

# Hypoxia induced upregulation of miR-301a/b contributes to increased cell autophagy and viability of prostate cancer cells by targeting NDRG2

Y.-J. GUO<sup>1</sup>, J.-X. LIU<sup>2</sup>, Y.-W. GUAN<sup>1</sup>

<sup>1</sup>Department of Urology, General Hospital of Beijing Military Region, PLA, Beijing, China

<sup>2</sup>Department of Urology, the Second People's Hospital of Shenzhen, Shenzhen, China

**Abstract. – OBJECTIVE:** Previous studies reported that NDRG2 might be a tumor suppressor of prostate cancer. In this study, we investigated the hypoxia-induced expression change of miR-301a/b in prostate cancer cells and explored its regulation on NDRG2 in autophagy and viability of prostate cancer cells.

**MATERIALS AND METHODS:** MiR-301a/b expression in hypoxia and normoxia cultured prostate cancer cells was measured. Its regulation on autophagy was measured by quantifying expression change of LC3B and p62. The direct binding between miR-301a/b and 3'UTR of NDRG2 was verified using dual luciferase, qRT-PCR and Western blot assay. The influence of miR-301a/b-NDRG2 axis on autophagy, viability and apoptosis of prostate cancer cells was further investigated.

**RESULTS:** Hypoxia induced a significant up-regulation of miR-301a/b in prostate cancer cells. Enhanced miR-301a/b expression significantly weakened autophagy of prostate cancer cells. Both miR-301a and miR-301b could directly target 3'UTR of NDRG2 and decrease its expression. Decreased NDRG2 expression directly resulted in increased autophagy and cell viability and reduced cell apoptosis.

**CONCLUSIONS:** Taken together, we demonstrated that miR-301a/b-NDRG2 might be an important axis modulating autophagy and viability of prostate cancer cells under hypoxia.

*Key Words:*

miR-301a, miR-301b, Hypoxia, Prostate cancer, NDRG2.

## Introduction

Prostate cancer is one of the leading causes of malignancy related death in men<sup>1,2</sup>. As a type of

solid tumor, one of the key environmental stressors is decreased oxygen supply, termed hypoxia<sup>3</sup>. A series of previous studies showed that the prostate cancer cells present multiple adaptive responses to hypoxia, such as changing cellular metabolism and increasing vascularization<sup>4</sup>, which contribute to enhanced migratory potential and cell survival. Higher level of hypoxia is also correlated to higher tumor aggressiveness, clinical stage and poor survival outcome of the prostate cancer patients<sup>5,6</sup>. Therefore, it is quite necessary to further explore the key players in hypoxic adaptation, which might be important to identify new therapeutic targets of prostate cancer<sup>7</sup>.

Hypoxia can also induce altered expression of miRNAs in prostate cancer, which contribute to the pathological process via modulating the expression of their downstream genes<sup>8</sup>. For example, one recent study found that hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) can promote the expression of miR-182, which directly target both prolyl hydroxylase domain enzymes (PHD) and factor inhibiting HIF-1 (FIH1), two negative regulators of HIF1 signaling<sup>9</sup>. Hypoxia induced higher expression of miR-106b~25 directly inhibit the expression of RE-1 Silencing Transcription Factor (REST), whose loss predict early recurrence of prostate cancer<sup>10,11</sup>. Some recent studies also showed that hypoxia responsive miRNAs are involved in the regulation of autophagy, a pro-survival mechanism of prostate cancer cells in hypoxia<sup>12-14</sup>. MiR-301a and miR-301b are two miRNAs clustered together. One recent study found that infiltrating pre-adipocytes in prostate tumor can induce miR-301a expression and promote metastasis through down-regulating androgen receptor (AR)<sup>15</sup>. In addition, in combination with miR-652/454/223/139, miR-301a can also strong-

ly predict metastasis of prostate cancer<sup>16</sup>. MiR-301b is also an oncomiR in pancreatic carcinoma<sup>17</sup> and breast cancer<sup>18</sup>. However, the expression change of these two miRNAs in hypoxia has not been reported yet. NDRG2 plays a tumor suppressor role in prostate cancer. NDRG2 overexpression can inhibit tumor growth and invasion of prostate cancer cells<sup>19,20</sup>. Its upstream regulation and its functional role in hypoxia in prostate cancer are not quite clear

In this study, we explored how hypoxia induced expression change of miR-301a/b and how these two hypoxia responsive miRNAs modulate autophagy.

## Materials and Methods

### Cell culture

Prostate cancer cell lines LNCaP, PC-3 and DU145 cell were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA) in an cell incubator with a humidified atmosphere, 5% CO<sub>2</sub> at 37°C under either normoxia or hypoxia (1% oxygen) environment.

### Reagents

pSELECT-GFP-LC3 plasmid was purchased from Invitrogen (Carlsbad, CA, USA). MiR-301a and miR-301b mimics, miR-301a and miR-301b inhibitors (IH), NDRG2 siRNA and the scrambled negative controls were purchased from RiBoBio (Shanghai, China). NDRG2 expression vector (pDONR-NDRG2, without 3'UTR sequence) was purchased from Futai Bio (Taizhou, Jiangsu, China). 3-methyladenine (3-MA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The cancer cells were transfected with 100 nM miR-301a or miR-301b for overexpression or transfected with 75 nM miR-301a inhibitor and 75 nM miR-301a inhibitor simultaneously for knockdown. To overexpress or knockdown of endogenous NDRG2, cells were transfected with pDONR-NDRG2 or 100 nM NDRG2 siRNA respectively. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to manufacturer's instruction. In some studies, cells were treated with 3-MA (5 mM) 1 hr before transfection, for a duration of 36 hrs.

### qRT-PCR Analysis

Total miRNA and RNA isolation, reverse transcription of cDNA and qRT-PCR analysis based on the prostate cancer cell samples followed the methods introduced in one previous study<sup>21</sup>. To quantify the change of miR-301a and miR-301b induced by hypoxia, the prostate cancer cells were subjected to hypoxia for 36 h. The primers used for qRT-PCR analysis of NDRG2 were: forward, 5'-CAGGACAAACACCCGAGACT-3'; reverse, 5'-AGCCATAAGGTGTCTC-CACAG-3'. The qRT-PCR was performed using an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) and the results were calculated using 2<sup>-ΔΔct</sup> methods.

### CCK-8 Assay of Cell viability and Flow Cytometry analysis of Cell Apoptosis

Cells after indicated transfection were subjected to hypoxia. Cell viability was assessed with Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) at 0, 24, 48 and 72h using a spectrophotometer. Cell apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit (V13241, Invitrogen, Carlsbad, CA, USA) using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

### Transfection and Selection of LNCaP Cells with Stable GFP-LC3 Expression

LNCaP cells transfected with pSELECT-GFP-LC3 were seeded into 96 well plates and were selected using 250 μg/ml Zeocin (Sigma-Aldrich, St. Louis, MO, USA) in RPMI 1640. Stable clones were selected after three weeks' screening. 24h after indicating transfection, cells subjected to hypoxia for another 36h, then the number of GFP-LC3 puncta per cell was quantified with Image J software.

### Western blot Analysis

Cells were lysed using a lysis buffer (Beyotime, Shanghai, China) with proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). The concentration of protein in different samples were quantified by a BCA protein assay kit (Beyotime, Shanghai, China). Then the samples were used for a conventional western blot analysis according to the methods introduced in one previous study<sup>13</sup>. Primary anti-LC3B (ab48394), anti-p62 (ab91526), anti-NDRG2 (ab57429) and anti-GAPDH (ab125247) and HRP conjugated secondary antibodies were purchased from Abcam (Cambridge, MA, USA). The protein

signals were detected using the BeyoECL Plus kit (Beyotime, Shanghai, China) according to manufacturer's instruction.

### **Dual Luciferase Assay**

To construct luciferase reporter vectors, two oligonucleotides containing wild-type (with 301a/b binding site) and mutant (without 301a/b binding site) 3'UTR of NDRG2 were chemically synthesized by Ribobio (Shanghai, China). The sequences were inserted into the downstream of the luciferase gene of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The reconstructed plasmids were named as pmirGLO-NDRG2-WT and pmirGLO-NDRG2-MT respectively. The insertion was confirmed using sequencing. Then, the LNCaP cells were co-transfected with 200 ng plasmids and 100 nM miR-301a or miR-301b mimics using Lipofectamine 2000 (Invitrogen). Relative luciferase activity was analyzed 24h after transfection, by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to manufacturer's protocol. Firefly luciferase activity was normalized to that of Renilla luciferase.

### **Statistical Analysis**

Data were reported as means  $\pm$  standard deviation (SD) based on at least three repeats of three times independent studies. Group comparison was performed using Student's *t*-test with SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). *p* value of  $<0.05$  was considered as statistically significant.

## **Results**

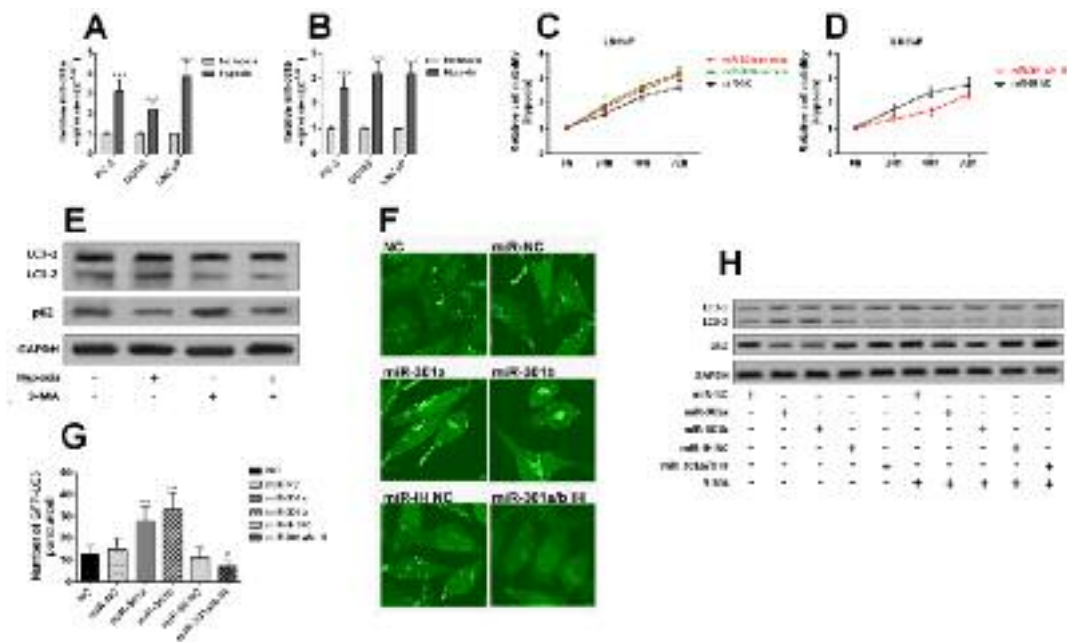
### **Hypoxia Induced Higher Expression of miR-301a and miR-301b Enhance Viability and autophagy of Prostate Cancer Cells**

Hypoxia can induce dynamic changes of cancer cells, acting as adaptive responses to the stressful environment. We firstly explored how hypoxia influences the expression of miR-301a and miR-301b, two oncomiRs of prostate cancer. QRT-PCR analysis showed that hypoxia induced at least two folds increase of miR-301a and miR-301b expression in PC-3, DU145 and LNCaP cells (Figure 1 A-B). Then, we investigated how these two miRNAs affected the viability of prostate cancer cells. MiR-301a and miR-301b

overexpression significantly increased cell viability in hypoxia (Figure 1C). Knockdown of endogenous miR-301a/b substantially decreased cell viability (Figure 1D). Induction of autophagy in hypoxia is a pro-survival physiological response. To investigate whether hypoxia induces autophagy in prostate cancer cells, LC3B and p62 level in LNCaP cells in hypoxia with or without 3-MA treatment was detected. Hypoxia treatment significantly increased LC3-II expression and facilitated degradation of p62, which suggest enhanced autophagy. In contrast, with the treatment of 3-MA, LNCaP cells in hypoxia has decreased LC3-II expression and partly restored p62, suggesting hypoxia induced autophagy was partly inhibited by 3-MA (Figure 1E). Then, we decided to further investigate the role of miR-301a/b in the autophagy under hypoxia. MiR-301a and miR-301b overexpression significantly enhanced LC3-GFP puncta accumulation in LNCaP cells. However, knockdown of endogenous miR-301a/b substantially attenuated the accumulation (Figure 1F-G). Western blot analysis was further performed to verify the influence of miR-301a/b on autophagy induced by hypoxia. In consistent with the GFP puncta results, miR-301a and miR-301b significantly promoted LC3-II expression and p62 degradation. MiR-301a/b inhibitor presented the reverse effects (Figure 1H). These results suggest that hypoxia induced higher expression of miR-301a and miR-301b enhanced viability and autophagy of prostate cancer cells.

### **MiR-301a and miR-301b Directly Target 3'UTR of NDRG2 and Decrease its Expression**

Online prediction using TargetScan 6.3 showed that NDRG2 is a common target of miR-301a and miR-301b (Figure 2A). In fact, NDRG2 is a potential tumor suppressor of prostate cancer<sup>19</sup>. In LNCaP cells, enforced expression of miR-301a or miR-301b significantly decreased NDRG2 expression at mRNA and protein level (Figure 2 B-C). Then, we constructed two dual luciferase reporter plasmids carrying either wild-type or mutant miR-301a/b targeting 3'UTR of NDRG2. Dual luciferase assay showed that both miR-301a and miR-301b could substantially suppress the relative luciferase activity of LNCaP cells transfected with the reporter plasmids carrying wild-type sequence, but not the cells transfected with mutant plasmids (Figure 2 D-E).



**Figure 1.** Hypoxia induced higher expression of miR-301a and miR-301b enhances viability and autophagy of prostate cancer cells. A and B, PC-3, DU145 and LNCaP cells were cultured in hypoxia for 36 h. miR-301a (A) and miR-301b (B) expression levels were determined by qPCR (n=3). C and D, LNCaP cells were transfected with 100 nM miR-301a or miR-301b mimics (C) or 75 nM miR-301a inhibitor and 75 nM miR-301b inhibitor (D) and, then, exposed to hypoxia up to 72 h. Cell viability were measured by CCK-8 assay at indicating time point. E, LNCaP cells were exposed to normoxia or hypoxia (1%) with or without 3-MA treatment (5 mM) for 36 h. LC3B, p62 and GAPDH were determined by Western blot. F, LNCaP cells with stable GFP-LC3 expression were transfected with miR-301a mimics, miR-301b mimics or miR-301a inhibitor and miR-301b inhibitor and then subjected to hypoxia for 36 h. The cells were then observed under confocal microscopy. G, Number of GFP-LC3 puncta/cell was calculated with Image J software. H, LNCaP cells with the same transfection as (F) were subjected to 3-MA treatment and hypoxia for 36 h. LC3B, p62 and GAPDH were determined by Western blot. \*comparison with normoxia or miR-NC. #comparison with miR-IH NC. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**MiR-301a and miR-301b Enhances Viability and autophagy of Prostate Cancer Cells in hypoxia via Decreasing NDRG2 Expression**

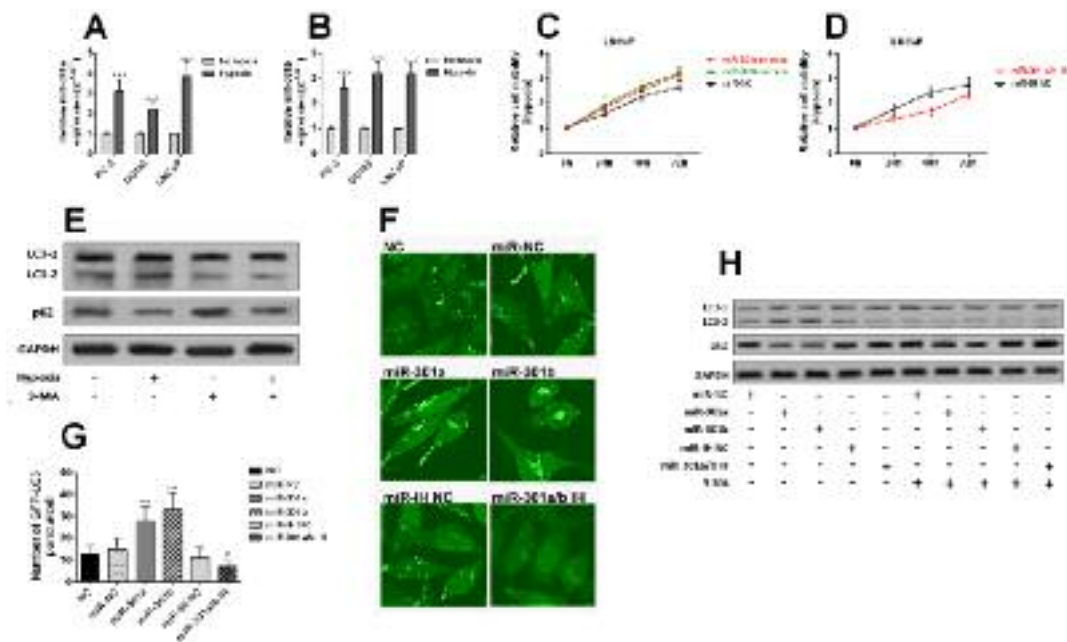
Since we verified the regulative role of miR-301a and miR-301b over NDRG2 expression, we further explored the function of miR-301a/b-NDRG2 axis in viability and autophagy of prostate cancer cells in hypoxia. Knockdown of NDRG2 significantly promoted autophagy in both normoxia and hypoxia (Figure 3A). In addition, NDRG2 knockdown also enhanced cell viability (Figure 3C) and reduced cell apoptosis (Figure 3E) in hypoxia. To further verify the functional role of NDRG2 and miR-301a/b, we used a NDRG2 expression vector without 3'UTR. Enforced NDRG2 expression using this vector remarkably attenuated autophagy (Figure 3B) and decreased cell viability (Figure 3D) under hypoxia. Besides, cells with enforced NDRG2 expression also had a higher rate of apoptosis (34.9% vs. 22.4%) in hypoxia (Figure

3E). However, neither miR-301a nor miR-301b could reverse the effect of this expression vector on autophagy, cell viability and apoptosis (Figure 3B, D-E).

**Discussion**

Both miR-301a and miR-301b function as tumor promoters contributing to tumor progression of multiple cancers, including hepatocellular carcinoma<sup>22</sup>, breast cancer<sup>18,22</sup>, colorectal cancer<sup>23</sup>, gastric cancer<sup>24</sup> and pancreatic cancer<sup>25</sup>. The underlying mechanism of miR-301a or miR-301b in promoting these cancers include directly targeting TP63<sup>25</sup>, TGFBR2<sup>23</sup>, Gax<sup>26</sup> and RUNX3<sup>24</sup>. In prostate cancer cells, one recent study demonstrated that pre-adipocytes, one of the basic components in the tumor microenvironment (TME) can induce significantly higher expression of miR-301a, which contribute to enhanced cancer metastasis by downregulating





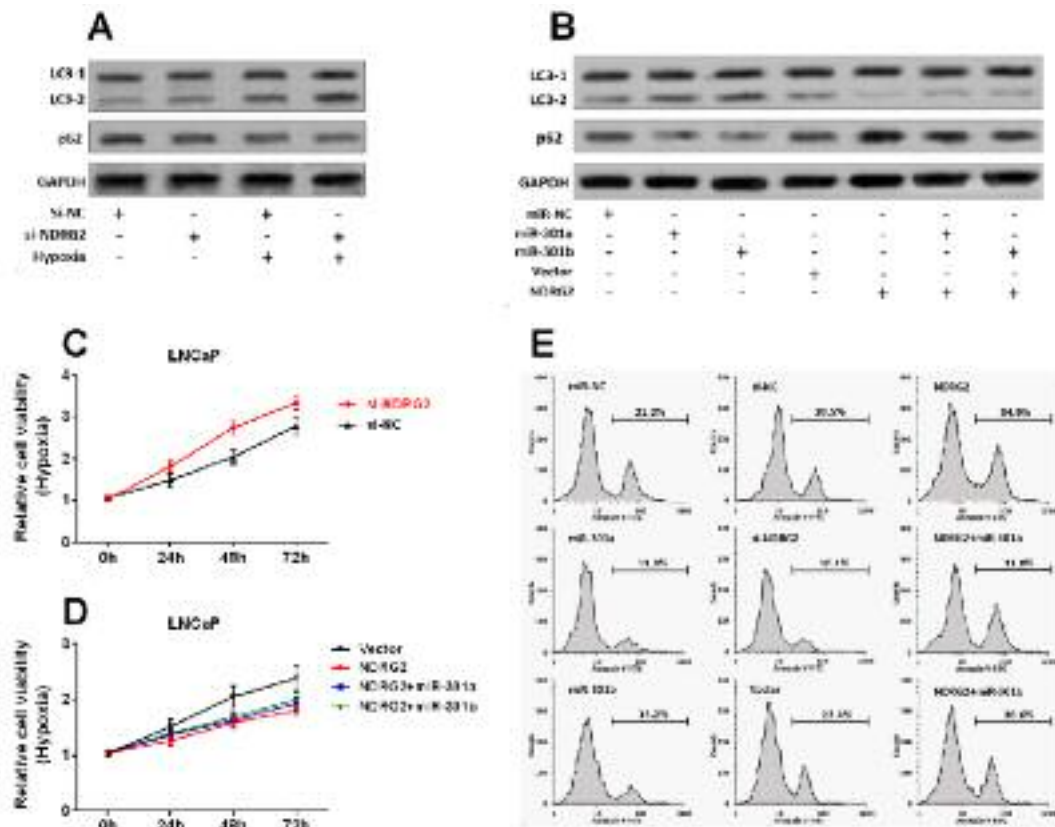
**Figure 2.** MiR-301a and miR-301b directly target 3'UTR of NDRG2 and decrease its expression. **A**, Predicted binding sites between miR-301a/b and NDRG2 3'UTR. The designed mutant sequence was also provided. **B** and **C**, LNCaP cells were transfected with 100 nM miR-301a mimics, miR-301b mimics or NDRG2 siRNA. NDRG2 mRNA (**B**) and protein (**C**) was measured by qRT-PCR and western blot respectively. **D** and **E**, LNCaP cells were co-transfected with 100 nM miR-301a mimics (**D**) or miR-301b mimics (**E**) and pmirGLO-NDRG2-WT or pmirGLO-NDRG2-MT. The relative luciferase activity was measured 24h after transfection. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

AR<sup>15</sup>. Another study found that prostate cancer cell lines overexpressing miR-301a had significantly higher tumor growth and metastasis in a xenograft mouse model<sup>16</sup>. Besides, in combination with miR-652/454/223/139, miR-301a strongly predict metastasis of prostate cancer<sup>16</sup>. Hypoxia is an important TME stressor forcing cancer cells make adaptive responses. In this study, we observed that hypoxia induced significantly higher miR-301a and miR-301b expression in prostate cancer cells. Considering the important oncogenic role of these two miRNAs, we decided to further investigate their functions in hypoxia.

Autophagy is a tightly controlled and evolutionarily conserved process that eliminates defective organelles and proteins to maintain cellular homeostasis<sup>27</sup>. In prostate cancer, autophagy is utilized by cancer cells as an important survival mechanism to androgen deprivation and hypoxia<sup>12,28</sup>. Inhibition of autophagy in prostate cancer can enhance tumor apoptosis in hypoxia<sup>29</sup>. However, the molecular mechanism involved in autophagy of prostate cancer cells in hypoxia has not been fully revealed. Some recent studies suggest that hypoxia responsive

miRNAs are involved in this process. For example, hypoxia increased the expression of miR-96 in prostate cancer cells, which stimulates autophagy through suppressing MTOR<sup>13</sup>. HIF1 $\alpha$  induced miR-182 can enhance HIF1 signaling through decreasing PHD and FIH1, two negative regulators of HIF1<sup>9</sup>. Hypoxia can also induce the expression of miR-21, a well-recognized oncomiR in prostate cancer<sup>5</sup>. In this study, we observed that hypoxia induced miR-301a and miR-301b can suppress autophagy and enhance cell viability. Through performing dual luciferase assay, we confirmed that both miR-301a and miR-301b could downregulate NDRG2, a prostate tumor suppressive gene. In addition, we also demonstrated that the effect of miR-301a and miR-301b in enhancing viability and autophagy of prostate cancer cells in hypoxia is through decreasing NDRG2 expression.

NDRG2 overexpression can inhibit tumor growth and invasion of prostate cancer cells<sup>19</sup>. The downregulation of NDRG2 in prostate cancer tissues was significantly correlated with advanced pathological stage, positive metastatic status and high Gleason score<sup>30</sup>. The loss of NDRG2 directly results in enhanced phosphory-



**Figure 3.** MiR-301a and miR-301b enhances viability and autophagy of prostate cancer cells in hypoxia via decreasing NDRG2 expression. **A**, LNCaP cells transfected with 100 nM NDRG2 were then exposed to normoxia or hypoxia for 36 h. LC3B, p62 and GAPDH were determined by Western blot. **B**, LNCaP cells transfected with NDRG2 expression vector with or without co-transfection of miR-301a mimics or miR-301b mimics were exposed hypoxia for 36 h. LC3B, p62 and GAPDH were determined by Western blot. **C** and **D**, Cell viability of LNCaP cells with indicating transfection in Figure A (**C**) and Figure B (**D**) were then exposed to hypoxia for 72 h. Cell viability were measured by CCK-8 assay. **E**, LNCaP cells with indicating transfection in Figure A and Figure B were incubated under hypoxia for 36 h. Apoptosis was examined by flow cytometer (n=3).

lation of PTEN<sup>31,32</sup>. In fact, PTEN phosphorylation can promote autophagy in response to DNA-damaging agents in cancer cells<sup>33</sup>. This might help to explain the how miR-301a/ miR-301b-NDRG2 axis modulate autophagy in prostate cancer in hypoxia.

### Conclusions

Hypoxia can induce significant upregulation of miR-301a/b, which contributes to increased cell viability and reduced cell apoptosis through decreasing autophagy by targeting NDRG2. This axis represents a potential therapeutic target for the treatment of prostate cancer.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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