



TGF- β 1 can be regulated by NDRG2 via the NF- κ B pathway in hypoxia-induced liver fibrosis

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Background: The identification of the important elements that control hepatic stellate cell (HSC) activation will expand our understanding of the mechanism of liver fibrosis induced by hypoxia and affect the outcome of clinical treatment. A previous research demonstrated that N-Myc downstream-regulated gene 2 (*NDRG2*) is a potential regulator of fibrosis and a downstream target gene of hypoxia-inducible factor 1 (*HIF-1*). In this research, we studied the expression and function of *NDRG2* in liver fibrosis induced by hypoxia.

Methods: LX-2 cells/NF- κ B-silenced LX-2 cells were exposed to hypoxic conditions (1% O₂) to activate HSCs *in vitro*. The protein and mRNA expression levels of *NDRG2*, α -SMA and transforming growth factor beta 1 (*TGF- β 1*) were evaluated by western blotting and real-time polymerase chain reaction (RT-PCR), respectively. Functional studies were performed using adenovirus-mediated gene upregulation.

Results: The *NDRG2* mRNA and protein levels were reduced under hypoxic conditions in LX-2 cells and overexpression of *NDRG2* resulted in a decrease in the expression of *TGF- β 1* and α -SMA. Interestingly, no relationship was observed between *NDRG2* and *TGF- β 1* when the NF- κ B pathway was blocked, which indicates that *NDRG2* can regulate the expression of *TGF- β 1* in LX-2 cells via the NF- κ B pathway under hypoxic conditions.

Conclusions: *NDRG2* may regulate the expression of *TGF- β 1* via the NF- κ B pathway and may be a novel therapeutic target for liver fibrosis induced by hypoxia.

Keywords: Liver fibrosis; N-Myc downstream-regulated gene 2 (*NDRG2*); hypoxia; therapeutic target

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Introduction

Liver fibrosis is the liver's reaction to chronic injury and a major cause of fatal liver disease worldwide, especially in Asian countries. There are many causes of liver fibrosis, including metabolic disorders, cholestatic diseases, chronic hepatitis, and others (1,2). A hypoxia response system may

contribute to the pathophysiology of liver fibrosis (3), and hypoxia may be a prominent determinant of liver fibrosis in pathological situations, Cellular hypoxia is an important feature of liver injury (4). Studies have shown that hypoxia regulates liver fibrosis by activating hepatic stellate cells (HSCs), and that HSC activation may be a key event in extracellular matrix (ECM) production (5), the biosynthetic

changes of ECM production present as interstitial and perivascular fibrosis of liver. Despite advances in the characterization of liver fibrosis, the exact molecular mechanisms underlying hypoxia-induced liver fibrosis are still poorly understood. Therefore, clarification of these mechanisms and identification of potential therapies that could reduce the progression of hypoxia-induced fibrosis are urgently needed.

Transforming growth factor beta 1 (TGF- β 1) is an important molecule involved in the progress of liver fibrosis through the activation of its downstream Smad signaling pathway (6,7). The signaling pathway of TGF- β 1/Smad is integrally involved in the activation of HSCs, but the connection between hypoxia and the TGF- β 1 signaling pathway is still unclear.

N-Myc downstream-regulated gene 2 (*NDRG2*) participates in the differentiation and growth of the liver cell and in hormonal responses (8,9). It has been demonstrated that *NDRG2* is involved in liver histogenesis; the expression levels of *NDRG2* mRNA and protein are markedly low in the early phases of histogenesis and are significantly higher in the later phases of histogenesis in the fetal livers of human (10,11). One study has shown that *NDRG2* may be involved in the activation of LX-2 cells, which are HSCs (12). *NDRG2* is a target of hypoxia-inducible factor 1 (*HIF-1*), which is a key mediator of the signaling pathway of hypoxia (13); however, the degree to which *NDRG2* participates in the liver fibrotic process under hypoxic conditions is still unclear. This study tried to analyse the role of *NDRG2* in hypoxia-induced liver fibrosis.

We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/atm-21-1298>).

Methods

Cell culture

LX-2 cells were kindly provided by Dr. Scott Friedman (The Icahn School of Medicine at Mount Sinai, New York City, NY, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PeproTech, Rocky Hill, NJ, USA) supplemented with 10% fetal bovine serum (FBS; PeproTech,) at 37 °C in an incubator (normoxic conditions 21% O₂, 5% CO₂, and 74% N₂).

TGF- β 1 or hypoxia treatment

The cells were plated in 60 mm dishes and cultured in

DMEM containing 10% FBS and 0.2% bovine serum albumin (BSA) and serum starved for 48 h after they reached 70% confluence. Cells were then incubated with TGF- β 1 (2.5 ng/mL, Invitrogen Life Technologies, Carlsbad, CA, USA) for 0 or 24 h. Cells were placed in a humidified atmosphere normoxic conditions or in a hypoxic condition (37 °C, containing 1% O₂, 5% CO₂, and 94% N₂).

Adenovirus transduction

Adenoviral vectors expressing human *NDRG2* (Ad-*NDRG2*), β -galactosidase [Ad-LacZ, negative control (NC)], and enhanced green fluorescent protein (Ad-EGFP, NC) were purchased from Zhengyang Benyuan (Beijing, China). The cells were seeded in the 100 mm dishes and then incubated with Ad-*NDRG2*, Ad-LacZ, or Ad-EGFP in the serum-free DMEM for 2 h. The medium was then replaced with the fresh DMEM containing 10% FBS and cells were incubated for 48 h. The multiplicity of infection (MOI) was set at 40.

Production of lentiviral vector

NF- κ B small-interfering RNAs (siRNAs) or control siRNAs (scrambled sequence) were subcloned in lentiviral vectors, as previously reported (14,15). The NF- κ B siRNA sequence was 5'-GGACCTACGAGACCTTCAA-3'. The NC siRNA was 5'-TTCTCCGAACGTGTCACGT-3'. The final titer of the recombinant virus was 5×10⁸ TU/mL.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of LX-2 cells was isolated by the TRIzol reagent (Invitrogen) and quantified. Complementary DNA (cDNA) was synthesized with the TaqMan Reverse Transcriptase Reagent Kit (Invitrogen) primed with oligo(dT) from 5 μ g RNA and then analyzed using qRT-PCR. Then the mRNAs were detected with SYBR Green PCR Master Mix and an ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, USA), and relative quantification was performed with the comparative threshold cycle method. The PCR consisted of 10 pmol of the forward and reverse primers, 12.5 μ L SYBR Green PCR Master Mix, and 5 μ L of template cDNA in a total volume of 25 μ L. The conditions of thermal cycling comprised a step of initial denaturation at 95 °C for 10 s, followed by 45 cycles at 95 °C for 5 s and 60 °C for 34 s.

The following primers were used for the human genes:
NDRG2-F: 5'-GAGATATGCTCTTAACCACCCG-3',
NDRG2-R: 5'-GCTGCCCAATCCATCCAA-3';
 α -*SMA*-F: 5'-GACAATGGCTCTGGGCTCTGTAA-3',
 α -*SMA*-R: 5'-CTGTGCTTCGTCACCCACGTA-3';
TGF- β 1-F: 5'-CAATTCCTGGCGATACCTCAG-3',
TGF- β 1-R: 5'-GCACAACCTCCGGTGACATCAA-3';
 β -*actin*-F: 5'-AGCGAGCATCCCCAAAGTT-3',
 β -*actin*-R: 5'-GGGCACGAAGGCTCATCATT-3'.

Analysis of Western blot

The cells were harvested from the 60 mm culture dishes, and then lysed in 200 μ L RIPA buffer [0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4), 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 10 μ g/mL leupeptin, and 10 μ g/mL aprotinin]. Protein concentrations were measured by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The proteins were separated using SDS-PAGE and transferred to the nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were saturated using Tris-buffered saline mixed with 3% TBST-BSA and 0.1% Tween 20 and then probed using the appropriate antibodies for the following targets: *NDRG2* (1:2,000, Cell Signaling Technology, Danvers, MA, USA), β -*actin* (1:2,000, Cell Signaling Technology), α -*SMA* (1:1,000, Sigma-Aldrich, St. Louis, MO, USA), and *TGF- β 1* (1:1,000 Cell Signaling Technology). The membranes were then incubated using the species-matched secondary antibodies. The bands were detected with enhanced chemiluminescence (Pierce). The band intensities were quantified using Kodak Digital Science 1D software (version 3.0; Eastman Kodak, New Haven, CT, USA).

Statistical analysis

Statistical analyses were performed with the SPSS software (version 16.0; IBM Corp., Armonk, NY, USA). The *t*-test method was used to compare the differences between the two groups, and the variance method analysis was used to compare the differences of the groups. Statistical significance was based on a P value <0.05.

Result

NDRG2 was involved in liver hypoxia-induced fibrosis

qRT-PCR and western blotting analysis revealed that the

protein and mRNA levels of *NDRG2* significantly decreased under hypoxia condition in LX-2 cells in a time-dependent manner ($P < 0.05$) (Figure 1A,B).

Hypoxia-induced liver fibrosis

The spontaneously immortalized human HSC cell line, LX-2, was grown to confluence and exposed to hypoxia (1% O₂). The protein and mRNA expression levels of *TGF- β 1* and α -*SMA* increased as the duration of hypoxic exposure increased ($P < 0.05$) (Figure 1A,C,D). The analyses of western blot and qRT-PCR showed that the mRNA and protein levels of *TGF- β 1* and α -*SMA* were increased in a time-dependent manner following incubation of LX-2 cells under the hypoxic (1% O₂) conditions for 6 and 24 h (Figure 2A,B,C,D). These results demonstrated that HSC activation could be induced by hypoxia within 24 h.

NDRG2 inhibited the expression of TGF- β 1 in hypoxia-induced liver fibrosis

To determine whether *TGF- β 1* can be regulated by *NDRG2*, LX-2 cells were infected with Ad-*NDRG2* (10 μ M) under hypoxic conditions (1% O₂) for 24 h, and their effects were evaluated using qRT-PCR and western blot analysis. The increase in the mRNA and protein expression of α -*SMA* and *TGF- β 1* in LX-2 cells incubated under hypoxic (1% O₂) conditions for 24 h was greater than that in cells incubated with hypoxic (1% O₂) conditions for 6 h (Figure 2A,B,C,D). However, incubation of LX-2 cells treated with Ad-*NDRG2* (10 μ M) under hypoxic (1% O₂) conditions resulted in a time-dependent decrease in the levels of mRNA and protein expression of *TGF- β 1* and α -*SMA* ($P < 0.05$) (Figure 2A,B,C,D). Therefore, the overexpression of *NDRG2* can reduce the protein and mRNA expression levels of α -*SMA* and *TGF- β 1* in LX-2 cells subjected to hypoxic conditions, which indicates that under hypoxic conditions, *NDRG2* can reverse fibrosis through *TGF- β 1*.

NDRG2 could not inhibit the expression of TGF- β 1 in the absence of NF- κ B

To examine the role of NF- κ B, hypoxic NF- κ B-silenced LX-2 cells were treated with Ad-*NDRG2* (10 μ M). The analyses of western blotting and qRT-PCR revealed that the mRNA and protein expression of *NDRG2* decreased as the hypoxia exposure time increased (from 0 to 24 h) in NF- κ B-silenced cells (Figure 3A,B). However, the protein

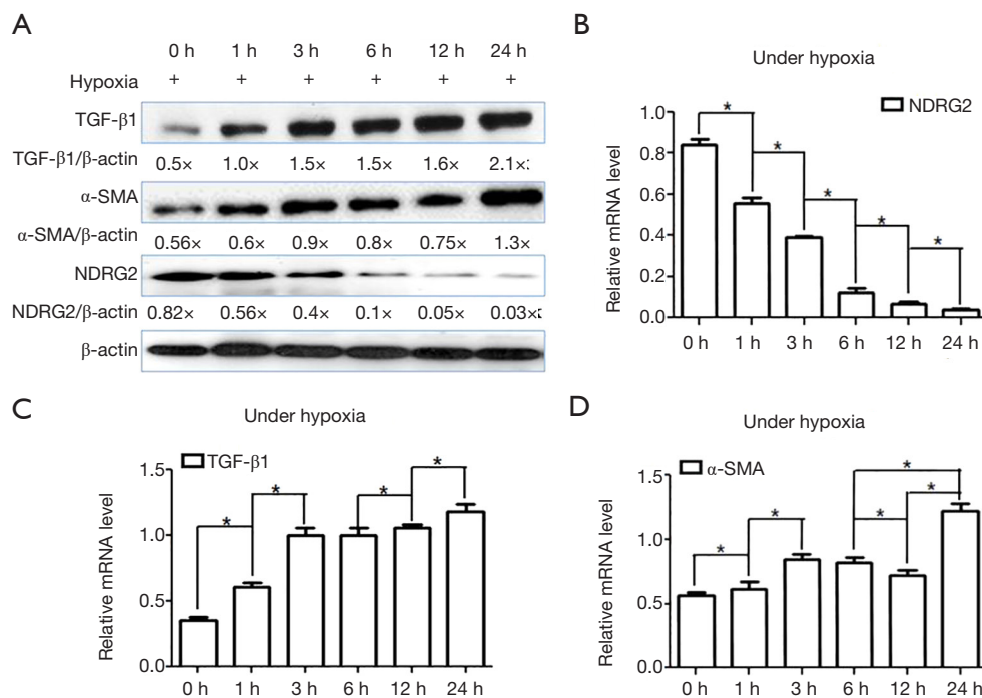


Figure 1 *NDRG2* expression was downregulated by hypoxia in HSCs (LX-2). (A) The protein levels of *NDRG2*, *TGF-β1*, and *α-SMA* in LX-2 cells at various time points (from 0 to 24 h) after hypoxic exposure were analysed by western blot. (B,C,D) The expression levels of the mRNAs encoding *NDRG2*, *α-SMA*, and *TGF-β1* in LX-2 cells at various time points (from 0 to 24 h) after hypoxic exposure were analysed by qRT-PCR, and β -actin served as a control to ensure equal loading. qRT-PCR and western blot analysis showed that the mRNA and protein levels of *α-SMA* and *TGF-β1* were significantly enhanced as the duration of hypoxic exposure increased in LX-2 cells ($P < 0.05$); however, the protein and mRNAs levels of *NDRG2* decreased significantly as the duration of hypoxic exposure increased ($P < 0.05$). *, $P < 0.05$.

and mRNA levels of *TGF-β1* and *α-SMA* expression did not significantly change under Ad-*NDRG2* treatment conditions (10 μ M) as the hypoxia exposure time increased in these NF- κ B-silenced LX-2 cells (Figure 3A,C,D), which indicated treatment with Ad-*NDRG2* results in overexpression and the hypoxic conditions result in *NDRG2* decrease had no effect on the mRNA and protein expression of *α-SMA* and *TGF-β1* in hypoxia-induced LX-2 cells when NF- κ B expression was blocked. Therefore, *NDRG2* could not inhibit the expression of *TGF-β1* in the absence of NF- κ B.

Discussion

Hypoxia is an important factor in cell damage, especially in acute and chronic liver injury, and may thus play an important role in the pathogenesis of liver fibrosis. The chronic injury may be related to the activation of HSCs in the hepatic fibrotic process (16). When stimulated, HSCs transition from a quiescent state to an activated

state (17), and the levels of *α-SMA* can be regarded as a marker of HSC activation. In this study, we investigated whether HSCs are activated by hypoxia *in vitro*. The results showed that the protein expression of *α-SMA* increased, reaching a peak at 24 h after the induction of hypoxia, indicating that HSC activation occurred within the first 24 h of exposure to hypoxic conditions. *NDRG2* has been found to be a downstream molecular target of *HIF-1* (18), *HIF-1* is thought to facilitate fibrosis through interaction with *TGF-β1* (19). In this study, a decrease in the levels of the protein and mRNA expression of *NDRG2* was found following exposure of LX-2 cells to hypoxic conditions (1% O_2), which indicates that *NDRG2* might be involved in hypoxia-induced liver fibrosis.

TGF-β1 may be the most potent fibrogenic cytokine in the liver (20,21), and a biologically inactive form of *TGF-β1* can convert into an active form of that from in response to injury. When activated, *TGF-β1* can regulate the transcription of profibrotic target genes via its cognate *TGF-β1* receptor in the nucleus (22). The signaling

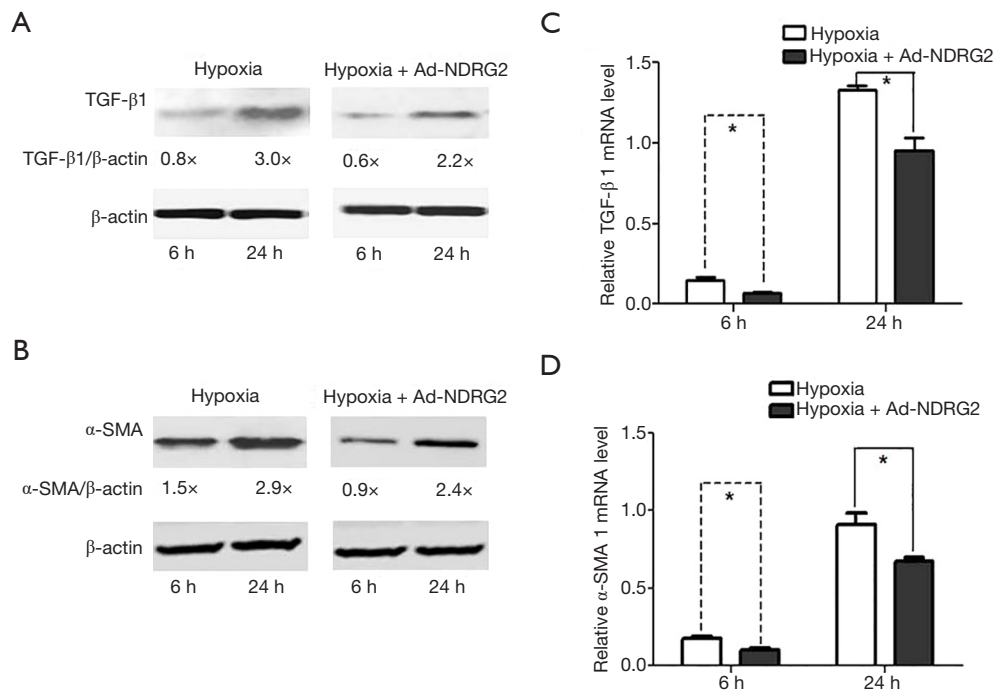


Figure 2 *NDRG2* could reverse fibrosis through *TGF-β1* under hypoxic conditions. (A,B,C,D) The results of western blot and qRT-PCR showed that the increase in the protein and mRNA levels of *TGF-β1* and α -*SMA* in LX-2 cells under hypoxic conditions (1% O_2) for 24 h was higher than that in the cells exposed to these conditions only for 6 h. qRT-PCR and western blot also revealed that the decrease in the mRNA and protein expression of α -*SMA* and *TGF-β1* in LX-2 cells after treatment with Ad-*NDRG2* (10 μ M) under hypoxic conditions (1% O_2) for 24 h or 6 h was larger than that in cells only exposed to hypoxic conditions (1% O_2) ($P < 0.05$). These results showed that the mRNA and protein expression levels of α -*SMA* and *TGF-β1* were increased as the duration of hypoxia increased, and that the overexpression of *NDRG2* could inhibit the mRNA and protein expression levels of α -*SMA* and *TGF-β1*. *, $P < 0.05$.

pathway of *TGF-β1* can influence aspects of the fibrogenic process, including activation of HSC, and the subsequent production of ECM during the process of liver injury (23,24), which present as the fibrosis of liver. We examined whether *NDRG2* links hypoxia with the *TGF-β1* signaling pathway in the fibrogenic process. In the present study, the analysis of qRT-PCR and western blot revealed an increased mRNA and protein expression of *TGF-β1* in LX-2 cells under hypoxic conditions (1% O_2), and compared to the control, treatment of LX-2 cells with Ad-*NDRG2* (10 μ M) under hypoxic conditions (1% O_2) for 24 h reduced *TGF-β1* and α -*SMA* expression, indicating that *NDRG2* can reverse fibrosis through *TGF-β1*. Western blotting and qRT-PCR also demonstrated that the protein and mRNA expression of *TGF-β1* and α -*SMA* did not significantly change after treatment with Ad-*NDRG2* (10 μ M) in NF- κ B-silenced LX-2 cells under hypoxic conditions (1% O_2), indicating that *NDRG2* cannot inhibit the expression of *TGF-β1*

in the absence of NF- κ B. Activation of NF- κ B has been found to attenuate *TGF-β1*-induced cell death in liver cells (25). In our study, *NDRG2* did not affect the activation of *TGF-β1* signaling pathways in NF- κ B-silenced LX-2 cells under hypoxic conditions (1% O_2). These data suggest that *NDRG2* can inhibit the expression of *TGF-β1* in hypoxia-induced liver fibrosis via the NF- κ B pathway.

So, we inferred that *TGF-β1* could be regulated by *NDRG2* via the NF- κ B pathway in hypoxia-induced liver fibrosis. Furthermore, adenovirus-mediated *NDRG2* overexpression may attenuate hypoxia-induced liver fibrosis.

Conclusions

Our findings demonstrate that *NDRG2* participates in the regulation of hypoxia-induced liver fibrosis. Overexpression of *NDRG2* can decrease the expression of *TGF-β1* via the NF- κ B pathway in LX-2 cells under hypoxic

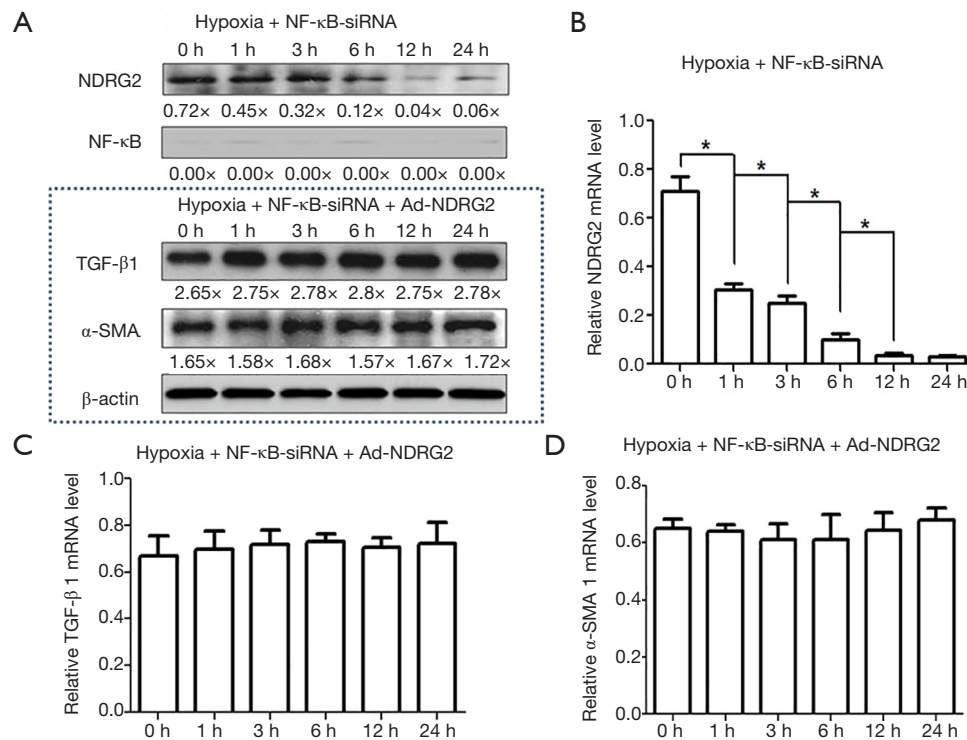


Figure 3 *NDRG2* could not inhibit the mRNA and protein expression of *TGF-β1* and *α-SMA* without NF-κB. (A,B,C,D) NF-κB protein expression was barely detected, indicating successful knockdown of the *NF-κB* gene. The results of western blot and qRT-PCR showed that the protein and mRNA expression of *NDRG2* were decreased as the hypoxia exposure time increased (from 0 to 24 h) in NF-κB-silenced cells; however, the level of *TGF-β1* and *α-SMA* expression did not significantly change when the cells were treated with Ad-*NDRG2* (10 μM) as the hypoxia exposure time increased in these NF-κB-silenced cells (LX-2HSCs). *, $P < 0.05$.

conditions. Therefore, *NDRG2* may be a novel target for the development of treatments for patients with hypoxia-induced liver fibrosis.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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