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microRNA-15a-5p suppresses hypoxia-induced tumor growth and chemoresistance in bladder cancer by binding to eIF5A2

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In various malignant tumors (including bladder cancer) poor prognosis is associated with hypoxia and therapeutic resistance. Evidence indicates that in bladder cancer, microRNAs (miRNAs) have vital functions in acquired drug resistance. However, the involvement of miRNAs in hypoxia-mediated bladder cancer doxorubicin (Dox) resistance is unknown. Herein, we showed that hypoxia and Dox treatment downregulated miR-15a-5p expression. Using UM-UC-3 and J82 bladder cancer cell lines and *in vivo* mouse models of bladder cancer, we confirmed that miR-15a-5p arrests tumor cell growth and Dox resistance *in vitro* and *in vivo*. Furthermore, we determined the interaction between miR-15a-5p and eukaryotic translation initiation factor 5A-2 (eIF5A2) using dual luciferase reporters and quantitative real-time reverse transcription polymerase chain reaction assays. We also showed that a miR-15a-5p agomir repressed EIF5A2 expression in bladder cancer cells, thereby inhibiting the epithelial-mesenchymal transition (EMT) induced by Dox or hypoxia. Moreover, ectopic expression of miR-15a-5p abrogated eIF5A2-mediated Dox resistance in bladder cancer cells. Collectively, these data indicated that hypoxia promotes tumor growth and chemoresistance through the HIF-1α/miR-15a-5p/eIFTA2/EMT pathway. This new finding not only has implications for improving our understanding of the Dox resistance process during bladder cancer progression but also indicates that the miR-15a-5p agomir is a promising tool to prevent Dox resistance in patients with bladder cancer.

Key words: microRNA-15a-5p; bladder cancer; doxorubicin resistance; eIF5A2; EMT

Bladder cancer is a heterogeneous malignant tumor with numerous histological subtypes that can originate from anywhere in the urinary tract, with the majority of tumors starting from the epithelial cells in the bladder lining (urethral epithelium). According to the latest GLOBOCAN data, 3% of all cancers and 2.1% of cancer deaths are attributed to bladder cancer worldwide [1, 2]. Moreover, patients with bladder cancer have a 70% 5-year survival rate, whereas in those with metastasis, the 5-year survival rate is only 5% [1, 2]. Doxorubicin (Dox) is a commonly used chemotherapy for bladder cancer; however, the development of drug resistance markedly limits its long-term curative effects [3]. Hypoxia is characterized by low oxygen and has critical roles in the angiogenesis, metastasis, and drug resistance in most solid tumors, including bladder cancer [4, 5, 6, 7]. The bladder carbogen and nicotinamide (BCON) phase III clinical trial showed that hypoxia modification increased the survival of bladder cancer patients expressing high levels of hypoxia-related genes [8]. Targeting hypoxia-mediated tumor metabolism is therefore a promising strategy to treat bladder cancer.

Hypoxia can regulate all the steps of metastasis, from the initial epithelial–mesenchymal transition (EMT) to the final organotropic colonization; therefore, it has been suggested

that hypoxia acts as a master regulator in metastasis [9]. Previous studies have demonstrated that a hypoxic environment could induce EMT during bladder cancer progression [10]. In addition, EMT participates in the promotion of Dox resistance in bladder cancer cells [11]. Therefore, chronic treatment with Dox induces hypoxia, which leads to EMT, causing Dox resistance in bladder cancer. However, the molecular mechanisms that link hypoxia and EMT-induced Dox resistance in bladder cancer remain elusive.

Recently, studies have reported that certain microRNAs (miRNAs) are directly regulated by hypoxia, participating in hypoxia-mediated survival signaling pathway [12, 13]. miRNAs (small, endogenous, and non-coding RNAs) are usually 20–22 nucleotides long. miRNAs regulate gene expression negatively at the post-transcriptional level by binding to their target mRNA via complementary sequences. miRNAs participate in many cancers' physiological and pathological processes, such as radioresistance, apoptosis, infiltration, proliferation, differentiation, and chemoresistance [14, 15]. In this study, we discovered that 20 miRNAs are downregulated by hypoxia in bladder cancer. Among them, miR-15a-5p was also reduced when bladder cancer cells were treated with Dox. Furthermore, we found that miR-15a-5p can improve hypoxia-induced Dox resistance and inhibit tumor growth in bladder cancer by inhibiting EMT. Therefore, we believe that in bladder cancer, miR-15a-5p has a vital function in the development of Dox sensitivity. Our findings provide new insights into therapeutic targets for enhancing Dox clinical efficacy in treating patients with bladder cancer.

Materials and methods

Cell culture. The ATCC (Rockville, MD, USA) provided the bladder cancer cell lines UM-UC-3 and J82. RPMI 1640 medium (Lonza, Switzerland) containing fetal bovine serum (10% (v/v)), penicillin (1%, Gibco, Grand Island, NY, USA), and streptomycin (1%; Gibco) were used to culture the cells under standard conditions. Dox was obtained from Sigma-Aldrich (Merck, Darmstadt, Germany) and was diluted in dimethyl sulfoxide.

Cell transfection. Cells (2×10^5) were plated evenly on each well of a 6-well plate and allowed to attach to the bottom of the well. Then, we mixed the short interfering RNA (siRNA), miRNA mimics, or their inhibitors with Lipofectamine 2000, and then the mixture was added to cells grown in a serum-free medium. Six hours later, the medium was replaced with a normal medium for subsequent experiments. RiboBio (Guangzhou, China), Fulengen (Guangzhou, China), and Thermo Scientific (Waltham, MA, USA) provided the above reagents.

The miR-15a-5p mimics comprised: 5'-UAGCAGCACAUAAUGGUUUGUG-3'; 5'-CAAACCAUUAUGUGCUGCUAUU-3'; The miR-15a-5p inhibitor comprised: 5'-CACAAACCAUUAUGUGCUGCUA-3'; The negative control comprised: 5'-CAGUACUUUUGUGUAGUACAA-3'.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay for cell viability was carried out following the procedures detailed by Wang et al. [16].

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The expression levels of mRNAs in bladder cancer cells were assessed using qRT-PCR according to the procedures detailed by Wang et al. [16]. The following primers were used: *EIF5A2* (encoding eukaryotic translation initiation factor 5A-2): Forward: 5'-TATGCAGTGCTCGGCCTTG-3'; Reverse: 5'-TTGGAACATCCATGTTGTGAGTAGA-3'; *CDH1* (encoding E-cadherin): Forward: 5'-TACACTGCCCAGGAGCCAGA-3'; Reverse: 5'-TGGCACCAGTGTCCGGATTA-3'; *VIM* (encoding Vimentin): Forward: 5'-TGAGTACCGGAGACAGGTGCAG-3'; Reverse: 5'-TAGCAGCTTCAACGGCAAAGTTC-3'; miR-15a-5p: 5'-GTGTTTGGTAATACACGACGAT-3'.

Dual-luciferase reporter gene assay. J82 cells were co-transfected with negative control mimics or miR-15a-5p mimics and wild-type (WT) (pGL3-WT-*EIF5A2*-3' untranslated region [UTR]) or mutant (mut) (pGL3-mut-*EIF5A2*-3' UTR) reporter plasmids (GenePharma, Shanghai, China). The cells were incubated with the transfection mixtures for 48 h, collected by centrifugation, and lysed. The luciferase assays were then carried out following the instructions of the luciferase assay kit (Biovision, Milpitas, CA, USA) and the luciferase reporter assay system (Promega, Madison, WI, USA). The ratio of firefly luciferase activity to Renilla luciferase activity determined the relative luciferase activity. The WT-*EIF5A2*-3' UTR and mut-*EIF5A2*-3' UTR were 5'-AAAATTATTAATCCGTGCTGCTT-3' and 5'-AAAATTATTAATCCGACGACGAT-3', respectively.

Western blotting. Western blotting was carried out following the procedures detailed by Wang et al. [16]. Primary antibodies against the following proteins were used: eIF5A2 (1:1000; Abcam, Cambridge, MA, USA; ab227537), HIF-1α (1:1000; Cell Signaling Technology (CST), Beverly, MA, USA; 36169S), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000; CST; 2118S). Goat anti-rabbit IgG was used as the secondary antibody (1:2000, CST; 7074S).

Immunofluorescence analysis. The cells were seeded into 48-well plates at a density of 3×10^3 cells/well, fixed with 4% formaldehyde for 15 min, washed with PBS, treated with 5% BSA for 30 min at room temperature, and incubated with primary antibodies anti-E-cadherin (1:200; CST; 3195S) or anti-Vimentin (1:200; CST; 5741S) at 4 °C overnight. The cells were incubated with a FITC-conjugated anti-rabbit secondary antibody (Invitrogen; A27034 and A32732) at 4 °C for 2 h. Nuclear staining was performed with DAPI (Invitrogen; D1306) at room temperature for 5 min. Following two washes with PBS, cells were observed using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Cell proliferation analysis. Cell proliferation was determined using a Click-iT® EdU Imaging kit according to the manufacturer's (Invitrogen) protocols.

Tumor xenograft study. The Experimental Animal Center of Sun Yat-sen University (Guangzhou, China) provided BALB/c nude mice (male, 3–4 weeks old). Cells were collected by centrifugation and suspended in serum-free medium at 1×10^7 cells/0.2 ml. UM-UC-3 cells were inoculated subcutaneously into the right flank of each mouse. Every 2 days, the tumor size was determined and mice were humanely killed after 4 weeks. For the *in vivo* chemosensitivity assays, Dox or saline were delivered to the mice via intraperitoneal injection (2 mg/kg body weight Dox [once every 2 days]). miR-15a-5p agomir (GenePharma, China) treatment was achieved via intratumor injection (30 µl/mouse [once every 3 days]). All animal experiments were approved by the Ethics Committee of Tongde Hospital of Zhejiang province in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications, No. 8023, revised 1978). We confirmed that all methods were performed in accordance with ARRIVE guidelines.

TUNEL and immunohistochemistry staining. Formalin-fixed, paraffin-embedded tumor specimens were sliced into 4 µm thick sections and mounted on glass slides. Terminal deoxynucleotidyl transferase nick-end-labeling (TUNEL) (Roche, Basel, Switzerland; 11684817910) and Ki-67 (AiFang biological; China; SAF008) staining were performed on three independent tumors from each group. The ratio of TUNEL- or Ki-67-positive nuclei among total nuclei was used to express the results. ImageJ (for Windows, NIH, Bethesda, MD, USA) was used to carry out the quantitative analysis of the positive cells.

Statistical analyses. SPSS v18.0 (IBM, Armonk, NY, USA) was used to carry out all the statistical analyses. The mean ± standard deviation (SD) was used to present all the data. Differences between the two groups were analyzed using two-tailed Student's t-tests. One-way analysis of variance (ANOVA) was used to determine the statistically significant differences among multiple samples. Statistical significance was indicated by a p-value <0.05. All experiments were repeated three times.

Results

miR-15a-5p is transcriptionally inhibited by HIF-1α under hypoxia. Hypoxia, a salient feature of advanced-stage bladder cancer, correlates with poor prognosis [17]. To identify the miRNA signature regulated by hypoxia, we evaluated the expression of 20 different drug resistance-related miRNAs in

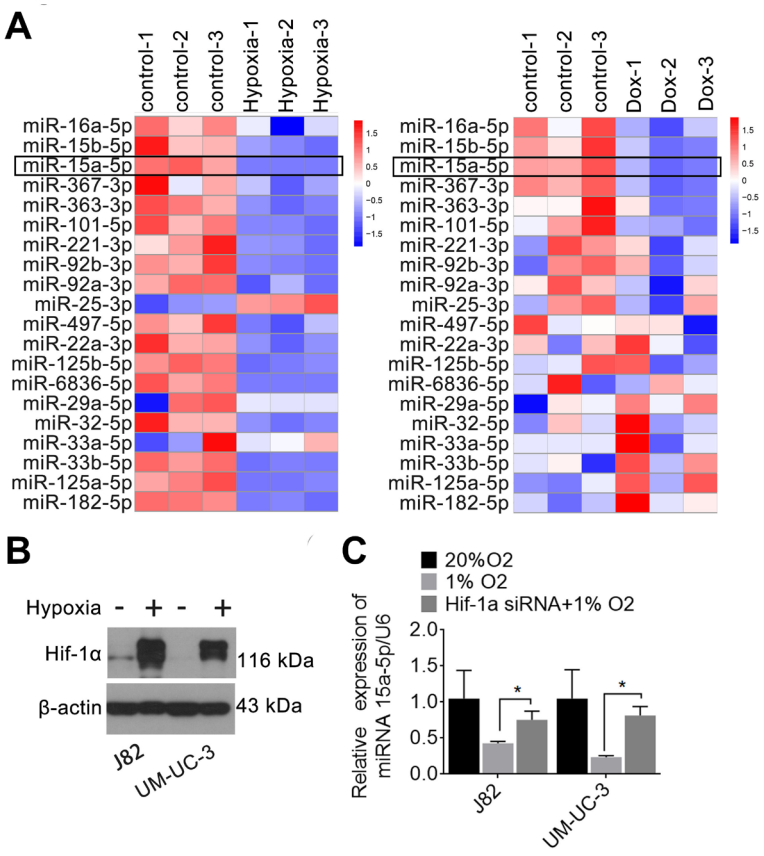

Figure 1. miR-15a-5p is transcriptionally inhibited by HIF-1α under hypoxia. A) qRT-PCR analysis of miRNA expression levels after hypoxia or Dox treatment. B) Levels of the HIF-1α protein in UM-UC-3 and J82 cells after hypoxia. C) miR-15a-5p expression in bladder cancer cell lines transfected with HIF-1α siRNA or negative control and treated with hypoxia.

J82 bladder cancer cells treated with hypoxia. The majority of the selected miRNAs were downregulated by hypoxia treatment (left panel of Figure 1A). We then measured the expression of these 20 miRNAs in UM-UC-3 bladder cancer cells treated with Dox and found that miR-15a-5p expression was significantly reduced under both Dox and hypoxic conditions (Figure 1A). Next, we explored whether the transcription of miR-15a-5p is regulated by hypoxia-inducible factor (HIF). The HIF-1a levels were greatly increased in UM-UC-3 and J82 cells under hypoxic conditions (Figure 1B). Reducing HIF-1a levels using HIF-1α-specific siRNAs partially recovered the levels of miR-15a-5p after hypoxia (Figure 1C). These results suggest that hypoxia, which is involved in the progression and chemoresistance of bladder cancer, activates HIF-1a, leading to miR-15a-5p downregulation.

miR-15a-5p suppresses tumor growth in bladder cancer. Previous studies have suggested that hypoxia plays a major role in bladder cancer chemoresistance, angiogenesis, and progression [18, 19]. We first investigated whether miR-15a-5p is involved in tumor growth in bladder cancer. The expression of miR-15a-5p was evaluated using the online prediction software starBase (version 3.0), which indicated high expression of miR-15a-5p in bladder cancer (Figure 2A). A bladder cancer model was then established by inoculating 3×10^6 cells (UM-UC-3) subcutaneously into athymic nude mice. The results showed that compared with those in the saline-treated control group, intratumoral administration of the miR-15a-5p agomir decreased the tumor sizes (Figures 2B, 2C). Treatment with miR-15a-5p significantly slowed down tumor growth (Figure 2D). We then examined cell proliferation using Ki-67 staining and apoptosis using TUNEL staining in the extracted tumor tissues. miR-15a-5p agomir treatment inhibited tumor cell proliferation and increased apoptosis (Figures 2E–2G). These results indicate that high miR-15a-5p expression arrests bladder cancer growth.

miR-15a-5p enhances the Dox sensitivity of bladder cancer cells. Next, we examined the involvement of miR-15a-5p in hypoxia-mediated Dox resistance in UM-UC-3 and J82 cells. miR-15a-5p expression was highest in UM-UC-3 cells and lowest in J82 cells (Figure 3B). CCK-8 assays determined the viability of bladder cancer cells treated with different concentrations of Dox for 48 h. This result indicated that increased Dox concentrations reduced the viability of both cell lines in a dose-dependent manner, with different sensitivity to Dox in different bladder cancer cells (Figure 3A), and the value of IC50 was expressed in Supplementary Figure S1A.

To test whether miR-15a-5p suppresses Dox resistance, the effect on Dox resistance of miR-15a-5p expression was studied in the two bladder cancer cells: miR-15a-5p mimic transfection significantly enhanced miR-15a-5p levels (Figure 3C), while transfection with the miR-15a-5p inhibitor reduced miR-15a-5p levels in both cell lines (Figure 3D). miR-15a-5p overexpression enhanced bladder cancer cell sensitivity to Dox (Figure 3E). By contrast, miR-15a-5p downregulation using the miR-15a-5p inhibitor enhanced Dox resistance in UM-UC-3 and J82 cells (Figure 3F), which were consistent with the results of EdU (Supplementary Figures S1B, S1C).

Next, the involvement of miR-15A-5p in hypoxia-mediated Dox resistance was investigated. miR-15a-5p upregulation using the miR-15a-5p mimic under hypoxia decreased Dox resistance to a similar level in both cell lines (Figure 3G) and the value of IC50 was shown in Supplementary Figure S1D. These results suggested that hypoxia-induced Dox resistance is mediated through downregulation of miR-15a-5p and the miR-15a-5p mimic could raise the sensitivity of bladder cancer cells to Dox.

miR-15a-5p reverses the hypoxia-induced EMT. EMT is recognized as a critical regulator of chemoresistance in cancer [20, 21]; therefore, we explored whether in bladder cancer, miR-15a-5p suppresses Dox resistance by modulating the EMT process. Transfection of UM-UC-3 and J82 cells with the miR-15a-5p mimics significantly elevated miR-15a-5p levels (Figure 4A). Overexpression of miR-15a-5p in both cell lines decreased the level of Vimentin (a mesenchymal marker) but increased the level of E-cadherin (an epithelial marker) at both the protein and mRNA levels (Figures 4B, 4C), suggesting that miR-15a-5p can suppress EMT. Both Dox treatment and hypoxia facilitate EMT, as indicated by increased expression of Vimentin and decreased expression of E-cadherin (Figure 4D). miR-15a-5p upregulation reversed the hypoxia and Dox-mediated changes in Vimentin and E-cadherin expression (Figure 4D). We also determined E-cadherin and Vimentin using immunofluorescence and the results were consistent with western blot (Figures 4E, 4F). Collectively, these results indicated that miR-15a-5p inhibits EMT of bladder cancer cells and reverses the increase in EMT caused by hypoxia and Dox treatment.

EIF5A2 is the downstream target of the miR-15a-5p. Previously, we showed that eIF5A2 promotes EMT and increases Dox resistance in bladder cancer cells [22]. Consistent with our previous findings, in bladder cancer cells, overexpression of *EIF5A2* enhanced Dox resistance, while *EIF5A2* knockdown inhibited Dox resistance (Supplementary Figures S2A, S2B). In addition, in bladder cancer cells, overexpression of *EIF5A2* promoted EMT, while *EIF5A2* knockdown inhibited EMT (Supplementary Figures S2C, S2D). We further demonstrated that hypoxia increased eIF5A2 expression by 2- to 4-fold in J82 cells (Supplementary Figure S3A). *EIF5A2* knockdown inhibited hypoxia-induced EMT and thus rescued hypoxia-mediated Dox resistance (Supplementary Figures S3B, S3C).

We next attempted to determine whether eIF5A2 lies downstream of miR-15a-5p during EMT regulation and Dox resistance. Using Target Scan, we found that there is a consensus-binding site for miR-15a-5p in the *EIF5A2* mRNA (Figure 5A). In addition, eIF5A2 showed a weak negative correlation with the expression of miR-15a-5p (Figure 5A).

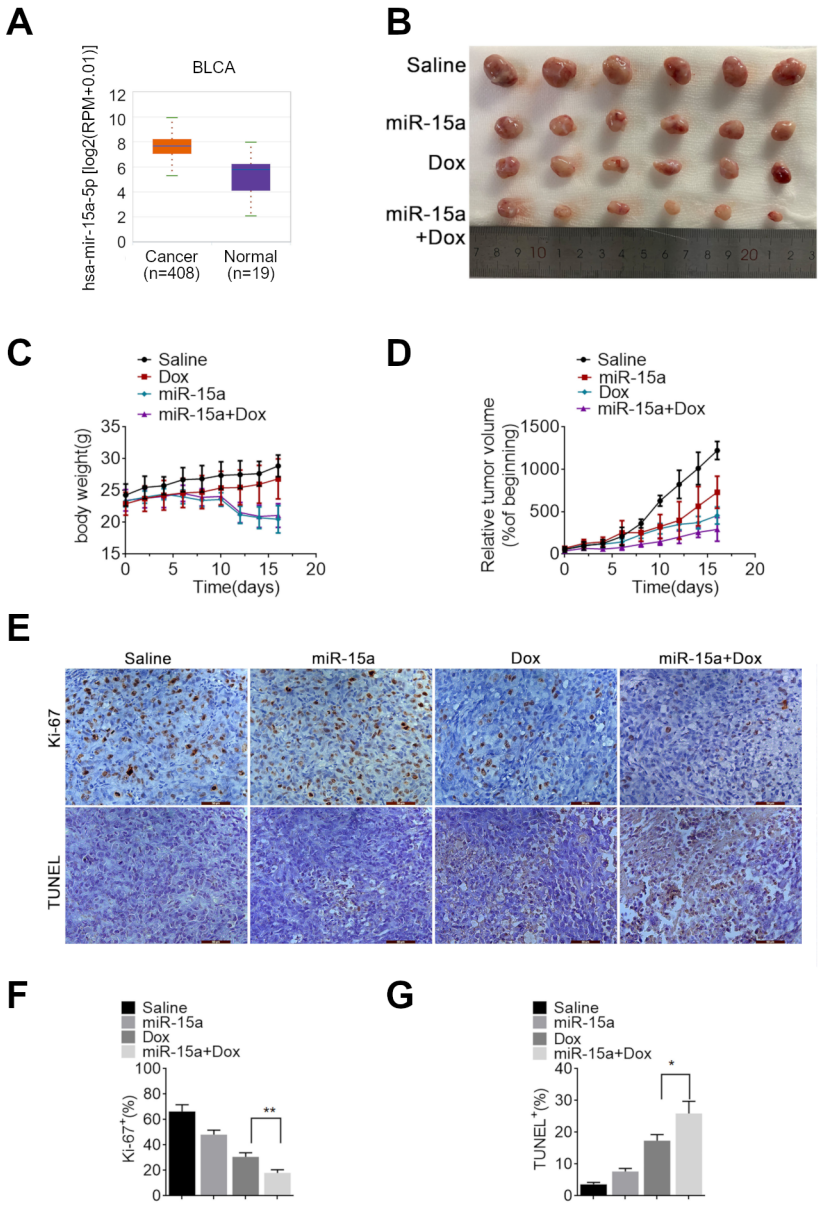

Figure 2. miR-15a-5p suppresses bladder cancer tumor growth. A) starBase predicted the high bladder cancer expression of miR-15a-5p. B) Representative images of excised tumors in the indicated groups. C) Determined tumor weights. *p<0.05, vs. the negative control group, #p<0.05, vs. the Dox group. D) Growth curve of tumor volumes. E) miR-15a-5p expression increases the number of Ki-67-positive cells and decreases the number of TUNEL-positive cells. F, G) The % of Ki67-or TUNEL-positive cells. *p<0.05, Dox group vs. the miR-15a-5p agomir + Dox group.

Furthermore, eIF5A2 protein and mRNA levels were downregulated significantly in miR-15a-5p mimic-transfected UM-UC-3 and J82 cells compared to those in cells transfected with the negative control miRNA (Figures 5B, 5C). In addition, eIF5A2 protein and mRNA levels were markedly elevated in bladder cancer cells transfected with the miR-15a-5p inhibitor (Figures 5B, 5C).

Next, the effect of miR-15a-5p on eIF5A2 expression was assessed using the psiCK-eIF5A2 luciferase reporter system. miR-15a-5p mimic co-transfection with the psiCK-wt-eIF5A2 reporter significantly downregulated the luciferase activity; however, the miR-15a-5p mimic had no effect on luciferase activity when it was co-transfected with the reporter vector expressing the mutated *EIF5A2* sequence that cannot be recognized by miR-15a-5p (Figures 5D, 5E). Thus, miR-15a-5p targets *EIF5A2* and reduces its expression.

We then studied whether overexpression of miR-15-5p with eIF5A2 would affect bladder cancer cell Dox resis-

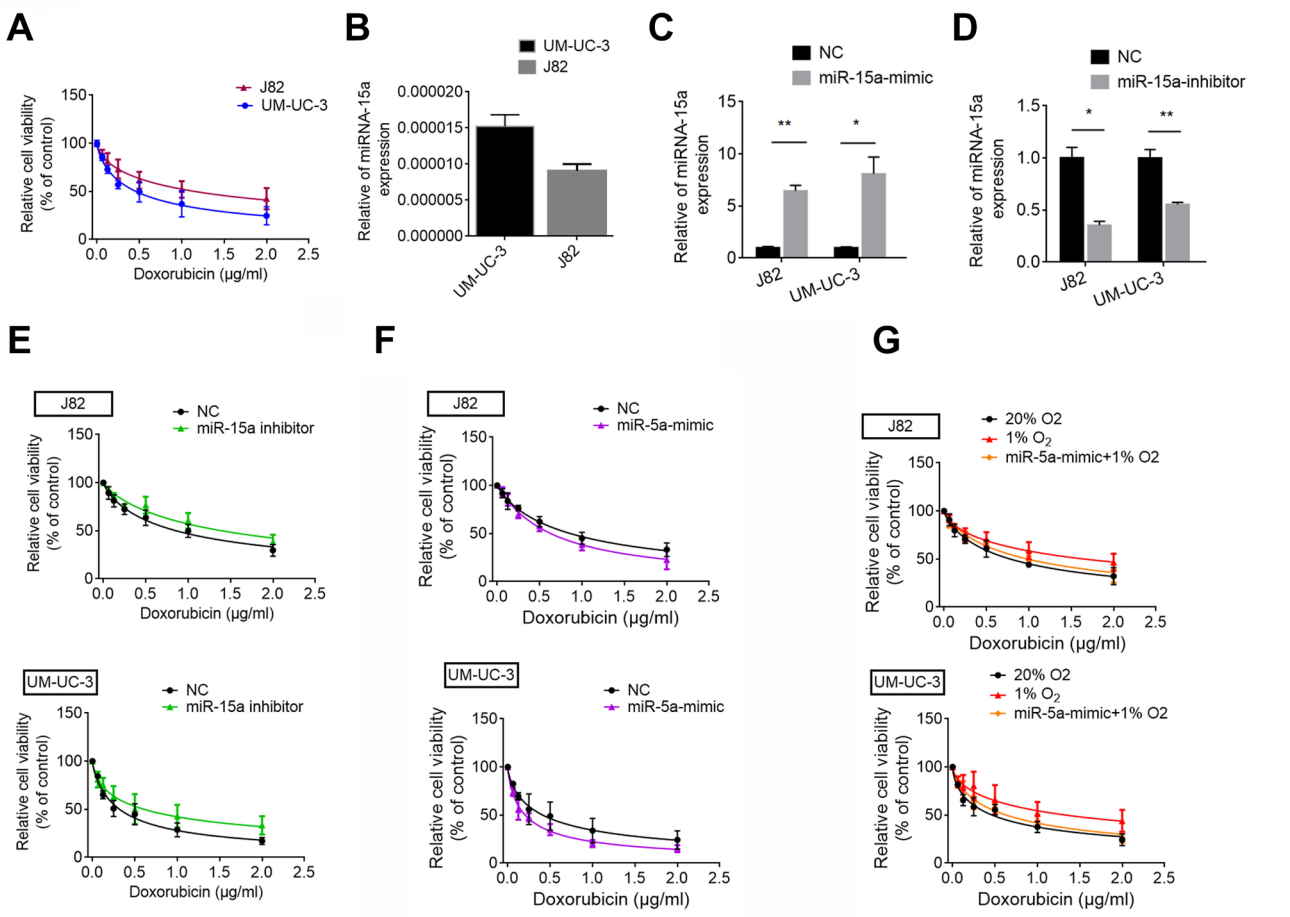

Figure 3. The EMT-promoting effect of hypoxia is miR-15a-5p-dependent. A) Effects of various Dox concentrations on bladder cancer cell line viability (CCK-8 assay). B) Effect of Dox on the relative expression of miR-15a-5p in bladder cancer cell lines. C) miR-15a-5p mimic transfection efficacies in bladder cancer cell lines, *p<0.05, **p<0.01, miR-15a-5p mimics group vs. the negative control group. D) miR-15a-5p inhibitor transfection efficacies. *p<0.05, **p<0.01, miR-15a-5p inhibitor group vs. negative control group. E) Effects of various Dox concentrations on the viability of UM-UC-3 and J82 cells transfected with miR-15a-5p mimics or negative control (CCK8 assay). F) Effects of various Dox concentrations on the viability of UM-UC-3 and J82 cells transfected with miR-15a-5p inhibitor or negative control. G) Effects of various Dox concentrations on the viability of hypoxia-treated bladder cancer cell lines transfected with miR-15a-5p mimics a or a negative control oligo (CCK-8 assay.)

tance. Transfection of the miR-15a-5p mimic into bladder cancer cells ameliorated eIF5A2-induced Dox resistance (Figure 5F). The miR-15a-5p mimic only slightly reduced eIF5A2-induced Dox resistance in UM-UC-3 and J82 cells (Figure 5F). Collectively, these findings showed that miR-15a-5p might inhibit the hypoxia-induced Dox resistance by specifically downregulating *EIF5A2* expression.

Discussion

In the advanced stage of bladder cancer, hypoxia stimulates HIF-1α-mediated signaling pathways, permitting cancer cell survival under hypoxic conditions by promoting tumor growth, metastasis, drug resistance, and radioresistance [23, 24]. Generally, intratumoral hypoxia-induced HIF-1α is considered an oncoprotein [25]. Experimental and clinical evidence supports the view that hypoxia adaptation contributes to chemotherapy resistance development in bladder cancer, and overexpression of HIF-1α is associated with poor patient prognosis [18, 26]. Our findings not only provide further evidence supporting the key roles of hypoxia in bladder cancer progression and chemoresistance, but also reveal a new molecular mechanism by which hypoxia activates the miR-15a-5p/eIF5A2/EMT signaling pathway to facilitate tumor growth and Dox resistance in bladder cancer.

Recently, miR-15a-5p was identified as a cancer-associated miRNA that participates in cancer progression. For example, miR-15a-5p overexpression was reported to be oncogenic in hepatocellular carcinoma and renal cell carcinoma [27, 28]. According to the microRNA target gene database starBase, in bladder cancer, miR-15a-5p functions as an oncogene (Figure 2A). However, in this paper, we found that the roles of miR-15a-5p in bladder cancer are opposite to previously reported findings. Our results revealed that miR-15a-5p

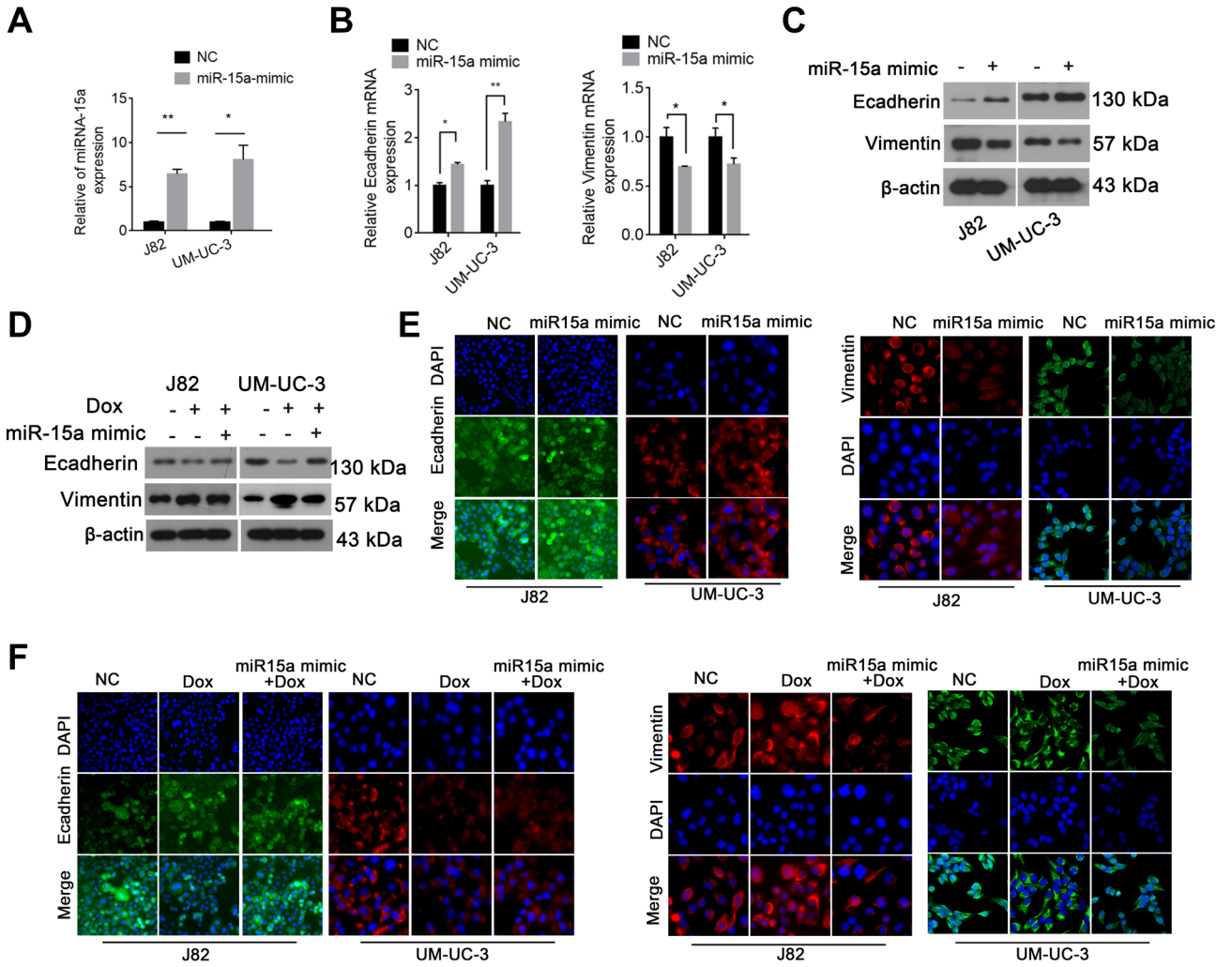

Figure 4. miR-15a-5p reverses the hypoxia-induced EMT. A) miR-15a-5p mimic transfection efficacies in bladder cancer cell lines, *p<0.05, **p<0.01, miR-15a-5p mimics group vs. the negative control group. B) miR-15a-5p upregulates E-cadherin and downregulates Vimentin. qRT-PCR analysis of CDH1 (encoding E-cadherin) (left) and VIM (encoding Vimentin) (right) expression. *p<0.05, **p<0.01, miR-15a-5p mimics group vs. the negative control group. C) Vimentin and E-cadherin levels in UM-UC-3 and J82 cells transfected with miR-15a-5p mimics or the negative control oligonucleotide. D) miR-15a-5p overexpression attenuates hypoxia and Dox-induced E-cadherin inhibition and Vimentin increase. E, F) Immunofluorescence was used to detect the expression of E-cadherin and Vimentin.

expression was downregulated by both hypoxia and Dox treatment (Figure 1). In bladder cancer, miR-15a-5p knockdown conferred *in vivo* and *in vitro* chemotherapeutic resistance, while the overexpression of miR-15a-5p decreased hypoxia-induced tumor growth and enhanced Dox resistance. Thus, our results indicated that miR-15a-5p is a therapeutic target for enhancing Dox cytotoxicity in patients with bladder cancer.

miRNAs exert their functions by repressing gene expression post-transcriptionally by binding to the 3¢ UTR of their target mRNAs [29]. Herein, we determined that *EIF5A2* is the target of miR-15a-5p: miR-15a-5p binding to the *EIF5A2* 3¢ UTR decreased its expression. Previously, high expression of eIF5A2 was reported in bladder cancer, which is capable of elevating Dox resistance [22]; therefore, we speculated that miR-15a-5p-mediated downregulation of *EIF5A2* would decrease Dox resistance in bladder cancer.

EMT plays a pivotal role in the induction of Dox resistance under hypoxia in bladder cancer [30, 31]. In the present study, we found that rescue of miR-15a-5p expression in hypoxia-treated bladder cancer cells disturbed the EMT phenotype induced by hypoxia, i.e., increased HIF-1α and Vimentin (a mesenchymal marker) levels and decreased E-cadherin levels (an epithelial marker). Additionally, knockdown of *EIF5A2* prevented the EMT phenotype, while high expression of *EIF5A2* induced EMT, in hypoxic bladder cancer cells. Furthermore, the miR-15a-5p mimic inhibited eIF5A2-mediated EMT. Notably, EIF5A2 overexpression in

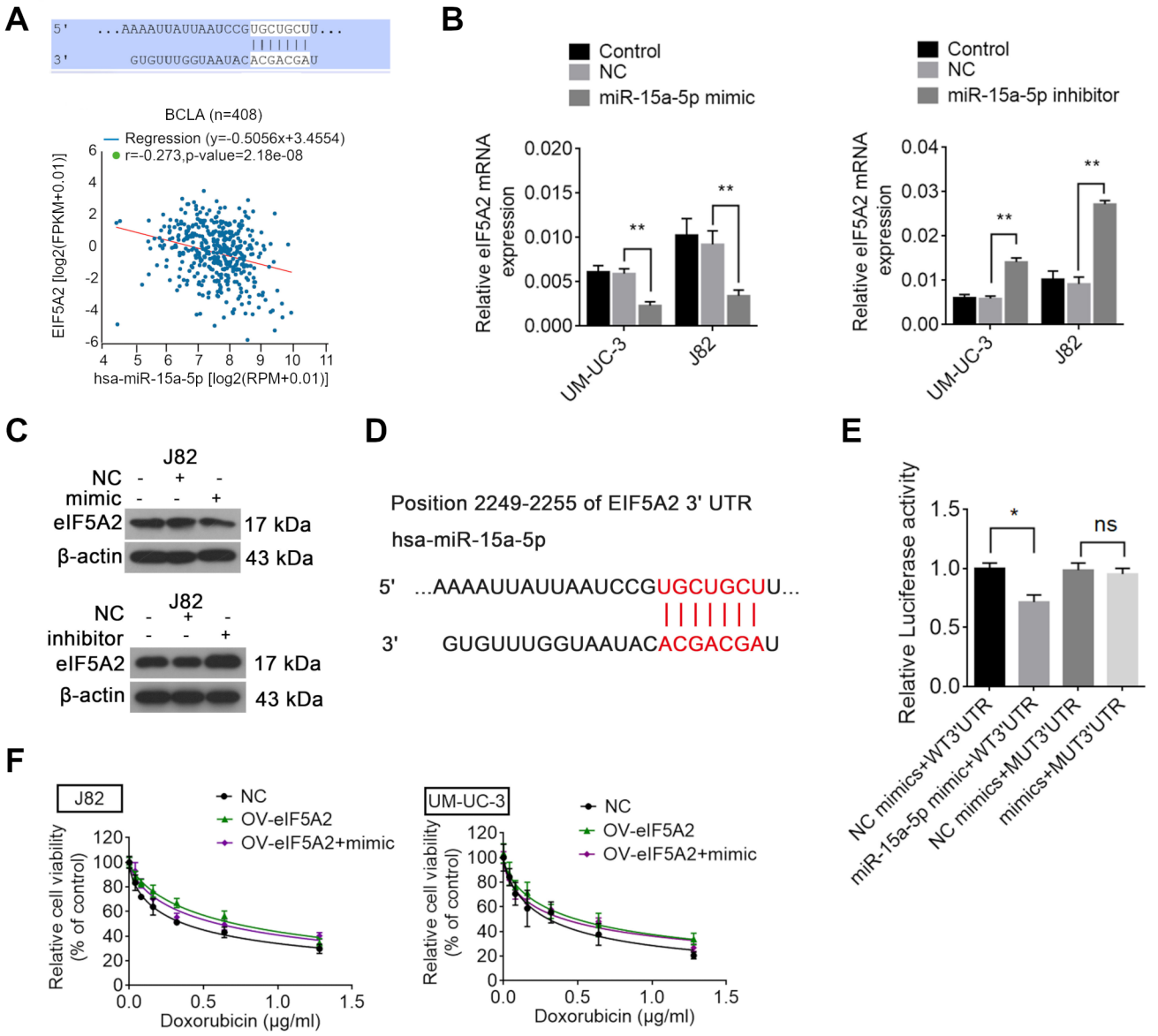

Figure 5. EIF5A2 is the downstream target of the miR-15a-5p. A) StarBase predicted binding between miR-15a-5p and eIF5A2. B) eIF5A2 levels in cells transfected with miR-15a-5p mimics (left) or the miR-15a-5p inhibitor (right). **p<0.01, vs. the control group. C) eIF5A2 protein levels in J82 cells transfected with miR-15a-5p mimics or the miR-15a-5p inhibitor. D) The binding site sequences of eIF5A2 and miR-15a-5p. (E) The miR-15a-5p WT group showed decreased luciferase activity. F) miR-15a-5p mimic transduction of UM-UC-3 and J82 cells significantly suppressed the Dox sensitivity inhibited by the EIF5A2 overexpressed plasmid (CCK-8 assay).

UM-UC-3 and J82 cells only slightly increases Dox resistance (Figure 5F), because the control cells also have high endogenous eIF5A2 expression (Figure 5B). Consequently, miR-15a-5p cannot effectively repress *EIF5A2* expression and reduce Dox resistance in these cell lines (Figure 5F). Our results suggest that inhibiting the downregulation of miR-15a-5p in hypoxic tumor regions could reduce HIF signaling and EMT in bladder cancer.

In conclusion, our findings identified miR-15a-5p as a new molecular player involved in hypoxia-induced tumor growth and Dox resistance in bladder cancer. We revealed that hypoxia, which occurs during tumor progression and chemoresistance, activates HIF-1α expression, resulting in the downregulation of miR-15a-5p. This causes a reversal of miR-15a-5p-mediated inhibition of *EIF5A2* expression and EMT, thereby promoting tumor growth and decreasing Dox sensitivity. Therefore, specific upregulation of miR-15a-5p could represent a therapeutic strategy to ameliorate hypoxia-induced Dox resistance in the treatment of bladder cancer.

Supplementary information is available in the online version of the paper.

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microRNA-15a-5p suppresses hypoxia-induced tumor growth and chemoresistance in bladder cancer by binding to eIF5A2

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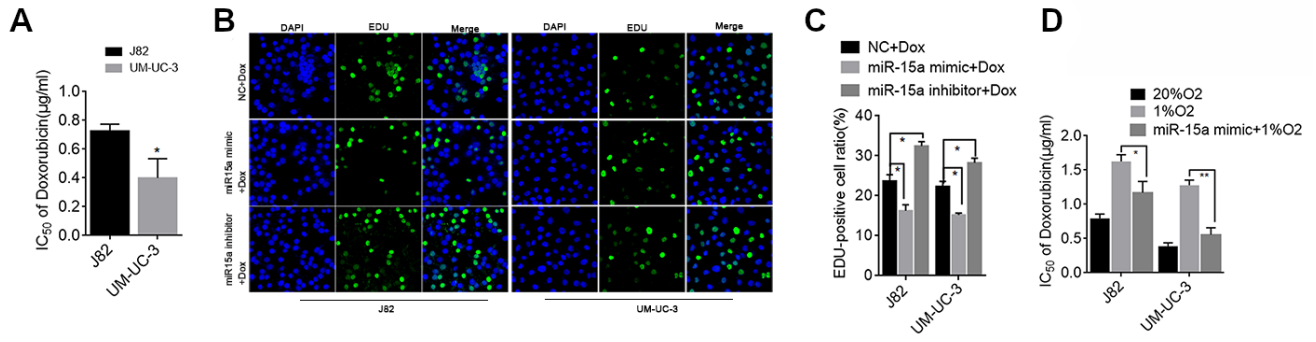
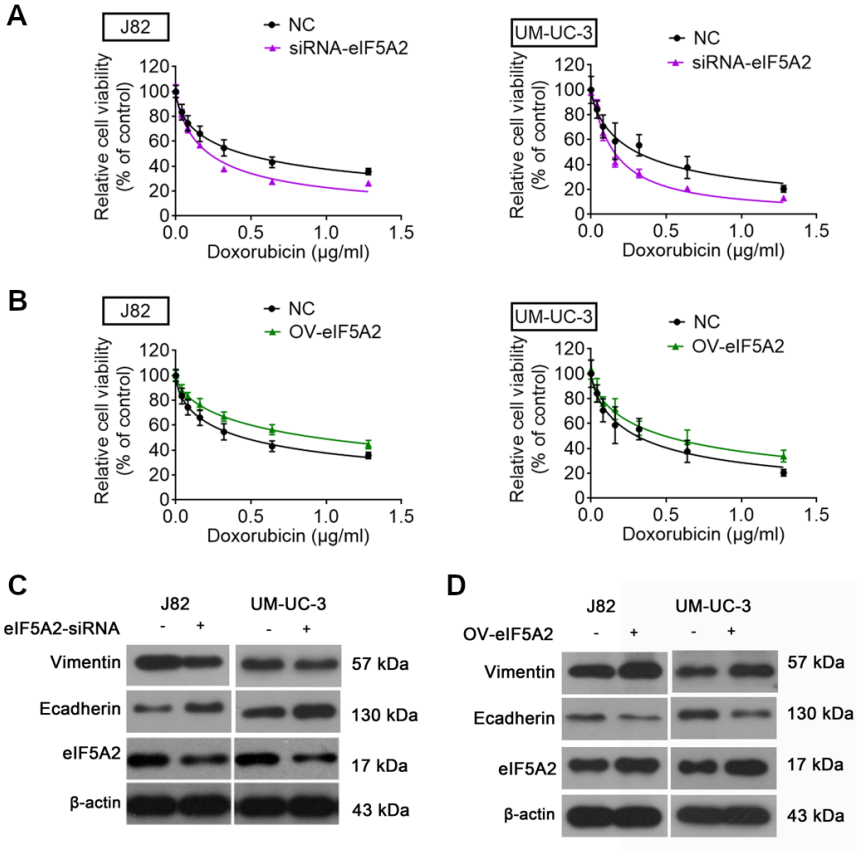
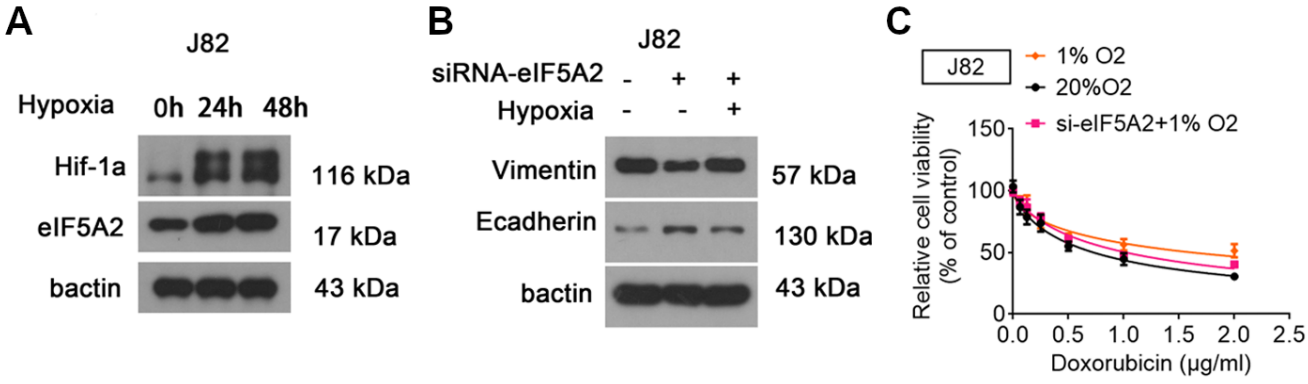
Supplementary Information



Supplementary Figure S1. A, D) The IC50 was determined in different groups. B, C). EdU determined cell proliferation in different groups.



Supplementary Figure S2. A, B) CCK-8 assay the viability of Dox-treated cells transfected with an *EIF5A2* siRNA or OV-eIF5A2. C, D) Vimentin, E-cadherin and eIF5A2 expression levels in cells transfected with eIF5A2 siRNA or OV-eIF5A2.



Supplementary Figure S3. A, B) Vimentin, E-cadherin, and eIF5A2 expression levels examined following transfection with or without an *EIF5A2* siRNA under hypoxic condition. C) CCK-8 assay of cell viability following transfection with or without the *EIF5A2* siRNA under hypoxic condition.