

Silencing Hypoxia-inducible Factor-1 α gene Transcription on Effects of Human hepG2 cell Proliferation and Angiogenesis Factors

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Hypoxia-inducible factor (HIF)-1 α is a key transcription regulator for multiple angiogenic factors and appealing target and HIF-1 α promotes angiogenesis are not fully understood. Therefore, we investigated the effects of silencing HIF-1 α on proliferation of HepG2 cells and angiogenesis. After the cells transfected with HIF-1 α miRNA at 72 h, HIF-1 α expression was down to 23 % at mRNA level by quantitative real time PCR and 44 % at protein level by Western blotting. The expressions of the down-stream vascular endothelial growth factor and angiopoietin-2 were decreased 54 % and 34 % by enzyme-linked immunosorbent assay, respectively. The alteration of cell cycle proportion was 61.49 % in G1 phase, 22.40 % in S phase, and not in G2/M phase. The apoptotic ratio of HepG2 cells increased from 22.46 % to 36.99 % with 65.68 % of G1 phase and 19.47 % of S phase when HIF-1 α activation interfering with miRNA plus doxorubicin by flow cytometry or annexin Annexin-V-FLUOS assay. The down-regulated HIF-1 α expression resulted in decreasing angiogenic factors, inhibition of HepG2 cell growth, and inducing apoptosis. Therefore, we conclude that HIF-1 α may serve as a useful molecular target for miRNA-based liver cancer therapy.

Key words: HCC, HIF-1 α , Ang-2, SiRNA, VEGF, Real-time PCR, Gene silencing.

Hypoxia enhances cell proliferation, angiogenesis, and suppresses differentiation and apoptosis of hepatocellular carcinoma (HCC) consequently leads to resistance of transarterial embolization with or without chemotherapy (Li, *et al.*, 2011; Aravalli, *et al.*, 2008). Hepatic hypoxia-inducible factor-1 (HIF-1) is a heterodimer consisting of subunits HIF-1 α (tightly regulated by changes in oxygen regimes) and HIF-1 β (constitutively expressed), whereas HIF-1 α is a master regulator of the transcriptional response of angiogenesis by multiple mechanisms such as

oncogene activation, inactivation of tumor suppressor genes, and activation of growth factors, although it primarily involves protein ubiquitination (Simon, *et al.*, 2010; Dai, *et al.*, 2009). Once activated, HIF-1 α regulates a repertoire of key angiogenic genes, including vascular endothelial growth factor (VEGF, Xiang, *et al.*, 2011), insulin-like growth factor-I (Freise, *et al.*, 2011), and angiopoietin-2 (ANG-2, Coulon, *et al.*, 2011). Given HIF-1 α central role in angiogenesis, some angiogenic growth factors have become prime targets for therapeutic angiogenesis of HCC (Choi, *et al.*, 2010; Lau, *et al.*, 2009).

Anti-angiogenic therapy is beneficial to HCC patients following surgical resection of tumor (Keith, *et al.*, 2011). Satisfactory results have not been achieved due to HIF-1 α over-expression, and stimulate the required angiogenic growth factors endogenously. The abnormal activation of hepatic

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HIF-1 α may influence HCC biological behaviors and act in concert with signal pathways (Li, *et al.*, 2011). Hypoxia leads to the escape of HCC cells from transarterial embolization and anti-angiogenic therapy (Tung, *et al.*, 2011). Thus combined therapy that induces and targets hypoxia may be of benefit to HCC patients. HIF-1 α was activated at the early stages during rat hepatoma formation, and its up-regulated expression might be associated with the occurrence and development of HCC, suggesting that it should be a potential molecular target for HCC gene therapy (Yao, *et al.*, 2009) The objective of this study was to establish a functional inactivation of HIF-1 α via miRNA and investigate the effect of specific HIF-1 α miRNA on HepG2 cell cycle, proliferation, and angiogenesis.

METHODS

miRNA and plasmid DNA (pDNA)

Short hairpin-expressing pDNAs targeting HIF-1 α (NM_001530) were constructed from pcDNATM6.2- GW/EmGFPmiR vector using BLOCK-iTTM Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen). Target sites in HIF-1 α genes are as follows: 5'-TGCTGTAAAGCATCAGGTTCTTCTTGTGTTGGCCACTGACTGACAAGAAGGACTGATGCTTTA-3' and 5'-CTGTAAAGCATCAGTCCCTTCTGTCAGTCA GTGGCCAAAACAAGAAGGAACCTGATGCTTTAC-3'. The amplified fragments were purified and sequenced by MegaBACE 1000 Sequencing and Analysis System (Amersham Biosciences, England) using DY EnamicTM ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Little Chalfont, England) according to the manufacturer's instructions.

Cell culture

HepG2 cells were cultured in RPMI-1640 (Gibco BRL, Gaithersburg, MD, USA) containing 10 % FCS (Gibco BRL, USA), 2.0 mM L-Glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin in a constant environment (37°C, 10 % CO₂ and 10 % humidity). Doxorubicin was diluted into the medium to a final concentration (0.5 μ M/L) after transfection for 24h if necessary.

In vitro transfection

HepG2 cells were plated on six-well plates. After an overnight incubation, transfection

of HIF-1 α miRNA and negative miRNA were carried out using transfection reagent (Roche, Germany) according to the manufacturer's instructions. In brief, 2 μ g miRNA was mixed with 8 μ L FuGENE HD at a final volume of 100 μ L RPMI-1640. Incubate the transfection reagent: DNA complex for 15 minutes at room temperature. The resulting complex was added to the cells and the cells were incubated with the complex in 2 mL total medium.

Amplification of nested RT-PCR

The primers were designed using Premier Primer 5.0 Software based on the human HIF-1 α sequence (NM_001530). The primers were synthesized using a synthesizer (Invitrogen, USA). The sequences of the 2 external primer pairs used for the initial PCR amplification were HIF-1 α -P1 (sense): 5'-ATACTCAA AGTCGGACAGC-3' (nucleotide [nt] 2386-2404) and HIF-1 α -P2 (antisense): 5'-TTCACCCTGCAGTAGGTC-3' (nt 2833-2852), and the size of the amplified gene fragment was 467 bp. The sequences of the 2 internal primer pairs used for the second PCR amplification were HIF-1 α -P3 (sense): 5'-CTCATCC AAGAAGCCCTAAC-3' (nt 2452-2471) and HIF-1 α -P4 (antisense): 5'-TCATAACTGGTCAGCTGTGG-3' (nt 2781-2800). The final size of the amplified gene fragment was 349 bp. The internal control primers were from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, GAPDH-P1: 5'-GGCTGCCA TTGCACTGGCAA-3' and 5'-TGCCDTGAGTGGAGTCATACTG-3', and the amplified size was 595 bp. PCR amplification consisted of initial denaturation at 94 °C for 5 min, followed by 94 °C for 25 s, 55°C for 30 s, and 72°C for 90 s for 30 cycles. The PCR products were electrophoresed on 2% agarose gels with ethidium bromide staining. The fragment sizes were evaluated using PCR markers (Promega) as molecular weight standards.

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (MRC, Cincinnati, OH, USA) as previously described. 1 μ g of total RNA was reverse-transcribed into cDNA with reverse transcriptase using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) in a 25- μ L final reaction volume. The ICycler (BIO-RAD) was used for qPCR amplification and detection. qPCR was prepared in 25 μ L reaction mixture. Each reaction

well contains 2 μ l template DNA, 9.5 μ l ddH₂O, 12.5 μ l SYBR Premix Ex Taq (TaKaRa, Japan), 0.5 μ l HIF-1 α (NM_001530) forward (5'-CCACTGCCAC CACTGATGAA-3', nt 2254-2273) and 0.5 μ l reverse (5'-TTGGTGAGGCTGTCCGACTT-3', nt 2412-2431) primers to generate an amplification of 178 bp. The negative control was included in experimental runs. The qPCR cycling program was 2 min at 95 $^{\circ}$ C for activation of the hot-start enzyme, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 10s, annealing at 60 $^{\circ}$ C for 30 s and elongation at 72 $^{\circ}$ C for 45s. Melting curves analysis was performed after completed Q-PCR collecting fluorescence between 70 and 95 $^{\circ}$ C at 1 $^{\circ}$ C increments.

Western blotting

At 24h, 48h, and 72h after transfection, total proteins were collected from HepG2 cells. For total protein extraction, cells were lysed in a lysis buffer with 1 mM PMSF. 50 μ g of protein was mixed with SDS sample buffer, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Beyotime Institute of Biotechnology, China) and blocked with 0.1% Tween 20 and 5% skim milk for 1h. The membranes were incubated with anti-HIF-1 α antibody (1:500, Abcam, England) overnight at 4 $^{\circ}$ C. Anti-human β -actin antibody (1:500, Beyotime, China) was selected as a control. The membranes were stained with HRP-conjugated secondary antibodies for 2h at room temperature. The bound HRP was detected using BeyoECL Plus (Beyotime, China), and the image was taken using gel imaging scanning system (BioRad, USA).

Enzyme-linked immunosorbent assay

At 24 h, 48h, and 72h after transfection, the culture supernatant were collected to detect VEGF (R&D systems, Abingdon, UK) and ANG-2 (ADL Biotech Dev Co., USA) production by ELISA in accordance with the manufacturer's instructions. During the procedure, washing the plate was according to the ELISA routine method. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. Inter and intra-assay variances were lower than 10 %.

Cell cycle analysis

After transfection, the cells with or without doxo- rubicin were harvested at the designed time points by trypsinization, collected by centrifugation, and fixed in 70 % ethanol (-20 $^{\circ}$ C) for 3 days, then suspended in propidium iodide

mixed buffer (Trisodium, USA) were detected by ELISA in accordance with the Citrate 0.25 g, Triton X-100 0.75 g, Propidium iodide 0.025 g, RNase A 0.005 g, ddH₂O 250 mL). After 30 min at 4 $^{\circ}$ C, the cells were filtrated with cell strainers (50 μ m), then analyzed with a FACS Calibur cytofluoro- meter (Becton Dickinson, San Jose, CA, USA) and the percentage of cells in different cell cycle was determined.

Cell apoptosis assay

The apoptosis of HepG2 cells was determined by Annexin-V-FLUOS Staining Kit (Roche, Germany). At the designed time point, cells and their culture fluid were harvested by trypsinization in the same tubes. After centrifugation, cell pellets were resuspend in 100 μ L Annexin-V-FLUOS labeling solution (pre- dilute 20 the reagent in 1 mL incubation buffer and add 20 μ L of propidium iodide solution), incubated for 10-15 min at room temperature in the dark, finally analyzed by a flow cytometry (Becton Dickinson, USA) as soon as possible.

Statistical analysis

Data were expressed as mean \pm standard deviation ($\bar{x} \pm SD$). ANOVA were performed on the means. A P value of less than 0.05 was considered to be statistically significant.

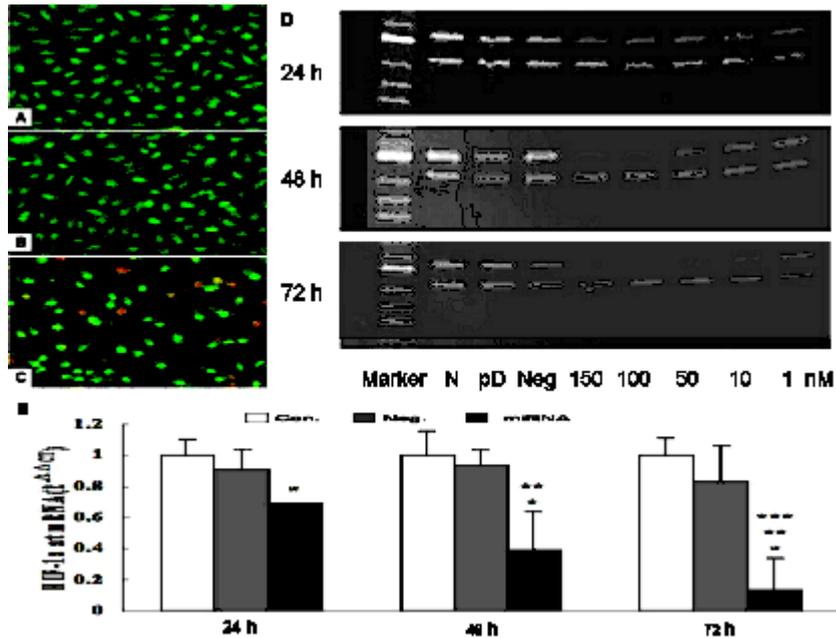
RESULTS AND DISCUSSION

miRNA inhibited HIF-1 α at mRNA level

The alteration of HIF-1 α expression at mRNA level in HepG2 cells with miRNA transfection is shown in Figure 1. HIF-1 α overexpression was confirmed during malignant transformation of hepatocytes and HCC progress (Wang, *et al.*, 2009). HIF-1 α with pivotal function were intervened at gene transcription level, and the related genes could be downregulated and inhibited the cell growth and metastasis of HCC (Liu, *et al.*, 2008). After HepG2 cells transfected with miRNA at 72 h, the down-regulation of HIF-1 α expression was 87% at mRNA (Figure 1.) or 56% at protein (Figure 2.) level, respectively, indicated that the intervention of specific HIF-1 α miRNA should be an effective method for regulating HIF-1 α expression.

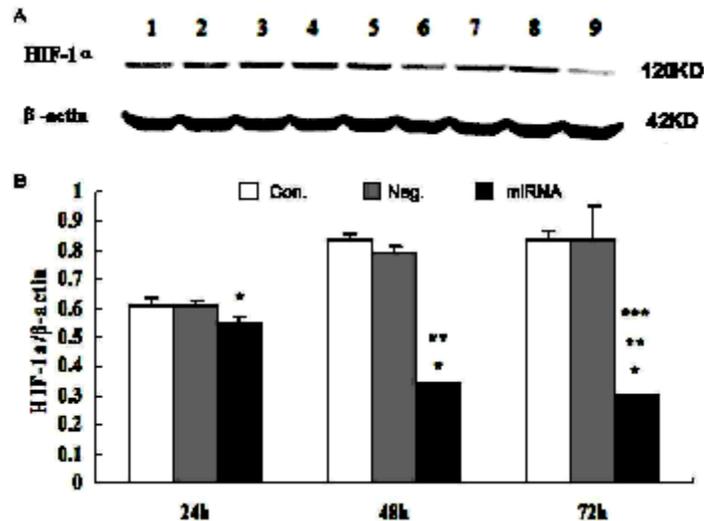
miRNA inhibited HIF-1 α at protein level

The level of HIF-1 α expression in HepG2 cells after miRNA transfection is shown in Figure 2. HCC cell lines were transfected with the specific



A, the HepG2 cells as the control group, B, the HepG2 cells transfected with the negative miRNA; C, the HepG2 cells transfected with the miRNA (original magnification $\times 40$). D, the down-regulation of HIF-1 α mRNA expression in the HepG2 cells depended on the dose of HIF-1 α miRNA and the time after transfection. HIF-1 α mRNA (up,349 bp), GAPDH (down, 595 bp) were separated on 2% agarose gel. Marker, DNA, N, the control, pD, pDNA, Neg, neg-control, miRNA (1~150 nMol/L). E, Quantitative analysis of HIF-1 α mRNA at miRNA (150 nMol/L). *Compared with the control group without miRNA, **Compared with the transfected group at 24 h, and ***Compared with the transfected group at 48 h, P < 0.05.

Fig. 1. HepG2 cells transfected with HIF-1 α miRNA and inhibition of HIF-1 α expression at mRNA level



A, HIF-1 α expression was analyzed in HepG2 cells by Western blotting. Lane 1, 4, and 7 were the control group; Lane 2, 5, and 8 were transfected with negative miRNA at 24 h, 48 h, and 72 h; and Lane 3, 6, and 9 were transfected with miRNA at 24 h, 48 h, and 72 h. B, The ratio of HIF-1 α to β -actin after miRNA transfection. *Compared with the control group, **Compared with the transfection group at 24 h with miRNA, ***Compared with the transfection group at 48 h with miRNA, P < 0.05.

Fig. 2. Level of HIF-1 α expression in HepG2 cells after miRNA transfection.

HepG2 cells were transfected with negative miRNA or HIF-1 α miRNA

HIF-1 α miRNA to investigate the effectiveness of cell growth and angiogenesis at gene transcription or protein level. After HepG2 cells transfected with miRNA at 72 h, the down-regulation of HIF-1 α expression was 87% at mRNA or protein level, respectively, indicated that the intervention of specific HIF-1 α miRNA should be an effective method for regulating HIF-1 α expression.

miRNA down-regulation of VEGF and ANG-2 expression

The deregulations of VEGF and ANG-2 expression after HepG2 cells with HIF-1 α miRNA transfection are shown in Table 1. To further estimate the effect of HIF-1 α miRNA transfection on the expression of VEGF and ANG-2, an endogenous gene product of HIF-1 α miRNA transcription activity in the culture media of HepG2 cells were analyzed at 24 h, 48 h, and 72 h after miRNA transfection. Angiogenesis is thought to depend on a perfectly coordinated balance between endogenous-positive and negative regulatory factors (Coulon, *et al.*, 2011). Of these factors, VEGF and ANG-2 seem to play an essential role. HIF-1 α knockout mice demonstrated attenuated overproduction of VEGF and intercellular adhesion molecule-1, reduced vascular leakage and alleviated neovascularization. Regulation of ANG-2 is HIF-dependent. HIF-1 α binds to an evolutionary conserved HRE located in the first intron of the ANG-2 and ANG-2 is up-regulated, expressed only at sites of vascular

remodeling and plays a crucial role in destabilizing vessels for normal or pathological angiogenesis. In this study, miRNA decreased HIF-1 α expression and suppressed angiogenesis in HepG2 cells by down-regulating VEGF and ANG-2 (Table 1.). However, it is noteworthy that VEGF expression be more significantly than ANG-2.

miRNA down-regulated HIF-1 α promote HepG2 cell Apoptosis

The comparative analysis of the apoptosis rate in HepG2 cells transfected with miRNA or doxorubicin at 48 h is shown in Table 2 and HIF-1 α inhibition could augment doxorubicin-induced cell apoptosis, sensitize chemoresistant HCC cells to doxorubicin and result in cell cycle arrest (Table 3, Figure 3), suggesting that specific HIF-1 α miRNA effect the cell cycle and proliferation of HepG2 cells.

miRNA on effect of HepG2 cell cycles

The alteration of HepG2 cell cycles after miRNA transfection at 72 h is shown in Table 3. The HIF-1 α expression in HepG2 cells was inhibited by doxorubicin plus miRNA in Figure 3. To our knowledge, HIF-1 α was identified as the major positive modifier for chemoresistance acquisition in HCC, and it represents a prime molecular target for overcoming chemoresistance. HIF-1 α miRNA sheds light on a possible role for combating HCC and is warranted validation as adjuvant therapeutic agents in clinical studies of human HCC.

Table 1. Down-regulation of VEGF and ANG-2 expression after HepG2 cells transfected with HIF-1 α miRNA

Group	n	24 h	48 h	72 h
VEGF (pg/mL)				
Control	3	920.5 \pm 62.4	1516.7 \pm 39.4	5861.8 \pm 69.7
Neg-miRNA	3	903.5 \pm 5.0	1465.6 \pm 10.1	5769.4 \pm 51.4
HIF-1 α miRNA	3	688.8 \pm 22.3*	820.0 \pm 19.3**	2691.3 \pm 100.5**
ANG-2 (pg/mL)				
Control	3	525.4 \pm 23.2	764.3 \pm 82.1	1613.6 \pm 97.2
Neg-miRNA	3	509.4 \pm 42.4	787.3 \pm 44.0	1672.7 \pm 71.9
HIF-1 α miRNA	3	450.6 \pm 21.4*	571.6 \pm 58.4**	1027.6 \pm 115.0**

*Compared with the control group or the Neg-miRNA group, P < 0.05.

**Compared with the control group or the Neg-miRNA group, P<0.001.

Table 2. Apoptosis increase of HepG2 cells at 48 h after HIF-1 α miRNA transfection(\pm SD)

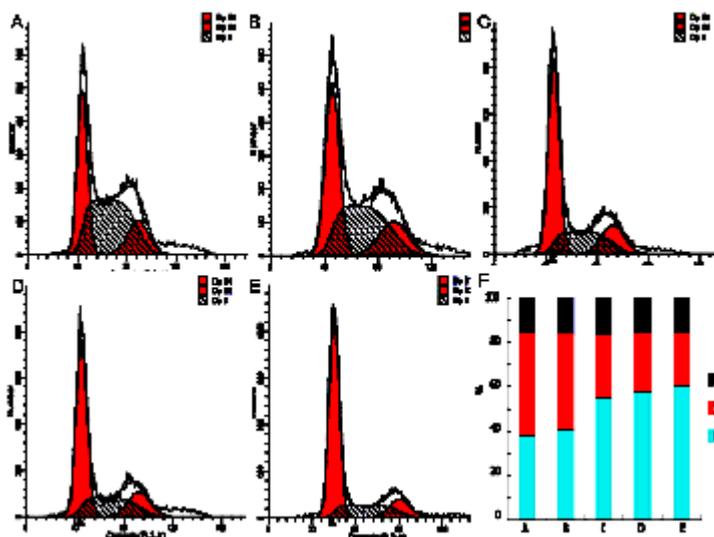
Group	Apoptosis (%)	q value	P value
Control	3.80 \pm 0.12		
Neg-miRNA	4.84 \pm 0.23*	2.10	0.062
HIF-1 α miRNA	15.49 \pm 0.99*	23.65	0.000
Doxorubicin	15.91 \pm 0.37*	24.50	0.000
Dox. puls HIF-1 α miRNA	27.33 \pm 0.94* ^{ab}	47.40	0.000

*Compared with the control group; a Compared with the HIF-1 α miRNA group, q=23.75, P<0.000; bCompared with the doxorubicin group, q=22.90, P<0.001; Dox. plus HIF-1 α miRNA, Doxorubicin (0.5 μ M/L) plus HIF-1 α miRNA

Table 3. Alteration of cell cycles after HepG2 transfected with HIF-1 α miRNA (\pm SD) at 72 h

Group	G ₀ /G ₁ phase			S phase		
	Ratio (%)	q	P	Ratio (%)	q	P
Control	56.8 \pm 1.1		26.1 \pm 0.8			
Neg-miRNA	57.4 \pm 1.3*	0.82	0.432	25.6 \pm 1.2*	0.67	0.516
HIF-1 α miRNA	61.5 \pm 1.1*	5.72	0.000	22.4 \pm 0.6*	4.31	0.002
Doxorubicin	62.0 \pm 0.4*	6.27	0.000	21.6 \pm 1.2*	5.22	0.000
Dox.+HIF-1 α miRNA	65.7 \pm 0.9* ^{ab}	10.77	0.000	19.5 \pm 1.3* ^{ab}	7.70	0.000

*Compared with the control group; a, Compared with the HIF-1 α miRNA group, G₀/G₁ phase q = 5.50, P<0.000, S phase q = 0.903, P = 0.007; b, Compared with the doxorubicin group (0.5 μ M/L), G₀/G₁ phase q = 4.50, P<0.000, S phase q = 2.48, P = 0.033. Neg-miRNA, Negative miRNA; Dox + HIF-1 α miRNA, Doxorubicin (0.5 μ M/L) plus HIF-1 α miRNA.



A, the control group, B, the negative miRNA group, C, the HIF-1 α miRNA group, D, the doxorubicin group, E, the doxorubicin combined with HIF-1 α miRNA group and F, the distribution of the cell cycle. The G₁ phase was 54.78 \pm 1.64 (%) in the HIF-1 α miRNA group, 57.48 \pm 0.71 (%) in the doxorubicin group, 60.32 \pm 1.20 (%) in the doxorubicin combined with HIF-1 α miRNA group.

Fig. 3. The cell cycle of HepG2 cells at 48 h after transfection of miRNA expressing plasmid DNA (pDNA).

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