miR-29c is downregulated in renal interstitial fibrosis in humans and rats and restored by HIF-α activation

Yi Fang,1 Xiaofang Yu,1 Yong Liu,2 Alison J. Kriebel,2 Yanyan Heng,1 Xialian Xu,1 Mingyu Liang,2 and Xiaoliang Ding1

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Fang Y, Yu X, Liu Y, Kriebel AJ, Heng Y, Xu X, Liang M, Ding X. miR-29c is downregulated in renal interstitial fibrosis in humans and rats and restored by HIF-α activation. Am J Physiol Renal Physiol 304:F1274–F1282, 2013. First published March 6, 2013; doi:10.1152/ajprenal.00287.2012.—Treatment with L-mimosine, which activates hypoxia-inducible factor-α (HIF-α), attenuates renal tubulointerstitial injury and improves renal function in a rat remnant kidney model. The miR-29 family of microRNAs directly targets a large number of extracellular matrix genes and reduces renal interstitial fibrosis. We analyzed microRNA expression profiles in rat remnant kidneys with or without treatment with L-mimosine. The expression of miR-29c was downregulated in rat remnant kidneys compared with sham control and significantly restored by the l-mimosine treatment. In cultured human kidney epithelial HK2 cells, cobalt chloride activated HIF-α and upregulated miR-29c expression. The upregulation of miR-29c expression was significantly attenuated by knockdown of HIF-1α or HIF-2α. Downregulation of miR-29c was associated with significant increases in interstitial fibrosis, collagen type II α1 (COL2A1) protein, and tropomyosin 1α (TPM1) protein in rat remnant kidneys and in kidneys from IgA nephropathy patients. The increases in rat remnant kidneys were attenuated by the l-mimosine treatment. COL2A1 and TPM1 were confirmed to be new, direct targets of miR-29c. In conclusion, miR-29c, an antifibrotic microRNA, is upregulated by HIF-α activation. MiR-29c is downregulated in renal interstitial fibrosis in humans and rats and restored by activation of HIF-α that attenuates fibrosis.

HIF; miR-29c; tubulointerstitial fibrosis; tropomyosin 1α; collagen type II α1

TUBULINTERSTITIAL FIBROSIS (TIF) is the common pathway in the progression of chronic kidney disease (CKD), leading to functional deterioration and eventual loss of renal function, irrespective of the nature of the initial renal injury (5, 10, 19, 36). Tubulointerstitial ischemia or hypoxia has been proposed as a pivotal microenvironmental factor in the development of renal fibrosis (47). The molecular mechanisms mediating responses to hypoxia are complex, with hypoxia-inducible factor (HIF) playing a key role (32). Regulation of HIF occurs mainly through oxygen-dependent destruction of its α-subunit. In the presence of oxygen, two α-prolyl residues undergo enzymatic hydroxylation, which is required for its proteasomal degradation. Pharmacological activation of HIF-α could be achieved in an oxygen-independent manner by using prolyl 4-hydroxylase (PHD) inhibitors such as l-mimosine (l-Mim) (23).

MicroRNAs (miRNAs) are small regulatory RNA molecules encoded by specific genes in plant and animal genomes (8, 9, 24, 29, 33). Typically, miRNAs bind to the 3′-untranslated region (UTR) of target miRNAs and decrease the abundance of target proteins. The effect may be mediated by translational repression and/or decreases in mRNA abundance. Several miRNAs have been shown to be involved in the development of kidney disease, including renal interstitial fibrosis (2, 20, 28). HIF plays an important role in regulating the expression of a subset of hypoxia-related microRNAs (HRM), providing additional links between HIF and gene expression control. However, it is not known whether HIF regulation of miRNAs is involved in kidney disease, including renal interstitial fibrosis.

We have shown in previous studies that pimonidazole, which serves as an indicator of hypoxic cells, was detected in remnant kidneys soon after renal mass reduction, from the outer medulla to the cortex. The addition value reached the peak at weeks 4 and 6 and decreased thereafter. Simultaneously, there was transient HIF-α activation in the remnant kidney of rats at the early stage following subtotal nephrectomy, which also peaked at weeks 4 and 6. L-Mim administered in later stages reactivated HIF-α and ameliorated the progression of CKD and renal interstitial fibrosis (56, 57). In the present study, we found the abundance of a miRNA, miR-29c, which is known to suppress the expression of extracellular matrix genes (22), was decreased in the rat remnant kidney model as well as in human kidneys with TIF from IgA nephropathy patients. In vivo and in vitro experiments demonstrated a novel role of HIF-α in upregulating miR-29c expression.

MATERIALS AND METHODS

Experimental animal protocol. All experiments were performed in male Sprague-Dawley rats, weighing 180–200 g, obtained from the Animal Centre, Shanghai Medical College, Fudan University (Shanghai, China). Rats were allowed free access to water and rodent food. All protocols were approved by the Institutional Animal Care and Use Committee of Fudan University.

Approximately two-thirds of the left kidney were removed by abdominal resection of the upper and lower thirds of the kidney. One week later, a right nephrectomy was performed through a transumbilical route. All the surgical procedures were carried out under general anesthesia (pentobarbital sodium, 40 mg/kg ip). Four weeks after 5⁄6 nephrectomy, rats were treated with intraperitoneal injections of vehicle or L-Mim (Calbiochem), an inhibitor of PHD activity (12, 17), at a dosage of 50 mg/kg every other day. Rats without kidney resection served as the sham-operated group. At the end of week 12 after 5⁄6 nephrectomy, all rats (n = 8/group) were euthanized and blood samples were collected via cardiac puncture. Renal tissue was harvested, one piece of which was fixed in neutral formalin and then embedded in paraffin. The remaining kidney tissue was dissected in ice-cold PBS to remove the medulla and then snap-frozen in liquid nitrogen before transferance to storage at −80°C until further analysis.
Cell culture and transforming growth factor-β1 treatment. HK-2, a human kidney epithelial cell line, was obtained from and cultured as suggested by American Type Culture Collection (48, 49). Cells at ~40% confluence were treated with recombinant human transforming growth factor (TGF)-β1 (3 ng/ml, R&D Systems) or the vehicle control in DMEM for the indicated time period.

Patient selection and renal morphological analyses. We studied 10 patients with primary IgA nephropathy confirmed by kidney biopsy in Zhongshan Hospital, Fudan University, between 2009 and 2010, five with no TIF and five with moderate to severe TIF. Analysis of renal fibrosis was performed with 2-μm paraffin-embedded sections stained by periodic acid-Schiff (PAS) or Masson trichrome. The degree of renal fibrosis was scored by an experienced pathologist. The degree of glomerulosclerosis was represented by the percentage of sclerotic glomeruli in total glomeruli obtained from biopsy (42). For TIF, 10 microscopic fields were viewed at a magnification of ×200 and scored subjectively from 0 to 100% for each patient. The degree of TIF was graded on a scale of 0–4: grade 0, affected area 0% (normal); grade 1, affected area <10%; grade 2, affected area 10–25%; grade 3, affected area 25–75%; grade 4, affected area >75% (30). The resulting index in each slide was expressed as a mean of all scores obtained. The same method was applied in the analysis of rat renal pathology. The study was approved by the Clinical Research Ethical Committee of the Zhongshan Hospital, Fudan University. All patients provided informed consent. Clinical data including blood pressure, serum creatinine, and 24-h urine protein were recorded at the time of kidney biopsy.

RNA isolation. Total RNA was extracted using TRIzol (Invitrogen) from cells or tissue sections as previously described (3, 15, 21, 27, 48–50). Total RNA from formalin-fixed and paraffin-embedded (FFPE) tissue was extracted by using a RecoverALL total nucleic acid isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol.

For microRNA microarray experiments, total RNA was extracted from the tissue samples using a mirVana miRNA isolation kit (Applied Biosystems, Foster City, CA). The concentration was quantified by a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA). The quality check of RNA was performed using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA). Total RNA from formalin-fixed and paraffin-embedded (FFPE) tissue was extracted by using a RecoverALL total nucleic acid isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol.

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Table 1. 3′-UTR reporter constructs of TPM1 and COL2A1

<table>
<thead>
<tr>
<th>Target</th>
<th>miR-29c Target Site</th>
<th>P Value vs. Control</th>
<th>3-UTR Segment</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| TPM1   | 371–377             | 0.028             | -14 to 760    | Forward: 5-atata actagt taacacaacagtgtgaaacccctcct-3  
          |                     |                   |               | Reverse: 5-atcact aggtt tgtctcataagtgacctttttt-3 |
| COL2A1 | 348–355             | 0.014             | 1 to 700      | Forward: 5-taata actagt gcgaatagctgaagctacagc-3  
          |                     |                   |               | Reverse: 5-taata actagt gcgaatagctgaagctacagc-3  
          |                     |                   |               | Forward: 5-taata actagt gcgaatagctgaagctacagc-3  
          |                     |                   |               | Reverse: 5-taata actagt gcgaatagctgaagctacagc-3  

The position numbers are relative to the first nucleotide in the 3′-untranslated region (UTR) or the first nucleotide after TAA/TGA stop codon. TPM1, tropomyosin; COL2A1, collagen type II α1; miR, microRNA.
bled control were designed and synthesized by GenePharma (A01010, GenePharma, Shanghai, China). siRNAs were trans- 
ferred using DharmaFECT 1 (Dharmacon). In brief, cells were 
cultured in a six-well plate to 50% confluence. For each well, 100 μ 
siRNAs (2 μM) was added into 100 μl Opti-MEM medium (GIBCO 
BRL), 2 μl DharmaFECT 1 into 198 μl Opti-MEM medium, and then 
mixed siRNA with DharmaFECT 1 after a 5-min incubation. After 20 
min, the culture medium was removed from the wells of the six-well 
plate and the transfection mixture (400 μl) and another 1,600 μl 
Opti-MEM were added to each well, with the target 100 nM siRNAs. 
Total RNA and protein were prepared 24 or 48 h after transfection 
and were used for qRT-PCR or Western blot analysis.

Pre-miR oligonucleotides. Pre-miR oligonucleotides were obtained 
from Ambion. HK2 cells were transfected with pre-miR (100 nM) 
using Oligofectamine (Invitrogen).

Statistical analysis. Data were analyzed using Student’s t-test and 
multiple-group analysis of variance, except the microarray experiment 
described above. P < 0.05 was considered significant. Data are shown 
as means ± SD.

RESULTS

miRNA profiles in the renal cortex of 5/6 nephrectomized rats 
treated with L-Mim or vehicle. In our previous studies, we 
found that chronic renal damages developed progressively in 
rats with 5/6 nephrectomy and that L-Mim intervention from 
week 5 to week 12 resulted in additional accumulation of HIF-1α 
and -2α at week 12 and improved renal function (56). To explore the role of microRNAs in this renoprotective 
phenomenon, we compared microRNA expression profiles in 
rats with 5/6 nephrectomy treated with L-Mim or vehicle (GEO 
accession no. GSE28471). The abundance of 350 miRNAs in 
the remnant renal cortex was examined with miRNA microar-
rays in both groups. Nine miRNAs were considered differen-
tially expressed between the L-Mim-treated and the vehicle-
treated remnant kidneys 12 wk after surgery. Rno-miR-130b, 
rno-miR-450a, mo-872, rno-miR-24, and rno-miR-25 were 
expressed at lower levels in the L-Mim-treated group, while 
rno-miR-29c, rno-miR-29c*, rno-miR-22*, and miR-345–5p 
were higher in the L-Mim-treated group (Fig. 1A).

Taqman real-time PCR analysis in RNA samples independent 
of those used in the microarray experiment indicated that 
mir-29c was significantly downregulated in the remnant kid-
ney and significantly restored by the L-Mim treatment, the 
latter confirming the finding in the miRNA microarray exper-
iment (Fig. 1B). mir-29a and mir-29b, two other members of the 
miR-29 family, also tended to be downregulated in the 
remnant kidney and restored by L-Mim, but the differences did 
not reach statistical significance (Fig. 1B). Moreover, the 
L-Mim treatment resulted in significant upregulation of the 
primary transcript pri-miR-29b2/c (Fig. 1C).

The nuclear expression of HIF-α was upregulated in the 
early stage after 5/6 nephrectomy when renal function was 
stable and returned to the basal level later, accompanied by 
impaired renal function and interstitial fibrosis (56). During the 
same progression time course in the remnant kidney model, 
abundance of miR-29c was decreased at week 2 and gradually 
restored from week 4 to week 6, coinciding with the peak of 
HIF-α upregulation. miR-29c abundance remained lower than the 
baseline level from week 8 to week 12, even though it did not 
decline again as HIF-α did (Fig. 1D).

Activation of HIF-α upregulated miR-29c expression. We 
examined in cultured human kidney epithelial HK2 cells 
whether miR-29c was regulated by HIF-α. Treatment of HK2 
cells with CoCl2, a potent stimulator of HIF-α, significantly 
upregulated miR-29c (Fig. 2A). The upregulation of miR-29c 
was significantly attenuated when the cells were transfected with 
siRNA targeting HIF-1α or HIF-2α compared with negative 
control siRNA (Fig. 2A). mRNA and protein levels of HIF-1α and
miR-29c expression was inversely correlated with renal interstitial fibrosis in the rat remnant kidney and in humans. The miR-29 family has been shown to suppress the expression of a large number of collagens and genes related to the extra-

Fig. 2. Activation of HIF-α upregulated miR-29c. HK2 cells were preincubated with CoCl₂ (200 µM; negative control group); HIF-1α small interfering (si) RNA group; HIF-2α siRNA group; and CoCl₂ alone group) or vehicle (vehicle control group) for 24 h before siRNA treatment. Samples were collected 24 h (for RT-PCR) or 48 h (for Western blotting) after siRNA treatment. siRNA sequences against HIF-1α, HIF-2α, and HIF-α scrambled control (negative control) were designed and synthesized by GenePharma (Shanghai, China) and used at a final concentration of 100 nM. A: miR-29c was upregulated by CoCl₂, and the upregulation was blunted by siRNA targeting HIF-1α or HIF-2α (n = 6). B: mRNA levels of HIF-1α and HIF-2α were increased by CoCl₂ and knocked down by respective siRNAs (n = 3). C: protein levels of HIF-1α and HIF-2α were increased by CoCl₂ and knocked down by respective siRNAs. D: quantitation of Western blotting results (n = 3). E: relative abundance of pri-miR-29b2/c in HK2 cells was upregulated 24 h after CoCl₂ treatment (200 µM; n = 6). Negative control group: pretreated with CoCl₂ and the nonspecific siRNA; HIF-1α siRNA group: pretreated with CoCl₂ and the HIF-1α siRNA; HIF-2α siRNA group: pretreated with CoCl₂ and the HIF-2α siRNA; CoCl₂ alone group: pretreated with CoCl₂, no siRNA; vehicle control group: pretreated with saline and nonspecific siRNA. *P < 0.05 vs. negative control. #P < 0.01 vs. negative control. ΔP < 0.01 vs. CoCl₂ alone. ▲P < 0.05 vs. CoCl₂ alone. §P < 0.01 vs. vehicle control. □P < 0.05 vs. HIF-2α siRNA.

HIF-2α, which were increased substantially by the CoCl₂ treatment, were significantly and substantially reduced by corresponding siRNA (Fig. 2, B–D). In addition, pretreating cells with CoCl₂ upregulated the abundance of pri-miR-29b2/c (Fig. 2E).

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Fig. 3. Interstitial fibrosis in rat remnant kidneys was attenuated by i-Mim. The relative abundance of collagens was analyzed using Masson’s trichrome staining. The positive-stained area of collagens was quantitatively measured using a computer-aided image system (IMS; ShentengIT, Shanghai, China) on digitized images that were transformed from analog images taken by a video camera (Panasonic, MV-CP410). Each field was 72,800 µm². Magnification ×200. A: sham-operated rats. B: remnant kidneys from i-Mim-treated rats. C: remnant kidneys from vehicle-treated rats. D: summary of the quantitation (n = 5). *P < 0.01 vs. A. ΔP < 0.01 vs. B.
Table 2. Demographic and baseline clinical data of IgA nephropathy patients

<table>
<thead>
<tr>
<th>Patients with Grade 3-4 TIF (n = 5)</th>
<th>Patients with No TIF (n = 5)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M:F</td>
<td>3:2</td>
<td>2:3</td>
</tr>
<tr>
<td>Age, yr</td>
<td>34.12 ± 5.87</td>
<td>38.74 ± 8.45</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>1.54 ± 0.88</td>
<td>2.31 ± 1.96</td>
</tr>
<tr>
<td>Serum creatinine, µmol/l</td>
<td>253.27 ± 78.24</td>
<td>89.4 ± 24.86</td>
</tr>
<tr>
<td>Estimated GFR, ml·min⁻¹·1.73⁻²</td>
<td>38.61 ± 19.35</td>
<td>78.41 ± 25.32</td>
</tr>
</tbody>
</table>

Values are means ± SD. GFR, glomerular filtration rate; NS, not significant.

Patients with primary IgA nephropathy were confirmed by kidney biopsy. Five had no significant tubulointerstitial fibrosis (TIF), and the other five had moderate to severe TIF. Analysis of renal fibrosis was determined in 2-µm paraffin-embedded sections stained by periodic acid-Schiff or Masson trichrome.

cellular matrix (ECM) in several diseases (22), including renal interstitial fibrosis (27, 39). Substantial renal interstitial fibrosis developed in the rat remnant kidney, in which miR-29c was suppressed (Fig. 3). The L-Mim treatment significantly attenuated interstitial fibrosis in the remnant kidney, concomitant with restoration of miR-29c expression (Fig. 3).

The inverse relationship between miR-29c expression and renal interstitial fibrosis also exists in patients. A group of IgA nephropathy patients was selected to include cases with minimal or substantial renal interstitial fibrosis (Table 2 and Fig. 4, A and C). RNA samples were extracted from paraffin blocks of the patients’ biopsies. Real-time PCR analysis showed that miR-29c abundance was significantly lower in IgA nephropathy patients with substantial interstitial fibrosis (Fig. 4E). HIF-1α protein was clearly present in both groups (IgAN with TIF and IgAN without TIF), and nuclear staining of HIF-1α was comparatively weak or negative in most proximal tubules and atrophied distal tubules. The staining was upregulated in the dilated tubules or tubules surrounded by inflammatory cells, suggesting a potential hypoxia stimulus existing in the involved live tubules. The comparative abundance of positive HIF-1α staining was higher in the group of IgAN without TIF compared with IgAN with TIF (Fig. 4, B and D, Table 2), which might be attributed to the decreased number of tubules in the TIF area.

Identification of TPM1 and COL2A1 as new targets of miR-29c. Identification of target genes is the most critical step in understanding the mechanism mediating the effect of a miRNA. miR-29c is known to target and suppress several extracellular matrix genes (22). We set out to identify additional targets of miR-29c. TPM1 and COL2A1 were predicted targets of miR-29c based on TargetScan v5.1 (http://www.targetscan.org/) and MicroCosm Targets v5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), but were not yet confirmed. Western blot analysis of TPM1 showed that the renal cortical protein abundance of TPM1 was substantially upregulated in the rat remnant kidney and significantly attenuated by the L-Mim treatment (Fig. 5A). Immunohistochemical analyses showed that TPM1 staining was significantly higher in the kidneys of IgA nephropathy patients with substantial interstitial fibrosis (Fig. 5B). COL2A1-positive areas were significantly increased in the rat remnant kidney or in the kidneys of IgA nephropathy patients with substantial interstitial fibrosis (Fig. 6). Staining of COL2A1 was diffuse. We only chose renal interstitial regions for comparison, and the stained regions were thresholded using the computer-aided image system (IMS; ShentengIT, Shanghai, China). The L-Mim treatment reduced COL2A1-positive areas in the rat remnant kidney (Fig. 6). These changes in TPM1 and COL2A1 expression were inversely correlated with miR-29c expression as described above.

3′-UTR reporter analysis was performed to directly examine the 3′-UTR-mediated interaction between miR-29c and TPM1.
and COL2A1 (Table 1). The miR-29c mimic, compared with a control miRNA mimic, significantly reduced COL2A1 and TPM1 3’-UTR activity by 39.5 and 31.5%, respectively. The miR-29c mimic did not have significant effects on mutated TPM1 3’-UTR activity by 39.5 and 31.5%, respectively. The control miRNA mimic, significantly reduced COL2A1 and TPM1 (−17.9 and −19.6%, respectively) (Fig. 7).

miR-29c suppressed TPM1 expression in cultured HK2 cells treated with TGF-β1. We performed additional studies on TPM1 because TPM1 is known to be involved in stress fiber function, and in our previous study in HK2 cells protein expression of TPM1 was found to be upregulated in a time-dependent manner after TGF-β1 treatment (21). TGF-β1 significantly reduced miR-29c expression in HK2 cells (Fig. 8A), which was restored by pretreatment with CoCl2 (200 µM) (Fig. 8B). Real-time RT-PCR analysis indicated that TPM1 mRNA levels were not altered by the transfection of the miR-29c mimic. However, Western blot analysis showed that the miR-29c mimic, compared with control pre-miR, significantly reduced protein levels of TPM1 by 19.6% (n = 5, P < 0.05) (Fig. 8C).

**DISCUSSION**

In the present study, we identified a novel role of HIF-α in upregulating miR-29c expression. We found that the expression of miR-29c was downregulated in renal interstitial fibrosis in rats and humans and restored by HIF-α activation. Moreover, we identified TPM1 and COL2A1 as new target genes for miR-29c.

The experimental model of 5⁄6 nephrectomy or the remnant kidney model represents one of the most widely used animal models of progressive renal failure by reduced nephron number best characterized in rats (55). In our previous studies, we found that activation of HIF-α was dynamic during the disease progression in the remnant kidney model of CKD, which was activated transiently in the early stage and suppressed in the later stage; inhibition of PHDs using 1-Mim activated HIF-α and attenuated the progression of CKD in the 5⁄6 nephrectomy model in a time-dependent manner (56). The renoprotective effect of the 1-Mim treatment might be attributed to selective upregulation of renoprotective HIF target genes and/or down-regulation of injurious genes at appropriate time points during disease progression. The number of reported HIF target genes is >100. The mechanism underlying “selective” regulation of renoprotective or injurious genes is not clear (11, 14, 18, 32, 41, 47). Since selectively activation of HIF-α isoforms might partially contribute to the complex regulation of HIF-targeted genes (2), hypoxia-regulated microRNAs appeared particularly
suitable to serve as hubs of regulatory networks underlying these complex processes (25, 26, 53). The result of the present study suggests that downregulation of miR-29c could be an important part of the molecular mechanism underlying the development of TIF and CKD in humans and rats and that partial restoration of miR-29c might contribute to 1-Mim-induced attenuation of CKD progression in the 5⁄6 nephrectomy model. This notion is supported by in vivo studies showing that knockdown of a miR-29 family member exacerbated, while overexpression attenuated, renal interstitial fibrosis in rats or mice (27, 39). It is clearly possible that miR-29c can be regulated by multiple factors and pathways especially in vivo. In this study, the correlation between HIF-α and miR-29c in 1-Mim-treated rats justified our focus on the HIF-α pathway. HIF-1α acts by binding to HIF-responsive elements (HREs) in promoters that contain the sequence NCGTG (4, 22). As shown in Supplemental Fig. 1 (all supplemental material for this article is accessible on the journal website.), in the 2.3-kb promoter region for the rat miR-29b/c gene cluster, there are five NCGTGs, and two of them are very close to the transcription start site (TSS). It would be interesting to further investigate these HIF-responsive elements in future studies.

A renoprotective effect of miR-29c would be consistent with the well-established effect of the miR-29 family in targeting extracellular matrix genes (22). Members of the miR-29 family have been shown to target extracellular matrix genes in the rat kidney, hepatic stellate cells, cardiomyocytes, nasopharyngeal carcinomas, and other tissues (27, 31, 40, 46, 51, 54). These studies support a key role of the miR-29 family in the control of tissue fibrosis. Targeting of extracellular matrix (ECM) genes by miR-29 is indeed one of the most dramatic and compelling examples of a single microRNA family targeting a large group of functionally related genes (22, 25). Since only miR-29c reached statistical significance in both our microarray data and real-time PCR results, we focused on miR-29c in this paper. However, the effect of the PHD inhibitor can certainly involve mechanisms other than the miR-29 family.

Besides ECM genes, TPM1 was identified as a new target gene of miR-29c. Tropomyosins are actin-stabilizing proteins consisting of 2 α-helical chains arranged as a coiled-coil that bind along the actin filaments and protect them from the binding of actin-destabilizing proteins (38, 44, 46). Four genes encode multiple isoforms of tropomyosin in a tissue-specific manner with at least 20 different isoforms expressed widely in vertebrates. Fibroblasts and epithelial cells express high-mo-

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**Fig. 7. MiR-29c interacted with the 3′-untranslated region (UTR) of TPM1 and COL2A1.** HeLa cells at 80–90% confluency were cotransfected with a firefly luciferase reporter construct containing the 3′-UTR of TPM1 or COL2A1, a pRL-TK internal control plasmid, and control pre-miR oligonucleotides or the miR-29c mimic. In additional experiments, mutations were introduced into the miR-29c seed region binding site within the 3′-UTR of TPM1 and COL2A1 in the reporter construct. The miR-29c mimic decreased luciferase activity when the reporter gene was linked to a 3′-UTR segment of COL2A1 (A) or TPM1 (B). The effect of miR-29c mimics was not significant when the binding site was mutated. *P < 0.01 vs. pre-miR negative control. **P < 0.05 vs. pre-miR negative control; n = 3.

**Fig. 8. Downregulation of miR-29c contributes to transforming growth factor (TGF-β1-induced upregulation of TPM1 in HK2 cells.** A: TGF-β1 downregulated miR-29c. HK2 cells were treated with TGF-β1 (3 ng/ml) or vehicle for 24 h. B: CoCl2 restored miR-29c abundance that was suppressed by TGF-β1 treatment. HK2 cells were preincubated with or without CoCl2 (200 μM) for 24 h before TGF-β1 (3 ng/ml) treatment; n = 6. *P < 0.05 vs. TGF-β1 alone. C: miR-29c mimics reduced TPM1 protein levels in HK2 cells treated with TGF-β1. The density of the TM band was normalized by Coomassie blue staining of the entire lane; n = 5. *P < 0.05 vs. vehicle-treated. **P < 0.05 vs. pre-miR negative control.

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**Fig. 8. Downregulation of miR-29c contributes to transforming growth factor (TGF-β1-induced upregulation of TPM1 in HK2 cells.** A: TGF-β1 downregulated miR-29c. HK2 cells were treated with TGF-β1 (3 ng/ml) or vehicle for 24 h. B: CoCl2 restored miR-29c abundance that was suppressed by TGF-β1 treatment. HK2 cells were preincubated with or without CoCl2 (200 μM) for 24 h before TGF-β1 (3 ng/ml) treatment; n = 6. *P < 0.05 vs. TGF-β1 alone. C: miR-29c mimics reduced TPM1 protein levels in HK2 cells treated with TGF-β1. The density of the TM band was normalized by Coomassie blue staining of the entire lane; n = 5. *P < 0.05 vs. vehicle-treated. **P < 0.05 vs. pre-miR negative control.
REFERENCES


