Na/K-ATPase signaling regulates collagen synthesis through microRNA-29b-3p in cardiac fibroblasts

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Na/K-ATPase signaling regulates collagen synthesis through microRNA-29b-3p in cardiac fibroblasts. Physiol Genomics 48: 220–229, 2016. First published December 23, 2015; doi:10.1152/physiolgenomics.00116.2015.—Chronic kidney disease (CKD) is accompanied by cardiac fibrosis, hypertrophy, and dysfunction, which are commonly referred to as uremic cardiomyopathy. Our previous studies found that Na/K-ATPase ligands or 5/6th partial nephrectomy (PNx) induces cardiac fibrosis in rats and mice. The current study used in vitro and in vivo models to explore novel roles for microRNA in this mechanism of cardiac fibrosis formation. To accomplish this, we performed microRNA profiling with RT-qPCR based arrays on cardiac tissue from rats subjected to marinobufagenin (MBG) infusion or PNx. The analysis showed that a series of fibrosis-related microRNAs were dysregulated. Among the dysregulated microRNAs, microRNA (miR)-29b-3p, which directly targets mRNA of collagen, was consistently reduced in both PNx and MBG-infused animals. In vitro experiments demonstrated that treatment of primary cultures of adult rat cardiac fibroblasts with Na/K-ATPase ligands induced significant increases in the fibrosis marker, collagen protein, and miR-29b-3p expression both in vivo and in vitro. Additionally, these data indicate that miR-29b-3p expression plays an important role in the formation of cardiac fibrosis in CKD.

microRNA (miRNA); cardiovascular disease; Na/K-ATPase; fibrosis; chronic kidney disease

CARDIAC FIBROSIS IS A COMMON pathological process in different cardiac diseases (31). Formation of fibrotic scars in the myocardium increases cardiac stiffness, whereas regression of fibrosis improves cardiac function (2, 17). Chronic kidney disease (CKD) worsens congestive heart failure (CHF) and is directly associated with morbidity and mortality in CHF patients (32). Our previous studies have shown that formation of cardiac fibrosis in experimental CKD models involves increases in endogenous circulating cardiotoxic steroids (CTS) and activation of Na/K-ATPase signaling (9, 18). Na/K-ATPase is an important cell membrane protein enriched in heart and kidney tissues. The Na/K-ATPase signaling pathway involves Src, Akt, PKC, and other signaling proteins (45). Activation of Na/K-ATPase signaling in experimental CKD models induces left ventricular hypertrophy and cardiac fibrosis, while neutralization of CTS like marinobufagenin (MBG), by active or passive immunization, attenuated Na/K-ATPase signaling and cardiac fibrosis (15, 18).

During the past decade, microRNA (miRNA) was discovered to be associated with cardiac disease, and regulation of miRNA became part of important therapeutic interventions for treatment of fibrosis (40, 41). Strategies that regulate miRNAs are being developed and evaluated in several settings including cardiovascular diseases (30, 51). These small, 18–25 bp miRNAs play a role in the progression of many diseases that have fibrotic, hypertrophic, and apoptotic phenotypes (19). Recently, several studies have focused on the ability of miRNA-29b-3p (miR-29b-3p) to regulate fibrosis in different organs, including heart, lung, liver, skin, and kidney (27, 29, 38, 41, 53). Transforming growth factor (TGF)-β/Smad signaling is a major pathway that regulates miR-29b-3p and other miRNAs (6, 27, 29, 53), while other studies suggest that miRNA expression can also be altered by Akt and NF-κB signaling (30, 51).

We have previously shown that in experimental CKD, 5/6th partial nephrectomy (PNx) regulates collagen mRNA levels by activation of protein kinase C δ (PKCδ) and degradation of Friend leukemia integration 1 (Fli-1) (10). Interestingly, no increases in TGF-β/Smad signaling were observed in these studies, although inhibition of TGF-β blocked stimulation of MBG-induced collagen production (9, 10). Our previous findings, the emerging importance of miRNAs, and our need to develop better therapeutic targets to combat uremia-induced cardiac fibrosis led us to examine the role of Na/K-ATPase mediated signaling in regulating miRNA expression in fibrosis development using in vivo and in vitro models of uremia induced cardiac fibrosis.

MATERIALS AND METHODS

Animals. Animal experiments were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of
Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee at the University of Toledo. Eight-week-old male Sprague-Dawley rats weighing 250–300 g were used for experiments obtained from Charles River (Spencerville, OH). All animals were reared under a 12 h dark/light cycle, fed standard chow (Teklad standard maintenance diet), and were provided water ad libitum. These conditions were utilized for the entire duration of the experiment. Rats were subjected to PNx surgery or MBG infusion for 4 wk by the same minipump. Animals were killed at the end of the fourth week, and cardiac tissue was collected and weighed for use in morphometric analysis; portions were flash-frozen in liquid nitrogen for later use in histological and biochemical processing.

Isolation, culture, and treatment of cardiac fibroblasts. Isolation of cardiac fibroblasts was carried out as described previously (9). Hearts of adult male Sprague-Dawley rats were used to obtain fibroblasts. The rats were anesthetized with Ketamine-Xylazine (100/10 mg/kg), and their hearts were then removed and perfused under sterile conditions via the ascending aorta with Joklik’s medium (cat no. M0518; Sigma-Aldrich, St. Louis, MO) on a modified Langendorff apparatus. After 5 min, perfusate was switched to Joklik’s medium containing 0.1% collagenase type 1 (C0130-1G, Sigma-Aldrich) and 0.1% bovine serum albumin (BSA), which was circulated for 25 min until the heart became flaccid. After perfusion the heart was removed from the apparatus and the ventricles were excised, finely cut, and shaken in Joklik’s modified medium with 0.1% collagenase and 0.1% BSA for 30 min at 37°C with constant agitation. The resulting cell/tissue suspension was centrifuged at 600 g for 10 min. The resulting supernatant from the first spin was then centrifuged at 1,500 rpm for 15 min. The resulting fibroblast enriched cellular pellet was suspended in Dulbecco’s modified Eagle’s medium (DMEM, cat no. D1152, Sigma-Aldrich) supplemented with antibiotics (penicillin-streptomycin-fungizone, cat no. 30-002-CI; Mediatech, Manassas, VA) and 15% fetal bovine serum (FBS, cat no. 10437-028; Life Technologies, Grand Island, NY) and then seeded onto plates and incubated for 1 h. Unattached cells were removed by changing the media, and remaining attached fibroblast cells were allowed to grow to confluence. Subsequently, they were trypsinized and passaged once at 1:6 dilution. Cells were then allowed to grow to confluence before experiments.

Primary cultures of rat cardiac fibroblasts were treated as follows, cells were first serum-starved for 18–24 h and then treated with ouabain or MBG at 1, 10, 100 nM in serum-depleted medium for 24 h prior to collection.
Transfection of primary cultures of adult rat cardiac fibroblasts. Cells at passage 2 were seeded at 2.5 × 10^5 cells per well of a six-well plate in 2.3 ml of DMEM with antibiotics and 15% FBS. Cells were then incubated overnight under normal growth conditions (37°C, 5% CO2). Next, Syn-RNO-miR-29b-3p mimic (cat. no. MSY0000801-219600-S1; Qiagen, Carol Stream, IL) was diluted to 1, 5, or 25 nM in 100 µl DMEM without FBS, and 12 µl of HiPerFect Transfection Reagent (cat. no. 301705, Qiagen) was added to the diluted miRNA mimic followed by mixing via vortex. MiRNA mimic containing samples were then kept at room temperature for 5–10 min prior to use to ensure formation of transfection complexes. The transfection complexes and mock reagent alone (serving as control) were added drop-wise to the cells, and the plate was swirled gently to ensure uniform distribution of the transfection complexes. Cells were incubated under normal growth conditions for 48 h prior to collection of cell lysates for miRNA, mRNA, and protein quantitation. Control transfection with fluorescently tagged AllStars Negative Control-Alexa Flour 488 (cat. no. 1027284, Qiagen) was performed to determine the transfection efficiency. Transfection with reagent only was used as negative control.

RNA isolation and reverse transcription-quantitative polymerase chain reaction. Left ventricular tissue or cardiac fibroblasts were homogenized in Qiazol, and total RNA including small RNAs was isolated using the miRNeasy mini kit (cat. no. 217004, Qiagen) according to the manufacturer’s instructions. Immediately following RNA isolation two sets of cDNA were synthesized: First for mRNA (to evaluate changes in collagen Iα1 mRNA expression), cDNA synthesis was performed using the RT2 First Strand cDNA Synthesis Kit from Qiagen according to the manufacturer’s protocol (cat. no. 330404, Qiagen). Briefly, 1 µg of total RNA was incubated with 2 µl of Buffer GE and RNase-free water to bring the total reaction volume to 10 µl. The above-mentioned mix was incubated at 42°C for 5 min using a T-100 Thermocycler (Bio-Rad, Hercules, CA) and then placed immediately on ice for at least 1 min; this step ensured the efficient and complete removal of contaminating genomic DNA from the reaction mix. Following genomic DNA removal reverse transcription was carried out with each resulting genomic DNA-free RNA fraction receiving the following: 4 µl of 5X Buffer BC3, 1 µl of Control P2, 2 µl of RE3 Reverse Transcriptase Mix, and 3 µl of RNase-free water. Following addition of the reverse transcription reaction components we performed cDNA synthesis on a T-100 thermocycler (Bio-Rad) by incubating the reaction mix at 42°C for exactly 15 min and then immediately stopping the reverse transcription reaction by incubating at 95°C for 5 min. Following reverse transcription cDNA was diluted according to the manufacturer’s protocol for storage at −20°C and later use in quantitative real-time polymerase chain reaction (qPCR). For synthesis of cDNA for miRNA, 200 ng of total RNA from the total RNA isolated above was used in the miScript II RT kit (cat. no. 218160, Qiagen) following the manufacturer’s protocol.

To determine changes in gene expression, primers for collagen Iα1 and GAPDH (cat. no. PPR42922A and PPR06557B, respectively) were purchased from Qiagen and were used in the RT2 SYBR Green qPCR reaction mix (cat. no. 330529, Qiagen) according to the manufacturer’s protocol. For initial determinations of changes in rat left ventricular or cardiac fibroblast miRNAs, miFinder array plates from Qiagen (cat. no. MIRN-001Z) or the primer assay for miR-29b-3p or the control small RNAs SNORD96A, SNORD95 (cat. no. MS00005544, MS00033733, MS00033726, respectively; Qiagen) were used in the miScript Sybr Green PCR kit (cat. no. 218161, Qiagen) according to the manufacturer’s protocol. Reactions were carried out on an ABI 7500 Fast platform (Life Technologies, Boston, MA) using the following cycling program: 10 min incubation at 95°C [95°C for 15 s, 60°C for 1 min (fluorescence detection performed)] repeated 40 times. Relative cycle thresholds (CT) for exponential amplification were determined, and the fold changes calculated by the Delta-Delta CT method.

Western blot analysis. Cells were lysed in ice-cold RIPA lysis buffer (pH 7.0) from Santa Cruz Biotechnology (cat no. SC-24948; Santa Cruz Biotechnology, Santa Cruz, CA). Aliquots were then made and frozen at −80°C for future use. For Western blot, protein from cell lysates or 40 µl of medium (for the Western blots of collagen secreted into the medium of treated cells) from cells treated as described were separated by sodium dodecyl sulfate-polyacrylamide

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**Fig. 2.** Cardiotonic steroids (CTS) regulate miR-29b-3p and collagen synthesis in primary cultures of adult rat cardiac fibroblasts. Cells were treated with MBG (A, C) or ouabain (Oua; B, D) for 24 h, and cell lysates were collected, and total RNA including small RNA was extracted for measurement of miR-29b-3p or collagen expression (n = 6 per treatment). *P < 0.05, **P < 0.01 vs. control.
gel electrophoresis. Procollagen expression was probed using a goat antibody from Southern Biotech (1:1,000 dilution, cat. no. 1310-01; Southern Biotech, Birmingham, AL). Actin (1:1,000 dilution, cat. no. sc-1616, Santa Cruz Biotechnology) and GAPDH (1:1,000 dilution, cat. no. sc-25778, Santa Cruz Biotechnology) were probed as internal loading controls. Phospho-Src at Tyrosine 418 (pY418) pSRC was probed using a rabbit polyclonal antibody from Invitrogen (1:500 dilution, cat. no. 44660G, Grand Island, NY). Total SRC was probed using an antibody from Santa Cruz Biotechnology at 1:1,000 dilution (cat. no. sc-8056). All appropriate horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology and used at a dilution of 1:1,000.

Small RNA sequencing methodology (MiSeq). Small RNA sequencing of cardiac tissue was performed at the University of Mississippi Medical Center (UMMC) Molecular and Genomics Core Facility. Sample information was entered into a laboratory information management system to track sample progress through the small RNA sequencing pipeline. Subsequently, samples underwent an initial quality control step to determine RNA concentration and integrity (Bio-Rad Experion-High Sensitivity Chip). Samples that passed quality parameters were used to develop small RNA libraries (n = 3–6 for each sample) using the Illumina TruSeq Small RNA Sample Preparation Kit according to the Manufacturer’s protocol (Illumina, San Diego, CA). This produced on average 600,000–750,000 reads per sample for n = 3–6 or 1.5 million to 3.0 million reads in total. The libraries were sequenced using the MiSeq Reagent Kit v2 (50 cycles) carried out on an Illumina MiSeq platform. The sequencing reads were automatically uploaded and evaluated for quality using the Illumina BaseSpace computing platform. Subsequently, Fastq files were uploaded and run through small RNA/bowtie v3 pipeline using the commercially available GeneSifter software platform (http://www.genesifter.net). Each indexed sample generated >600,000 reads (QC30 >97%), with ~40% mapping to the rat reference genome, including known miRNA (miRBase) and piRNA annotated sequences.

Statistical analyses. Data are presented as means ± SE. Data obtained were analyzed by t-test or by one-way ANOVA followed by Tukey’s post hoc comparison for differences between groups where appropriate.

RESULTS

miRNA profiling in cardiac left ventricle tissue from PNx and MBG-infused rats. To establish a better understanding of the miRNA-related changes that lead to cardiac fibrosis in animal models with CKD, we performed miRNA profiling of cardiac left ventricular tissue in rats that were subjected to PNx surgery or MBG infusion. Changes in expression of miRNAs were evaluated using a commercially available qPCR panel of 84 miRNAs (Rat miFinder, Qiagen) that are commonly dysregulated in various disease states. The most significant changes in miRNA expression are shown in Table 1. Significant changes were defined as having a change in expression of more than twofold vs. sham (control) animals and having a P value <0.05. We found that 18 miRNAs were significantly dysregulated by PNx or MBG infusion. Among these, nine antifibrotic miRNAs were downregulated. This is strong evidence that decreases of antifibrotic miRNAs may contribute to cardiac fibrosis in these animals. Expression changes vs. sham (controls) for all 84 miRNAs screened are shown in Supplemental Table S1.1.

Downregulation of miR-29b-3p in PNx and MBG-infused rats coincides with increased collagen expression and cardiac fibrosis in vivo. Based on the miRNA profiling data in cardiac tissue discussed above, we focused on miR-29b-3p expression because it directly targets and disrupts expression of the mRNA of collagen, a major component of fibrosis. Analyzing miRNA expression with qPCR in left ventricular tissue we found that MBG infusion led to an over 50% decrease in...
miR-29b expression ($P < 0.01$), while PNx led to an over 60% decrease ($P < 0.01$) in miR-29b-3p expression (Fig. 1A). This coincided with a 6.4 ± 0.4-fold increase in collagen 1A1 mRNA expression ($P < 0.01$) in tissues from MBG-infused animals and a 7.7 ± 0.5-fold increase ($P < 0.01$) in tissues from PNx-operated animals (Fig. 1B). Importantly these changes also were consistent with significant increases in left ventricular fibrosis as observed in our previous studies with these models of uremic cardiomyopathy (7, 8, 14, 15).

Analysis of left ventricular tissue with small-RNA sequencing also found significant decreases of miR-29b-3p in MBG-infused (~3.7-fold, $P < 0.001$) and in PNx-operated animals (~5.8-fold, $P < 0.001$) vs. sham (Fig. 1A), which is consistent with the data from our qPCR analysis. These data further support the role of Na/K-ATPase induced signaling in the specific downregulation of miR-29b-3p in vivo.

CTS regulate miR-29b-3p expression in vitro. To further study the mechanism underlying uremia-induced cardiac fibrosis, specifically, whether induction of Na/K-ATPase signaling regulates expression of miR-29b-3p and collagen, we isolated cardiac fibroblasts from adult male Sprague-Dawley rats and treated them with different concentrations of two different Na/K-ATPase ligands, MBG or ouabain, for 24 h. As shown in Fig. 2A, following 24 h treatment with 1 or 10 nM MBG, the expression of miR-29b-3p significantly decreased (4.9-fold and 5.65-fold, $P < 0.001$, respectively). In addition, ouabain, another commonly used Na/K-ATPase ligand, also significantly decreased the expression of miR-29b-3p by 27.6 ± 7.0% at 1 nM ($P < 0.01$), 54.2 ± 4.6% at 10 nM ($P < 0.01$), and 70.4 ± 0.7% at 100 nM ($P < 0.001$) (Fig. 2B). In turn, treatment with ouabain or MBG significantly increased protein expression of collagen, a fibrosis marker, in these cells (Fig. 2, C and D). These data indicate that CTS-induced Na/K-ATPase signaling mediates miR-29b-3p expression in cardiac fibroblasts and provide further evidence that decreases in miR-29b-3p contribute to the regulation of fibrosis in these cells and plays a role in uremia-induced cardiomyopathy.

Transfection of miR-29b-3p mimic into primary cultures of cardiac fibroblasts increases miR-29b-3p expression and reverses ouabain-induced miR-29b-3p expression reduction and collagen synthesis in cardiac fibroblasts. To determine whether ouabain regulates collagen production through miR-29b-3p in primary cultures of cardiac fibroblasts, miR-29b-3p mimic was transfected into the cells and cultured for 48 h; mock transfection was used as control. Transfected cells were then treated with ouabain for additional 24 h. Control experiments were conducted to test the efficiency of miRNA mimic transfection using FITC-tagged siRNA. As shown in Fig. 3A, when cells were transfected with 5 nM tagged siRNA, the siRNA was efficiently taken into the cells, and neither the siRNA nor the transfection reagent alone resulted in significant cell death or morphological changes. This indicates that these
primary cells were capable of internalizing the miRNA mimics and that they would not be deleterious to the survival of the cells at the concentrations used in the experiments to follow.

To show that the transfection protocol did not affect the ability Na/K-ATPase signaling in these cells to reduce the expression of miR-29b-3p and increase collagen 1A1 mRNA expression, we treated cardiac fibroblasts with 100 nM ouabain for 24 h following 48 h of mock transfection. As expected, simply using the transfection conditions without mimic resulted in the expected decrease in miR-29b-3p expression by 59.3 ± 8.5% (P < 0.001) vs. controls. Next we tested the ability of the miR-29b-3p mimics to prevent Na/K-ATPase signaling-induced changes in miRNA and mRNA expression as detailed above. The results show that transfection of miR-29b-3p mimics at 1, 5, and 25 nM increased cellular miR-29b-3p significantly compared with control at all concentrations used (Fig. 3B). Collagen 1A1 mRNA expression increased by 2.9 ± 0.4-fold (P < 0.001) in ouabain-treated cells following mock miRNA transfection, whereas transfection with miR-29b-3p mimics at 1, 5, and 25 nM for 48 h, prevented ouabain-induced increased in collagen 1A1 mRNA expression (P < 0.01) (Fig. 3C). These data demonstrate the ability of miR-29b-3p mimic to compensate for Na/K-ATPase signaling-induced decreases in miR-29b-3p expression and increases in collagen 1A1 mRNA expression at the transcriptional level.

To determine if the regulation of miR-29b-3p occurred at the translational level, cell lysates were used to probe for collagen protein expression with Western blotting. The results showed that treatment with 10 or 100 nM ouabain significantly increased collagen 1 protein expression by >50%, and transfection with different concentrations of miR-29b-3p mimics ameliorated the increase in collagen protein seen with ouabain alone in cardiac fibroblasts (Fig. 4A). We also probed collagen produced and secreted into the culture medium after ouabain treatment with or without miR-29b-3p mimic transfection. As shown in Fig. 4B, ouabain treatment alone increased collagen production in the culture media, whereas transfection with miR-29b-3p mimic significantly reduced ouabain-induced collagen production in culture media. These data suggest that miR-29b-3p is a mediator of ouabain-induced collagen synthesis.

Inhibition of Na/K-ATPase/src signaling by pNaKtide prevents ouabain-induced miR-29b-3p reduction and collagen expression both in vitro and in vivo. The primary step when CTS bind to and activate Na/K-ATPase-induced signaling is the activation of membrane-associated Src kinase (35). This mode of signaling does not occur in TGF-β-induced fibrosis and, as such, provides a novel signaling node by which to manipulate downstream signaling to evaluate specific Na/K-ATPase-related changes in miRNA expression. To accomplish this, we used pNaKtide, a specific peptide inhibitor of Src that is derived from the sequence of Na/K-ATPase (21), to inhibit CTS-induced Na/K-ATPase signaling in vitro followed by analysis of miR-29b-3p and collagen mRNA and protein expression. Ouabain alone activates Src phosphorylation at tyrosine 418 in cardiac fibroblasts, and that phosphorylation was blocked by pNaKtide (Fig. 5A). As shown in Fig. 5B, pNaKtide alone did not significantly alter miR-29b-3p expression compared with nontreated controls. However, pNaKtide treatment prevented ouabain-induced decreases in miR-29b-3p (P < 0.01). Consistently, pNaKtide blocked ouabain-induced increases in mRNA of collagen 1A1 (Fig. 5C). As shown in Fig. 6, pNaKtide also blocked ouabain-induced collagen expression in cell lysates as well as collagen production in culture media from these cells. Collectively, these data indicate that Na/K-ATPase/Src signaling mediates miR-29b-3p and collagen 1

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Fig. 5. Inhibition of Na/K-ATPase signaling by pNaKtide prevents ouabain-induced miR-29b-3p reduction and collagen synthesis. Primary cultures of adult rat cardiac fibroblasts were pretreated with 1 μM pNaKtide for 15 min followed by ouabain treatment for 24 h. Nontreated or ouabain treatment alone without pNaKtide was used as control. Cell lysates were collected in RIPA buffer for Western blot or in Qiazol for RNA extraction. A: expression of phospho-Src at Y418 (pSrc Y418) was measured by Western blot. Total c-Src and GAPDH were used as loading control. B: miR-29b-3p was measured by qPCR. C: collagen 1A1 mRNA expression was measured by qPCR. **P < 0.01 vs. control, ***P < 0.001; !P < 0.05, !!P < 0.01 vs. ouabain treatment alone.
expression in cardiac fibroblasts and implicate Na/K-ATPase signaling in the development of uremia-induced cardiac fibrosis.

DISCUSSION

In this study we have revealed through qPCR miRNA expression measurements, that significant downregulation of at least 18 miRNAs is found in the heart of two related experimental models of CKD and that miRNAs known to be involved in regulation of fibrosis were affected. We also show that the regulation of these specific miRNAs is highly consistent in terms of magnitude and direction of expression changes between the two different CKD models, suggesting that CTS-induced Na/K-ATPase signaling is an important regulator of miRNAs in the development and etiology of uremic cardiomyopathy. Additionally, we demonstrate that in vitro, CTS-induced Na/K-ATPase signaling resulted in significant decreases in miR-29b-3p and that transfection of miR-29b mimics prevented CTS-induced decreases in miR-29b and collagen 1 increases in mRNA and protein expression. Finally, we demonstrate that inhibition of Na/K-ATPase signaling with the specific Src inhibitor pNaKtide prevented CTS infusion and PNx-induced increases in cardiac fibrosis marker collagen 1 mRNA and protein expression in vitro; our data revealed that Na/K-ATPase signaling is a novel method for regulating miR-29b-3p expression in the setting of uremia. Although we focused on collagen 1 in this study, other isoforms of collagen may also play important roles in the process of fibrosis as described in the literature (41). Overall our results indicate that regulation of miR-29b-3p through Na/K-ATPase-mediated signaling is important to the development of cardiac fibrosis in the setting of renal failure.

In addition to miR-29b-3p we found that the expression of at least 18 out of 84 miRNAs screened was significantly changed in cardiac tissue from animals subjected to PNx surgery or MBG infusion. These miRNA changes were similar in PNx and MBG-infused left ventricular tissue with the notable exception of miR-122-5p. As shown in Table 1 these miRNA changes aligned with expected changes in fibrotic and apoptotic phenotype.

Fig. 6. pNaKtide prevents ouabain-induced collagen 1 in cell lysates and culture media. Primary cultures of adult rat cardiac fibroblasts were pretreated with 1 μM pNaKtide for 15 min followed by ouabain (10 or 100 nM) treatment for 24 h. Nontreated or ouabain treatment alone without pNaKtide was used as control. After treatment, culture media as well as cell lysates were collected. Expression of collagen 1 was measured by Western blot in cell lysates (A) and in culture media (B); n = 5–6 independent experiments. *P < 0.05, **P < 0.01 vs. nontreated control; †P < 0.05, ‡P < 0.01 vs. ouabain treatment alone.

Fig. 7. Representative signaling pathways evaluated in this work.
notypes found in the literature. We focused on the regulation of miR-29b-3p by CTS-induced Na/K-ATPase signaling changes due to its recognized role in fibrosis. Further studies are needed to elucidate the role of other miRNAs in CKD induced cardiac fibrosis.

When evaluating patterns of miRNA expression that are induced by Na/K-ATPase signaling we found that changes of miRNA levels in samples from PNx and from MBG-infused animals were consistent with respect to magnitude and direction of expression changes across both treatments. The only significant outlier to this trend was miR-122-5p, which increased over 20-fold in MBG-infused animals, while its expression decreased by more than twofold in PNx animals (Supplemental Table S1). Since miR-122-5p was first discovered as a liver-specific microRNA (12), the dramatic increase of miR-122-5p in cardiac tissue from animals subjected to MBG infusion is not well understood. The observation of highly congruent changes in miRNA expression following both PNx (a more general model of uremic cardiomyopathy) and MBG infusion (an Na/K-ATPase signaling-specific model of uremic cardiomyopathy) reveals a specific role for Na/K-ATPase signaling in regulating tissue levels of important miRNAs including antifibrotic miRNAs.

Regulation of miR-29b-3p is typically through the TGF-β/Smad signaling pathway (6, 27, 29, 53). However, studies also suggest that miRNA expression can be altered by Akt and NF-κB signaling pathways (30, 51). Multiple studies from our group and others have revealed important structural features of the Na/K-ATPase complex and how it functions as a receptor to mediate cellular signaling mechanisms (22, 35). Activation of the Na/K-ATPase receptor complex stimulates phosphoinositol-3-kinase (PI3K), which leads to activation of Akt and works in concert with PKCδ (45). Common to both the Na/K-ATPase and TGF-β/Smad, Akt activation of NF-κB may be an important interaction node for different pathways that link profibrotic signaling and miR-29b-3p expression. The significance of the effects of Na/K-ATPase activation in our previous studies indicated that while TGF-β/Smad signaling is permissive of increased fibrosis, increases of in vitro or in vivo TGF-β/Smad regulated signaling were not observed when Na/K-ATPase-regulated signaling was induced in the setting of CKD (9, 10). These data lead us to conclude that Na/K-ATPase signaling regulates an additional and significant source of miR-29b-3p regulation in uremia-induced cardiac fibrosis.

Previously, studies have focused on miR-29b-3p’s ability to regulate fibrosis in several organs and cell types, including the heart, lung, liver, skin, and kidney (6, 27, 29, 30, 41, 53). The antifibrotic ability of miR-29b-3p resides in its ability to directly target type I, III, IV, and V collagen (29); matrix metalloproteases 2, 3, and 9 (MMP2, MMP3, and MMP9); insulin-like growth factor 1 (IGF1); pentraxin 3; fibrillin 1 (FBN1); vascular endothelial growth factor A (VEGFA); platelet-derived growth factor (PDGF); lysyl oxidase (LOX); and integrin beta 1 (ITGB1) (29). Both in vitro and in vivo studies illustrate the ability of miR-29b-3p mimicry to inhibit pulmonary and cardiac fibrosis in relatively acute phenotypes (25, 38). However, studies linking miR-29b-3p in the progression of fibrosis in general or cardiac tissues specifically in the setting of CKD, a chronic condition, are lacking. Our current study used models of CKD in which CTS-induced signaling led to significant decreases in miR-29b-3p expression in vivo and in vitro to establish that link. Moreover, our transfection experiments revealed a capacity for miR-29b to act against fibrosis formation in vitro; experiments are currently under way in our lab to determine whether these effects occur in vivo. Additionally, inhibition of Na/K-ATPase signaling prevented significant decreases miR-29b-3p in vitro, and pNaKtide prevented fibrosis increases both in vitro and in vivo. Figure 7 shows the effects of signaling identified in this work. Taken together these studies illustrate the significant role of both Na/K-ATPase signaling to regulate miRNA in uremia-induced fibrosis and the ability of the regulated miRNA to affect fibrosis formation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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