European Review for Medical and Pharmacological Sciences 2016; 20: 3051-3059

Hypoxia-induced miR-17-5p ameliorates the viability reduction of astrocytes via targeting p21

J.-F. LI[1], P.-M. WANG[2], N.-N. ZHANG[3], X. ZHANG[1], J. GUAN[4], Z. YE[4], J.-Y. YAN[1]

[1]Department of Rehabilitation, the Second Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China
[2]Department of Orthopedics, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China
[3]Department of Cancer Minimally Invasive Interventional Radiology, Second People's Hospital of Tianjin, Tianjin, China
[4]Inner Mongolia Medical University, Hohhot, China

Jianfeng Li and *Peimin Wang* contributed equally to this work

Abstract. – OBJECTIVE: Glial scars are widely seen as a mechanical barrier to central nervous system regeneration. Up to now, several studies have addressed and clarified how different lesion microenvironment properties affect astrogliosis. In particular, hypoxia induces the astrocyte astrogliosis, and thus promotes the formation of glial scars. However, little is known about the mechanism underlining such process. In the present study, we investigated the regulation by the miR-17-5p on the hypoxia-induced viability via targeting p21.

MATERIALS AND METHODS: We examined the expression of miR-15a, miR-16, miR-17-5p, hypoxia inducible factor-1α (HIF-1α) and p21 in the astrocytes under hypoxia, with quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting (WB) methods. Then investigated the regulatory role of miR-17-5p on the level of HIF-1α and p21, with qRT-PCR, WB and luciferase reporting assay, and examined the activity of astrocytes under normoxia or hypoxia.

RESULTS: Results demonstrated that miR-15a, miR-16, miR-17-5p were significantly upregulated, while HIF-1α and p21 were markedly downregulated in the hypoxia-treated astrocytes. And the transfection with miR-17-5p mimics significantly downregulated the expression of HIF-1α and p21 in such cells. And the luciferase reporter assay confirmed the targeting inhibiting of p21 by miR-17-5p in astrocytes. Moreover, the viability of astrocytes was significantly upregulated by the miR-17-5p mimics transfection under the hypoxia condition.

CONCLUSIONS: Our novel data suggest that the upregulated miR-17-5p contributes to the proliferation of astrocytes, in response to hypoxia, implying the potential role of miR-17-5p in the formation of glial scars.

Key Words:
Hypoxia, miR-17-5p, Astrocytes, HIF-1α, p21.

Introduction

Damages to central nervous system (CNS), including the brain injury, stroke, and neurodegenerative diseases, cause regional neuronal death and forms a barrier at the edge of the damaged region, thus inhibiting the regeneration of new neurons and affecting the recovery of brain function[1]. Glial scar formed by CNS injury is the main inhibitory barrier of nerve regeneration, composed of astrocytes, microglia, macrophages, extracellular matrix and connective tissue elements[2-5]. Astrogliosis, also known as astrocytosis, is an abnormal increase in the number of astrocytes due to the destruction of nearby neurons[6-8]. It has been reported that hypoxia induces the astrocyte astrogliosis and promotes the formation of glial scars[9-12], but the mechanism is still not clear. Hypoxia-inducible factor-1 (HIF-1, two sub-units: HIF-1α and HIF-1β) is a transcription factor in response to hypoxia, plays an important role in hypoxia response[13-15].

microRNAs (miRNAs) are small non-coding RNAs that could regulate gene expression in a wide variety of physiological process. The expression levels of lots of miRNAs are regulated by hypoxia[16-18]. miR-17 cluster has been confirmed to involve in the biological development, and to play an important role in malignant tumor growth and death[19]. miR-17-5p, one member of the miR-17 cluster could regulate the cell proliferation and migration in various cancers[20-22]. p21 is a potent cyclin-dependent kinase inhibitor (CDI) which could inhibit the activity of cyclin-dependent kinase (CDKs) and regulate the cell

Corresponding Author: Jinyu Yan, MD; e-mail: jinyu1973yan@163.com

cycle progression. It is closely related to the tumor inhibition[23,24]. It has been reported that the miR-17-5p could repress p21 expression in cancers[25,26], but the interaction between the miR-17-5p and p21 in astrocytes were not reported.

In the present study, we investigated the expression level of miR-15a, miR-16, and miR-17-5p, HIF-1α, HIF-1β and p21 in astrocyte DI TNC1 cells under normoxia or hypoxia, analyzed the regulation of miR-17-5p, HIF-1α and p21. Then, determined in details the regulation and mechanism of miR-17-5p with p21. We also performed that the effect of miR-17-5p to the viability of DI TNC1 cells under hypoxia. Our study implied the function of miR-17-5p in astrocyte cells and the mechanism of the miR-17-5p-induced astrocyte astrogliosis.

Materials and Methods

Cell Culture and Hypoxia (Or Normoxia) Treatment

The DI TNC1 cells were bought from American Type Culture Collection (ATCC) (Rockville, MD, USA). The culture medium was Dulbecco's Modified Eagle Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 □g/ml of streptomycin and 100 U/ml of penicillin. The cells were cultured in an incubator at 37 °C with 5% CO_2. For hypoxia treatment, the DI TNC1 cells were cultured in a hypoxia incubator with 5% CO_2 and 2% oxygen, and the oxygen concentration was monitored continuously (Forma 3130; Thermo Scientific, Rockford, IL, USA).

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

To investigate the expression level of miR-15a, miR-16, and miR-17-5p, hypoxia-inducible factor-1 (HIF-1α and HIF-1β) and p21, we extracted the total mRNA of the DI TNC1 cells with PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol manual. The extraction of mRNA samples was stored at -70 °C before utilize. For the qRT-PCR assay, One Step SYBR PrimeScript PLUS RT-PCT Kit (Takara, Tokyo, Japan) was used to quantity the relative level of miR-15a, miR-16, miR-17-5p, HIF-1α, HIF-1β and p21 for each sample according to the product's manual. The results were calculated and presented as a relative level by ΔΔCt method. The expression levels of miR-15a, miR-16, miR-17-5p were presented as a relative level to U6 (as control), while the relative level of HIF-1α, HIF-1β and p21 were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All the experiments were performed independently in triplicate.

To further confirm the role of miR17-15p in regulating the express of HIF-1α and p21 in DI TNC1 cells. The DI TNC1 cells were transfected with the 30 nM or 60 nM miR-17-5p mimics or Scramble miRNA for 12 hours, then extracted the total mRNA of the DI TNC1 cells and examined the relative level of miR-17-5p (to U6), the mRNA level of HIF-1α and p21 (to GAPDH) by the qRT-PCR assay.

Western Blot Analysis

Western blot was utilized to investigate the protein level of HIF-1α and p21. The DI TNC1 cells were collected and lysed in cell lysis buffer (Bio-Rad, Hercules, CA, USA) post transfecting with 30 nM or 60 nM miR-17-5p mimics or Scramble miRNA for 12 hours. The cell lysis solution was centrifuged at 12000 x g for 15 min at 4 °C; therefore, the supernatant was the protein samples. The protein samples were boiled in 5× SDS/β-mercaptoethanol buffer and separated in a 10% SDS-PAGE gel. After the protein electrophoresis, the proteins were transferred to the nitrocellulose membrane (Millipore, Bedford, MA, USA) by the semi-dry transfer unit. The membrane was blocked with 7.5% skimmed milk powder overnight at 4 °C and washed with TBST for three times. Polyclonal mouse antibodies against HIF-1α (Abcam, Cambridge, UK) and p21 (Abcam, Cambridge, UK) were diluted in TBST, and were utilized to neutralize HIF-1α and p21 on the membrane for 1 h at 37 °C. At last, the membrane was incubated with the HRP-linked secondary anti-mouse antibody (New England Biolabs, Ipswich, UK) for 30 min at 37 °C. Immobilon Western chemiluminescent HRP substrate (Millipore, Bedford, MA, USA) was used to image the protein band, and the protein bands were scanned by a Smart Chemi™ lamp Analysis System (Life Science, USA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Relative protein levels of HIF-1α or p21 were quantified according to the band density by Quantity One software with GAPDH as a loading control.

Construction of the Recombinant Plasmid and Transfection

The sequence of miR17-15p and the 3‘ UTR of p21 from *Mus musculus* were download from Genebank (NCBI) and aligned by Megalign (DNASTAR). The sequence of the 3‘ UTR of p21 and the mutant 3‘ UTR of p21 were synthesized by GenePharma Technology (Shanghai, China). The pMIR-Luc vector with a luciferase reporter was chosen, and we inserted the sequence of the 3‘ UTR of p21 and the mutant 3‘ UTR of p21 into the pMIR-Luc vector just behind the Cytomegalovirus promote, and constructed the recombinant plasmid pMIR-Luc-p21-3’UTR and pMIR-Luc-p21-3’UTR^{mut}. To investigate the regulation by miR17-15p on p21, the recombinant reporter plasmid and miR17-15p mimics (30 nM or 60 nM) were co-transfected into the DI TNC1 cells under normoxia or hypoxia, with Lipofectamine® 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s specification. Then the transfected cells were lysed for the luciferase assay. Renilla luciferase assay system (Promega, Madison, WI, USA) was utilized to measure the luciferase activity of the cell lysate samples.

MTT Assay

The viability of the DI TNC1 cells was performed by methyl thiazol tetrazolium (MTT) assay (Invitrogen, Carlsbad, CA, USA). The DI TNC1 cells with more than 85% confluence in a 96-wells plate were prepared before the experiment. Cells were transfected with 0, 30 or 60 nM miR-17-5p mimics or Scramble miRNA for 12 hours. After that, each well was added with 20 □l MTT (5 mg/ml) and further cultured for 4 hours at 37 °C. Post the medium removal, each well was added 200 □l of DMSO and the plate was shaken for 15 min to dissolve the crystals. Then, the plate was put into the enzyme-linked immunometric meter and the absorbance was measured at 550 nM. The result was presented as a relative level of the group under normoxia. The experiments were performed respectively in triplicate.

Statistical Analysis

Statistical analyses were performed by the GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The difference between two groups was analyzed by Student’s *t*-test or by one-way ANOVA test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Expression of miR-15a, miR-16, and miR-17-5p, Hypoxia-Inducible Factor-1 (HIF-1α and HIF-1β) and p21 in the Hypoxia-treated Astrocyte DI TNC1 Cells

To investigate the mechanism of the hypoxia-induced astrocyte astrogliosis, we measured the expression level of miR-15a, miR-16, and miR-17-5p in DI TNC1 cells under normoxia or hypoxia by qRT-PCR with U6 as an internal control. The results were shown in Figure 1. The relative level of miR-15a was up-regulated in DI TNC1 cells under hypoxia for 6 and 12 hours (*$p < 0.05$, **$p < 0.01$). But the difference between the normoxia and hypoxia was disappeared in DI TNC1 cells for 24 h (Figure 1A). The relative level of miR-16 and miR-17-5p were up-regulated in DI TNC1 cells under hypoxia than the cells under normoxia for 6, 12 or 24 h with U6 as control (Figure 1B, **$p < 0.01$), especially for miR-17-5p. Figure 1C showed a significant high level of miR-17-5p expression in DI TNC1 cells under hypoxia (***$p < 0.001$).

The expression of HIF-1 (HIF-1α and HIF-1β) and p21 on mRNA level were also measured by qRT-PCR in the hypoxia-treated astrocyte DI TNC1 cells, and the expression level was expressed as a relative level to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with the ΔΔCt method. The results were depicted in Figure 2. The mRNA level of HIF-1α was decreased in DI TNC1 cells under hypoxia for 24h, but there was no significant difference for 6 or 12h (Figure 2A, *$p < 0.05$). Figure 2B showed that the expression level of HIF-1β was no statistical significance in DI TNC1cells under normoxia or hypoxia (ns: no significance), while the relative mRNA level of p21 was significantly down-regulated in DI TNC1 cells under hypoxia for 12h (Figure 2C, *$p < 0.05$).

miR-17-5p Mimics Transfection Down-Regulates HIF-1α and p21 in both mRNA and Protein Levels in DI TNC1 Cells

Based on the above results, we figured out that the miR17-15p was significantly up-regulated in DI TNC1 cells under hypoxia, while the expression level of HIF-1α and p21 were down-regulated. To further confirm the regulatory role of miR17-15p in the DI TNC1 cells with HIF-1α and p21, we manipulated the expression level of HIF-1α and p21 in DI TNC1 cells via transfection with miR-17-5p mimics. The ex-

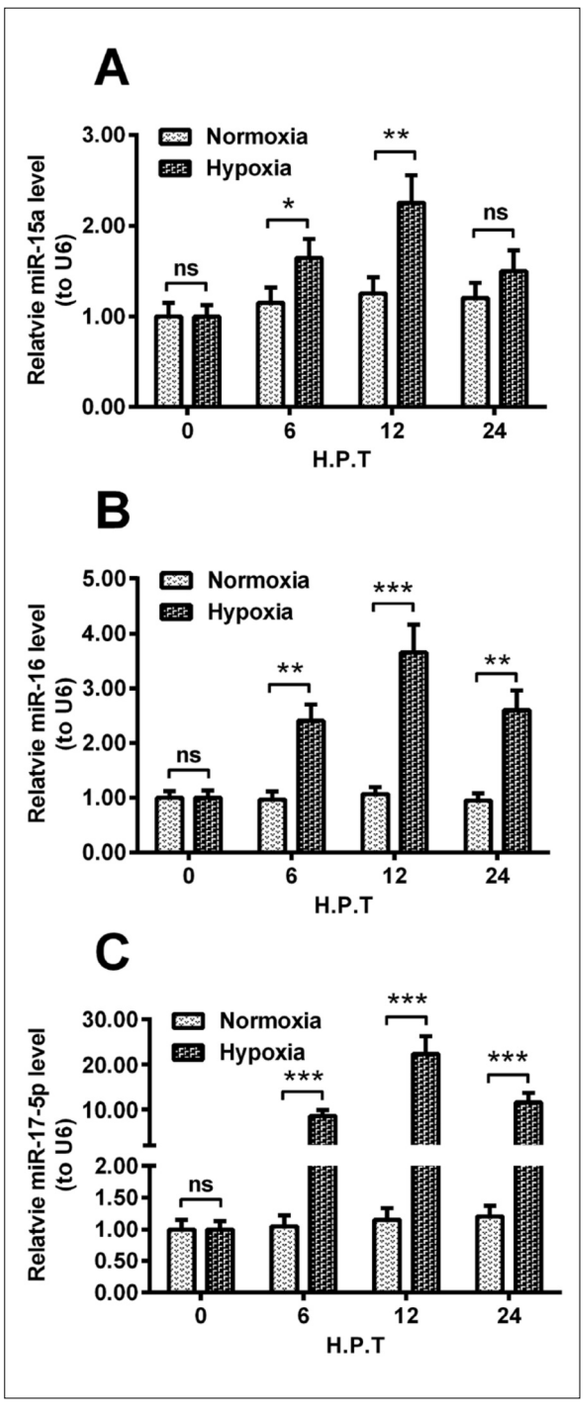

Figure 1. Hypoxia upregulates miR-15a, miR-16, and miR-17-5p in astrocyte DI TNC1 cells. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure miR-15a, miR-16, and miR-17-5p expression levels in DI TNC1 cells under normoxia or hypoxia for 0, 6, 12 or 24 hours. miR-15a ***(A)*** miR-16 ***(B)*** or miR-17-5p (C) was expressed as a relative level to U6 with the ΔΔCt method. Each data was averaged for triple independent results. Statistical significance was shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns: no significance.

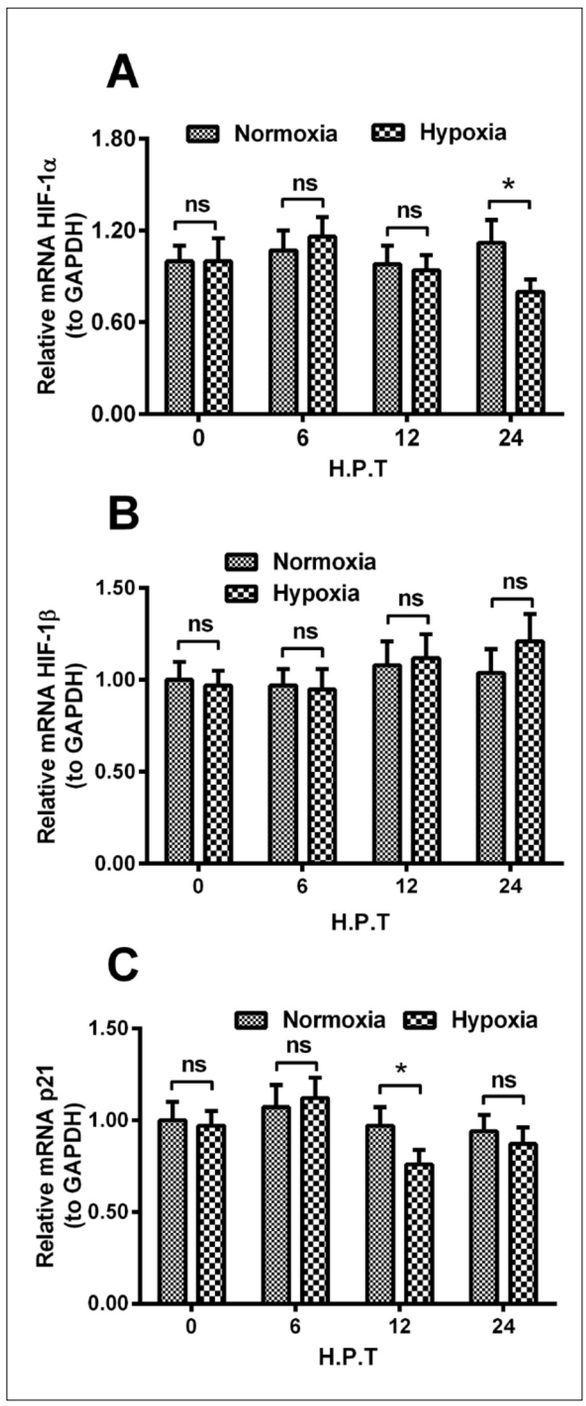

Figure 2. Expression of hypoxia-inducible factor-1 (HIF-1α and HIF-1β) and p21 in the hypoxia-treated astrocyte DI TNC1 cells. The mRNA levels of HIF-1α, HIF-1β and p21 were assayed with quantitative real-time polymerase chain reaction (qRT-PCR) in the astrocytes under normoxia or hypoxia for 0, 6, 12, or 24 hours. The HIF-1α ***(A)***, HIF-1β ***(B)*** or p21 ***(C)*** mRNA was expressed as a relative level to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with the ΔΔCt method. All experiments were performed independently in triplicate. Statistical significance was shown as $*p < 0.05$ ns: no significance.

pression of HIF-1α and p21 were determined in both mRNA and protein levels. As Figure 3A showed, miR-17-5p mimics can effectively improve the relative level of miR-17-5p (***p < 0.001), compared to the control group. The mRNA level of HIF-1α and p21 were both down-regulated in DI TNC1 cells transfected with miR-17-5p mimics (Figure 3B and 3C, *p <0.05, **p < 0.01). After that, we used WB assay to detect the expression of HIF-1α and p21 on protein levels. It was demonstrated that the levels of HIF-1α and p21 were significantly increased post transfection with 30 nM or 60 nM miR-17-5p mimics (Figure 3D), compared with the scramble control. Figure 3E indicate that the protein level of HIF-1α was decreased in DI TNC1 cells transfected with 60 nM miR-17-5p mimics (*p < 0.05). Figure 3F indicate that the protein level of p21 was down-regulated in DI TNC1 cells transfected with 30 nM or 60 nM miR-17-5p mimics (**p < 0.01).

miR-17-5p Down-Regulates the Luciferase Activity of the Reporter with the 3′ UTR of p21 in DI TNC1 Cells

It has been reported that miR-17 could repress p21 expression by targeting 3' UTR of p21 mRNA directly[27,28], to further confirm the interaction mechanism of miR-17-5p and p21, we aligned the sequence of miR-17-5p and p21 mRNA, there is a complementary sequence within the 3‘ UTR of p21. The alignment of *Mus musculus* miR-17-5p with the target sequences within the 3‘ UTR of *Mus musculus* p21 was shown in Figure 4A. We constructed the recombinant plasmid pMIR-Luc-p21 with the 3‘ UTR of p21 or with the mutant 3‘ UTR of p21 inserted behind the Cytomegalovirus promoter (CMV). The recombinant plasmid and the miR17-5p mimics were co-transfected into the DI TNC1 cells, and the luciferase activity could reflect the expression level of p21 indirectly. The relative luciferase of the reporter with the 3‘ UTR of p21 was decreased in DI TNC1 cells post the transfection with 30 nM or 60 nM miR-17-5p mimics, compared with the group of the Scramble miRNA (Figure 4B, **p < 0.01). In addition, the relative luciferase of the reporter with the mutant 3‘ UTR of p21 was no difference between the two groups (Figure 4C, ns: no significance). The result indicated that the miR-17-5p could down-regulate the expression of p21 by targeting the 3‘ UTR of p21.

miR-17-5p Mimics Transfection Ameliorates the Hypoxia-Induced Viability Reduction of DI TNC1 Cells

To investigate the effect of miR-17-5p to the viability of DI TNC1 cells under hypoxia, we examined the cellular viability by MTT assay. The DI TNC1 cells were treated under hypoxia for 12 or 24h, and the cellular viability was significantly decreased than those under normoxia, the result was shown in Figure 5A (*p < 0.05, **p < 0.01). Then we tested the cellular viability of DI TNC1 cells under normoxia or hypoxia, post the transfection with 0, 30 or 60 nM miR-17-5p mimics or with Scramble miRNA for 12 hours. Figure 5B showed us that the cellular viability was no difference between the cells transfected with miR-17-5p mimics and the cells transfected with Scramble miRNA under normoxia. Furthermore, under hypoxia, the cellular viability was significantly increased in the cells post-transfection with 30 or 60 nM miR-17-5p mimics under hypoxia, compared to the control (Figure 5C, *p < 0.05, **p < 0.01), and the higher of the concentration, the stronger ability of cellular viability. In conclusion, the above results implied that the miR-17-5p mimics ameliorated the hypoxia-induced viability reduction of DI TNC1 cells.

Discussion

miRNAs are essentials regulatory element in cells, the expression level of miRNAs reflect the physiological state of cells[16,17]. In this study, we examined the expression level of miR-15a, miR-16, and miR-17-5p in astrocyte DI TNC1 cells under normoxia and hypoxia. Our results indicated that the hypoxia could up-regulate the expression level of miR-15a, miR-16, and miR-17-5p in astrocyte DI TNC1 cells. Particularly, we found that the maximum elevated expression level was miR-17-5p. It implied that hypoxia could induce the expression of miR-17-5p. We also found that the relative expression level HIF-1α and p21 were increased under hypoxia. However, relative expression level of HIF-1β was no significant difference between the normoxia and hypoxia.

Based on the above results, we speculated that the miR-17-5p inhibited the expression of HIF-1α and p21. And followed experiments also confirmed that the expression of both markers were downregulated by the manipulated miR-17-5p in the astrocytes. Therefore, our results implied that miR-17-5p was promoted to downregulate p21

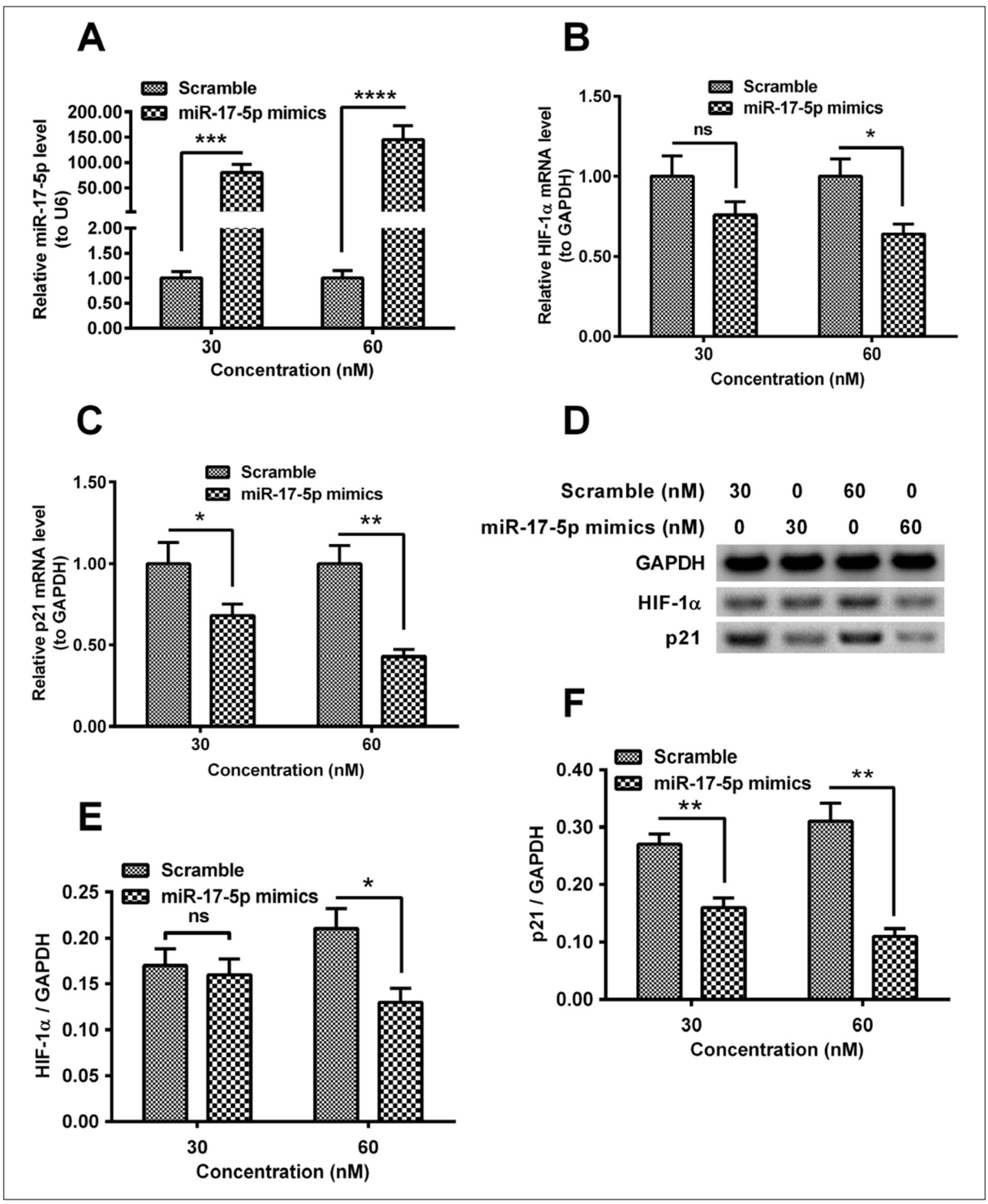

Figure 3. miR-17-5p mimics transfection downregulates HIF-1α and p21 in both mRNA and protein levels in DI TNC1 cells. ***A,*** Relative miR-17-5p level to U6 in the DI TNC1 cells, which were transfected with 30 or 60 nM miR-17-5p mimics or control miRNA (Scramble) for 12 hours. ***B*** and ***C,*** Relative mRNA level of HIF-1α ***(B)*** or p21 ***(C)*** to GAPDH in the DI TNC1 cells which were transfected with 30 or 60 nM miR-17-5p mimics or Scramble miRNA for 12 hours. D, Western blot analysis of HIF-1α and p21 in the DI TNC1 cells which were transfected with 30 or 60 nM iR-17-5p mimics or Scramble miRNA for 24 hours; ***E*** and ***F,*** Relative protein level of HIF-1α ***(E)*** or p21 ***(F)*** to GAPDH in the miR-17-5p mimics- or Scramble miRNA-transfected DI TNC1 cells. Each data was averaged for triple independent results, Statistical significance was considered when $p < 0.05$ or less, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ or $****p < 0.0001$, ns: no significance.

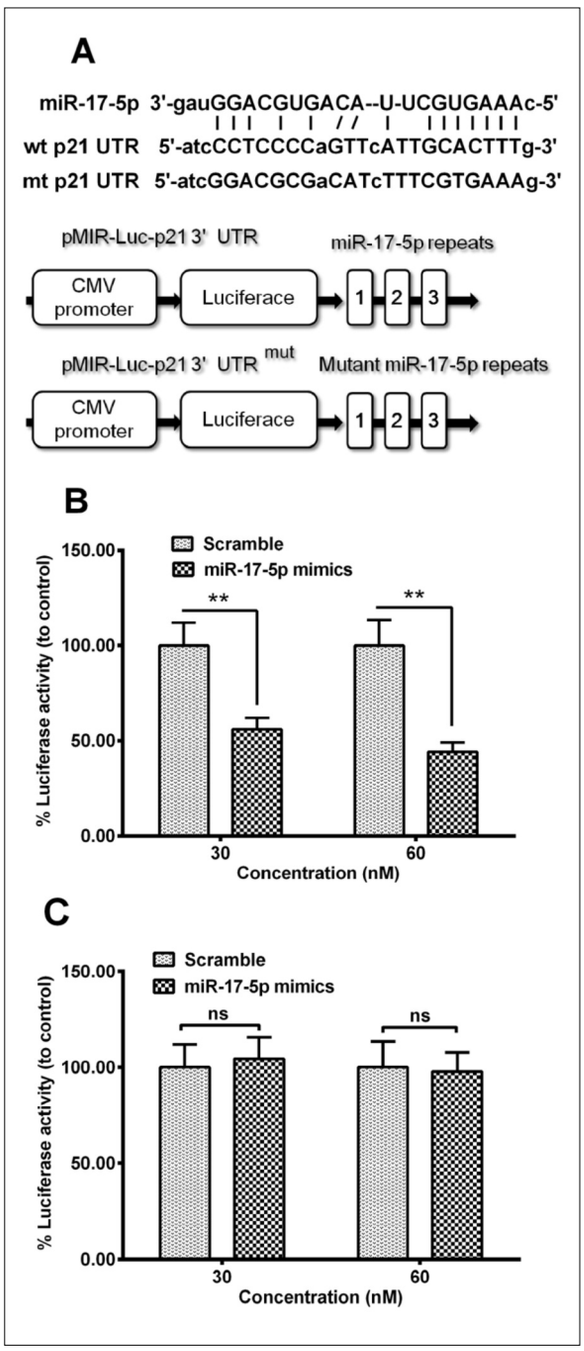

Figure 4. miR-17-5p downregulates the luciferase activity of the reporter with the 3' UTR of p21 in DI TNC1 cells. ***A,*** The alignment of Mus musculus miR-17-5p with the target sequences within the 3' UTR of Mus musculus p21 and the sketch of a luciferase reporter with the 3' UTR of p21 or with the mutant 3' UTR of p21, with the 3' UTR or with the mutant 3' UTR of p21 inserted behind the Cytomegalovirus promoter. ***B*** and ***C,*** Relative luciferase of the reporter with the 3' UTR of p21 ***(B)*** or the mutant 3' UTR of p21 ***(C)***, with renilla luciferase as internal control, in the DI TNC1 cells, post the transfection with miR-17-5p mimics or with Scramble miRNA. Each data was averaged for triple independent results, ns: no significance, $^{**}p < 0.01$

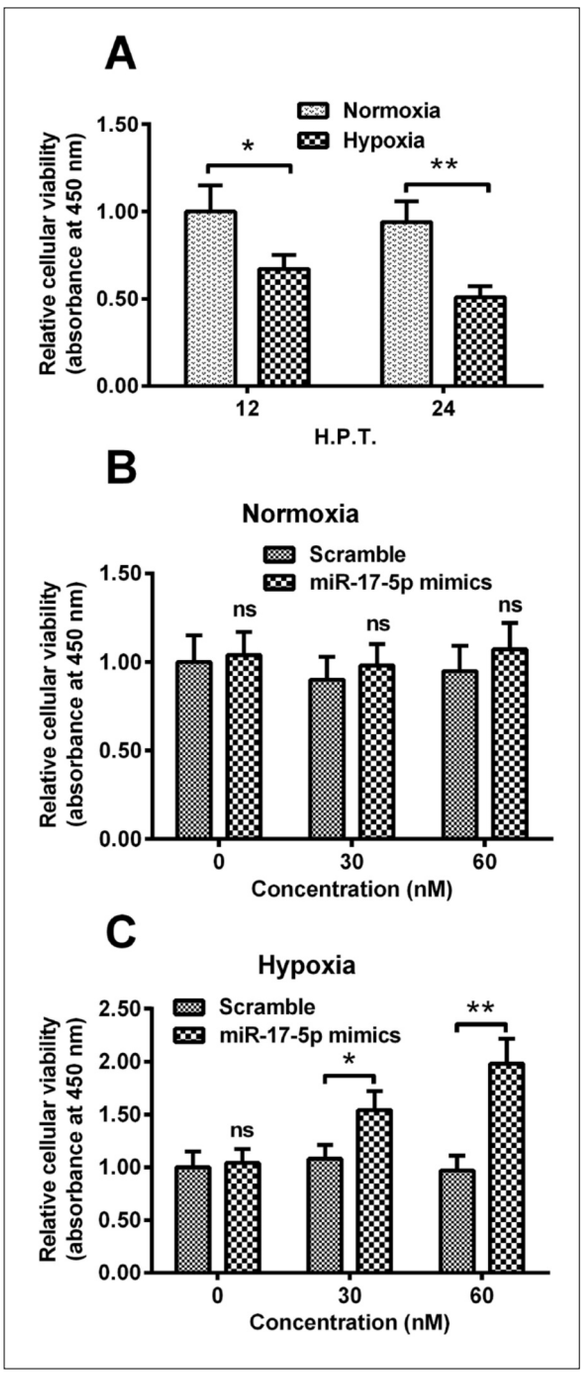

Figure 5. miR-17-5p mimics transfection ameliorates the hypoxia-induced viability reduction of DI TNC1 cells. ***A,*** Cellular viability of DI TNC1 cells under normoxia or hypoxia for 12 or 24 hours. The cellular viability was examined with MTT assay and was presented as a relative level to the group under normoxia for 12 hours. ***B*** and ***C,*** Cellular viability of DI TNC1 cells under normoxia ***(B)*** or hypoxia ***(C)***, post the transfection with 0, 30 or 60 nM miR-17-5p mimics or with Scramble miRNA for 12 hours. The experiments were performed respectively in triplicate. Statistical significance was shown as $^{*}p < 0.05$, $^{**}p < 0.01$, or ns: no significance.

(and HIF-1α) in response to hypoxia in astrocytes. The targeting regulation by miR-17-5p on HIF-1α and p21 has never been reported in astrocytes, though there are some studies showed that the miR-17 could target the 3‘ UTR of *p21*[28] in tumor cells. Our results firstly confirmed the targeting regulation by miR-17-5p on the 3‘ UTR of p21 by the luciferase reporting assay with the wild/mutant 3‘ UTR of *p21*. Basing on the reporting results, we deduced that the miR-17-5p could down-regulate the express of p21 by targeting the 3‘ UTR of p21 in astrocytes.

Our findings also confirmed the protective role of miR-17-5p against the hypoxia-mediated cellular viability reduction of astrocytes. Hypoxia could significantly reduce the viability of the astrocyte cells. However, the viability was significantly increased in the DI TNC1 cells post the miR-17-5p upregulation under hypoxia condition. The results implied that the miR-17-5p could ameliorate the hypoxia-induced viability reduction of astrocytes cells.

The role of p21 in the hypoxia-mediated damage varies according to cell types. Inhibition of p21/HIF-1α axis has been indicated to restore the therapeutic potential of old human endothelial progenitor cells[29]. p21 mediates the hypoxia-induced Cdc25A phosphorylation and S-phase arrest in colon, prostate, kidney, liver and lung cancer cell lines[30,31]. However, p21 was not necessary for the hypoxia-induced cell cycle in fibroblasts[32]. In the present report, the correlated miR-17-5p upregulation and p21 downregulation were found in the hypoxia-treated astrocytes. And the manipulated upregulation of miR-17-5p ameliorated the hypoxia-induced cellular viability reduction. It implies that the targeting inhibition of p21 protects astrocytes. However, there were several questions open about the amelioration by miR-17-5p on the hypoxia-induced viability reduction of astrocytes, such as to what degree the hypoxia-induced viability reduction was influenced by miR-17-5p, what roles of other biomarkers in the cellular response to hypoxia in astrocytes.

Conclusions

Our investigation demonstrated that the miR-17-5p was up-regulated in astrocytes under hypoxia and downregulated the p21 expression targeting the 3‘ UTR of *p21*. And the miR-17-5p ameliorated the hypoxia-induced viability reduction of astrocytes cells. Our study implied the protective role of miR-17-5p against hypoxia-induced damage in astrocytes.

Acknowledgements

The present study was supported by the grant from National Natural Science Foundation (81560212) and from Inner Mongolia Science Foundation (2015MS0898).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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