miR-20b negatively regulates VEGF expression by targeting STAT3 in H22 hepatocellular carcinoma cells

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Received March 5, 2018; Accepted August 13, 2018

DOI: 10.3892/or.2018.6651

Abstract. Vascular endothelial growth factor (VEGF) promotes angiogenesis during tumor growth, and its expression involves multiple signaling pathways and transcription factors. In the present study, transforming growth factor (TGF)-β1 promoted upregulation of VEGF and downregulation of microRNA (miR)-20b expression in mouse H22 hepatocellular carcinoma cells. miR-20b negatively regulated both constitutive VEGF expression and TGF-β1-induced VEGF expression. The miRanda algorithm predicted that a binding site of the miR-20b GCAAUCUGGGCACUUU sequence was present in the signal transducer and activator of transcription (STAT)3 3'-untranslated region. Following transfection of miR-20b mimics into H22 cells, expression of STAT3 protein was downregulated. A dual-luciferase activity assay revealed that miR-20b directly targeted STAT3 to regulate its expression, and that interference with STAT3 expression significantly downregulated VEGF mRNA and protein expression. Interference with STAT3 expression resulted in increased VEGF expression in H22 cells overexpressing miR-20b, but expression was lower than that in quiescent H22 cells. This indicated that STAT3 was involved in the negative regulation of VEGF expression in H22 cells by miR-20b. The data demonstrated that miR-20b negatively regulated VEGF expression by directly targeting STAT3 in H22 cells.

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Key words: microRNA-20b, vascular endothelial growth factor, signal transducer and activator of transcription 3, H22 cells, transforming growth factor-β1

Introduction

Vascular endothelial growth factor (VEGF) is a homologous glycoprotein dimer consisting of two identical polypeptide chains linked by disulfide bonds (1). The VEGF family includes seven members, VEGF-A, B, C, D, E, F and placenta growth factor (PIGF). VEGF-A was the first identified, and was initially referred to simply as VEGF (2,3). VEGF is widely distributed in endothelial and epithelial cells, macrophages, smooth muscle and tumor cells (4), is a key physiological and pathological angiogenesis promoting factor, and a potential target of tumor therapy (5). VEGF expression can be regulated at either the transcriptional or post-transcriptional level, and involves multiple intracellular signaling pathways and a variety of specific transcription factors (6).

Micro (mi)RNAs are a highly conserved class of noncoding RNA in eukaryotes and are ~20 nucleotides in length (7). They mediate cell growth, differentiation, proliferation, and apoptosis (8). miR-20b is a member of the miR-106a-363 gene cluster located in the mammalian X chromosome, the larger miR-17 family, and the miR-17-92 and miR-106b-25 gene clusters (9,10). miR-20b is highly homologous to miR-20a from the miR-17-92 gene cluster and miR-93 from the miR-106b-25 gene cluster. miR-20a and miR-93 have been reported to regulate VEGF production (11,12). The present study investigated miR-20b and VEGF expression in H22 mouse hepatocellular carcinoma cells.

Materials and methods

Cell culture. H22 cells were obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI-1640 complete medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Tianhang Biotechnology Co., Ltd., Hangzhou, China) at 37˚C in an environment containing 5% CO_2. Cells were subcultured until the density reached $3x10^6$ cells/ml, and were passaged every 3-4 days. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) high glucose cell culture medium containing 10% fetal bovine serum, subcultured at 85-90% confluency, and passaged every 2-3 days.

Cell transfection. One day prior to transfection, $3x10^5$ H22 cells/well were inoculated into 24-well plates containing RPMI-1640 complete medium and transfected when 70-90% confluent. Cells were transfected using Lipofectamine® 2000 following the manufacturer's protocol. Briefly, 3 μl miR-20b mimics or scramble control stock solution diluted with 250 μl of Opti-MEM I (V1), and 3 μl Lipo2000 diluted with 100 μl of Opti-MEM I (V2), V1 and V2 solutions were mixed and incubated at room temperature for 20 min. Transfection complexes (V1+V2) were added to 500 μl of suspended H22 cells and cultured for 6 h. The H22 cells were then resuspended in RPMI-1640 medium supplemented with 10% serum and cultured for use in the experimental procedures. The miR-20b sequences used were as follows: Forward, 5'-CAAAGUGCU CAUAGUGCAGGUAG-3' and reverse, 5'-ACCUGCACUAUG AGCACUUUGUU-3' for miR-20b mimics; forward, 5'-UUC UCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGA CACGUUCGGAGAATT-3' for miR-20b scrambled control.

Identification of miR-20b binding site. The miRanda algorithm (www.microrna.org/microrna/home.do) was used to predict binding sites of miR-20b.

Construction of pmiR-RB-ReportTM-signal transducer and activator of transcription (STAT)3 3'-untranslated region (UTR) dual-luciferase reporter recombinant plasmids. Total RNA was extracted from RAW 264.7 cells using TRIzol® (Thermo Fisher Scientific Inc.) reagent. cDNA synthesis was performed following the TransScript First-Strand cDNA Synthesis Super Mix kit protocol (TransGen Biotech Co., Ltd, Beijing, China). Polymerase chain reaction (PCR) amplification of the STAT3 gene 3'-UTR sequence was then performed using 5'-CCGCTCGAGCGGTGCCCCGTGCTCCACCCC TA-3' upstream and 5'-GAATGCGGCCGCTCCCCTTGC TCCCTTCTGCT-3' downstream primers. The PCR reaction was carried out following the TransStart FastPfu DNA Polymerase kit protocol (TransGen Biotech Co., Ltd). Reaction conditions were 95˚C predenaturation for 1 min; 95˚C denaturation for 20 sec; 57˚C annealing for 20 sec; 72˚C extension for 30 sec, for a total of 40 cycles. The pmiR-RB-Report plasmid (Guangzhou RiboBio Co., Ltd, Guangzhou, China) and purified STAT3 gene 3'-UTR sequence PCR amplification products were digested using *Xho*I and *Not*I. The digested product was separated using agarose gel electrophoresis, and the desired band was removed for purification. The ligation reaction was carried out following the TaKaRa DNA Ligation Ver.2.0 Kit protocol (Takara Bio, Inc., Otsu, Japan) and the ligation product was transformed into competent *Escherichia coli* DH5α cells for amplification. The recombinant plasmids were extracted from concentrated bacterial culture supernatant, identified after *Xho*I and *Not*I digestion, and sequenced by Shanghai Kehua Bio-engineering Co., Ltd. (Shanghai, China).

Detection of luciferase activity. Luciferase activity was assayed in four experimental groups: i) pmiR-RB-ReportTM empty vector+miR-20b mimics; ii) pmiR-RB-ReportTM empty vector+miR-20b scramble; iii) pmiR-RB-ReportTM-STAT3 3'-UTR recombinant vector+miR-20b mimics; and iv) pmiR-RB-ReportTM-STAT3 3'-UTR recombinant vector+miR-20b scramble. Luciferase activity was detected with a Promega Dual-Luciferase Activity Assay kit (Promega Corporation, Madison, WI, USA). Transfected HeLa tool cells were washed 2-3 times with 1xPBS before assay. Cells in each well of a 24-well plate were fully lysed by adding 150 μl of fresh lysis buffer with repeated agitation. Luciferase activity was read in 96-well nontransparent plates with 20 μl lysis supernatant with 100 μl LAR II added to detect firefly luciferase fluorescence followed by 100 μl Stop & Glo reagent to detect *Renilla* luciferase fluorescence. Transfection reagent used was Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and luciferase activity was measured 24 h following transfection.

Reverse transcription (RT)-quantitative (q)PCR. Total cell RNA was extracted using Invitrogen TRIzol® (Thermo Fisher Scientific Inc.) reagent, the quality and amount of RNA were determined with a Smart Spec3000 Nucleic Acid Protein Analyzer, and 1 μg aliquots were removed from each sample for cDNA synthesis. The RT reaction was carried out using an RT TransScript First-Strand cDNA Synthesis SuperMix kit, composed of reverse transcriptase, buffer dNTPs and primers purchased from TransGen Biotech Co., Ltd., with 20 μl reaction systems, at 42˚C for 30 min followed by 85˚C for 5 min. PCR products were amplified using SYBRGreen TransStart Top Green qPCR SuperMix (TransGen Biotech Co., Ltd.) as a detection system. The PCR two-step method reaction conditions were 94˚C 30 sec; 94˚C 5 sec, 60˚C 30 sec, 40 cycles. Gene expression was analyzed by the $2^{-\Delta\Delta Cq}$ method (13). The VEGF gene assay included *GAPDH* as the internal reference, and the miR-20b assay included U6 as the internal reference.

Primer sequences were as follows: Forward, 5'-CTACTG CCGTCCGATTGAGA-3' and reverse, 5'-CCTATGTGCTGG CTTTGGTG-3' for VEGF; forward, 5'-GGAGCAGAGATG TGGGAATGGA-3' and reverse, 5'-GCAAGGAGTGGGTCT CTAGGTCAA-3' for STAT3; forward, 5'-GGCAAATTC AACGGCACA-3' and reverse, 5'-TCCACGACATACTCA GCACC-3' for GAPDH; forward, 5'-ATGCCAAAGTGC TCATAGTG-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3' for miR-20b; forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3' for U6.

Western blot assay. Cells were harvested and rinsed twice with precooled PBS before lysing with precooled NP-40 lysis buffer with added PMSF at 100:1. The protein concentration of the cell lysates was determined with the bicinchoninic acid assay. The extracted proteins (20 μg per lane) were separated by 10% SDS-PAGE gel electrophoresis, followed by 350 mA constant transfer for 120 min to nitrocellulose membranes. The membranes were blocked at room temperature with 5% BSA (Beyotime Institute of Biotechnology, Haimen, China) for 2 h and incubated with anti-VEGF (cat. no. ab46154; Abcam, Cambridge, UK) or anti-STAT3 antibody (cat. no. ab76315; Abcam) with anti-GAPDH antibody (cat. no. AF1186; Beyotime Institute of Biotechnology), all 1:1,000 dilution at 4˚C overnight. The membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (cat. no. A0208; Beyotime Institute of Biotechnology; 1:1,000 dilution) for 2 h at room temperature. Membranes were visualized with a BeyoECL

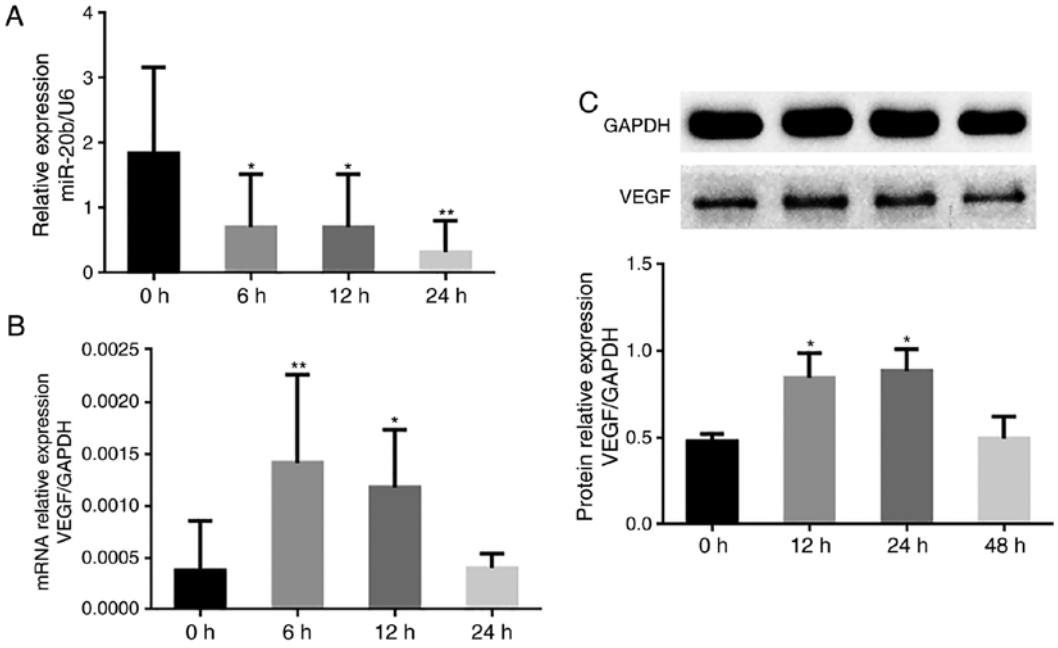

Figure 1. Expression of miR-20b and VEGF in H22 cells stimulated by TGF-β1. H22 cells were stimulated with TGF-β1 for 6, 12, or 24 h, and the relative expression of (A) miR-20b and (B) VEGF were assayed using reverse transcription-quantitative polymerase chain reaction. GAPDH and U6 were used as internal references. (C) VEGF protein expression in TGF-β1-stimulated H22 cells at 12, 24, and 48 h, was assayed using western blotting. GAPDH was used as an internal reference. Top, representative immunoblot; Bottom, densitometric analysis of VEGF relative to GAPDH. *P<0.05, **P<0.01 vs. 0 h controls. miR-20b, microRNA-20b; VEGF, vascular endothelial growth factor.

Plus kit (Beyotime Institute of Biotechnology, Haimen, China) and read with a multifunctional Image-Pro Plus software, version 6.0. digital gel imaging system (Media Cybernetics Inc., Rockville, MD, USA). GAPDH was used as an internal control.

RNA interference of STAT3. Small interference RNAs (siRNA) were designed and obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with 30 nM siRNA or control using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). H22 cells were transfected with STAT3-siRNA or its negative control for 6 h and then subjected to subsequent experiments according to the manufacturer's protocol. siRNA sequences used were as follows: Forward, 5'-CCCGCCAACAAAUUAAGAATT-3' and reverse, 5'-UUCUUAAUUUGUUGGCGGGTT-3' for STAT3-siRNA-1; forward, 5'-GAGGAGGCAUUUGGAAAG UTT-3' and reverse, 5'-ACUUUCCAAAUGCCUCCUCTT-3' for STAT3-siRNA-2; forward, 5'-GGGUCUCGGAAAUUU AACATT-3' and reverse, 5'-UGUUAAAUUUCCGAGACC CTT-3' for STAT3-siRNA-3; forward, 5'-CCCGCCAACAAA UUAAGAATT-3' and reverse, 5'-UUCUUAAUUUGUUGG CGGGTT-3' for STAT3-siRNA negative control.

Statistical analysis. Data were expressed as the mean ± standard deviation. An independent sample t-test was used to compare differences between groups, and analysis of variance followed by the least significant difference test was used for multiple comparisons. All experiments were repeated at least 3 times. The statistical analysis was performed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-20b negatively regulates VEGF expression in H22 cells stimulated by TGF-β1. Transforming growth factor (TGF)-β1 has previously been reported to upregulate VEGF expression (14) and the relative expression of miR-20b and VEGF in H22 cells in response to TGF-β1 is presented in Fig. 1. The relative expression of miR-20b was downregulated. The relative expression of VEGF mRNA was upregulated at 6 and 12 h, but after 24 h of stimulation, the relative expression was not significantly different from that in control group cells. VEGF protein expression in H22 cells was upregulated after 12 and 24 h of TGF-β1 stimulation compared with control group cells (P<0.05). TGF-β1 thus had opposite effects, as it downregulated miR-20b expression and upregulated VEGF expression. The effect of miR-20b on the upregulation of VEGF expression in H22 cells induced by TGF-β1 is presented in Fig. 2. Compared with the TGF-β1 only group, VEGF gene and protein expression was significantly downregulated in TGF-β1-stimulated H22 cells transfected with miR-20b mimics (P<0.05). This indicated that miR-20b negatively regulated TGF-β1-induced increase of VEGF expression.

miR-20b negatively regulates the production of VEGF in quiescent H22 cells. To further investigate the association of miR-20b with VEGF expression, untreated H22 cells were transfected with miR-20b mimics or miR-20b scramble controls. miR-20b transfection efficiency results are presented in Fig. 3A. The results revealed that the expression of miR-20b increased ~20 fold in H22 cells transfected with miR-20b mimics. The VEGF expression results are shown in Fig. 3B and C. VEGF mRNA expression in cells transfected

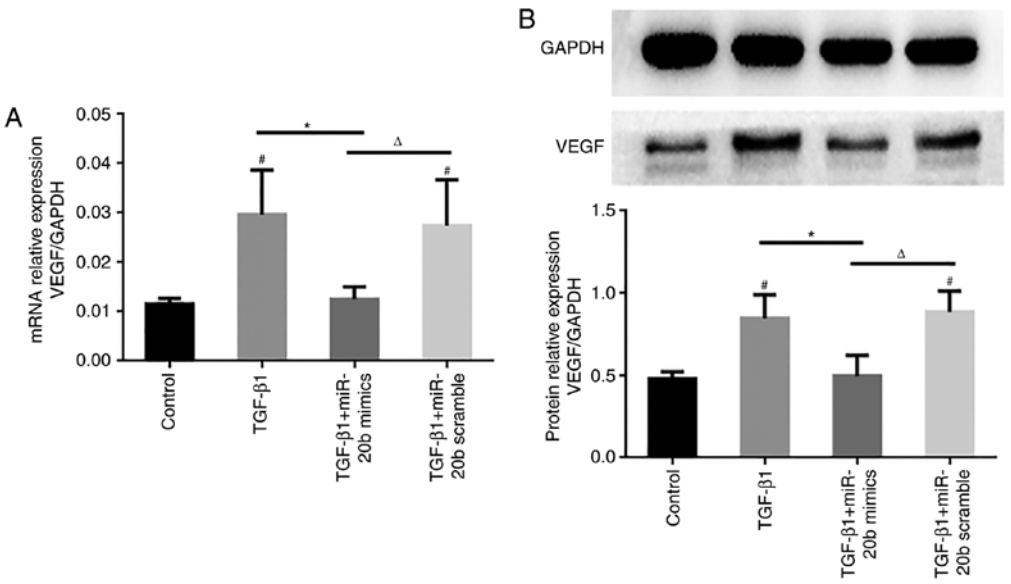

Figure 2. miR-20b negatively regulates the production of VEGF in H22 cells stimulated with TGF-β1. H22 cells were transfected with miR-20b mimics or a scramble control. After 24 h, H22 cells were (A) stimulated for 6 h with TGF-β1, and VEGF gene expression was measured using reverse transcription-quantitative polymerase chain reaction or (B) were stimulated for 24 h with TGF-β1 and VEGF protein expression was assayed using western blotting. Top, representative blot; Bottom, mean densitometric analysis. #P<0.05 vs. controls; *P<0.05 vs. TGF-β1; ΔP<0.05 vs. TGF-β1+miR-20b mimics. miR-20b, microRNA-20b; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1.

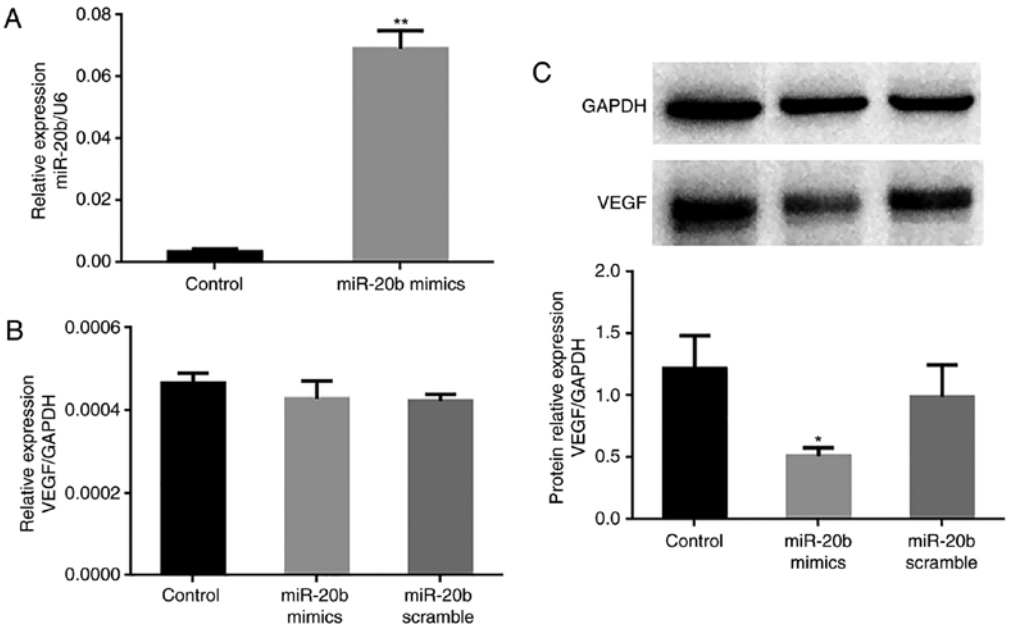

Figure 3. miR-20b negatively regulates VEGF expression in quiescent H22 cells. H22 cells were transfected with miR-20b mimics or miR-20b scramble controls, and 24 h later (A) miR-20b and (B) VEGF mRNA expression was assayed using reverse transcription-quantitative polymerase chain reaction. A total of 48 h later (C) VEGF protein expression was assayed using western blotting. Top, representative blot; Bottom, mean densitometric analysis. *P<0.05, **P<0.01 vs. control cells. miR-20b, microRNA-20b; VEGF, vascular endothelial growth factor.

with miR-20b mimics was not significantly different from that in the H22 controls, but VEGF protein expression was downregulated (P<0.05). This indicated that miR-20b negatively regulated VEGF protein expression.

miR-20b decreases STAT3 protein expression in H22 cells. STAT3 is involved in the occurrence and progression of liver cancer (15) and has been shown to bind directly to a VEGF promoter to upregulate its expression (16) The miRanda algorithm demonstrated that an miR-20b binding site was present in the STAT3 3'-UTR region (Fig. 4A). It was thus hypothesized that miR-20b influenced VEGF production in H22 cells by regulating STAT3 expression. STAT3 expression in H22 cells transfected with miR-20b mimics or miR-20b scramble controls is presented in Fig. 4B and C. STAT3 mRNA expression did not change significantly compared with controls, but STAT3 protein expression in miR-20b mimics was downregulated (P<0.05). This indicated that miR-20b

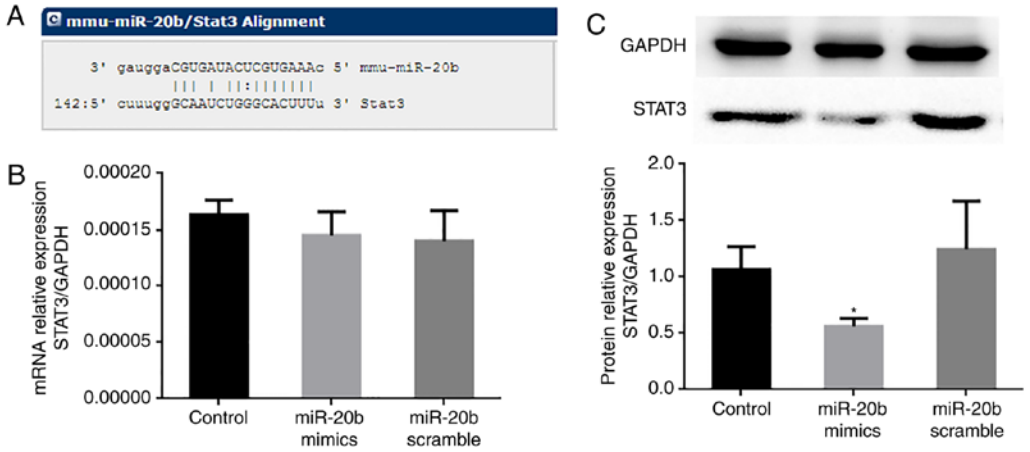

Figure 4. Effect of miR-20b on STAT3 expression in H22 cells. (A) The miR-20b binding site in the STAT3 3'-UTR region sequence was predicted by the miRanda algorithm. H22 cells were transfected with miR-20b mimics or miR-20b scramble controls, and (B) 24 h later STAT3 mRNA expression was assayed using reverse transcription-quantitative polymerase chain reaction and (C) 48 h later, STAT3 protein expression was assayed using western blotting. Top, representative blot; Bottom, mean densitometric analysis. *P<0.05 vs. controls. miR-20b, microRNA-20b; STAT3, signal transducer and activator of transcription 3.

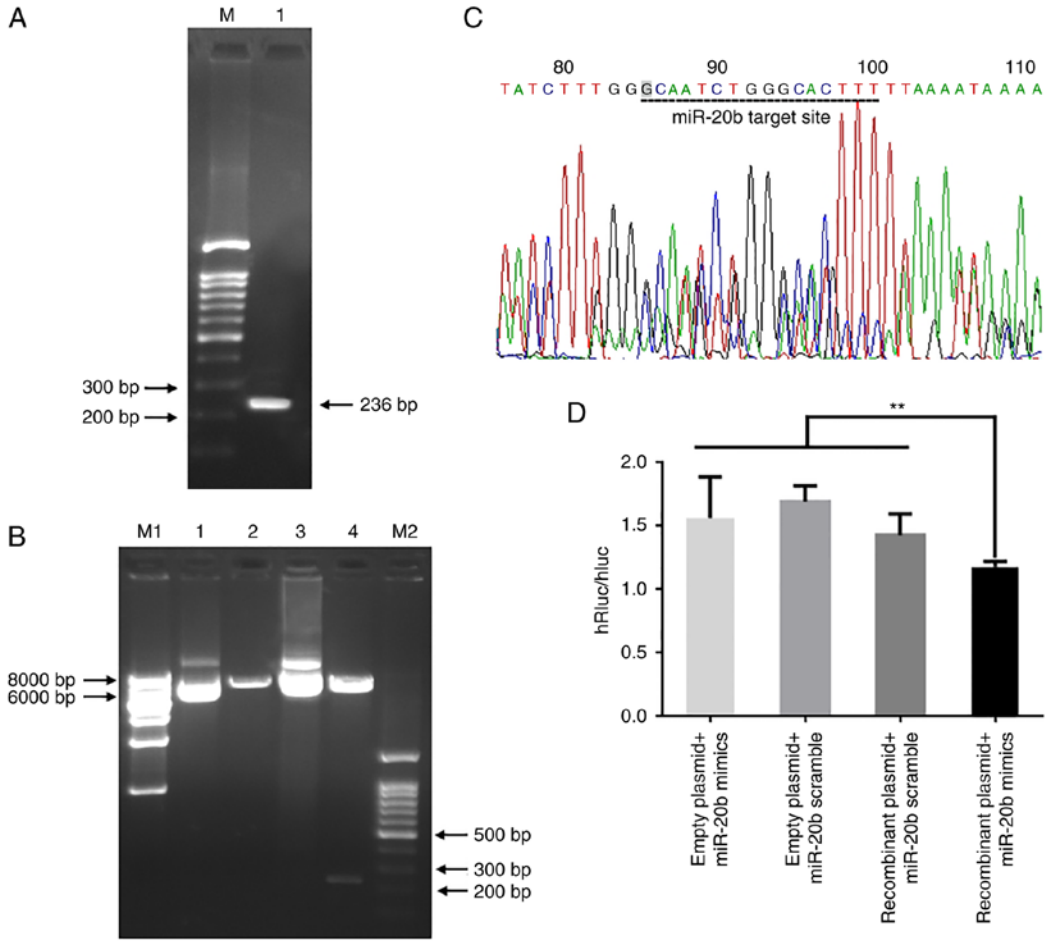

Figure 5. miR-20b directly targets STAT3 to regulate its expression in H22 cells (A) The STAT3 3'-UTR sequence was amplified with polymerase chain reaction using RAW264.7 cDNA as a template, and the 236 bp amplification product was detected using 1% agarose gel electrophoresis. (B) The recombinant pmiR-RB-ReportTM-STAT3 3'-UTR or empty plasmids were cut by *Xho*I and *Not*I enzymes, and the product was verified by electrophoresis. M1, 1,000-10,000 bp marker; lane 1, empty plasmid; lane 2, empty plasmid, double digestion; lane 3, recombinant plasmid; lane 4, recombinant plasmid, double digestion; M2, 100-1,000 bp marker. (C) Partial sequencing of recombinant plasmids, including the miR-20b binding site. (D) The recombinant plasmid or empty plasmid with miR-20b mimics or a scramble control were cotransfected into HeLa cells; 24 h later, the luciferase activity was detected with a dual-luciferase reporter assay. **P<0.01. miR-20b, microRNA-20b; STAT3, signal transducer and activator of transcription 3; UTR, untranslated region.

negatively regulated STAT3 expression; miR-20b affected the translation of STAT3 mRNA rather than its degradation.

miR-20b directly targets STAT3 to regulate its expression in H22 cells. Direct STAT3 targeting by miR-20b was

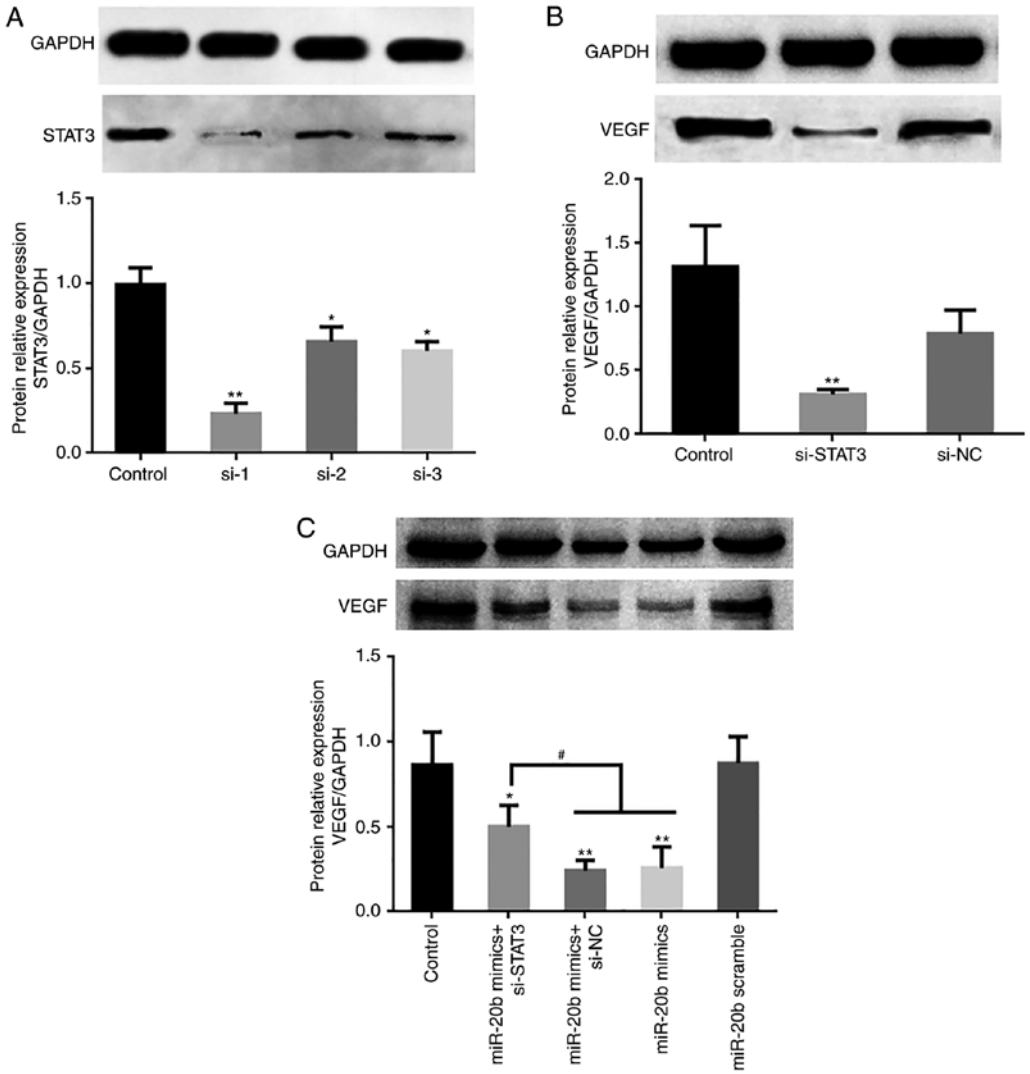

Figure 6. Involvement of STAT3 in the negative regulation of VEGF expression by miR-20b in H22 cells (A) STAT3-specific siRNA was evaluated in H22 cells transfected with si-1, or si-2, or si-3 si-STAT3 fragments. STAT3 protein expression was assayed in western blots 48 h after transfection; Top, representative blot; Bottom, mean densitometric analysis. *P<0.05, **P<0.01 vs. controls. (B) Western blot of VEGF protein expression 48 h after STAT3 RNA interference. Top, representative VEGF and GAPDH blots; Bottom, mean densitometric analysis. **P<0.01 vs. controls. (C) H22 cells were transfected with miR-20b mimics or miR-20b scramble controls and with si-STAT3 or si-NC fragments. VEGF protein expression was assayed in western blots after 48 h. Top, representative blot; Bottom, mean densitometric analysis. *P<0.05 **P<0.01 vs. controls. $^{\#}$P<0.05 vs. miR-20b mimics+si-STAT3. miR-20b, microRNA-20b; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; si, small interfering.

verified with a STAT3 3'-UTR dual-luciferase reporter gene vector. Luciferase activity was detected using the pmiR-RB-ReportTM plasmid vector system in which the reporter gene is *Renilla* luciferase (hRluc), the reference gene is firefly luciferase (hluc), and the final test results are expressed as hRluc/hluc fluorescence. The STAT3 gene 3'-UTR primers were designed according to the location of miR-20b binding, and the STAT3 3'-UTR sequence was amplified by PCR as presented in Fig. 5A. The 236 bp PCR amplification product was digested and ligated downstream of the *Renilla* luciferase in pmiR-RB-ReportTM plasmid to obtain the pmiR-RB-ReportTM-STAT3 3'-UTR dual-luciferase reporter vector. The recombinant plasmid was verified by electrophoresis after double enzyme digestion (Fig. 5B) and DNA sequencing (Fig. 5C). Luciferase activity was detected using a dual-luciferase activity assay (Fig. 5D). The luciferase activity in cells cotransfected with the recombinant plasmid+miR-20b mimic was significantly lower than that in those cotransfected with the empty plasmid+miR-20b mimics or empty plasmid+miR-20b scramble control (P<0.05). This indicated that miR-20b directly targeted the STAT3 3'-UTR and negatively modulated its expression.

STAT3 is involved in the regulation of VEGF expression by miR-20b in H22 cells. As miR-20b directly targets STAT3 3'-UTR and inhibits its expression, and because STAT3 is a transcriptional regulator of VEGF expression, the involvement of STAT3 in the regulation of VEGF expression by miR-20b was investigated. Three small interfering (si)RNA fragments of the STAT3 gene (si-1, si-2, and si-3) were designed and synthesized and transfected into H22 cells. Total protein was extracted from the siRNA-transfected cells and STAT3 expression was assayed to select the most efficient siRNA fragment. As presented in Fig. 6A, the

strongest interference was observed with si-1. The effects of si-1 and its negative control, si-NC, on VEGF expression in H22 cells are presented in Fig. 6B, which reveals significant downregulation of VEGF expression in H22 cells transfected with the si-1 siRNA fragment. To determine the involvement of STAT3 in the regulation of VEGF by miR-20b, H22 cells were transfected with miR-20b mimics and si-STAT3 was used to interfere with STAT3 gene expression, and VEGF protein expression was assayed in western blots. As shown in Fig. 6C, VEGF protein expression was significantly downregulated in H22 cells transfected with miR-20b mimics, miR-20b mimics+si-STAT3, and miR-20b mimics+si-NC. The decrease in VEGF expression was greater in the miR-20b mimics+si-NC and miR-20b mimics group ($P<0.01$) and although VEGF expression was higher in the miR-20b mimics+si-STAT3 group than in the miR-20b mimics group, it was still lower than that in control group. This indicated that in addition to the STAT3 pathway, miR-20b may also regulate VEGF expression by other signals and that STAT3 is involved in the downregulation of VEGF expression by miR-20b.

Discussion

In the present study, miR-20b was demonstrated to negatively regulate both TGF-β1-induced and constitutive VEGF expression in H22 cells. miRNAs are a type of noncoding regulatory RNA, have about 20 nucleotides, and are expressed in many cell types (17). miRNAs that are partially complementary with the 3'-UTR of the target gene mRNA can interfere with RNA polymerase II to inhibit translation or promote the degradation of the target gene mRNA to inhibit gene expression (18). In mammals, 20-30% of genes are regulated by miRNAs; frequently an miRNA can regulate hundreds of target genes. Consequently, the activity of miRNAs has been described in many biological processes, including signal transduction, cell growth and development, tumor development and immune response (19,20). Involvement of miRNAs in angiogenesis and regulation of VEGF expression has been reported. Li *et al* (21) suggested that miR-377 negatively targets VEGF expression in esophageal cancer to inhibit the proliferation of esophageal cancer cells and tumor growth in nude mice. miR-29c, miR-15a-5p, miR-195, and miR-205, all of which target the VEGF 3'-UTR region in a number of different tumor cells, inhibit both VEGF production and tumor development (22-25). miR-20b is a member of the miR-106a-363 family and is highly homologous to miR-20a (from the miR-17-92 gene cluster) and miR-93 (from the miR-106b-25 gene cluster) (9,10). Recently, Hu *et al* (11) reported that miR-20a regulates VEGF production in placental cells, and Saito *et al* (12) reported that miR-93 regulates the production of VEGF in the peripheral blood monocytes of Kawasaki disease patients. There are few studies investigating miR-20b regulation of VEGF. Mukhopadhyay *et al* (26) observed a negative correlation between miR-20b and VEGF expression in myocardial ischemia, but did not describe the regulatory mechanism. In the present study, miR-20b significantly downregulated VEGF protein expression in H22 cells, but had no significant effect on the expression of VEGF mRNA. The negative regulation of VEGF expression may have resulted from suppression of VEGF mRNA translation and not VEGF mRNA degradation.

In the present study, STAT3 expression in H22 cells was negatively regulated by direct targeting of miR-20b, and STAT3 positively regulated VEGF expression. As part of the Janus Kinase (JAK)/STAT signaling pathway, STAT is a JAK target protein, and a member of a family of transcription factors present in the cytoplasm (27). STAT3 is a bifunctional protein including 750 to 850 amino acids and is associated with promotion of cytokine regulation of cell growth, differentiation and malignant transformation (28,29). STAT3 has also been associated with the expression of proteins related to cell proliferation and apoptosis (30). miRNAs reported to regulate STAT3 expression include miR-9600, which was shown to regulate non-small cell lung cancer progression (31), miR-125a, which promotes the susceptibility of cervical cancer to paclitaxel (32) and miR-20b that regulates Th17-cell differentiation (33). STAT3 is thus a target of multiple miRNAs, including miR-20b, and there is a STAT3 binding site -842 bp to -849 bp upstream of the VEGF gene, and STAT3 can directly bind the VEGF promoter region to upregulate the expression of VEGF protein (34). The findings of this study are consistent with those of Mu *et al* (35) which suggested that STAT3 upregulates VEGF expression to promote angiogenesis, and Pan *et al* (36) that demonstrated that STAT3 upregulates VEGF expression in non-small cell lung cancer.

In conclusion, it was demonstrated that VEGF expression was decreased in H22 cells that overexpressed miR-20b, and after STAT3 RNA interference, it remained lower than that in resting H22 cells, i.e., in those not overexpressing miR-20b. In addition, miR-20b may regulate VEGF expression in ways other than indirectly through STAT3, and STAT3 was only partially involved in the miR-20b negative regulation of VEGF expression. miR-20b has been reported to influence VEGF expression by targeting various signaling proteins or transcription factors, including AKT336 and HIF-1. Negative regulation of VEGF production by miR-20b can undoubtedly occur in a number different of ways. In the present study, TGF-β1 induced upregulation of VEGF and downregulation of miR-20b expression, and miR-20b directly targeted STAT3 3'-UTR to modulate STAT3 expression. STAT3 was thus involved in the downregulation of VEGF in H22 cells by miR-20b.

Acknowledgements

Not applicable.

Funding

The present study was supported by National Science Foundation of China (grant no. 81273273), Anhui Provincial Natural Science Foundation (grant no. 1708085MH218) and the Scientific Research Innovation Team Project of Anhui Colleges and Universities (grant no. 2016-40).

Availability of data and materials

The datasets used during the present study are included in this published article.

Authors' contributions

CS and SG designed this study; YL, JH and XT performed experiments; HW and QF analyzed data; CS wrote the manuscript; CS and SG made manuscript revisions. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Bengbu Medical College Ethics Committee (Bengbu, China).

Patient consent for publication

Not applicable

Competing interests

The authors state that they have no competing interests.

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