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## Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes

Yong Liu<sup>1</sup>, Norman E. Taylor<sup>1</sup>, Limin Lu<sup>1,2</sup>, Kristie Usa<sup>1</sup>, Allen W. Cowley Jr.<sup>1</sup>, Nicholas R. Ferreri<sup>3</sup>, Nan Cher Yeo<sup>1</sup>, and Mingyu Liang<sup>1</sup>

<sup>1</sup>Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

<sup>2</sup>Department of Physiology and Pathophysiology, Fudan University Shanghai Medical College, Shanghai, P.R. China

<sup>3</sup>Department of Pharmacology, New York Medical College, Valhalla, New York 10595

### Abstract

MicroRNAs are endogenous repressors of gene expression. We examined microRNAs in the renal medulla of Dahl salt-sensitive rats and consomic SS-13<sup>BN</sup> rats. Salt-induced hypertension and renal injury in Dahl salt-sensitive rats, particularly medullary interstitial fibrosis, had been previously shown to be substantially attenuated in SS-13<sup>BN</sup> rats. Of 377 microRNAs examined, 5 were found differentially expressed between Dahl salt-sensitive rats and consomic SS-13<sup>BN</sup> rats receiving a high-salt diet. Real-time PCR analysis demonstrated that high-salt diets induced substantial up-regulation of miR-29b in the renal medulla of SS-13<sup>BN</sup> rats, but not in SS rats. miR-29b was predicted to regulate 20 collagen genes, matrix metalloproteinase-2 *Mmp2*, integrin  $\beta$ 1 *Itgb1*, and other genes related to extracellular matrix. Expression of 9 collagen genes and *Mmp2* was up-regulated by a high-salt diet in the renal medulla of SS rats, but not in SS-13<sup>BN</sup> rats, an expression pattern opposite to miR-29b. Knockdown of miR-29b in the kidneys of SS-13<sup>BN</sup> rats resulted in up-regulation of several collagen genes. miR-29b reduced expression levels of several collagen genes and *Itgb1* in cultured rat renal medullary epithelial cells. Moreover, miR-29b suppressed the activity of luciferase when the reporter gene was linked to a 3'-untranslated segment of collagen genes *Coll1a1*, *Col3a1*, *Col4a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col7a1*, *Col8a1*, *Mmp2*, or *Itgb1*, but not *Coll12a1*. The result demonstrated broad effects of miR-29b on a large number of collagens and genes related to extracellular matrix, and suggested involvement of miR-29b in the protection from renal medullary injury in SS-13<sup>BN</sup> rats.

### Keywords

gene expression; renal injury; hypertension; extracellular matrix; kidney

### Introduction

A mammalian genome encodes several hundred microRNAs (miRNA). Each miRNA is computationally predicted to regulate dozens to hundreds of protein-coding genes.<sup>1,2</sup> In most

Correspondence: Mingyu Liang, M.B., Ph.D., Tel: 414 955-8539, Fax: 414 955-6546, mliang@mcw.edu .

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cases, miRNAs decrease the expression level of target genes by suppressing translation or reducing mRNA abundance.<sup>3,4,5</sup> MiRNAs have been shown in recent years to be important regulators of several physiological processes and diseases in human and mammalian model organisms.

Evidence is beginning to emerge that suggests a role for miRNAs in the development of hypertension and renal injury.<sup>6</sup> For example, the miRNA miR-155 has been shown to be involved in angiotensin II signaling.<sup>7,8</sup> miR-192 and miR-377 may contribute to diabetic glomerular injury.<sup>9,10</sup> MiRNAs in general appears to be critical for the development of podocytes.<sup>11,12,13</sup>

The role of miRNAs in salt-sensitive hypertension and renal injury remains unknown. The Dahl salt-sensitive (SS) rat is the most widely used genetic model of human salt-sensitive forms of hypertension and renal injury.<sup>14,15,16</sup> SS-13<sup>BN</sup> rats are consomic rats in which chromosome 13 has been introgressed from Brown Norway rats into the SS genome.<sup>17</sup> Salt-induced hypertension and renal injury are substantially attenuated in SS-13<sup>BN</sup> rats.<sup>17,18,19,20</sup> Comparison of SS and SS-13<sup>BN</sup> rats has led to the discovery of several pathways, such as renal medullary oxidative stress,<sup>20</sup> local glucocorticoid excess,<sup>21</sup> and deficiencies in fumarate metabolism,<sup>22,23</sup> that may contribute to salt-induced hypertension and renal injury. It appears that a tree-like regulatory network might be underlying the disease phenotypes in SS rats.<sup>18,21</sup> An exciting possibility is that miRNAs could play an important role in this complex regulatory network.<sup>6,24</sup>

In the present study, we found that a miRNA, miR-29b, was elevated in the renal medulla of SS-13<sup>BN</sup> rats by a high-salt diet, but not in SS rats. We discovered evidence suggesting that miR-29 might be a master regulator of a large number of collagens and other genes related to extracellular matrix, which might be relevant to the development of prominent medullary interstitial fibrosis in SS rats.

## Methods and Materials

### Rats

Inbred SS and consomic SS-13<sup>BN</sup> rats, produced at the Medical College of Wisconsin,<sup>17</sup> were maintained on the AIN-76A diet containing 0.4% NaCl (Dyets) as described previously.<sup>17,21,23,25,26</sup> Male rats were used for experiments at approximately 6 weeks of age. The animal study was approved by the Institutional Animal Care and Use Committee at Medical College of Wisconsin.

### MiRNA Isolation and Expression Profiling

Expression levels of 377 miRNAs were analyzed with a custom-made miRNA microarray as described previously.<sup>27</sup> The renal medulla of SS and SS-13<sup>BN</sup> rats on the 0.4% NaCl diet or after 3 days on a 4% NaCl diet were examined with a total of 16 microarrays (n=4). Signal intensity data were processed and normalized as described previously.<sup>27</sup> Criteria of differential expression were set according to permutation-based false positive rate as previously described.<sup>19,27,28</sup>

### Measurement of Individual MiRNAs Using Modified Real-time PCR

Expression levels of individual miRNAs were quantified using modified real-time PCR with Taqman (Applied Biosystems) chemistry as described previously.<sup>27</sup> Total RNA samples used for the real-time PCR analysis were independent of those used in the microarray experiment. 5S rRNA was used as an internal normalizer.

## Bioinformatic Analysis

MiRNA target predictions were retrieved from three commonly used algorithms, TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.bio.nyu.edu/>), and miRanda (<http://microrna.sanger.ac.uk/targets/v4/>). Sequence analysis of *Col8a1* was performed using DNASTAR.

## 3'-Untranslated Region (UTR) Reporter Constructs

Reporter gene constructs containing the 3'-UTR region of a miRNA target gene were generated using molecular cloning techniques that we described previously.<sup>21,23,29,30</sup> Briefly, a segment, typically a few hundred bases, of the 3'-UTR that included the binding sites for miR-29b was amplified from DNA samples extracted from rat tissues using PCR primers containing restriction endonuclease recognition sequences. Locations of the segments and primer sequences are shown in Supplemental Table S1 (please see <http://hyper.ahajournals.org>). The PCR product with flanking restriction nuclease sites was double-digested and inserted into the multiple cloning site of the pMIR-REPORT luciferase plasmid (Ambion) downstream from a firefly luciferase gene. The chimeric 3'-UTR-luciferase reporter construct was transformed into competent *E. Coli* (Invitrogen). Colonies that were confirmed by PCR to contain an insert of the expected size were cultured. The plasmids were extracted using a QIAprep Spin Miniprep kit (Qiagen) and sequenced to verify the insertion of the 3'-UTR region. Transfection-quality plasmids were prepared using a PureLink HiPure Plasmid Filter Purification Kit (Invitrogen).

## Transfection and Analysis of 3'-UTR Reporter Constructs

HeLa cells were cultured in 96-well CELLSTAR white  $\mu$ Clear plates (Greiner Bio-One) with DMEM plus 5% FBS. Cells at 80–90% confluency were co-transfected with a 3'-UTR reporter construct (100 ng per well), a pMIR-REPORT  $\beta$ -galactosidase plasmid (50 ng per well), and control pre-miR oligonucleotides or the miR-29b mimic pre-miR-29b (10 pmol per well, from Ambion), using Lipofectamine 2000 (Invitrogen) following Invitrogen's protocol.<sup>21,22,23,29,30</sup> At 24 h after the transfection, luciferase and  $\beta$ -galactosidase activities in each well were measured using the Dual-Light system (ABI) following the manufacturer's protocol for microplate assay. The light signals were acquired using the high-throughput Analyst HT 96.384 microplate reader (Molecular Devices).  $\beta$ -galactosidase activity was used to normalize luciferase activity to control for transfection efficiency and cell density.

## Culture and transfection of rat medullary thick ascending limb cells

Rat medullary thick ascending limb (mTAL) cells were cultured as described previously.<sup>31</sup> Transfection of the miR-29b mimic pre-miR-29b or the control pre-miR (Ambion) was performed using Lipofectamine 2000 (Invitrogen) as described previously.<sup>21,22,23,29,30,32</sup>

## Real-time PCR

Real-time PCR using the SYBR Green chemistry was performed as described previously.<sup>28</sup> 18S rRNA was used as the internal normalizer. Sequences of the primers used were shown in Supplemental Table S2 (please see <http://hyper.ahajournals.org>).

## Western blot

Western blot was performed as we described previously.<sup>21,22,23,27,29,30</sup> Antibodies were from Santa Cruz Biotechnology. The density of a specific band was normalized by Coomassie blue staining of the entire lane.

## In vivo administration of locked nucleic acid (LNA)-modified anti-miR in chronically instrumented rats

LNA-modified anti-miR has been recently reported to efficiently knock down specific microRNAs in mice and monkeys.<sup>33</sup> We adopted the method for use in rats. SS-13<sup>BN</sup> rats were instrumented for chronic infusion through the femoral vein and blood pressure measurement through the femoral artery as we described previously.<sup>21,22,23,29</sup> Following recovery from surgery and stable baseline recording of blood pressure, LNA anti-miR-29b or LNA scrambled anti-miR was injected intravenously (10 mg/kg body weight, dissolved in 2 ml of normal saline, and injected over 3 min). The rats were switched to the 4% NaCl diet after the injection, and tissue collected 14 days later for expression analysis.

### Statistics

Data were analyzed using student t-test or multiple-group analysis of variance.  $P < 0.05$  was considered significant unless otherwise indicated. Data are shown as mean  $\pm$  SEM.

## Results

### MiRNA expression profiles in the renal medulla of SS and SS-13<sup>BN</sup> rats

The abundance of 377 miRNAs in the renal medulla of 6 weeks old SS and SS-13<sup>BN</sup> rats was examined with miRNA microarrays. Five miRNAs were considered differentially expressed between the two rat strains after 3 days on the 4% NaCl diet (Figure 1A). Rno-miR-29b and hsa-miR-302d were expressed at lower levels in SS rats, while rno-miR-365, hsa-miR-211, and rno-miR-15b were higher in SS rats. The criteria of differential expression were  $P < 0.05$ , signal difference  $> 150$ , and fold change  $> 50\%$ . The overall, permutation-based false positive rate was 20%.

In rats maintained on the 0.4% NaCl diet, no criteria for differential expression could be set that would yield an overall, array-wide false positive rate of less than 50%. Interestingly, a microRNA located on rat chromosome 13, miR-214, was readily detectable in SS rats, but barely so in SS-13<sup>BN</sup> rats.

Signal intensity data from all 16 microarrays, prior to any normalization or data processing, are shown in Supplemental Table S3 (please see <http://hyper.ahajournals.org>).

### MiR-29 was predicted to regulate 20 collagens and several related genes

miR-29b, one of the miRNAs expressed at lower levels in the renal medulla of SS rats than in SS-13<sup>BN</sup> rats, is a member of the miR-29 family that also includes miR-29a and miR-29c. The mature sequences of the three isoforms differ at nucleotide positions 10, 18, 21, 22, or 23, but have identical seed regions in nucleotides 2 to 8. Consequently, the three isoforms have largely overlapping sets of predicted target genes.

As shown in Table 1, a highly prominent group of predicted targets of miR-29 was 20 collagens and several other genes related to extracellular matrix, such as *Mmp2*, *Itgb1*, *Eln*, *Fbn1*, *Lamc1*, and *Sparc*. The large number of collagens as predicted targets is unique to miR-29 as no other miRNA was predicted to target more than 11 of the 20 collagen genes.

The predicted role of miR-29b in the regulation of collagens and other genes related to extracellular matrix is very interesting in the context of Dahl salt-sensitive rats. Extracellular matrix formation and renal medullary interstitial fibrosis are prominent features of SS rats, which are substantially attenuated in SS-13<sup>BN</sup> rats.<sup>17,19,20</sup> The subsequent studies focused on assessing changes in collagens and related genes in Dahl salt-sensitive rats and the interaction between miR-29b and those genes.

### Reciprocal expression of miR-29b and several collagens and related genes in the medulla

Real-time PCR analysis confirmed that the renal medullary abundance of miR-29b in SS-13<sup>BN</sup> rats was increased by more than 4 fold after 3 days on the 4% NaCl diet (Figure 1B). miR-29b levels in SS rats were not significantly altered by the high-salt intake. Similar changes were observed when rats were exposed to an 8% NaCl diet for 3 days ( $1.1 \pm 0.2$  fold of 0.4% NaCl for SS, n=4, not significant;  $2.1 \pm 0.3$  fold for SS-13<sup>BN</sup>, n=4, P<0.05). Response of miR-29a or miR-29c in the renal medulla to 3 days of 8% NaCl diet was not different between SS and SS-13<sup>BN</sup> rats (n=4, not significant in any of the following comparisons). miR-29a levels after 3 days of 8% NaCl diet, compared to 0.4% NaCl diet, were  $1.2 \pm 0.2$  fold in SS rats and  $1.3 \pm 0.2$  fold in SS-13<sup>BN</sup> rats. miR-29c levels were  $1.8 \pm 0.3$  fold in SS rats and  $1.7 \pm 0.3$  fold in SS-13<sup>BN</sup> rats.

In the renal cortex, miR-29b was not significantly altered by 3 days of 8% NaCl diet in SS rats ( $1.2 \pm 0.4$  fold of 0.4% NaCl, n=6, not significant) or in SS-13<sup>BN</sup> rats ( $0.8 \pm 0.2$  fold of 0.4% NaCl, n=3, not significant).

Taqman real-time PCR was performed to analyze the renal outer medullary mRNA abundance of 12 collagens as well as *Mmp2* and *Itgb1*, which were predicted to be miR-29 targets, and 4 collagens not predicted to be miR-29 targets. Of the predicted targets, *Col1a1*, *Col3a1*, *Col4a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col7a1*, and *Mmp2* were found to be up-regulated in SS rats, but not in SS-13<sup>BN</sup> rats, after 3 or 7 days on the 8% NaCl diet (Figure 2A). *Col1a2*, *Col4a2*, *Col6a3*, *Col15a1*, *Col16a1*, and *Itgb1* were not significantly altered, although several of them tended to be up-regulated in SS rats. Of the genes not predicted to be targets of miR-29b, *Col8a1* and *Col12a1* were significantly up-regulated in SS rats after 7 days on the 8% NaCl diet, while *Col6a1* and *Col6a2* were not (Figure 2A). See the section “3’-UTR Reporter Analysis” for additional information about a target site in *Col8a1*.

Exposure to the 4%, instead of 8%, NaCl diet significantly increased the renal medullary mRNA levels of *Col1a2* ( $1.7 \pm 0.2$  fold, 7 days), *Col4a1* ( $1.6 \pm 0.2$  fold, 3 days), and *Col7a1* ( $2.3 \pm 0.3$  fold, 7 days) in SS rats (n=3–6, P<0.05 vs. 0.4% NaCl diet), but did not significantly affect these genes in SS-13<sup>BN</sup> rats. The 4% NaCl diet for 3 or 7 days increased *Col1a1* mRNA in both SS and SS-13<sup>BN</sup> rats and did not cause significant changes in *Col3a1*, *Col4a2*, *Col5a1*, *Col5a2*, *Col5a3*, *Col6a3*, *Col15a1*, *Col16a1*, *Mmp2*, *Itgb1*, *Col6a1*, *Col6a2*, *Col8a1*, or *Col12a1* in either rat strain.

Western blot analysis showed that the renal medullary protein abundance of *Col1a1* was significantly higher in SS rats than in SS-13<sup>BN</sup> rats following 7 days of exposure to the 4% NaCl diet (Figure 2B).

### Knockdown of miR-29b up-regulated several ECM genes in the kidneys of SS-13<sup>BN</sup> rats

Intravenous administration of LNA anti-miR-29b (10 mg/kg body weight) in SS-13<sup>BN</sup> rats resulted in substantial and significant knockdown of miR-29b by 76% and 63% in the renal cortex and the renal inner medulla, respectively, compared to rats treated with a scrambled LNA anti-miR (Figure 3A). miR-29b levels in the outer medulla, however, were not significantly altered ( $1.1 \pm 0.2$  folds compared to rats treated with the scrambled, n=4 to 7, NS). In addition, LNA anti-miR-29b did not significantly suppress miR-29a ( $1.1 \pm 0.4$  folds in the cortex,  $0.8 \pm 0.1$  folds in the inner medulla, compared to the scrambled) or miR-29c ( $0.9 \pm 0.3$  folds in the cortex,  $0.8 \pm 0.2$  folds in the inner medulla) (n=4 to 7, NS).

mRNA levels of *Col1a1*, *Col3a1*, *Col4a1*, *Col4a2*, *Col5a1*, *Col5a2*, and *Col5a3* were examined. Knockdown of miR-29b was associated with significant up-regulation of *Col3a1*, *Col4a1*, *Col4a2*, and *Col5a1* in the renal cortex and *Col3a1* in the inner medulla (Figure 3B). *Col5a2* in the cortex and *Col1a1* in the inner medulla tended to be up-regulated, but did not

reach statistical significance ( $P=0.071$  and  $0.088$ , respectively). The up-regulation of ECM genes was not secondary to changes in arterial blood pressure since the treatment with LNA anti-miR-29b did not significantly affect mean arterial blood pressure (Figure 3C).

### Suppression of collagens and related genes by miR-29b in cultured cells

Cultured rat mTAL cells were treated with control pre-miR oligonucleotides or the miR-29b mimic pre-miR-29b. mRNA levels of 18 collagen genes, *Mmp2*, and *Itgb1* were measured with Taqman real-time PCR 48 hours later. The mTAL cells were used in this analysis because they were from the renal medulla of rats, although other cell types may also contribute to medullary interstitial fibrosis. The result showed that miR-29b, compared to control pre-miR, significantly reduced mRNA levels of *Col3a1*, *Col4a1*, *Col4a2*, *Col5a1*, *Col5a2*, *Col5a3*, *Col7a1*, and *Itgb1* (Figure 4). miR-29b did not affect the mRNA level of *Col18a1*, which did not contain a miR-29b binding site and could be viewed as a negative control. miR-29b did not significantly affect the mRNA level of *Col16a1* even though *Col16a1* has a predicted miR-29b binding site. mRNA levels of *Col1a1*, *Col1a2*, *Col6a1*, *Col6a2*, *Col6a3*, *Col12a1*, *Col14a1*, *Col15a1*, *Col17a1*, and *Mmp2* were not readily detectable in these cultured cells.

### 3'-UTR reporter analysis

3'-UTR reporter analysis (Figure 5A) was performed to directly examine any 3'-UTR-mediated interactions between miR-29b and 9 collagen genes as well as *Mmp2* and *Itgb1*. The miR-29b mimic, compared to a control miRNA mimic, significantly reduced luciferase activity when the reporter gene was linked to a 3'-UTR segment of *Col1a1*, *Col3a1*, *Col4a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col7a1*, *Col8a1*, *Mmp2*, or *Itgb1* (Figure 5B).

*Col8a1* is not a predicted target for miR-29b according to TargetScan, PicTar, or miRanda (Table 1). A 3'-UTR segment of 183 bases was used for rat *Col8a1* in those prediction algorithms. A segment of 2,655 bases, however, was used for mouse *Col8a1*. Bioinformatic analysis of rat genomic DNA indicated the presence of a putative 3'-UTR segment largely homologous to the mouse segment. Importantly, the 8-base sequence in the mouse 3'-UTR that is complementary to the seed region of miR-29b is also present in the putative rat 3'-UTR. Primers (Supplemental Table S1) were designed to clone a 673-base segment of the predicted 3'-UTR from rat *Col8a1* mRNA, which was used in the 3'-UTR reporter analysis. The result indicated that *Col8a1* was a target of miR-29b (Figure 5B).

*Col12a1* does not have a predicted binding site for miR-29b. The 3'-UTR analysis confirmed that *Col12a1* was not a target of miR-29b (Figure 5B). The result of *Col12a1* could be viewed as a negative control for the 3'-UTR analysis.

### MiR-29 as a “master” regulator of collagens and related genes

Table 1 summarized the data supporting a “master” regulatory role of miR-29 in the expression of collagens and other genes related to extracellular matrix in the renal medulla of SS and SS-13<sup>BN</sup> rats. It included computationally predicted interactions, up-regulation of collagens and *Mmp2* in the renal medulla in SS rats (as opposed to the up-regulation of miR-29b in SS-13<sup>BN</sup> rats) in response to the 8% NaCl diet, suppression of collagens and *Itgb1* in cultured renal medullary cells by a miR-29b mimic, and miR-29b-target interactions demonstrated by 3'-UTR reporter analysis.

### Discussion

The present study discovered the first miRNA possibly involved in the renal injury in Dahl salt-sensitive rats, and demonstrated a broad role of miR-29b in the regulation of a large number of collagens and other genes related to extracellular matrix.



Renal medullary interstitial fibrosis is a prominent pathological feature of SS rats, especially when the rats are on high-salt diets. Medullary interstitial fibrosis, along with salt-sensitive hypertension, is substantially attenuated in consomic SS-13<sup>BN</sup> rats.<sup>17,18,19,20</sup> Up-regulation of miR-29b may be an important part of the molecular mechanism underlying the attenuation of medullary fibrosis in SS-13<sup>BN</sup> rats. The notion is supported by reciprocal expression of miR-29b and a large number of collagens and *Mmp2* in vivo, as well as up-regulation of several collagens in vivo following miR-29b knockdown and suppression of several collagens by miR-29b in vitro. Several collagens targeted by miR-29b are components of, or associated with, basement membrane or intercellular matrix.<sup>34</sup> Matrix metalloproteinase 2 might be pro-fibrotic in the kidney.<sup>35</sup> Moreover, an anti-fibrotic role of miR-29b in the renal medulla would be consistent with the recently reported anti-fibrotic role of miR-29b in a mouse model of cardiac fibrosis.<sup>36</sup>

A previous study of miRNA expression in SS rats did not find differential expression of any miRNAs between SS and Lewis rats.<sup>37</sup> A subset of miRNAs was examined in that study using whole kidney extracts and mostly pooled samples. Although the present study also indicated that the differential expression of miRNAs associated with SS rats was limited, we were able to identify miRNAs that appeared relevant to the disease phenotypes in SS rats. This may be attributed to the use of consomic rats as control, a focus specifically on the renal medulla, a more complete coverage of miRNAs, or different techniques of expression analysis.

An important question that remains to be addressed is why miR-29b is up-regulated in SS-13<sup>BN</sup> rats, but not in SS rats. One copy of the miR-29b gene, miR-29b-2, is located on chromosome 13 of the rat genome, the only chromosome in SS-13<sup>BN</sup> that is derived from the Brown Norway rat. This could suggest that genetic differences might exist between SS and Brown Norway genomes in the regulatory sequence for the miR-29b-2 gene. However, the miR-29b-2 gene is separated on the chromosome from miR-29c only by 448 base pairs. The other copy of the miR-29b gene, miR-29b-1, is located on chromosome 4, separated from miR-29a by 292 base pairs. But miR-29c or miR-29a did not seem to follow the same expression pattern as miR-29b. Together, this suggests that the differential regulation of miR-29b in SS and SS-13<sup>BN</sup> rats could involve post-transcriptional processing in addition to any transcriptional changes.

The regulation of extracellular matrix genes by miR-29b shown by the present study and other recent studies<sup>36,38,39</sup> is one of the first experimentally proven cases in which one miRNA acts as a master regulator of a large group of related genes. Each miRNA has been predicted in silico to regulate a large number of protein-coding genes.<sup>1,2</sup> It has led to the exciting theory that miRNAs may serve as hubs in complex regulatory networks by fine-tuning multiple components of a network.<sup>6,24</sup> But experimental evidence supporting the theory has been sparse. Recent studies of cardiac tissue, bone cells, and tumors have led to the findings that miR-29b targets *Coll1a1*, *Coll1a2*, *Col3a1*, *Col4a2*, *Col5a3*, elastin, and fibrillin 1,<sup>36,39</sup> and that miR-29c targets *COL1A1*, *COL1A2*, *COL3A1*, *COL4A1*, *COL4A2*, *COL15A1*, laminin  $\gamma$ 1, and osteonectin.<sup>38</sup> The present study, which was focused on the renal medulla of Dahl salt-sensitive rats, has shown that miR-29b targets *Col5a1*, *Col5a2*, *Col7a1*, *Col8a1*, *Mmp2*, and *Itgb1*, in addition to confirming the targeting of *Coll1a1*, *Col3a1*, *Col4a1*, and *Col5a3*. Together, these findings (see Table 1) support a master role of miR-29 in the regulation of extracellular matrix gene expression, which might be important in a number of diseases including hypertensive renal injury.

MiRNAs may suppress target expression through translational repression or induction of mRNA degradation. miR-29 decreased the mRNA abundance of collagens and other extracellular matrix genes as shown in the present study as well as previous publications.<sup>36,38</sup> It appears that induction of mRNA degradation is a major mode of action for miR-29b.

Despite the broad effect of miR-29 on collagens and related genes, it is clear from Table 1 that miR-29 is not the only regulator of these genes in the medulla of SS and SS-13<sup>BN</sup> rats. Collagen expression and medullary fibrosis in the SS model are likely determined by a number of antagonistic forces. miR-29b appears to be one of the forces opposing fibrosis, while other mechanisms may drive fibrosis. This may explain why up-regulation of miR-29b or knockdown of miR-29b in vivo can be associated with a mosaic of target ECM gene expression, depending on the state of the rest of the regulatory network. For example, *Colla2*, *Col4a2*, and *Coll5a1* are targets of miR-29 in vitro based on the present study and/or previous publications.<sup>36,38,39</sup> However, these genes were not significantly up-regulated in SS rats within 7 days of 8% NaCl exposure. *Coll2a1* was up-regulated in SS rats, but it is not a target of miR-29. Knockdown of miR-29b in the renal cortex of SS-13<sup>BN</sup> rats was associated with up-regulation of several collagen genes, even though miR-29b was not differentially regulated in the renal cortex as it was in the medulla, suggesting that baseline levels of miR-29b may already be important in controlling the expression of ECM genes. LNA anti-miR-29b remarkably suppressed miR-29b in the cortex and the inner medulla, but not in the outer medulla, preventing us from directly assessing the in vivo role of outer medullary miR-29b. The reason for the regional differences in the efficiency of knockdown is not clear. In most cases, the in vivo and in vitro data were consistent as *Coll1a1*, *Col3a1*, *Col4a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col7a1*, *Col8a1*, and *Mmp2* are targets of miR-29 in vitro and also up-regulated in SS rats in vivo. However, even in these cases, the lack of miR-29b up-regulation in SS rats likely played a permissive role in the up-regulation of those genes, instead of being a primary stimulus.

### Perspectives

The findings of the present study suggest that the function and regulation of miRNA may contribute to the molecular mechanisms underlying the development of tissue injury associated with hypertension. In addition, the “master” role of miR-29 as a regulator of collagens and genes related to extracellular matrix has significant implications for understanding the general biology of miRNAs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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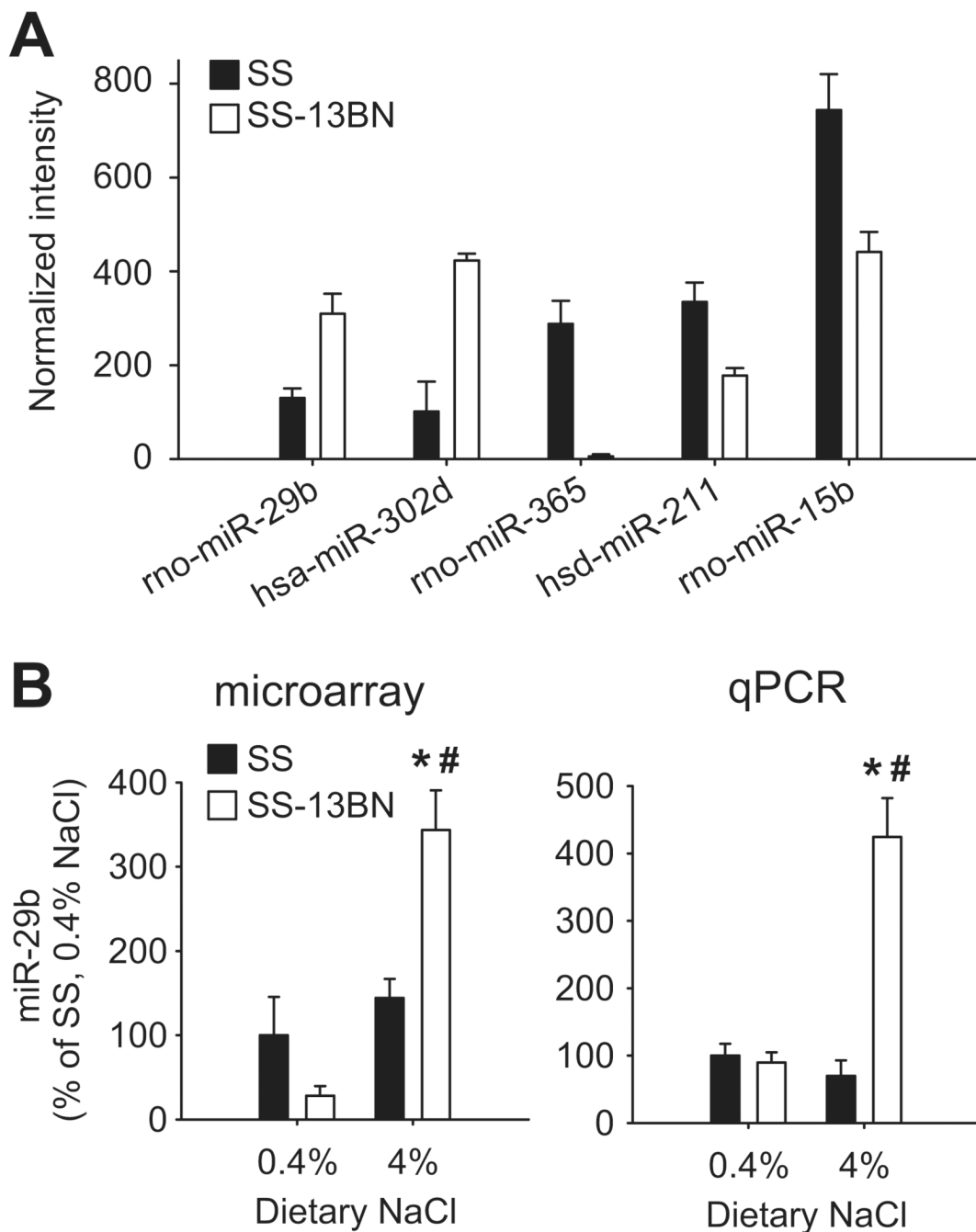
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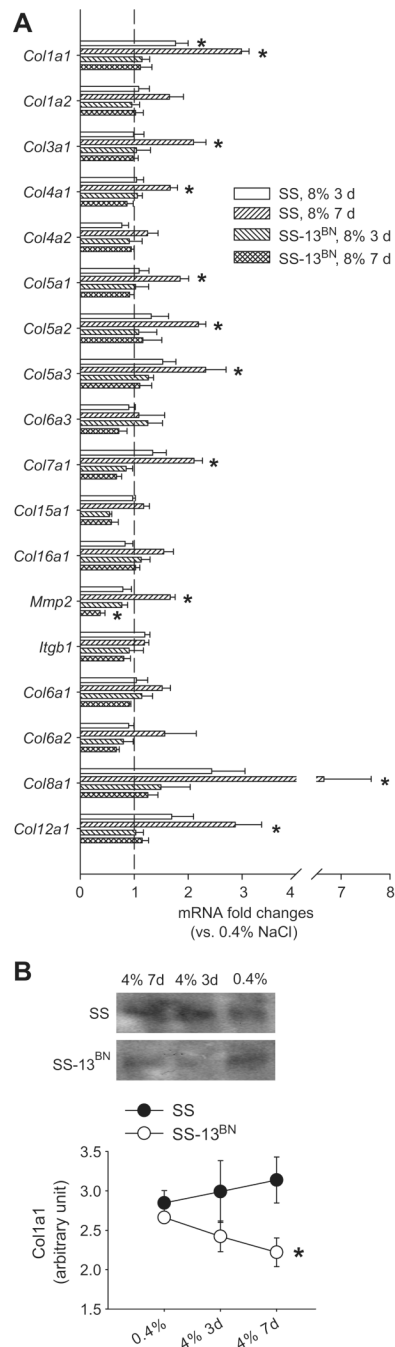
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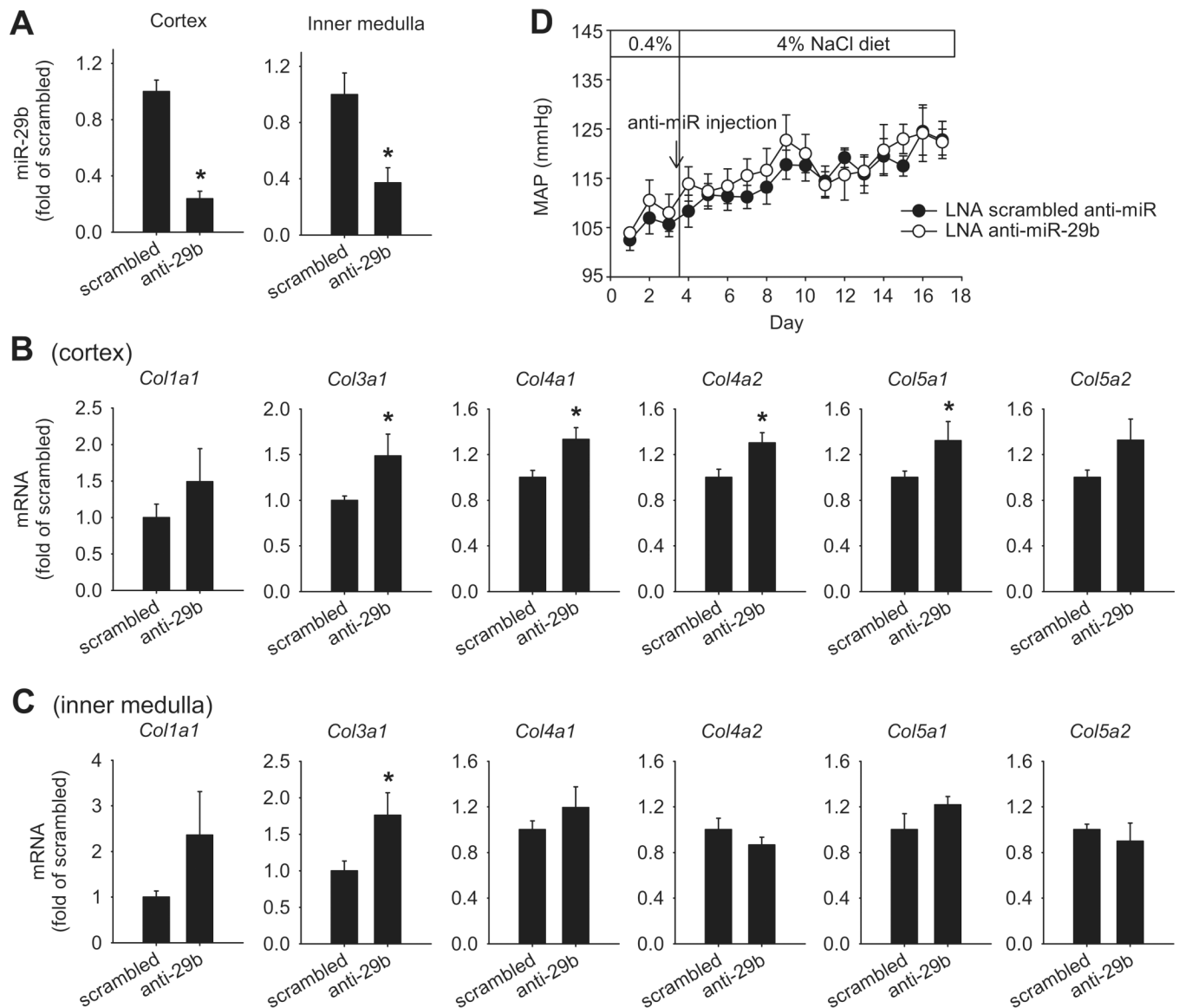
**Figure 1. MiRNA expression in the renal medulla of SS and SS-13<sup>BN</sup> rats**

**A.** Renal medullary miRNAs differentially expressed between SS and SS-13<sup>BN</sup> rats on a 4% NaCl diet. Expression levels of 377 miRNAs were analyzed with miRNA microarrays (n=4). Differentially expressed miRNAs were shown. See the text for criteria of differential expression. **B.** MiR-29b in the renal medulla of SS-13<sup>BN</sup> rats, but not SS rats, was up-regulated by a 4% NaCl diet. Data from microarray or real-time PCR analyses were shown. Samples used for the PCR experiment were independent of those used in the microarray experiment. N=6; \*, P<0.05 vs. SS-13<sup>BN</sup> 0.4%; #, P<0.05 vs. SS 4%.



**Figure 2. Effect of a high-salt diet on expression levels of 16 collagen genes, matrix metallopeptidase 2, and integrin  $\beta$ 1**

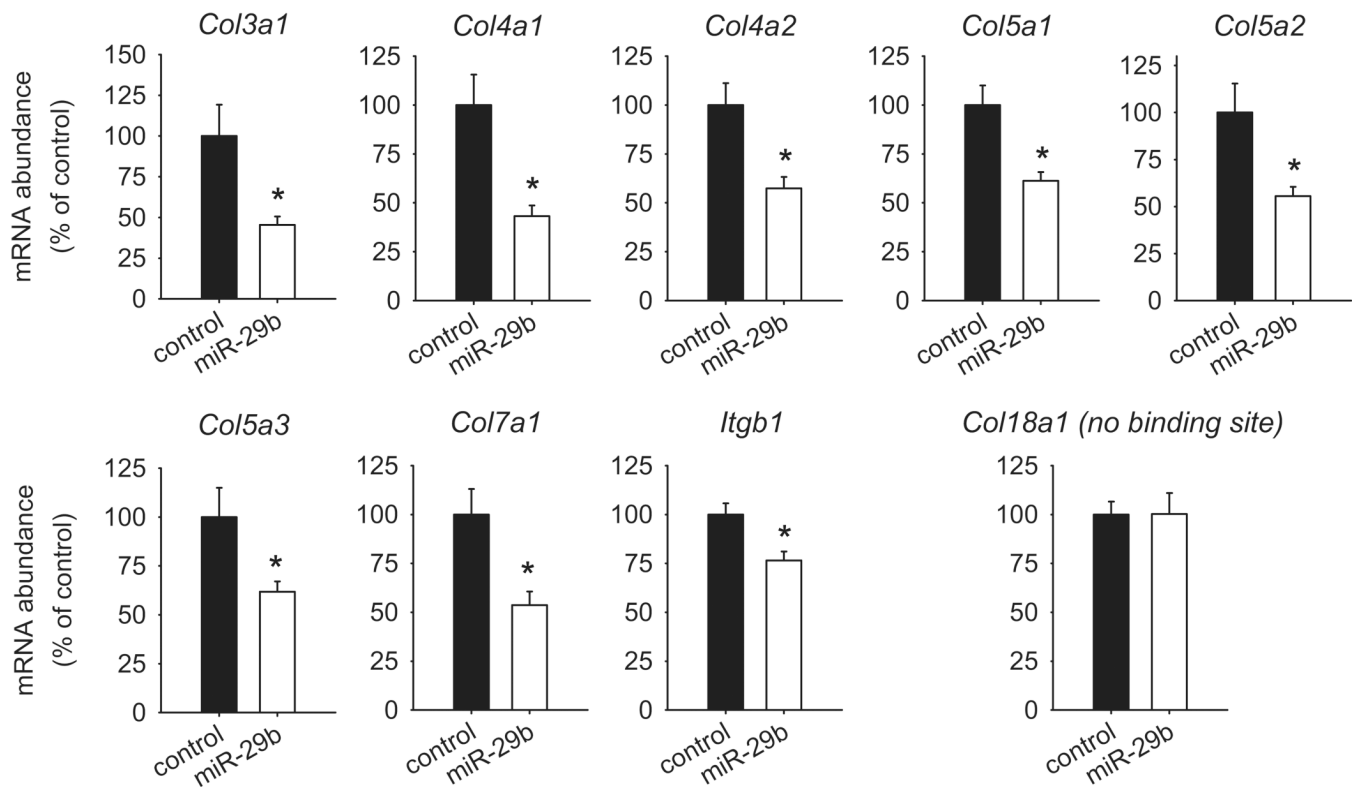
**A.** mRNA abundance was measured using real-time PCR in the renal outer medulla of male SS and SS-13<sup>BN</sup> rats. Rats were 6–7 weeks old and had been maintained on a 0.4% NaCl diet since birth or switched to an 8% NaCl diet for 3 or 7 days. Data were expressed as fold changes relative to the 0.4% NaCl group in each strain. N=6; \*, P<0.05 vs. 0.4% NaCl of the respective strain. **B.** Protein abundance in the renal medulla estimated by Western blot. Rats were 6–7 weeks old and had been maintained on a 0.4% NaCl diet since birth or switched to a 4% NaCl diet for 3 or 7 days. N=5–6; \*, P<0.05 vs. SS.



**Figure 3. Effect of LNA anti-miR-29b on SS-13<sup>BN</sup> rats in vivo**

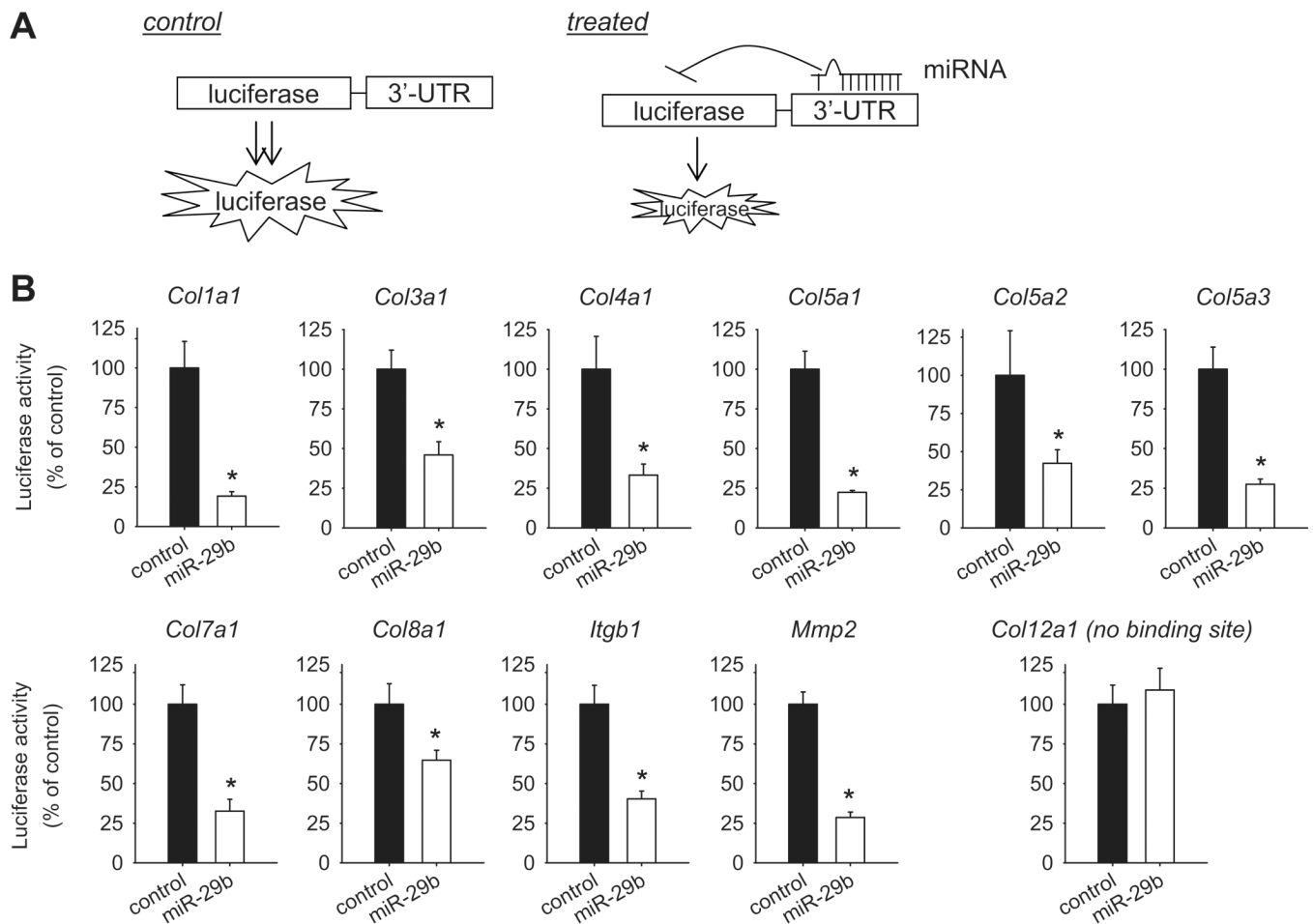
LNA anti-miR-29b (n=4) or LNA scrambled anti-miR (n=7) was administered intravenously to chronically instrumented, conscious rats at the dose of 10 mg/kg body weight. Rats were then switched to a 4% NaCl diet and gene expression analyzed 14 days later. **A.** miR-29b expression in kidney regions. **B.** Collagen gene expression in the cortex. **C.** Collagen gene expression in the inner medulla. **D.** Mean arterial blood pressure. \*, P<0.05 vs. scrambled.





**Figure 4. miR-29b suppressed the expression of several collagens and integrin  $\beta$ 1 in medullary epithelial cells**

Cultured rat medullary thick ascending limb cells were treated with pre-miR-29b or control scrambled pre-miR for 48 hours before mRNA abundance was measured using real-time PCR. N=7; \*, P<0.05 vs. cells treated with control pre-miR.



**Figure 5. miR-29b interacted with the 3'-UTR of several collagen genes, matrix metalloproteinase 2, and integrin  $\beta$ 1**

**A.** 3'-UTR reporter assay. A 3'-UTR segment was cloned into a gene expression construct downstream from a luciferase reporter gene. Interactions between a miRNA and the 3'-UTR segment would be expected to decrease luciferase activity. **B.** miR-29b decreased luciferase activity when the reporter gene was linked to a 3'-UTR segment of several collagens, matrix metalloproteinase 2, and integrin  $\beta$ 1. HeLa cells were transfected with a 3'-UTR reporter construct, a  $\beta$ -galactosidase construct (for normalization), and pre-miR-29b or control scrambled pre-miR. Luciferase and  $\beta$ -galactosidase activities were analyzed 24 hours later. N=4-8; \*, P<0.05 vs. cells treated with control pre-miR.

Table 1

miR-29 as a “master” regulator of several collagens and other genes related to extracellular matrix.

Gene name	protein name	TargetScan	PicTar	miRanda	up by salt in SS, not in SS-13 <sup>BN</sup>	down by miR-29b in mFAL	confirmed by 3'-UTR assay	previously confirmed*
<i>Coll1a1</i>	collagen type I alpha1	Yes	Yes	No	Yes		Yes	Yes
<i>Coll1a2</i>	collagen type I alpha2	Yes	Yes	No	No			Yes
<i>Col2a1</i>	collagen type II alpha1	Yes	Yes	Yes				
<i>Col3a1</i>	collagen type III alpha1	Yes	Yes	Yes	Yes	Yes	Yes	Yes
<i>Col4a1</i>	collagen type IV alpha1	Yes	Yes	No	Yes	Yes	Yes	Yes
<i>Col4a2</i>	collagen type IV alpha2	Yes	Yes	No	No	Yes		Yes
<i>Col4a3</i>	collagen type IV alpha3	Yes	Yes	No				
<i>Col4a4</i>	collagen type IV alpha4	Yes	Yes	Yes				
<i>Col4a5</i>	collagen type IV alpha5	Yes	Yes	Yes				
<i>Col5a1</i>	collagen type V alpha1	Yes	No	No	Yes	Yes	Yes	
<i>Col5a2</i>	collagen type V alpha2	Yes	Yes	No	Yes	Yes	Yes	
<i>Col5a3</i>	collagen type V alpha3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
<i>Col6a3</i>	collagen type VI alpha3	Yes	Yes	Yes	No			
<i>Col7a1</i>	collagen type VII alpha1	Yes	Yes	Yes	Yes	Yes	Yes	
<i>Col9a1</i>	collagen type IX alpha1	Yes	Yes	Yes				
<i>Col11a1</i>	collagen type XI alpha1	Yes	Yes	No				
<i>Col15a1</i>	collagen type XV alpha1	Yes	Yes	No	No			Yes

Gene name	protein name	TargetScan	PicTar	miRanda	up by salt in SS, not in SS-13 <sup>5N</sup>	down by miR-29b in mFAL	confirmed by 3'-UTR assay	previously confirmed*
<i>Col16a1</i>	collagen type XVI alpha1	Yes	Yes	Yes	No			
<i>Col19a1</i>	collagen type XIX alpha1	Yes	Yes	No				
<i>Col22a1</i>	collagen type XXII alpha1	Yes	Yes	No				
<i>Col6a1</i>	collagen type VI alpha1	No	No		No			
<i>Col6a2</i>	collagen type VI alpha2	No	No	Yes	No			
<i>Col8a1</i>	collagen type VIII alpha1	No <sup>†</sup>	No <sup>†</sup>	No <sup>†</sup>	Yes		Yes	
<i>Col9a2</i>	collagen type IX alpha2	No	No	No				
<i>Col10a1</i>	collagen type X alpha1	No	No	Yes				
<i>Col12a1</i>	collagen type XII alpha1	No	No	No	Yes		No	
<i>Col14a1</i>	collagen type XIV alpha1	No	No	No				
<i>Col17a1</i>	collagen type XVII alpha1	No	No	No				
<i>Col18a1</i>	collagen type XVIII alpha1	No	No	No		No		
<i>Col27a1</i>	collagen type XXVII alpha1	No	No	Yes				
<i>Mmp2</i>	matrix metalloproteinase 2	Yes	Yes	No	Yes		Yes	
<i>Igfb1</i>	integrin beta 1	Yes	Yes	No	No	Yes	Yes	Yes
<i>Eln</i>	elastin	Yes	No	Yes				Yes
<i>Fbn1</i>	fibrillin 1	Yes	Yes	No				Yes
<i>LAMC1</i>	laminin, gamma 1	Yes	Yes	No				Yes
<i>SPARC</i>	osteonectin	Yes	Yes	No				Yes

\* See references 36, 38, 39.

<sup>†</sup> See the text for additional information.