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Renal Protection Mediated by Hypoxia Inducible Factor-1α Depends on Proangiogenesis Function of miR-21 by Targeting Thrombospondin 1

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Background. Angiogenesis contributes to the repair process after renal ischemia/reperfusion (I/R) injury. In the present study, we tested the role of miR-21 in the angiogenesis induced by hypoxia inducible factor (HIF)-1α through inhibiting a predicted target gene thrombospondin 1 (TSP-1). **Methods.** To stabilize HIF-1α, hypoxia (1% O_2 for 24 hours) was performed in human umbilical vein endothelial cells and cobalt chloride ($CoCl_2$) was pretreated intraperitoneally 24 hours before renal I/R in mice. Locked nucleic acid modified anti-miR-21 and scrambled control was transfected with hypoxic cells or delivered into the mice via tail vein 1 hour before $CoCl_2$ injection. The kidneys and blood were collected at 24 hours after reperfusion. **Results.** HIF-1α induced by hypoxia and $CoCl_2$ upregulated vascular endothelial growth factor and miR-21, and increased angiogenesis. It was found that expression of TSP-1 was inversely related with miR-21 in vitro and in vivo. Targeting of TSP-1 by miR-21 was further confirmed in vitro. Furthermore, HIF-1α improved renal function, accompanied with increased angiogenesis after I/R injury in mice. The protective effect of HIF-1α was attenuated by inhibition of miR-21. **Conclusions.** HIF-1α induced angiogenesis by upregulating not only vascular endothelial growth factor but also miR-21 via inhibiting a novel target gene TSP-1. Both of them may contribute to the protective effect of HIF-1α on renal I/R injury.

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The pathophysiology of renal ischemia/reperfusion (I/R) injury has been considered to target principally tubular epithelial cells. Actually, injury or repair of microvascular endothelial cells could participate in the process of acute kidney injury.[1-5] Reduction of peritubular capillaries could result in persistent renal hypoxia after recovery from I/R injury, which may be important for progression of chronic kidney disease.[6] Angiogenesis is one of the compensatory response after renal I/R injury, which is out of balance with injury or apoptosis of microvascular endothelial cells. Promotion of angiogenesis after reperfusion could protect against renal I/R injury and improve the prognosis of acute kidney injury.[7,8] The current study focused on the role of angiogenesis and underlying mechanisms in the renal I/R injury.

Cells subjected to hypoxic stress engage in adaptive responses aimed at maintaining basal metabolic functions by activating a series of mediators, such as hypoxia-inducible factor-1α (HIF-1α). It has become clear that HIF plays an important role in angiogenesis by upregulating vascular endothelial growth factor (VEGF).[9] MiR-21 has been reported as one of the important hypoxia-regulated miRNAs, which could be induced by HIF in renal tubular cells.[10] In addition, miR-21 has been shown to play an important role in the process of vascular remodeling or angiogenesis in endothelial cells.[11,12] We hypothesized that miR-21 might play an important role in angiogenesis induced by HIF-1α.

Thrombospondin 1 (TSP-1), a homotrimeric 450-kDa matricellular glycoprotein, is a multifunctional protein and was initially recognized as an endogenous inhibitor of angiogenesis.[13] TSP-1 is rarely expressed in the healthy kidney and can be upregulated during renal injury.[14] There have been no published studies on the effect of TSP-1 on

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X.X. designed the experiments, performed in vivo experiments, and wrote the article. N.S. and X.J. performed in vitro experiments. X.Z. performed the 3′-UTR analysis. J. H. made the animal model. M.L. and J.T. revised the article. X.D. designed and supervised the study.

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angiogenesis after renal I/R injury. TSP-1 gene was shown as one of the miR-21 predicted targets in the MicroCosm Targets v5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). We identified TSP-1 as a novel miR-21 target and then determined the role of miR-21 in HIF-1α induced angiogenesis by inhibiting TSP-1 in the renal I/R injury.

MATERIALS AND METHODS

Cell Culture and Hypoxia Treatment

Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection and cultured in endothelial cell medium with 5% fetal bovine serum (Sciencell) under condition of 37°C and 95% air-5% CO_2. HUVEC with 60% to 70% confluency at passages 5 to 6 were exposed to hypoxia treatment (1% O_2) in a modular incubator chamber (Thermo Scientific, Waltham, MA) for 24 hours.[15]

In Vitro HUVEC Tube Formation Assay

Thawed growth factor-reduced BD Matrigel Matrix (BD Biosciences, USA) were placed into each well of a 24-well plate and hardened in an incubator for 30 minutes. Twelve thousand HUVECs/well were seeded on the prepared Matrigel and incubated for 24 hours. Cells were treated with hypoxia for 24 hours after attaching to the surface and then visualized under a light microscope. To quantify capillary-like structures, internodal points formed in 14 fields per well (magnification, 10×) were counted manually.

3′-UTR Reporter Analysis

A segment of the 3′-UTR region of the human TSP-1 mRNA including the predicted miR-21 binding site (1248-1269) was amplified (forward primer, 5′-CCGCTCGAGGGAAAACCTACCATCTCAGTGAGCA-3′; reverse primer, 5′-CCGGGATCCAGAAAAAAAGAATCCCATAACATCCAC-3′) for cloning into pGL3 basic plasmid downstream of the luciferase reporter gene. An inversion mutation was introduced to the seed region of the 3′-UTR. The assay was preformed as we described previously.[16] HUVEC cells with 80% to 90% confluency were cotransfected with 3′-UTR reporter or mutant construct (100 ng/well), a pRL-TK plasmid (internal normalizer, 50 ng/well, Giman, Shanghai), and miR-21 mimic or control oligonucleotides (10 pmol/well, GenePharma, Shanghai), using Lipofectamine 2000. Luciferase activities was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol at 24 hours after the transfection.

Mouse Models of I/R and Cobalt Chloride Pretreatment

Briefly, 6- to 7-week-old male C57BL/6 mice (20 to 23 g, animal lab, Fudan University) were anesthetized with intraperitoneal 1% pentobarbital (50 mg/kg). After performing a midline laparotomy, bilateral renal pedicles were clamped for 35 minutes using microserrefine clips (FST, Burlington, MA). Mice were maintained at 35-37 °C. The kidney was visually assessed for reperfusion after clamp removal. Mice in Sham group underwent the same surgical procedures, except that the renal pedicles were not clamped. For cobalt chloride ($CoCl_2$) (5 mg/mL, Sigma, St Louis, MO) pretreatment ($CoCl_2$ + IR group), $CoCl_2$ was administrated by intraperitoneal injection twice, at a dose of 30 mg/kg with a dosing interval of 12 hours, according to the Rosenberger et al study.[17] Mice underwent renal I/R procedure 12 hours after the second injection. The same volume of saline as $CoCl_2$ was injected in the NS + IR mice.

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Zhongshan Hospital of Fudan University.

The kidneys and blood were collected at 24 hours after reperfusion. Blood samples were taken through cardiac puncture. Serum creatinine (Scr) was measured as described previously.[10]

In Vitro and In Vivo Use of Locked Nucleic Acid–Modified Anti-miR

Locked nucleic acid (LNA) modified anti-miR-21 (Exiqon, Woburn, MA) were used for knocking down miR-21 in vitro and in vivo. HUVECs were transfected with anti-miR-21 and scrambled controls (at 100 nM), by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.[18] Briefly, LNA mix (150 pmol in Opti-MEM I Reduced Serum Media) was added to the Lipofectamine 2000 mix (8 μL in Opti-MEM media) and incubated for 20 minutes at room temperature before pouring into a 3.5-cm dish. The transfection medium was replaced with the regular medium 4 hours posttransfection. After 24 hours of transfection, cells were collected for analysis. The transfection efficiency was about 50% as determined using green fluorescent protein-siRNA.

LNA anti-miR-21 and scrambled control also were used for in vivo suppression and delivered by intravenous (tail vein) injection (10 mg/kg body weight) less than 1 hour before $CoCl_2$ pretreatment.[10]

Histological Analysis of Renal Injury and Immunohistochemical Staining

The kidney tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissues were sectioned at 4 μM thickness and stained with periodic acid–Schiff. Histopathological changes were examined in a blinded manner and scored according to the severity of changes on a semiquantitative scale: no injury, 0% (0); mild, less than 25% (1); moderate, less than 50% (2); severe, less than 75% (3); and very severe, more than 75% (4).

The kidney slices were incubated with primary antibodies against TSP-1 (mouse monoclonal, 1:50, Pierce, USA) and CD31 (rat monoclonal, 1:50, Abcam, USA) after dewaxation, dehydration, elimination of endogenous peroxidase activity by 3% H_2O_2, and blocking by normal goat serum. Antibody dilution and washing steps were performed with phosphate buffered saline. Staining was carried out by GTVision III Detection System/Mo&Rb (Gene Tech, Shanghai, China) according to the manufacturer's protocol. Semiquantitative analysis of CD31 and TSP-1 staining were done by the ImageJ software.

MiR-21 In Situ Hybridization

In situ hybridization (ISH) was completed on 4-μM-thick formalin-fixed paraffin-embedded kidney sections using 40 nM 5′- and 3′-DIG–labeled miR-21 probes or U6 (positive control) (Exiqon, Copenhagen, Denmark). The ISH was performed according to the manufacturer's instructions with minor modification. Deparaffinized sections were exposed to a

10-minute proteinase K (20 mg/mL) treatment at 37°C, dehydrated through ethanol solutions and then covered with hybridization buffer at room temperature for 30 minutes. The probe was diluted in hybridization buffer (40 nM, 50 μL/tissue section) and preheated at 90°C for 5 minutes to linearize. The probe was then added to the slides and incubated at 53°C for 2 hours. Slides were rinsed 2× 5 minutes in 5× saline sodium citrate (SSC), 1× SSC and 0.1× SSC at 53°C, followed by phosphate buffered saline with tween 20 washes and then blocked for 30 minutes followed by incubation with 1:800 antidigoxigenin alkaline phosphatase antibody for 1 hour at room temperature. Slides were incubated in NBT/BCIP (Roche) diluted in double-distilled H_2O for 2 hours at 34°C and then rinsed with AP stop solution, to stop the color development, before dehydration, clearing, and mounting. The tissue was photographed and analyzed.

Real-Time PCR

Total RNA from kidney tissue and cells was isolated using Trizol (Invitrogen). Expression of miR-21 and gene mRNAs (VEGF and TSP-1) was quantified in total RNA using real-time PCR, respectively, with the Taqman chemistry (Applied Biosystems, Carlsbad, CA) and SYBR Premix ExTaq (TaKaRa, Japan), as described previously. U6 snRNA and 18S rRNA were used as internal normalizer for miRNA and mRNAs, respectively. PCR primers (Sangon, Shanghai, China) were designed with sequences as follows:

VEGF (human): forward: 5′-TCGGGCCTCCGAAACCATGA-3′, reverse: 5′-CCTGGTGAGAGATCTGGTTC-3′;
VEGF (mouse) : forward: 5′-GCTTTACTGCTGTACCTCCAC-3′, reverse: 5′-TCCAGGGCTTCATCGTTA-3′;
TSP-1 (human): forward: 5′-GGCAAGGACTGCGTTGGT-3′, reverse: 5′-CACTTCACGCCGGCAAAG-3′;
TSP-1 (mouse) : forward: 5′-GACTCGGGACCCATCTATGA-3′, reverse: 5′-GGTTATGATTGGCAGCTGATG-3′;
18s : forward: 5′-CGGCTACCACATCCAAGGAA-3′, reverse: 5′-CCTGTATTGTTATTTTTCGTCACTACCT-3′.

Ambion (Thermo Fisher Scientific) RT and PCR primers of miR-21(has-miR-21 ID 000397) and U6 (U6 snRNA ID001973) are special for TaqMan MicroRNA Assays.

Western Blot

The nuclear extracts and cytosolic extracts were prepared by using Nuclear Extract Kit from Active Motif (Carlsbad, CA). Samples (40 μg total protein per lane) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk, followed by incubation with primary antibodies against HIF-1α (rabbit polyclonal, 1:1000, Novus, Littleton), TSP-1 (mouse monoclonal, 1:100, Pierce), GAPDH (rabbit polyclonal, 1:1000, Abcam), Histone H3 (mouse monoclonal, 1:1000, Cell Signaling Technology, Danvers, MA) overnight at 4°C. The secondary antibody was horseradish peroxidase-conjugated anti-rabbit or antimouse IgG from Santa Cruz. It was developed by ECL Western Blotting Detection Reagents (Thermo Fisher Scientific). The results were normalized to the protein levels of GAPDH except the nuclear HIF-1α.

Statistics

Statistical analysis was performed using the statistical software SPSS Version 20.0. For comparison of means between 2 groups, 2-tailed, unpaired t tests were used. Data were analyzed using the Student *t* test when comparing 2 groups. For comparison of means between 3 groups, 1-way analysis of variance with Bonferroni posttest was applied. Real-time PCR data were shown as a percentage of control because data from multiple PCR plates were combined. For these data, statistical analysis was performed on the original data before conversion to percentage values. A *P* value less than 0.05 was considered significant. Data are shown as mean ± SEM.

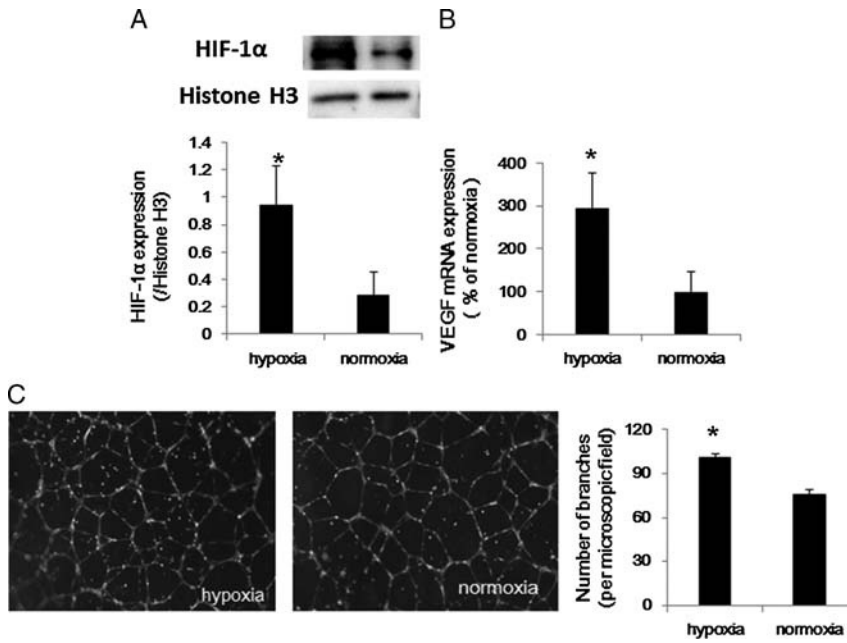

FIGURE 1. Hypoxia activated HIF-1α, upregulated VEGF and induced tube formation in HUVECs. The nuclear protein level of HIF-1α (A) and target gene VEGF mRNA (B) were upregulated significantly by 1% O_2 treatment for 24 hours in HUVEC. C, Tube-formation Assay showed increased capillary-like endothelial tube formation in hypoxic HUVECs. n = 3 per group, *$P < 0.05$ versus the normoxia group.

RESULTS

Hypoxia-Activated HIF-1α–Upregulated VEGF and Induced Tube Formation in HUVECs

Hypoxia is commonly used for activation of HIF-1α and resulting angiogenesis.[10,15] Treatment of HUVEC with 1% O_2 for 24 hours induced activation of HIF-1α in nuclear extracts (Figure 1A). Concomitant with activation of HIF-1α, the expression of VEGF mRNA was significantly higher in hypoxic cells than that in the normoxic cells (Figure 1B). Hypoxia increased capillary-like endothelial tube formation in HUVECs, consistent with the well-known proangiogenesis effect of HIF-1α through VEGF.

MiR-21 Targeted TSP-1 and Might Contribute to Hypoxia-Induced Tube Formation in HUVEC

Real-time PCR analysis showed that abundance of miR-21 was significantly increased in HUVECs 24 hours after hypoxia (Figure 2A). TSP-1 mRNA was found as a predicted target of miR-21 according to the MicroCosm database. As

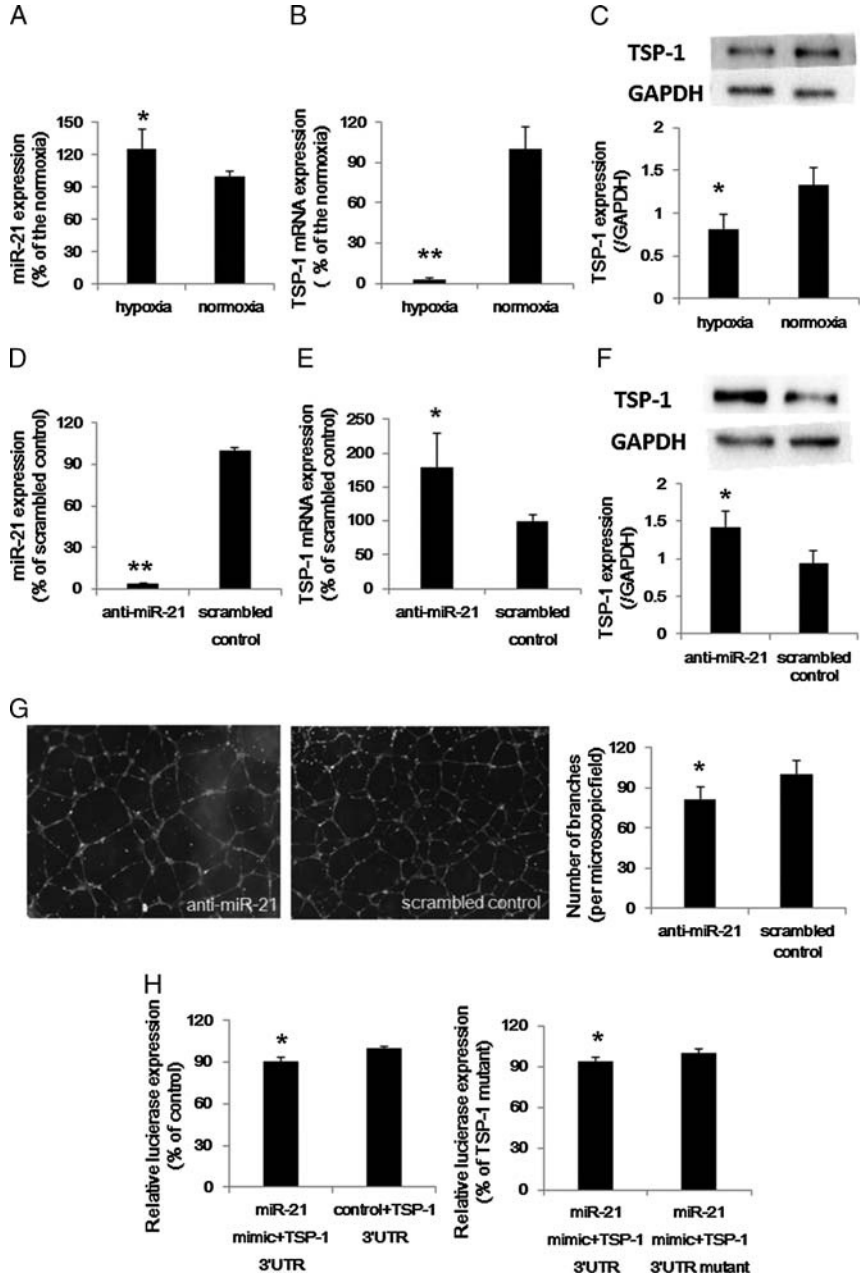

FIGURE 2. miR-21 targeted TSP-1 and might contribute to hypoxia-induced tube formation in HUVECs. miR-21 was upregulated by 1% O_2 treatment for 24 hours in HUVECs (A), with decreased expression of TSP-1 mRNA (B) and protein (C). n = 3 per group, $^{*}P < 0.05$ versus the normoxia group, $^{**}P < 0.01$ versus the normoxia group. D, Expression of miR-21 was suppressed by LNA anti-miR-21 effectively in hypoxic HUVEC. It was showed that LNA anti-miR-21 increased TSP-1 mRNA (E) and protein expression (F). G, Inhibition of miR-21 decreased capillary-like endothelial tube formation in hypoxic HUVECs, compared with the scrambled control. n = 4 per group, $^{*}P < 0.05$ versus the scrambled control group. H, Luciferase reporter assays analysis confirmed an interaction between miR-21 and a 3′-UTR of TSP-1 mRNA. This interaction did not occur when action with negative control or TSP-1 3′-UTR mutant construct. n = 8 per group,$^{*}P < 0.05$ versus control oligonucleotides or TSP-1 3′-UTR mutant construct.

shown in Figures 2B and C, expression of TSP-1 mRNA and protein was significantly downregulated in the hypoxic HUVECs.

Expression of miR-21 was significantly reduced in the hypoxic HUVECs receiving LNA anti-miR-21. Concomitantly, TSP-1 mRNA and protein levels were higher in the anti–miR-21 group than that in the antiscramble group (Figures 2E and F). Inhibition of miR-21 suppressed capillary-like endothelial tube formation in the hypoxic HUVECs, compared with the hypoxic cells treated with antiscrambled oligonucleotides (Figure 2G).

The 3′-UTR of TSP-1 contained the binding site of miR-21 as described in the MicroCosm software. The luciferase reporter assays were performed to test the direct interaction between miR-21 and TSP 3′-UTR. The miR-21 mimic significantly reduced TSP-1 3′-UTR luciferase activity, compared with a control miRNA mimic (Figure 2H, left panel). The miR-21 mimic did not have effects on mutated 3′-UTRs of TSP-1 (Figure 2H, right panel).These data indicated that miR-21 might inhibit TSP-1 expression by binding the 3′-UTR of TSP-1.

Increased Expression of HIF-1α Attenuated Renal I/R Injury

As shown in Figure 3A, HIF-1α expression in total protein was upregulated significantly in the kidney of mice treated with $CoCl_2$. Elevation of Scr and severe renal morphological damage were presented at 24 hours after I/R in the NS + IR

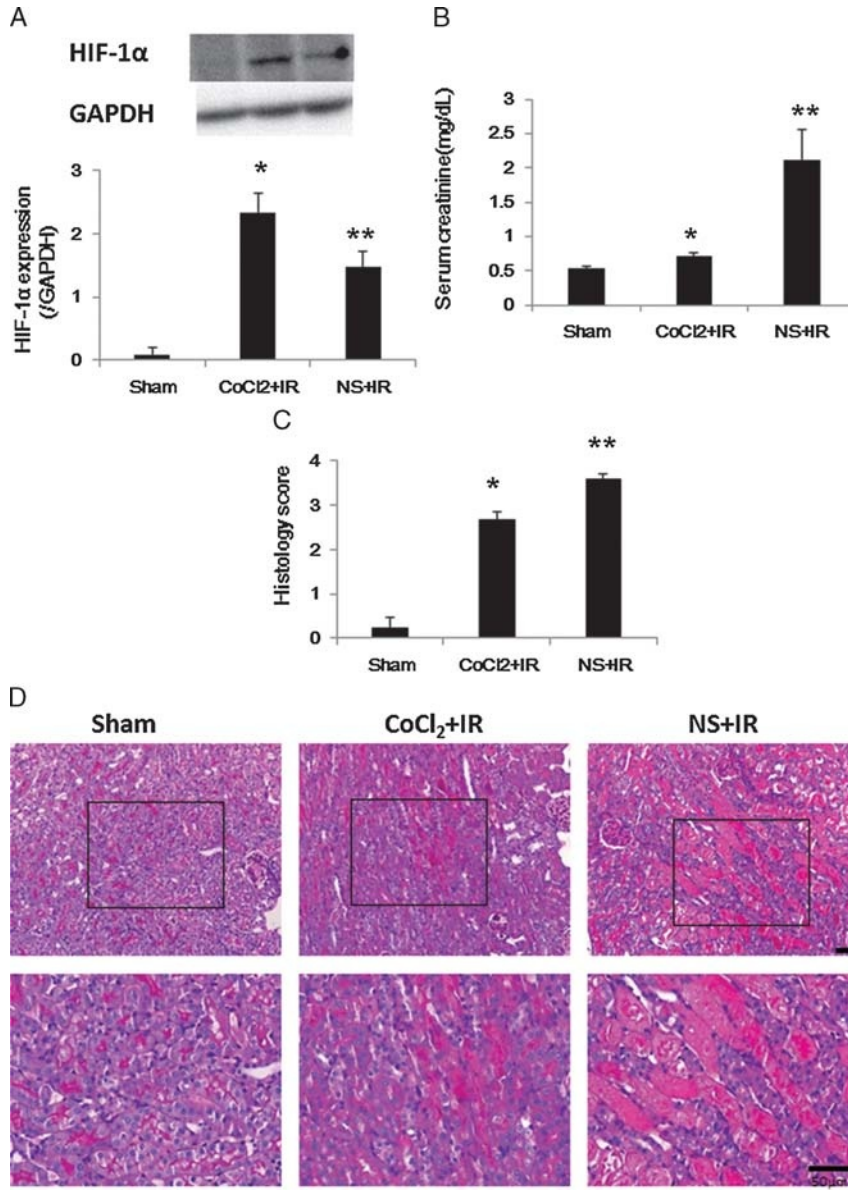

FIGURE 3. Increased expression of hypoxia inducible factor-1α (HIF-1α) attenuated renal ischemia/reperfusion (I/R) injury. A, In the kidneys of mice treated with $CoCl_2$, the total protein level of HIF-1α was upregulated significantly. $CoCl_2$ pretreatment protected mouse kidneys from I/R injury, shown in decreased serum creatinine concentration (B) and amelioration of renal acute tubulointerstitial injury score (C). D, Histopathological changes of mice kidney sections from each group. Representative periodic acid–Schiff (PAS)-stained micrographs in the corticomedullary junction (photographed at ×20 magnification). n = 6 per group. Bar = 50 μm. *$P < 0.05$ versus NS + I/R group, **$P < 0.01$ versus Sham + I/R group.

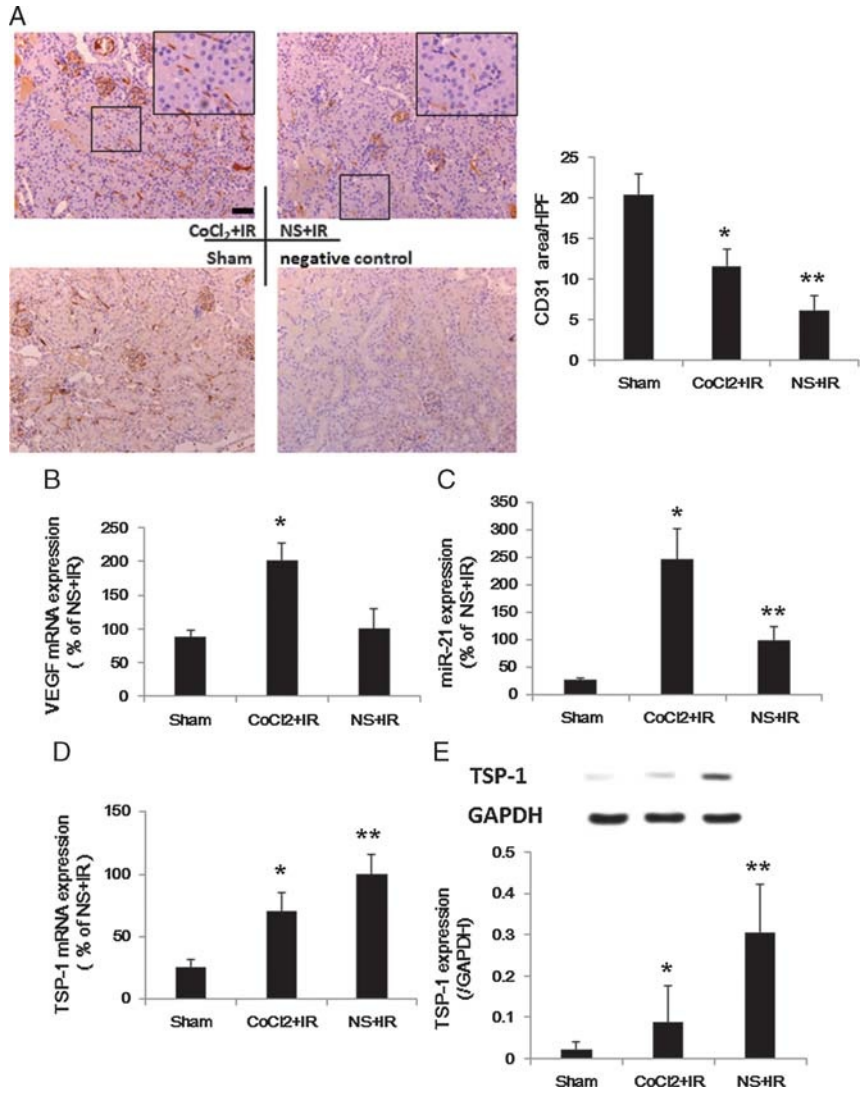

FIGURE 4. The renal protective effect of HIF-1α induced by $CoCl_2$ was associated with improved angiogenesis, upregulation of VEGF and miR-21, and downregulation of TSP-1. A, Immunohistochemical staining of CD31 in the renal sections and the CD31 positive area was analysis. Representative photomicrographs, ×10 magnification, with brown color indicating positive staining. Bar = 50 μm. VEGF mRNA (B) and miR-21 (C) expression was increased by the $CoCl_2$ treatment, companied with decreased expression of TSP-1 mRNA (D) and protein (E) in the mice I/R kidneys. n = 6 per group, *$P < 0.05$ versus NS + I/R group, **$P < 0.01$ versus Sham + I/R group.

mice, compared with the Sham mice. Pretreatment with $CoCl_2$ afforded striking renal functional improvement and marked amelioration of tubulointerstitial histological damage after the I/R injury (Figures 3B, C, and D).

The Renal Protective Effect of HIF-1α Induced by $CoCl_2$ Was Associated With Improved Angiogenesis, Upregulation of VEGF and miR-21, and Downregulation of TSP-1

Considering that increased HIF-1α expression led to significant functional and histological improvement in the I/R kidney, possible underlying mechanisms were examined. Microscopic views of renal peritubular capillary networks in each group are shown in Figure 4A. $CoCl_2$ treatment significantly increased the number of CD31-positive capillaries per HPF. Furthermore, the abundance of VEGF mRNA was significantly increased by $CoCl_2$ (Figure 4B), consistent with upregulated protein level of HIF-1α.

The increased expression of miR-21 in the kidney after I/R injury was similar with that reported before.[19] In addition, elevated abundance of miR-21 in the mice kidneys from $CoCl_2$ + IR group was more than that in the NS + IR mice (Figure 4C). As shown in Figures 4D and 4E, TSP-1 mRNA and protein expression were significantly downregulated in the $CoCl_2$ + IR mice. TSP-1 was not expressed in the kidneys from sham mice, consistent with the published result.[20]

Knockdown of miR-21 Attenuated Protection of HIF-1α Against I/R, Upregulated TSP-1 and Decreased Vascular Density in Mouse Kidneys

LNA-modified anti–miR-21 or antiscrambled oligonucleotides were administered to determine the functional role of miR-21 in the renal protection conferred by HIF-1α. As shown in Figure 5A, expression of renal miR-21 was significantly reduced in mice receiving LNA anti-miR-21. Knockdown of miR-21 attenuated HIF-1α protection conferred

by $CoCl_2$, which was shown as much higher Scr (Figure 5B) and more histological damage including tubular casts, moderate inflammatory infiltration, and cellular swelling (Figure 5C) in the $CoCl_2$ + IR mice with LNA anti–miR-21. Renal histology of $CoCl_2$ + IR mice with the scrambled anti-miR only showed less tubular casts and mild cellular swelling.

TSP-1 mRNA and protein expression were significantly upregulated by LNA anti–miR-21 treatment (Figures 5D and E). ISH and immunohistochemistry analyses also confirmed the inverse relationship between miR-21 and TSP-1 in the mice kidneys by inhibiting expression of miR-21 (Figure 5F). Importantly, LNA anti–miR-21 treatment significantly reduced the number of renal CD31-positive capillaries per HPF in the $CoCl_2$ + IR mice (Figure 5G). Immunohistochemical staining of CD31 and TSP-1 in serial sections suggested colocalization of positive staining of CD31 and TSP-1.

DISCUSSION

HIF-1α, one of the most important HIF subunits, is at the center of cellular hypoxia responses and could target a number of factors implicated in adaptive cytoprotection, such as VEGF, heme-oxygenase 1, and erythropoietin.[21] Optimal pretreatment, such as short-term ischemia (also known as ischemia preconditioning),[10] Xe,[22] or PHD inhibitor ($CoCl_2$),[23] could induce HIF-1α and related target genes for ischemic/hypoxic tolerance, which results in resistance against subsequent I/R injury. MiRNAs are critical in the maintenance of kidney homeostasis, and even in the incidence and progression of renal disease including posttransplanted kidney.[24-26] Current studies demonstrated that HIF-1α could also regulate miRNAs such as miR-210, miR-21, and miR-687[10,27,28] besides well-known downstream target genes. MiR-21 is an important multifunction miRNA and expresses in various cell types.[29] Our previous study was interested in the protective

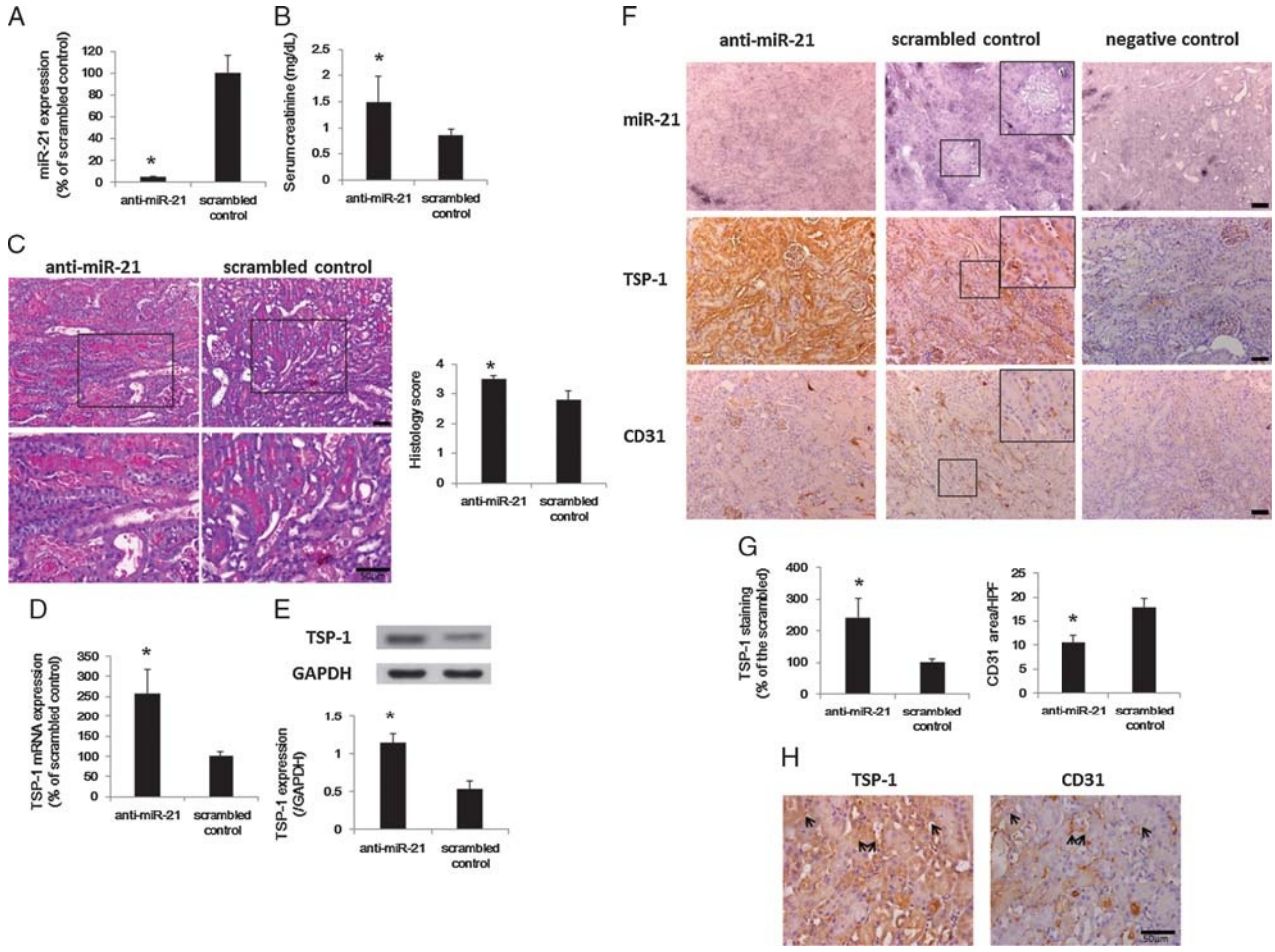

FIGURE 5. Knockdown of miR-21 attenuated HIF-1α protection against I/R, upregulated TSP-1, and decreased vascular density in mouse kidneys. A, LNA anti–miR-21, administered before $CoCl_2$ pretreatment inhibited miR-21 expression effectively in kidneys 24 hours after cobalt exposure. Knockdown of miR-21 in renal I/R mice exposed to $CoCl_2$ significantly increased serum creatinine (B) and worsened renal histological injury (C) 24 hours after I/R injury. Kidney sections were stained with periodic acid–Schiff and photographed at x20 magnification in the corticomedullary junction. D, E, TSP-1 real-time PCR and western blot showed that LNA anti-miR-21 increased TSP-1 mRNA and protein expression in mice kidneys 24 hours after I/R. F, As shown in representative images of miR-21 expression by ISH, TSP-1 and CD31 staining by immunohistochemistry in mouse kidney sections, increased miR-21 expression was significant in the vascular endothelial cells of anti–scrambled-treated mice besides in the renal tubular cells, accompanied with the decreased TSP-1 and CD31 positive staining. G, The semi-quantitative analysis shown anti-miR-21 treatment significantly restored renal TSP-1 abundance with reduced number of CD31-positive capillaries. H, TSP-1 expressed in the vascular endothelial cell which was marked by CD31 staining (the arrow). It was photographed at ×20 magnification with purple color indicating positive staining for miR-21 and brown color for TSP-1 and CD31.The arrow indicates vascular endothelial cells. n = 6 per group. Bar = 50 μm *$P < 0.05$ versus scrambled control group.

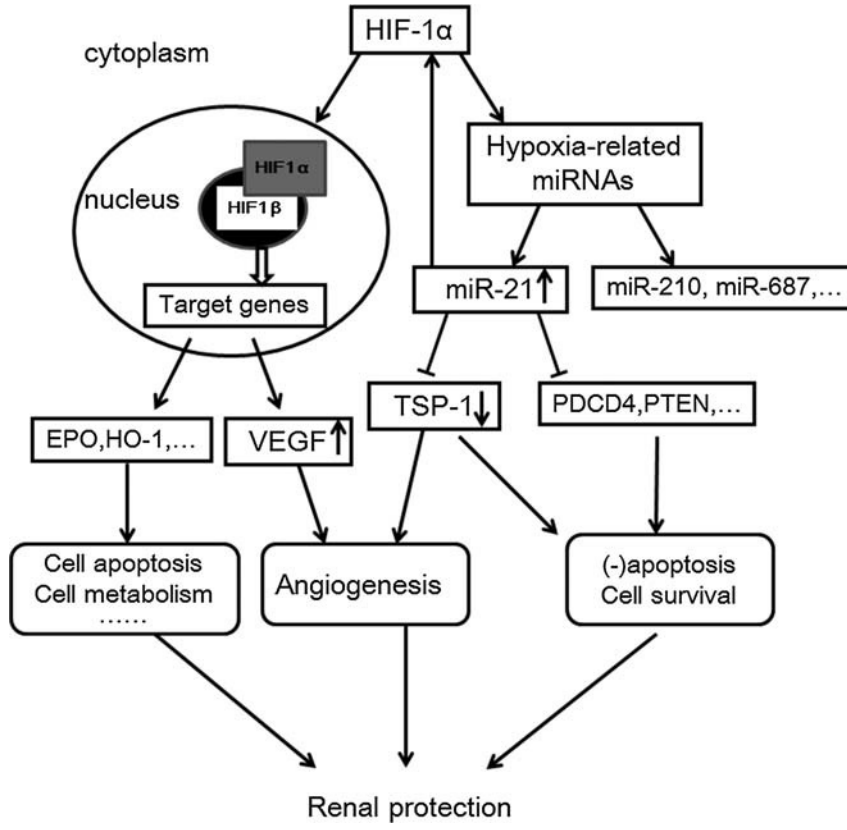

FIGURE 6. Schema of the pathways underlying renal protection conferred by HIF-1α. HIF-1α could upregulate expression of hypoxia-related miRNAs including miR-21. MiR-21 further increased expression of HIF-1α in a positive feedback loop, reinforcing the protective effect of HIF-1α. HIF-1α induced angiogenesis by directly increasing VEGF or indirectly inhibiting antiangiogenesis target TSP-1 of miR-21. Other HIF-1α downstream genes, such as heme-oxygenase 1, erythropoietin, and other miR-21 target genes including programmed cell death protein 4 and PTEN may also contribute to renal protection. Vertical arrows: "↓" means decrease, "↑" means increase.

effect of miR-21 on tubular epithelial cell after renal I/R injury.[10,22] High level of miR-21 was also found in the injured vascular endothelial cells.[11,30,31] In this study, miR-21 was elevated by HIF-1α activation in HUVEC after hypoxia and in the microvascular endothelial cell after renal I/R injury. We found that the protective effect of HIF-1α was abolished by knockdown of miR-21, which suggested that HIF-1α might contribute to renal protection against ischemic kidney failure by induction of miR-21.

It has been suggested that abnormal expression of miRNAs has an important impact on angiogenesis since key enzymes in the biogenesis of miRNAs Dicer and Drosha could affect generation and migration of endothelial cells and regulated angiogenesis.[32,33] Furthermore, it was found that miR-21 was involved in responses of endothelial cell to shear stress or hypoxia, which were related with vascular remodeling.[34] In a study by Ji et al,[11] knockdown of miR-21 decreased neointimal hyperplasia after angioplasty, suggesting a proangiogenic role of miR-21. However, the mechanism of miR-21 in angiogenesis is not well understood. Liu et al[35] found that miR-21 could enhance HIF-1α and VEGF expression and induced tumor angiogenesis by targeting Phosphatase and tensin homolog (PTEN) and resulting activation of Akt and ERK1/2 signaling pathways, even though HIF-1α may also regulate miR-21 expression under hypoxic condition. In our study, the novel findings showed that miR-21 targeted antiangiogenesis gene TSP-1 and proangiogenesis function of miR-21 could be achieved by suppressing TSP-1. Whereas miR-21 has also been reported to inhibit angiogenesis by targeting RhoB[31] in endothelial cells and transforming growth factor β receptor II in human adipose-derived stem cells.[36] The role of miR-21 in the angiogenesis may depend on not only the timing and context of injury but also the cell types.

TSP-1 is a multifunctional matricellular glycoprotein. It is reported that TSP-1 could inhibit angiogenesis by acting through its receptors on the endothelial cells or indirectly affecting expression and activation of some growth factors.[37] In addition, TSP-1 was reported expressed in various cell types with functions of profibrosis, proinflammatory,[14,38] and proapoptosis[20] during renal diseases besides antiangiogenic effect. Thakar et al[20] found that expression of TSP-1 predominantly in the renal proximal tubules peaked at 3 hours and declined gradually after renal I/R injury, which was conversely related with the renal expression trend of miR-21 in the Godwin et al study.[19] Because TSP-1 was consistent with renal tubules damage after I/R injury, other contributive roles of miR-21 on TSP-1 remain to be investigated. In addition, it is interesting that Stein et al[39] indicated an active role for miR-21 in TSP1- ERK1/2 cell signaling cascade based on the finding that treatment with hyperglycemia and TSP-1 could stimulate the expression of miR-21. They could not confirm however the mechanism by which TSP-1 regulated the expression of miR-21.

However, there are some limitations in this study. We did evaluate the effects of HIF-1α on the angiogenesis, but did not assess the role of HIF-2α because HIF-2α might also

contribute to angiogenesis and be important to protection against renal injury.[9] Another limitation of the study is that the measurements were assessed in 1 time point 24 hours after the reperfusion with $CoCl_2$ pretreatment, and it would be better to measure the time course of Scr levels up to 48 hours or more. Our data demonstrated that HIF-1α protected kidney against I/R-induced injury by proangiogenesis via VEGF and miR-21 target pathways, but it did not mean that there were no other pathways involved.

In summary, although the protective function of HIF-1α against I/R injury has been extensively studied in various organs,[23,40–42] angiogenesis induced by HIF-1α has only been examined in a small number of studies. The majority of these studies focused on well-known angiogenic growth factor VEGF. Our study indicated that angiogenesis of renal microvascular induced by HIF-1α represented one of the protective mechanisms against renal I/R injury, depending on upregulation of miR-21 and resulting inhibition of TSP-1 besides targeting VEGF (Figure 6).

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