

Exogenous miR-29a Attenuates Muscle Atrophy and Kidney Fibrosis in Unilateral Ureteral Obstruction Mice

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Renal fibrosis leads to end-stage renal disease, but antifibrotic drugs are difficult to develop. Chronic kidney disease often results in muscle wasting, and thereby increases morbidity and mortality. In this work, adeno-associated virus (AAV)-mediated overexpressing miR-29a was hypothesized to counteract renal fibrosis and muscle wasting through muscle–kidney crosstalk in unilateral ureteral obstruction (UO) mice. miR-29a level was downregulated in the kidney and skeletal muscle of UO mice. The secretion of exosome-encapsulated miR-29a increased in cultured skeletal muscle satellite cells and HEK293 renal cells after stimulation with serum from UO mice. This result was confirmed by qPCR and microRNA deep sequencing in the serum exosomes of mice with obstructed ureters. A recombinant AAV-miR-29a was generated to overexpress miR-29a and injected into the tibialis anterior muscle of the mice 2 weeks before UO surgery. AAV-miR-29a abrogated the UO-induced upregulation of YY1 and myostatin in skeletal muscles. Renal fibrosis was also partially improved in the UO mice with intramuscular AAV-miR-29a transduction. AAV-miR-29a overexpression reversed the increase in transforming growth factor β , fibronectin, alpha-smooth muscle actin, and collagen 1A1 and 4A1 levels in the kidney of UO mice. AAV-green fluorescent protein was applied to trace the AAV route *in vivo*, and fluorescence was significantly visible in the injected/uninjected muscles and in the kidneys. In conclusion, intramuscular AAV-miR-29a injection attenuates muscle wasting and ameliorates renal fibrosis by downregulating several fibrotic-related proteins in UO mice.

Keywords: kidney fibrosis, YY1, MuRF1, α SMA, TGF- β 3, TGF- β 1

INTRODUCTION

KIDNEY FIBROSIS IS a major common hallmark in the development and progression of organ damage in chronic kidney diseases (CKDs) and ultimately leads to end-stage renal failure.¹ Muscle mass loss is a consequence of CKD that leads to muscle wasting, and results in increased morbidity and mortality.^{2,3} Recent studies suggest that attenuating skeletal muscle atrophy improves kidney recovery after injury by reducing renal fibrosis.^{4,5} The mechanism underlying the benefits of these muscle-derived microRNAs remains unknown, and the adeno-associated virus (AAV)-mediated microRNA transfer from skeletal muscles to other organs is poorly understood.

Muscle crosstalk with other organs such as bone, brain, heart, kidney, and adipose tissues has been widely reported.^{4,6–11} Crosstalk occurs between the skeletal

muscle and the kidney in different mouse models.^{4,12} Muscle exercise has renoprotective effects, ameliorates cardiovascular parameters, improves clinical outcomes, and thereby can be a good model for muscle–organ crosstalk.¹³ Kokkinos *et al.* found that physical exercise attenuates the risk of developing CKD.¹⁴ Peng *et al.* found that muscle-specific peroxisome proliferator-activated receptor- γ coactivator-1 α overexpression in mice reduces renal damage and fibrosis, thereby providing myokine-mediated muscle–kidney crosstalk.¹⁵ Hanatani *et al.* presented that Akt1-mediated skeletal muscle growth attenuates renal damage in experimental kidney disease.⁴ The treatment of cardiac injury also improves muscle damage.¹⁶ Muscle wasting therapy can enhance the function of other organs and serve as a major advantage in treating systemic diseases.¹⁷ However, the

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mechanism underlying the benefits of muscle wasting treatment for other organs is not well known.

Several potential mediators of crosstalk, including interleukin 6 (IL-6), IGF-1, and transforming growth factor β (TGF- β), have been identified.^{18–20} Injured renal epithelial cells could increase the production of TGF- β -containing exosomes, which initiate kidney fibrosis through activating fibroblasts.²¹ An increased level of TGF- β /myostatin cascade can suppress the insulin-IGF1/PI3K/Akt signaling pathway, leading to insulin resistance and muscle wasting. We previously found that an increase in TGF- β inhibits miR-29 promoter activity,²² leading to decreased miR-29 in the skeletal muscles and resulting in muscle atrophy.²³ TGF- β is originally geared toward renal repair but could travel to the muscle and promote muscle atrophy. This proatrophic action might occur through Smad2/3 phosphorylation and/or by decreasing miR-29 and alter other fibrosis-related miRs. Our group is currently developing new therapeutic approaches to prevent muscle wasting through the inhibition of the transcription factor myostatin.^{20,24} This protein belongs to the TGF- β superfamily and inhibits muscle growth. An increased myostatin level is found in the muscles of diabetes mellitus mice and induces muscle atrophy.^{20,25}

In this work, AAV-mediated microRNA, which is commonly used for muscular dystrophy treatment, is hypothesized to also attenuate kidney injury through exosome-mediated microRNA delivery. miR-29a (a well-known antifibrotic microRNA) and unilateral ureteral obstruction (UUO) mice (a well-established renal fibrosis model) were used to test our hypothesis. The role of miR-29a in muscle atrophy was examined by evaluating myostatin and muscle regeneration in UUO mice. The effects of intramuscular injection of AAV-miR-29a on renal fibrosis were determined. Finally, the possible mechanism that occurs when circulating exosomal miR-29a transfers from the skeletal muscle to the kidney was identified. The results could serve as a basis for the treatment of muscle atrophy to attenuate kidney damage.

MATERIALS AND METHODS

Animals and UUO mouse model

Two-month-old male mice (C57BL/6J) were obtained from Jackson Laboratories (Bar Harbor, ME). For UUO surgery, the mice were anesthetized with intraperitoneal injection of a combination of 12 mg/kg xylazine and 60 mg/kg ketamine and placed in a prone position. An incision was made in the skin and the subcutaneous tissue along the length of the 11th rib using scissors. Muscles were divided, and pleura were carefully pushed upward, exposing the kidney. The left ureter was visualized and ligated with 4-0 silk at two points below the lower pole of the left kidney. Body weight and food intake were measured three times per week postsurgery. The mice were sacrificed at 7 and 14 days after UUO operation.

Virus and gene delivery

A type 9 recombinant AAV-expressing miR-29a gene (AAV-miR-29a) and a recombinant AAV encoding green fluorescent protein (GFP) (AAV-control) as the control vector were prepared by Emory Integrated Genomics Core. The stock virus titer for AAV-miR29a was 3.4×10^{14} and 6.5×10^{14} for AAV-control. Thereafter, 15 μ L of viral preparation (10^{10}) was injected into the tibialis anterior (TA) muscle of the left leg over 5 min at 2 weeks before UUO surgery.²⁶

Culture of primary muscle satellite cells and HEK293 cells

Satellite cell isolation and culture were performed as previously reported.¹² Fibroblast growth factor was removed for 2 days before the experiments to allow myotube formation.

miRNA sequencing

miRNA-Seq Library preparation and sequencing were performed as previously reported.¹¹

Western blot analysis and antibodies

Total proteins were extracted using radioimmunoprecipitation assay buffer containing protease inhibitor cocktail. Equal amounts (20–50 μ g/lane) were used for Western blot analysis.^{27,28} Primary antibodies: type I collagen (1:1,000), PTEN (1:1,000) were obtained from Santa Cruz; TGF- β (1:1,000) was purchased from R&D, and GAPDH (1:1,000) was from Millipore. TRIM63/MuRF1 (1:1,000) and Fibronectin (1:1,000) were from Sigma-Aldrich. Protein bands were detected using the Li-COR Odyssey infrared scanning system.

RNA extraction and quantitative real-time PCR

Total RNA from muscle and kidney were extracted using Tri-Reagent (Molecular Research, Inc., Cincinnati, OH). Real-time qPCR was used with the following PCR parameters: 94°C for 2 min, 45 cycles at 95°C for 15 s, and 60°C for 60 s. The mouse U6 gene and miR-103 were used as the standard for evaluating individual miRNA levels in the tissue and serum, respectively. miRNA expression was calculated as the difference between the threshold values of the two genes ($\Delta\Delta CT$).

Muscle and kidney histology

Skeletal muscle immunohistology was performed in accordance with an established procedure.²⁹ In brief, TA muscles were embedded in Tissue Freezing Media and cross-sections (10 μ m thick) stained with an antilaminin antibody (1:50 dilution; Sigma-Aldrich) to evaluate cross-sectional area. At least 500 individual myofibers per muscle were measured. Immunohistochemical stains for kidney were performed on formalin-fixed and paraffin-embedded 3 μ m sections. The ratio of primary antibodies

against fibronectin was 1:200. Masson's trichrome staining was used to assess collagen levels in the kidney. All immunohistochemical analyses were repeated at least three times, and representative images were presented.

Statistical analysis

Data were presented as mean \pm standard error. The statistical significance of the difference between the control and treatment groups was determined by simple ANOVA followed by Dunnett's multiple comparison tests. Student's *t*-test was used to identify significant differences

between two groups. Statistical significance was defined as *p*-values <0.05 .

RESULTS

Skeletal muscle and kidney release miR-29a into the serum through exosomes

miRs were measured in the serum exosome to identify whether kidney disease altered microRNA in the serum. Serum exosome was isolated from sham and 7 days UUO mice and tested using a NanoSight instrument (Fig. 1A). The exosome size was significantly increased in the UUO

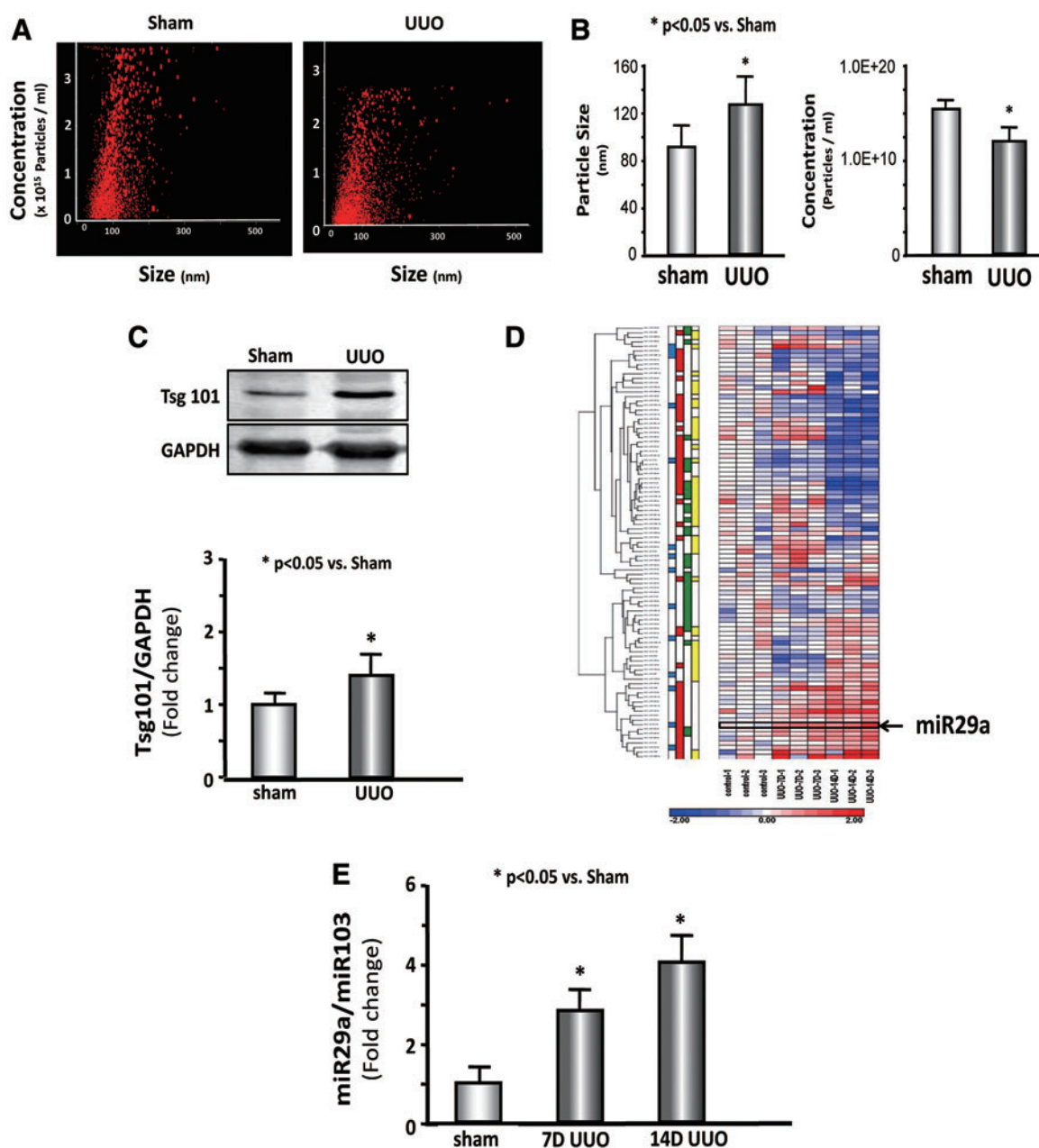


Figure 1. miR-29a-3p levels increased in the serum exosome of UUO mice. (A, B) Serum exosome size and concentration were measured by NanoSight Instruments in 7d UUO mice and sham mice ($n=5$ /group). (C) Expression and bar graph of Tsg101 in 7d UUO mice and sham mice ($n=9$ /group). (D) Heat map graph showing that miR-29a increased in serum exosomes from UUO mice compared with controls at 7 and 14 days. (E) Expression of miR-29a-3p/miR-103 ($n=9$ /group). 7d UUO, 7 days unilateral ureteral obstruction. Color images are available online.

serum; however, exosome concentration was decreased in UUO serum compared with shams (Fig. 1B). The exosome protein marker, Tsg101, was used to measure exosome abundance; Tsg101 was significantly increased in UUO serum exosome (Fig. 1C). miRNA deep sequencing was performed, and RNA was isolated from serum exosomes of 7-day and sham mice. A total of 53 miRs were altered in the serum exosomes from UUO mice compared with controls at 7 days (Fig. 1D and Supplementary Table S1). The expression of serum exosome miR-29a-3p was increased by 2.2-fold, miR-29c-3p was increased by 1.4-fold, and miR-29b was not statistically different. Real-time qPCR confirmed that the miR-29a-3p of serum exosome was increased 2.4-fold in 7 days and 4.1-fold in 14 days UUO mice versus sham mice (Fig. 1E).

miR-29a-3p was reduced in the kidney and skeletal muscle of UUO mice versus shams.¹² Skeletal muscle satellite cells and human embryonic kidney cells (HEK293) were cultured, and treated with 5% pooled serum from UUO mice and normal mice serum for control to investigate this opposing change in miR-29a. The blood urea nitrogen level of the pooled serum was ~60 mg/dL (vs. control levels of ~18 mg/dL). UUO serum was replaced with exosome-free serum 48 h before cell harvest, and exosomes were isolated from the culture medium. The expression of miR-29a-3p was decreased by 20% in the lysates of satellite cells (Supplementary Fig. S1A) and decreased by 49% in HEK293 cells treated with UUO serum (Supplementary Fig. S1B). However, miR-29a-3p was 1.5-fold increased in the exosomes isolated from the culture medium of satellite cells (Supplementary Fig. S1C) and 1.9-fold increased in the culture medium of HEK293 cells (Supplementary Fig. S1D). These data suggest the possibility that skeletal muscle and kidney release exosome-carried miR-29a into the circulation in UUO mice.

AAV-miR-29a attenuated the upregulation of muscle atrophy proteins in UUO mice

AAV-miR-29a and AAV-control (GFP) were injected into the TA muscle of mice. GFP was significantly visible in the TA muscle of AAV-control injected mice (Fig. 2A). In the muscle injected with AAV-miR29a, the expression of miR-29a-3p was increased 6-fold in the sham mice and 5.1-fold in the UUO muscle at 14 days (Fig. 2B). A time-dependent increase in miR-29a expression was observed. miR-29a expression also appeared to accumulate in the

uninjected muscle of UUO mice because miR-29a-3p was increased in the contralateral TA muscle, soleus, and extensor digitorum longus (EDL) of the UUO mice compared with the levels of the muscle of sham mice (Fig. 2C).

Intramuscular injection of AAV-miR29a into the TA muscle prevented body weight loss and reversed the decrease of the soleus and EDL muscle mass in UUO mice; however, this treatment had no effect on body weight and muscle mass in sham mice (Fig. 2D, E). The effects of Exo/miR-29a on muscle regeneration-related protein markers were examined through Western blot analysis.²³ The protein abundance of YY1, a transcription factor that inhibits muscle regeneration, was increased in UUO muscle. The muscle atrophy markers MuRF1 and PTEN were also increased. The muscle proliferation and differentiation markers myoD and myogenin were decreased in the muscle of UUO mice. miR-29a intervention retarded all these changes (Fig. 2F). Furthermore, the muscle cross-section area was decreased in UUO muscle; however, overexpressing miR-29a caused a shift to a large cross-sectional area compared with the results in UUO muscles (Fig. 2G). As such, injecting AAV-miR29a in TA muscle could attenuate muscle atrophy and body weight loss in UUO mice.

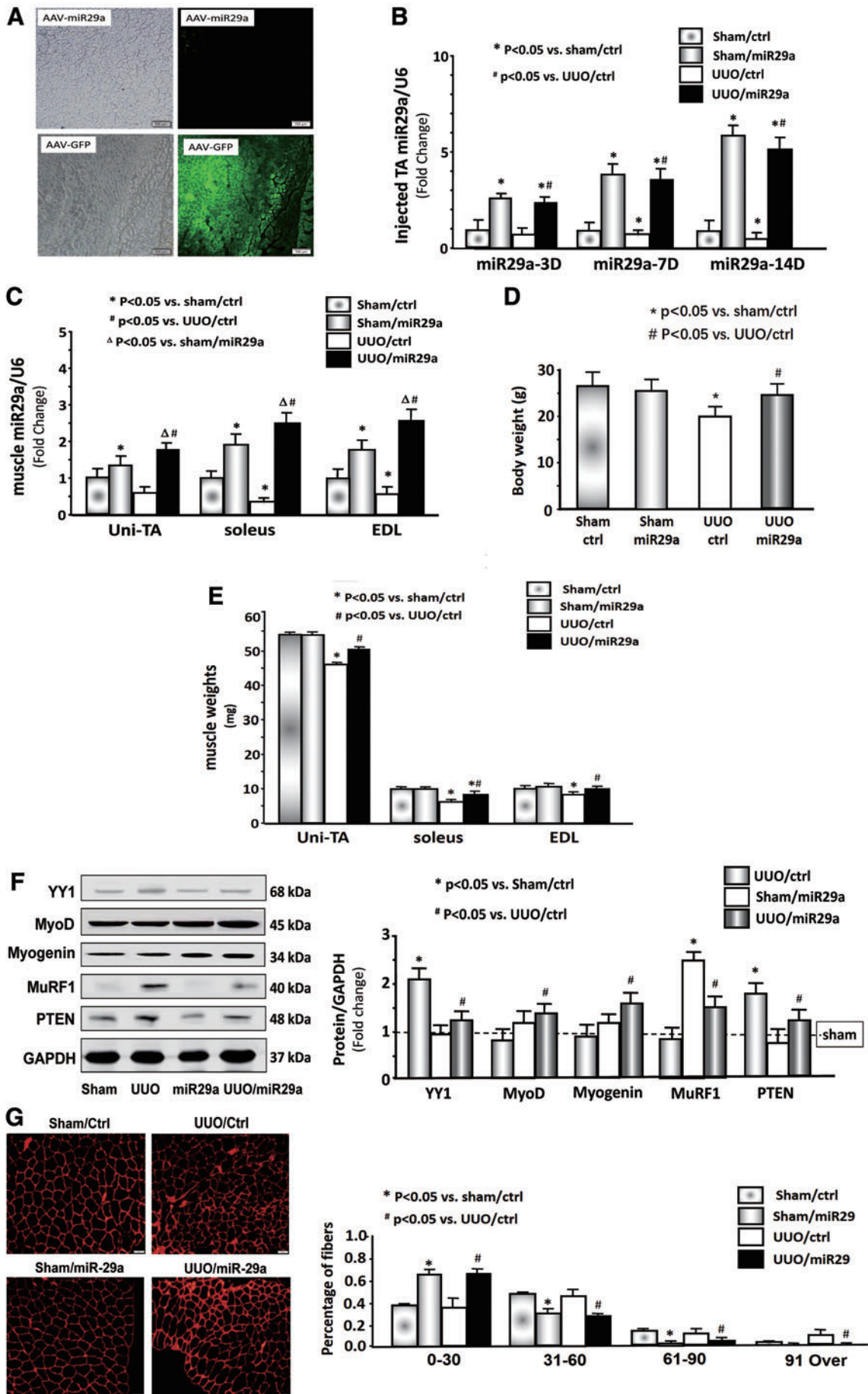
Intramuscular injection of AAV-miR-29a attenuated renal fibrosis in UUO mice

Collagen staining (indicated by blue color) as indicated by Masson's trichrome staining was sharply increased in the 14-day UUO kidney in the presence or absence of AAV-ctrl treatment. However, AAV-miR29a treatment significantly attenuated this change (Fig. 3A). Fibronectin is a glycoprotein of the extracellular matrix and closely associated with newly deposited collagen fibrils.³⁰ The amount of fibronectin is significantly increased in the kidney of UUO mice and attenuated through AAV-miR29a treatment (Fig. 3B). In addition, miR-29a limited the increase in fibrosis-related proteins,³¹ including TGF- β 1, TGF- β 3, and collagen 1A1. Transcription factor YY1 (a profibrotic protein)³² is increased in the kidney of UUO mice and reduced after the intramuscular injection of AAV-miR29a (Fig. 3C). These results suggest that miR-29a overexpression in the skeletal muscle can improve renal fibrosis in UUO mice.

Evidence of muscle-kidney crosstalk

In serum exosome, miR-29a was significantly increased in the sham and UUO mice after AAV-miR29a trans-

Figure 2. Provision of AAV-miR29a attenuated UUO-induced muscle loss. **(A)** Representative frozen cross-sections from TA muscles transduced with AAV in the normal control mice. The *right panels* show fluorescence microscopy images for detecting GFP expression; the *left panels* show bright-field microscopic images of muscle cross-section. The *top panels* show microscopy images in mice with AAV-miR-29 transduction. The *bottom panels* show microscopy images in mice with AAV-GFP transduction. **(B)** miR-29a/U6 expression in the TA muscle ($n=9$ /group). **(C)** miR-29a/U6 expression in the uninjected TA (Uni-TA), soleus, and EDL ($n=9$ /group). **(D)** Body weights and **(E)** muscle weights of sham-operated and UUO cohorts ($n=9$ /group). **(F)** Expression of YY1, myoD, myogenin, MuRF1, PTEN, and GAPDH. The bar graph shows the fold change of each protein band compared with the levels in sham mice (represented by a *line* at one fold) ($n=6$ /group). **(G)** The representative cross-sectional area of TA muscle of sham/ctr and UUO cohorts ($n=9$ /group). AAV, adeno-associated virus; EDL, extensor digitorum longus; GFP, green fluorescent protein; TA, tibialis anterior; UUO, unilateral ureteral obstruction. Color images are available online.



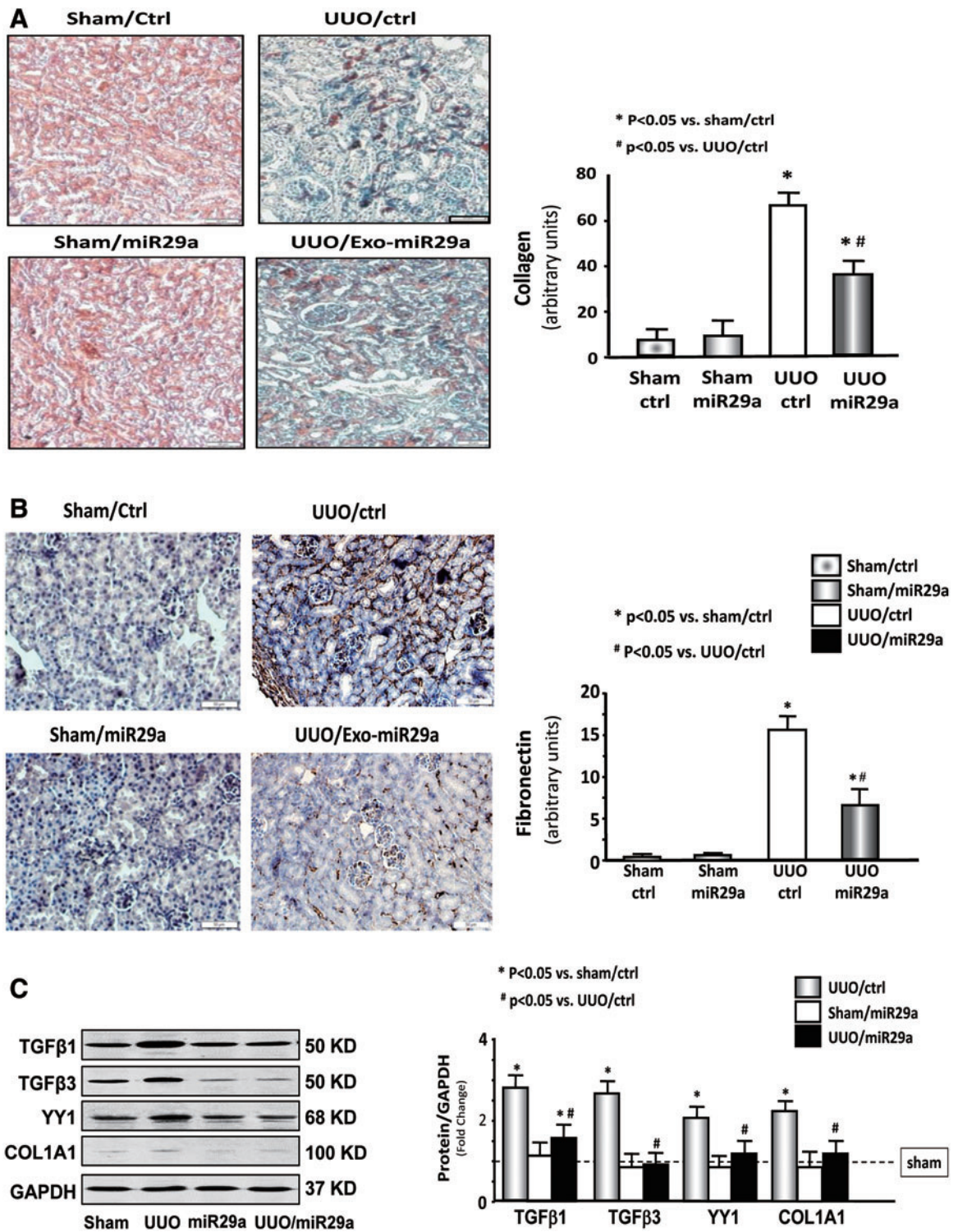


Figure 3. Exogenous miR-29a in skeletal muscle can attenuate fibrosis progression in the UUO kidney. **(A)** Masson's trichrome staining of paraffin sections from kidneys of various groups as indicated. The bar graph shows the collagen amount ($n=6$). **(B)** Immunostaining for fibronectin and bar graph showing the amounts of fibronectin in various groups as indicated ($n=6$ /group). **(C)** TGF- β 1, TGF- β 3, YY1, and collagen 1A1 expression in kidney lysates from different groups of mice. The bar graph shows the fold change of each protein band compared with levels in sham mice (represented by a line at onefold). ($n=9$ /group). TGF- β , transforming growth factor β ; UUO, unilateral ureteral obstruction. Color images are available online.

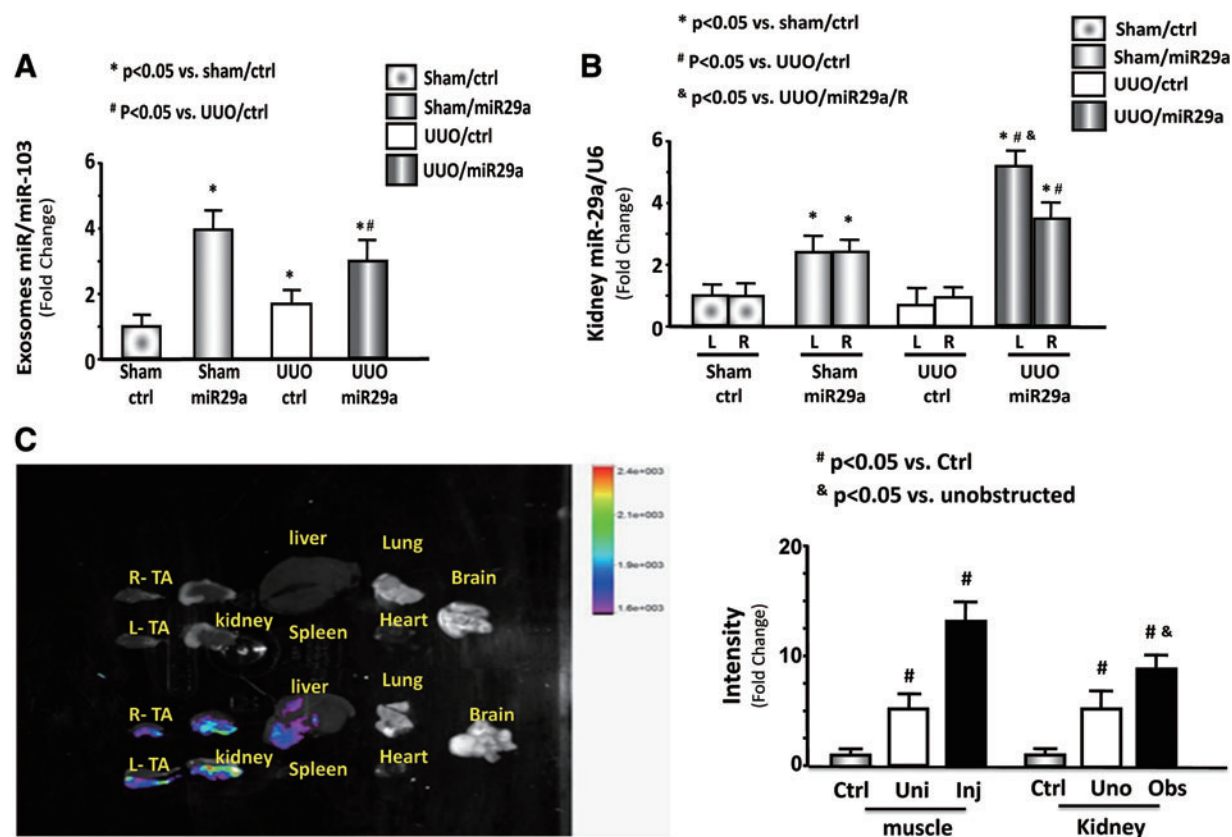


Figure 4. Evidence of miR-29a travel from the muscle to the kidney through exosome circulation of exosomes. **(A)** miR-29a/miR103 expression and bar graph showing miR expression in the serum exosomes of each group of mice. ($n=6/\text{group}$). **(B)** Expression and bar graph of miR-29a/U6 in the right and left kidneys of each group of mice ($n=6/\text{group}$). **(C)** Representative fluorescent organ images. Mice were injected in the left TA muscle with AAV/miR-ctrl (GFP positive). The fluorescence was assessed 14 days after injection. In each pair, the left kidney received UUO ligation (Obs=obstructed kidney), and the right was not obstructed (Uno=unobstructed kidney). The left TA muscle was transduced by AAV (Inj=injected muscle) and the right muscle was not (Uni=uninjected). Bar graph showing GFP intensity from AAV-GFP transduced mice compared with non-AAV injection mice ($n=3/\text{group}$). AAV, adeno-associated virus; GFP, green fluorescent protein; TA, tibialis anterior; UUO, unilateral ureteral obstruction. Color images are available online.

duction compared with AAV-ctrl (Fig. 4A). In the kidney, miR-29a expression was higher in the intramuscular injection of AAV-miR-29a than that of AAV-ctrl. The level of miR-29a was significantly higher in the obstructed kidney (left) than that in the unobstructed kidney (right) of the UUO mice (Fig. 4B). In addition, AAV-GFP was tracked with In-vivo Xtreme camera system. After TA injection with AAV-GFP, the fluorescence levels were increased in the kidney and noninjected muscle. Compared with other organs, UUO kidneys, including non-UUO kidney, showed the strongest fluorescence (Fig. 4C). These results suggest that miR-29a travels from the muscle to kidney possibly through exosome circulation.

DISCUSSION

This study aimed to determine whether AAV-mediated miR-29a transfer can suppress muscle wasting and renal fibrosis. AAV-miR-29a attenuated muscle wasting and limited renal fibrosis by inhibiting YY1 and PTEN in skeletal muscle and YY1, TGF- β 1, and TGF- β 3 in the

kidney in an UUO-induced kidney damage. Our observations provided a rationale that exogenous miR-29a has multiple functions in a kidney disease mouse model.

Treatment of muscle wasting can improve the function of other organs, thereby promising a major therapeutic advantage in the treatment of systemic diseases.¹⁷ The benefit of exercise in improving clinical outcomes in a wide range of diseases is a good example of muscle-organ crosstalk.¹⁴

The three members in the miR-29 family are a, b, and c. The effects of miR-29 on muscle atrophy remain controversial (subset dependent). The present group and other researchers found that miR-29a was decreased in muscle atrophy models; an increase in miR-29a could prevent muscle atrophy in CKD, cancer, and Duchenne muscular dystrophy animal models.^{23,33,34} However, Li *et al.* found that overexpressing miR-29b contributes to muscle atrophy in culture C2C12 cells and in denervation-induced muscle atrophy.³⁵ The different results are attributed to subset difference. In this study, miR-29a overexpression can ameliorate muscle wasting induced by UUO and attenuate renal fibrosis.

Our group found that miR-29 directly reduces TGF- β induced by targeting TGF- β 3, thereby upregulating fibrosis signaling.¹² In this study, the major benefit of miR-29a treatment for both muscle and kidney is the transcription factor YY1 inhibition. In the muscle, miR-29a decreases phosphatase and PTEN expression and YY1 protein levels. Both are negative regulators of myogenesis and muscle protein metabolism.³⁶ YY1 plays a negative role in myogenesis by directly repressing the synthesis of various genes, including skeletal alpha-actin (α -actin),³⁷ muscle creatine kinase (MCK), and myosin heavy chain IIb (MyHCIIb).^{38,39} The transcription factor YY1 occupies regulatory regions on these muscle-specific genes preventing transcription. Upon transcriptional activation, YY1 is displaced by serum response factor (SRF). SRF recruits the MyoD transcription factors, which initiate transcription of the muscle proteins (activated state).³⁹ Hence, YY1 inhibition of satellite cell proliferation is the main mechanism leading to muscle wasting. Restoration of miR-29a in the kidney, at least in part, is through YY1 because it directly upregulates alpha smooth muscle actin and collagen³² and limits renal fibrosis.

This study found that serum microRNA levels might not reflect the levels in the organs. Decreasing miR-29a levels in the skeletal muscle and kidney reflect miR-29a secretion from the tissue into circulation. In a cell culture model, UUO serum enhanced miR-29a release from satellite cells through exosomes. This phenomenon could explain miR-29a decrease in organs and its increase in serum exosomes (Supplementary Fig. S1). Chen *et al.* have revealed that TGF- β overexpression during the progression of kidney fibrosis downregulates miR-29 and is consistent with the findings of this study.⁴⁰ Conversely, miR-29 overexpression regulates TGF- β expression and attenuates fibrosis.²²

In conclusion, this study suggests that treatment of muscle wasting through AAV-mediated miR-29a transfer will inhibit kidney fibrosis, thereby offering a potential

treatment strategy to decrease morbidity and improve the quality of life of patients with multiple organ injury.

DISCLAIMER

The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH, the Department of Veterans Affairs, or the US Government.

COMPLIANCE WITH ETHICS REQUIREMENTS

All Institutional and National Guidelines for the care and use of animals were followed.

AUTHORS' CONTRIBUTIONS

H.W. and X.H.W. designed the study; B.W. and J.W. carried out experiments; W.H. and Y.Z. analyzed the data; A.Z., F.H., F.M., and Y.L. made the figures; J.D.K., X.H.W., and H.W. drafted and revised the article; all authors approved the final version of the article.

AUTHOR DISCLOSURE

No competing financial interests exist.

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Table S1

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