter system. The initiation of translation normally starts with the interaction of certain key proteins with the 5′-m7GpppN cap. However, cap-dependent translation is impaired under certain stress conditions, including viral infection, heat, UV irradiation or hypoxia. Even in such circumstances, a number of genes containing IRES are able to be translated through a cap-independent mechanism [19–22]. There are several studies addressing proteins associated with the 5′-UTR of *HIF1A* [11–13]. Schepens et al. reported that the poly-pyrimidine tract-binding protein (PTBP-1) bound to the 5′-UTR of *HIF1A* and that the knockdown of PTBP-1 impaired IRES activity of the *HIF1A* 5′-UTR. Galban et al. demonstrated that HuR bound to the *HIF1A* 5′-UTR whereas PTBP-1 bound principally with the 3′-UTR of *HIF1A*. Our present results suggested that PTBP-1 (but not HuR) enhanced translation. Because *HIF1A* expression by HIF C or HIF C3 constructs were not influenced by PTBP-1 over-expression, it seems likely that PTBP-1 does not regulate expression of *HIF1A* in a 3′-UTR-dependent fashion. It is also notable that PTBP-1 over-expressing HeLa cells showed more than a two-fold HRE-dependent luciferase activity compared with control cells under hypoxic conditions. It is possible that the cells in which 5′-UTR/PTBP-1-dependent translational machinery was activated might express elevated amount of HIF-1α under hypoxic conditions. It is highly controversial whether there are any consensus sequences that are universally essential to IRES activity. It is unclear whether IRES activity and translational enhancement are independent of one another because we did not find any translational enhancer activity with the IRES of foot-and-mouth disease virus (FMDV) under our experimental conditions. It should be noted that the poly-pyrimidine sequence of *HIF1A* 5′-UTR contains two typical PTBP-1 binding sequences (UCUUC). In contrast, FMDV-IRES lacks the binding consensus sequence.

The immigration and packaging of cytoplasmic mRNA into distinct RNA granules such as stress granules (SGs) and processing bodies (P-bodies or PBs) plays a central role in mRNA turnover [23]. In mammals, deadenylation of mRNA is the initial event and is followed by decapping and 5′- to 3′-degradation by exonucleases. PBs contain mRNA decapping (Dcp1 and Dcp2) and degradation components of the 5′- to 3′- decay machinery, whereas SGs act as an “antechamber” of PBs. It has been reported that some

unstable mRNAs, including growth factors, transcription factors and oncogenes contain pentatonic AU-rich elements (AREs: AUUUA) in the 3′-UTR and several ARE-binding proteins (ARE-BPs) are associated with either SGs or BPs. Hu/ELAV family proteins bind to this element and stabilize the mRNA while other ARE-binding proteins (such as AUF1, TTP and TIA-1) destabilize the mRNA [24]. However, a full explanation for the destabilizing activities of ARE-BPs is incomplete. There are at least eight AREs in the 3′-UTR of human, mouse and chicken *HIF1A* (NM 001530, NM 010431 and NM 204297, respectively).In the present study, we demonstrated that the HIF C3 plasmid, which contained the coding region and the 3′-UTR (devoid of the 5′-UTR) of *HIF1A*, expressed only modest levels of protein compared with HIF C (coding region only). We also found that the 3′-UTR-tagged luciferase construct (pmir HIF) transfected cells showed reduced (50%) luciferase activity (Fig. 3B). These results confirm the inhibitory effect of 3′-UTR for HIF-1α protein expression. Real time PCR data suggested the accelerated degradation of 3′-UTR-tagged luciferase mRNA (Fig. 3B and C).

HuR, an intensively studied protein, is a promising candidate ARE-BP that stabilizes cellular ARE-mRNAs. It has been reported that HuR and *HIF1A* 3′-UTR are apparently associated [14,25]. We observed that HuR had a down-regulatory effect on *HIF1A* expression, results that were unexpected. HuR inhibited *HIF1A* expression not only with the full-length *HIF1A* (HIF 5C3) but also with the HIF C (coding region only) construct (Fig. 4C). HuR-dependent stabilization of VHL might explain this phenomenon [25].

It is intriguing that each cancer cell line had a different relative ratio of UTR-tagged luciferase activity in a cis-element-dependent fashion. In the non-metastatic breast carcinoma cell line MCF7, the 5′-UTR-tagged luciferase/control luciferase ratio was 2.24 whereas the ratio for the U87MG malignant glioma cell line was 7.27. On the other hand, the 3′-UTR tagged luciferase/control luciferase ratio of MCF7 was 0.50 and that of U87MG was 0.95. We observed the same tendency between non-metastatic mouse melanoma B16-F0 cells and highly metastatic B16-F10 cells (data not shown). It seems likely that the products of the increased ratio of 5′-UTR-tagged luciferase activity and the reduced ratio of 3′-UTR-tagged luciferase activity (ex. MCF7: $2.24 \times 0.5 = 1.12$, U87MG: $7.27 \times 0.95 = 6.91$) might be a useful parameter to predict cancer cell malignancy. However, these immortalized cell lines have been maintained in culture for many years and might not be completely representative of fresh tissues. Thus, to substantiate this hypothesis, future analysis of fresh tumors and primary tissues will be required.

Acknowledgments

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