YMTHE, Volume *27*

Supplemental Information

Exosome-Mediated miR-29 Transfer Reduces Muscle Atrophy and Kidney Fibrosis in Mice

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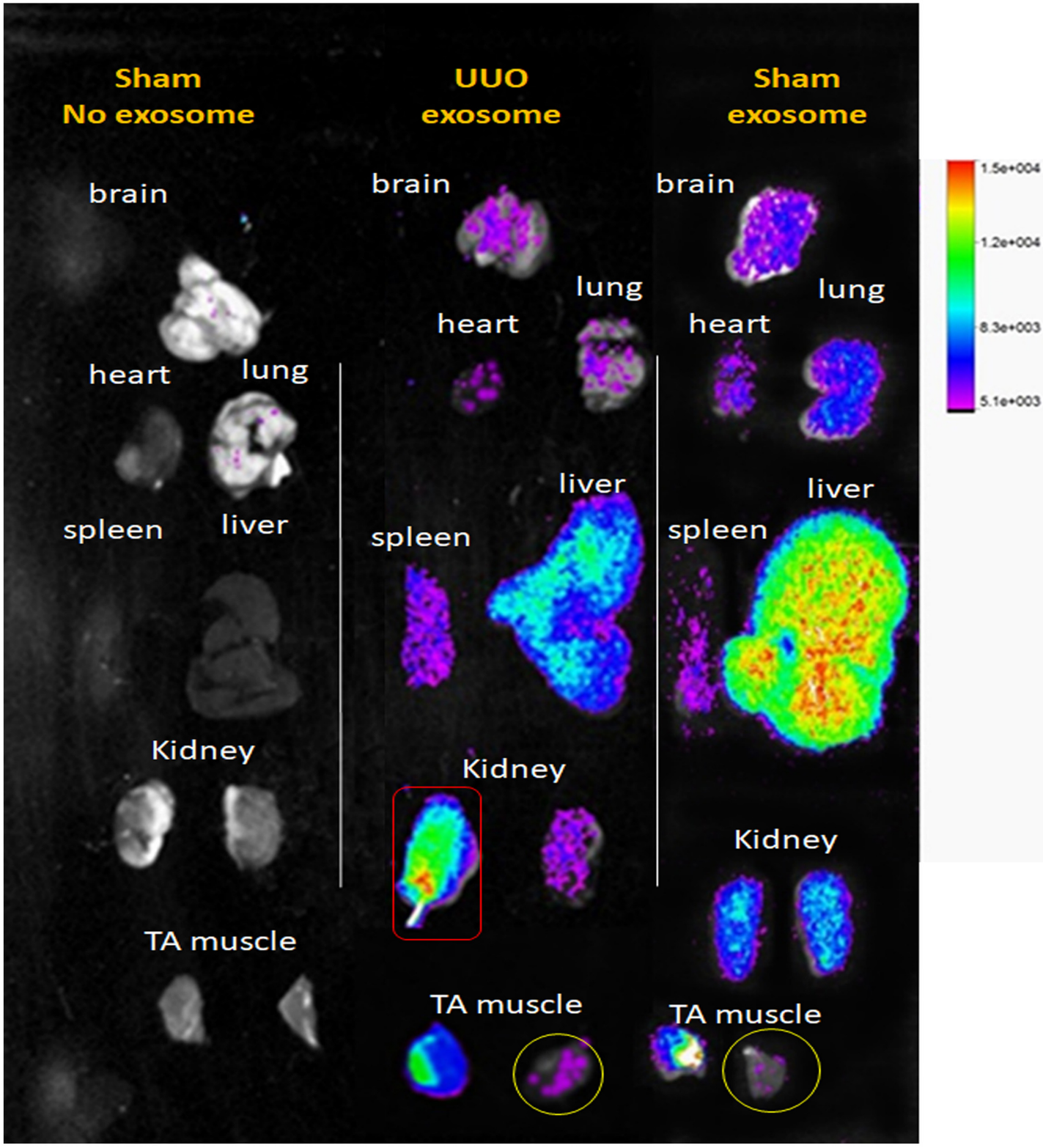
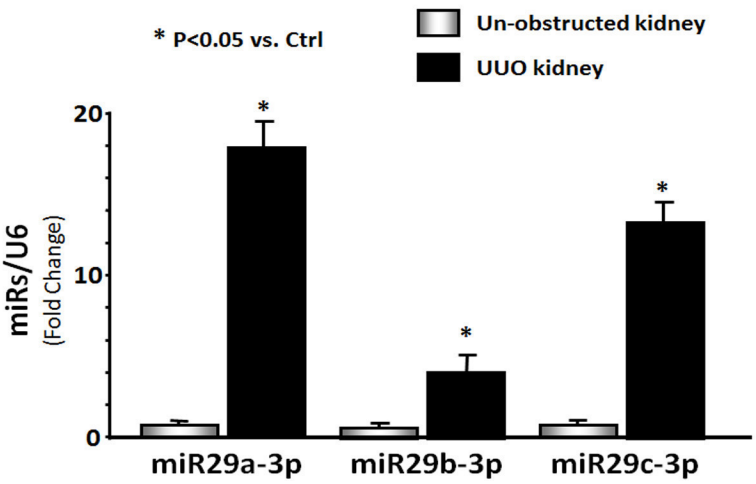
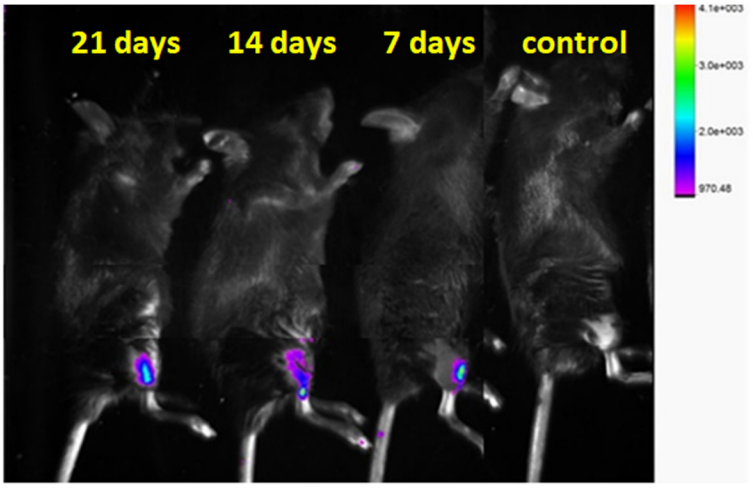


Supplement Figure 1: the fluorescently labeled Exo/miR -29 distribution in organs following intramuscular injection. Mice were injected in the left TA muscle with Exo/miR-29 labeled with1 μmol/l fluorescent lipophilic tracer DiR at the same time as UUO surgery. The injection was repeat once per week. Shown are representative fluorescent organs images at 14 -days that were acquired using a Bruker Small Animal Optical Imaging System. Panels from left to right: Sham operated with no exosome/DiR injection, UUO mouse with exosome/DiR injection at 14 -days and sham operated with exosome injection. The UUO kidney is in red square and the right TA that did not receive any injection is designated within a yellow circle.

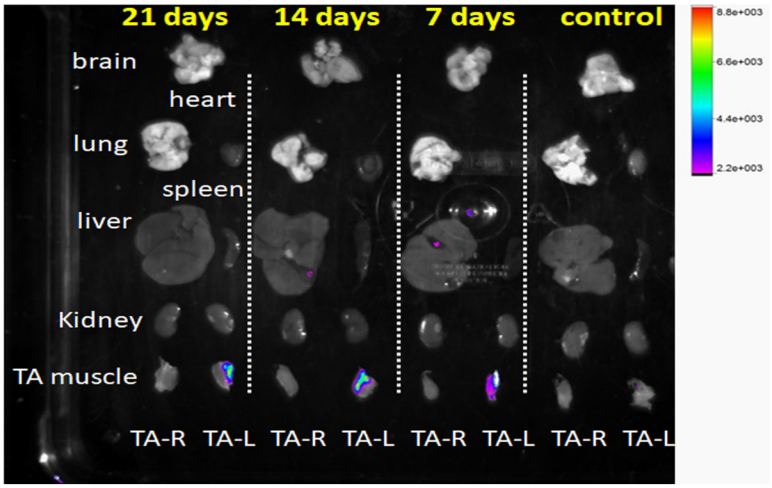


Supplement Figure 2: the increase of miR-29 in the obstructed kidney was more than in the unobstructed kidney. The expression of miR-29a-3p, miR-29b-3p and miR-29c-3p were assayed by real time qPCR in UUO mice. The bar graph shows miR-29s expression from the obstructed kidney compared with levels in non-obstructed kidney (represented by 1-fold). Results are normalized to U6. (Bars: mean ± s.e.; n=6/group; *=p<0.05 vs. Exo/ctrl). The expression of miR-29a, b and c was increased in the obstructed kidney by 18-, 4- and 13-fold, respectively.

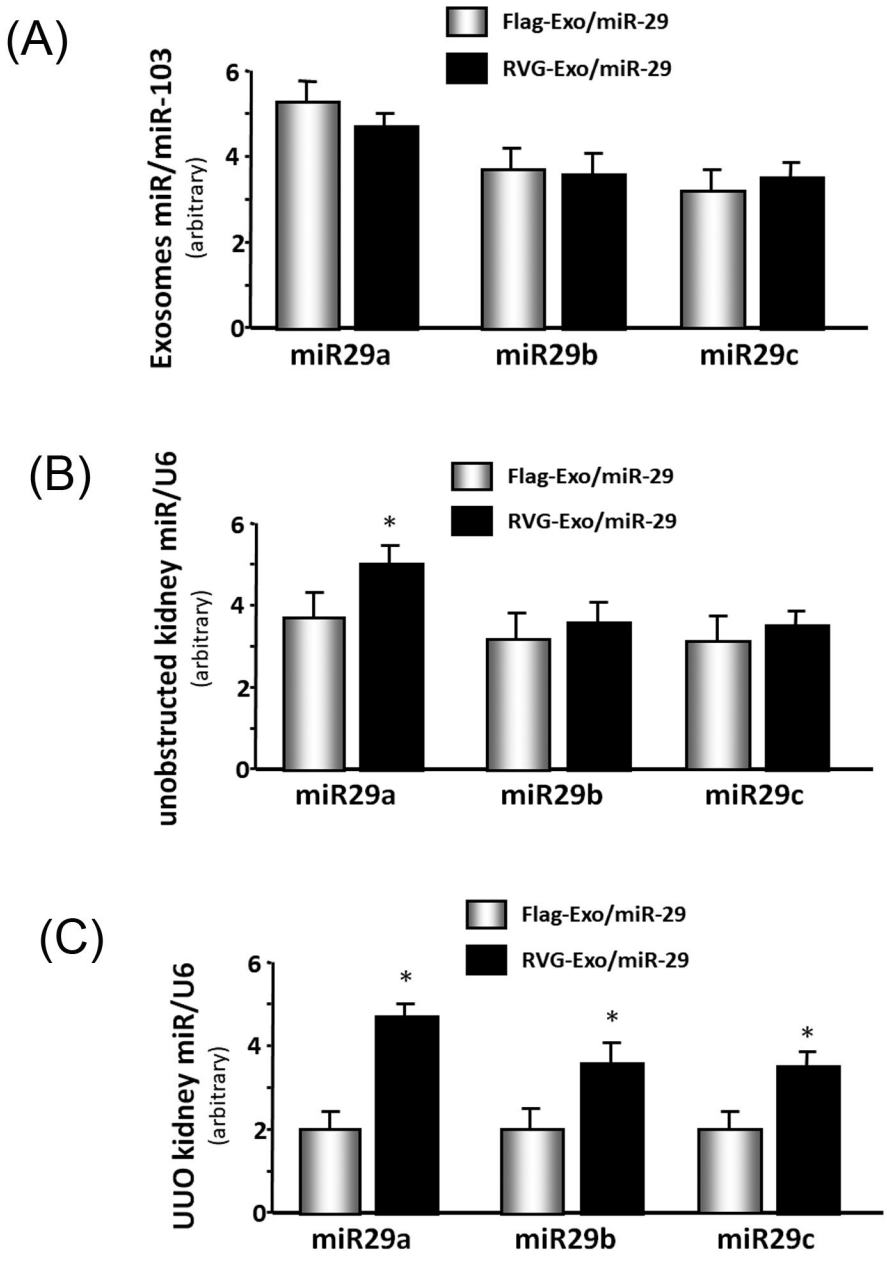
(A)



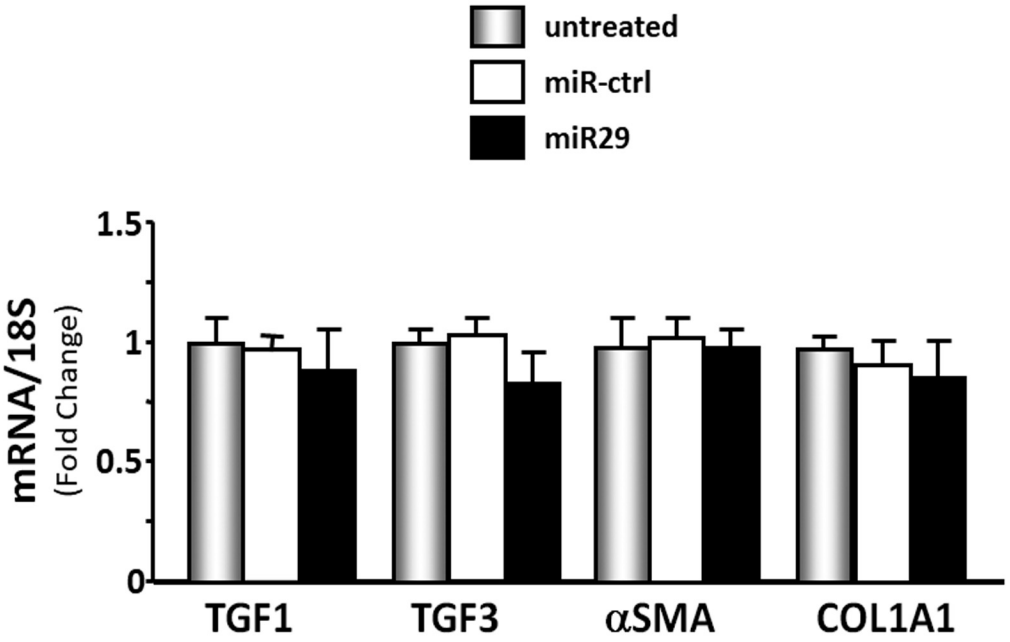
(B)



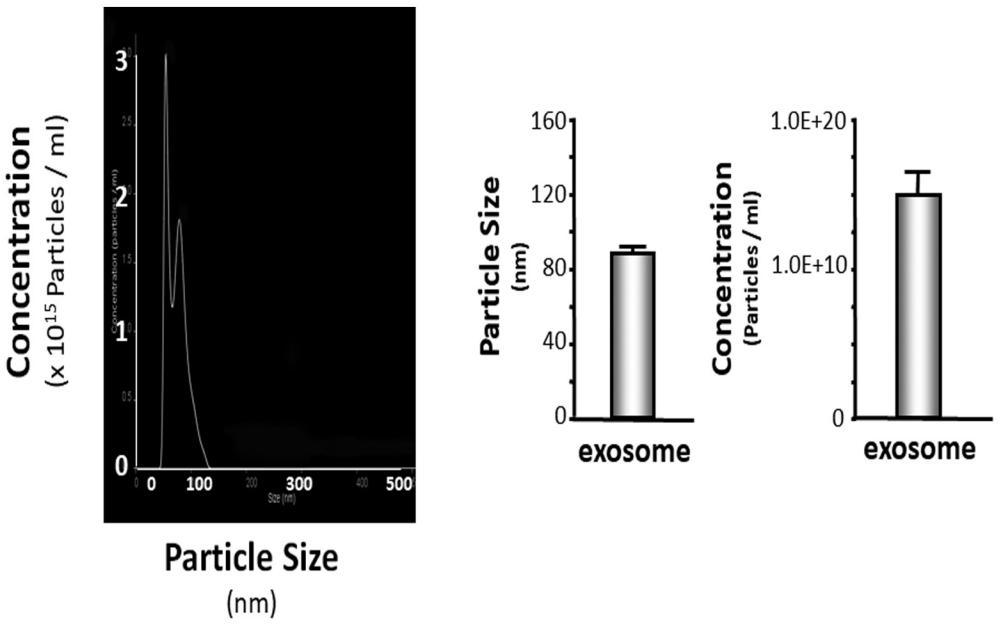
Supplement Figure 3: injection of the DiR only into the left TA muscle did not result in multiple organ distribution. Supplement Figure 3: injection of the DiR only into the left TA muscle did not result in multiple organ distribution. The fluorescence distribution was determined in muscle and organs at 7, 14 and 21 days after intramuscular injection of DiR without exosome and microRNA in normal mice. (A) Images were taken of whole mice; the right control mouse did not receive any DiR injection. (B) Each organ was removed from the mice that received intramuscular injection of DiR in the left TA muscle.



Supplement Figure 4: Compare the miR-29 expression in Flag-Exo/miR-29 and RVG-Exo/miR-29 injected mice. (A) RNA was isolated from serum exosome of UUO mice. (B) RNA was isolated from un-obstructive kidney of UUO mice. (C) RNA was isolated from obstructive kidney of UUO mice. The expression of miR-29a-3p, miR-29b-3p and miR-29c-3p were assayed by real time qPCR. The bar graph shows miR expression in an arbitrary unit. Results are normalized to miR-103 (serum) or U6 (kidney). (Bars: mean ± s.e.; n=6/group; *=$p<0.05$ vs. Flag-Exo/miR-29).



Supplementary Figure 5: Compare the mRNA expression in miR-control, miR-miR-29 and untreated HEK293 cells. Cultured cells were treated with Ad-miR-ctrl (miR-ctrl), Ad-miR29 (miR29), and untreated. Total RNA was extracted from the cells 24 hours after treatment. The expression of TGF-β1, TGF-β3, α-SMA and collagen 1A1 (COL1A1) were assayed by real time qPCR. The bar graph shows mRNA from the cells of each group compared with levels in untreated. Results are normalized to 18S (Bars: mean ± s.e.; n=4/group).



Supplementary Figure 6: The size and concentration of exosomes isolated from satellite cell culture medium. The exosome size and concentration was measured a NanoSight instrument. The bar graphs shows the size (x axis, nanometer diameter) and concentration (Y axis, Particles / ml) of exosomes from serum of 3 of mice (Bars: mean ± s.e.; n=3/group).

Supplementary Materials and Methods

Animals and unilateral ureteral obstruction (UUO) Model: These experiments were approved by the Emory University IACUC (protocol 141-2008). The mice (C57BL/6J) were from Jackson Laboratories (Bar Harbor, ME, USA) and were housed with a 12-hour light/12-hour dark cycle. For UUO surgery, mice were anesthetized with intraperitoneal injection of a combination of 12 mg/kg xylazine and 60 mg/kg ketamine and were placed in a prone position. An incision was made in skin and subcutaneous tissue along the length of the 11th rib using scissors. Muscles were divided and pleura was carefully pushed upward, exposing the kidney. The left ureter was visualized and ligated with 4-0 silk at two points just below the lower pole of the left kidney. The ureter was cut between the two ligatures in order to prevent retrograde urinary tract infection. The sham operation consisted of a similar incision and identification of the left ureter, but no ligation of the ureter was performed. The mice were terminated at 3, 7, 14 and 30 days after the UUO operation. Blood urea nitrogen (BUN) was measured with a BUN Kinetic Procedure Kit (Thermo Electron, Louisville, CO.).

Generation of exosome encapsulated miR-29a: Satellite cells were grown to 60% confluence in DMEM/F12 culture medium containing 20% fetal bovine serum. The Lamp2b-RVG vector was transfected into satellite cells using the Effectene transfection reagent (Qiagen, Valencia, CA). Six hours after transfection, the cells were transduced with Ad-miR29abc (adenovirus containing miR-29ab1 and -b2c precursor sequences)[1]. Control cells were transduced with Ad-empty for production of RVG-exosome-control (Exo/ctrl). 24 hours after transfection/transduction the culture medium was exchanged for a medium with extracellular vesicle free serum (EVFS) and cultured for an additional 48 hours to allow exosomes to be released into the medium. The RVG-exosomes enriched with miR-29abc (Exo/miR29) were harvested from culture medium and re-suspended in PBS (supplement Figure 4). The increased expression of miR-29a, b and c in the exosomes was assayed by qPCR (Figure 2A). The yield of exosomes from the culture medium, approximately 1.5×10^{15} particles/ml, was determined using a NanoSight instrument measurement (Figure 2A). Previously study showed that RVG is located on external exosome membrane[37].

Exosome isolation, NanoSight measurement and in vivo imaging: To isolate exosomes, cell debris and non-exosome organelles were removed from either serum (diluted 5x with PBS) or culture medium (undiluted) by centrifugation at 1,000 g for 10 min, 4^{o}C. The supernatant fraction

was further centrifuged at 16,000 g for 30 min, 4°C. This second supernatant was sterile filtered through a 0.22 μm filter. Exosomes were pelleted from this filtrate at 120,000 g for 90 min at 4°C (L8-70M ultracentrifuge, Beckman-Coulter, Indianapolis IN). Exosome pellets were re-suspended in PBS and protein was quantified using a Bradford protein assay (Bio-Rad, Hercules, CA). Exosome concentration and size (Figure 2B) were measured using nanoparticle tracking analysis (NanoSight NS300, Malvern Instruments, Inc. Westborough, MA). The exosomal protein marker, TSG101, was assessed by Western blot (Figure 2C). *In vivo* exosome distribution was determined with the Bruker Small Animal Optical Imaging System (In-Vivo Xtreme II; Billerica, MA).

Culture of primary muscle satellite cells and HEK293 cells: Satellite cells were isolated from the hindlimb muscles of 4-month old mice. A Skeletal Muscle Dissociation Kit (130-098-305; MACS, Miltenyi Biotec, Inc. Auburn, CA) was used to dissociate mouse skeletal muscle tissue into cell suspensions and a Satellite Cell Isolation Kit (130-104-267, MACS) was used to isolate satellite cells. Cells were cultured in Ham's F-10 Nutrient Mixture medium (Invitrogen) with 20% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin (growth medium). Isolated satellite cells were passaged a maximum of 5 times. Myotube differentiation was inhibited by the addition of 5ng/ml human β-fibroblast growth factor (FGF, Atlanta Biologicals, Atlanta, GA) to the media. FGF was removed for 2 days before the experiments to allow myotubes formation. Satellite cells were stained with anti-eMyHC or anti-α-smooth muscle actin (Sigma) to verify the purity of the culture [2]. To stimulate fibrosis in cultured cells, cells were treated with 10ng/ml recombinant human TGF-β purchased from R&D Systems (Cat# 240-B/CF) for 48h. We re-probe every single western blot membrane with GAPDH. In general, we did not strip the membrane. Primary antibody protein bands were scanned and quantified using the Li-COR Odyssey infrared scanning system (Li-COR Biosciences, Lincoln, Nebraska). Then the blot is reprobed with GAPDH antibody and GAPDH density is determined, again using the Li-COR system. When the primary protein has a very different molecular weight from GAPDH there is no overlap and no adjustments are needed. Usually even if the first antibody is close in molecular weight to GAPDH, the density of the first antibody is tiny compared with the response of GAPDH, so any underlying density if minimal, but since we have the signal of the underlying band, we can subtract it from the GAPDH band which allows us to use GAPDH as a loading control. In the event of an unusually strong first antibody response, we 1) strip the membrane and then probe for GAPDH, or 2) use a different host antibody (ie, rabbit Ab first, mouse Ab GAPDH) which allows

us to use a secondary antibody with a different wavelength fluorescent moiety so the overlap does not contribute to the signal. We showed a representative GAPDH response that we routinely see, but think it too cumbersome to provide the GAPDH bands for each protein probed.

Western blot and antibodies: Hind limb muscle were homogenized in Gentle Lysis Buffer (10 mM Tris-HCl, 10 mM NaCl, 2 mM EDTA, 0.5 % NP-40, 1 % glycerol, and fresh added: 1 mM Na3VO4; 10μg/ml PMSF; 5μg/ml Aprotinin; 1μg/ml Leupeptin) with phosphatase inhibitors cocktail 1 and 2 (Sigma) [3, 4]. Protein concentration was measured using a RC-PC protein assay kit (Bio-Rad). Equal amounts of protein were loaded on the acrylamide/bis SDS-PAGE gel. We used 50 micrograms for exosome protein loading, and 20 micrograms for the skeletal muscle and kidney. Protein was transferred to a PVDF membrane and blotted with a specific primary antibody. The secondary antibodies that we used included Alexa Fluor® 680 goat anti-Rabbit IgG or goat anti-mouse IgG are from Invitrogen (Carlsbad, CA). Protein bands were scanned and quantified using the Li-cor Odyssey infrared scanning system (Li-COR Biosciences, Lincoin, Nebraska).

RNA extraction and quantitative real-time PCR: Total RNA from muscle and kidney were extracted using Tri-Reagent (Molecular Research Inc., Cincinnati, OH). Exosomal RNA was isolated using a miRNAeasy kit (217004, Qiagen Sciences, Germantown, MD) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). For mRNA expression, total RNA (1-2 μg) was reverse transcribed using a Thermoscript RT-PCR kit (Invitrogen Carlsbad, CA). Real-time qPCR was performed with the SYBR Green PCR reagent (Bio-Rad) and the following PCR parameters: 94°C for 2 minutes and 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds with final extension at 72°C for 10 minutes[5, 6]. The Cq (threshold cycle) was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Individual mRNA expression was standardized to 18S gene and expression was calculated as the difference between the threshold values of the two genes (ΔΔcq). Melting curve analysis was always performed during real-time qPCR to analyze and verify the specificity of the reaction. Primers for mRNA are listed in Table 2. For microRNA, RNA was reverse transcribed using a universal cDNA synthesis kit II (cat #203301 Exiqon, Wobum, MA). The primers were purchased from Exiqon. Real-time qPCR was performed with the ExiLENT SYBR green master mix (Exiqon cat# 203421). Expression of individual miR-29a-3p, miR-29b-3p and miR-29c-3p in muscle and kidney tissue were normalized to the mouse U6

mRNA and calculated as the difference between the threshold values of the two genes (ΔΔcq). The expression of individual miRs in serum exosomes was normalized to miR-103a.

Muscle and kidney histology: Tissues were fixed in 3.7% formaldehyde/PBS (pH 7.4), and dehydrated, paraffin embedded and sectioned. Masson trichromatic staining was performed with a Masson modified IMEB stain kit (K7298, IMEB Inc. San Marcos, CA). Images were visualized with an Olympus 1X 51 inverted microscopes and captured by DP73-1-51-17MP color camera. Collagen (blue color) in kidney was measured using the CellSens Dimension 1.9 Software (Olympus, Melville, NY, USA) and color density calculated as the average from 10 individual fields.

For skeletal muscle immunohistology: muscles were embedded in Tissue Freezing Media (Cat# H-TFM; Fisher, Pittsburgh, PA, USA) by immersing in isopentane cooled in dry ice. Cross sections (10 mm) from the mid-belly of different muscles were mounted on gelatin-coated slides and fixed in 4% paraformaldehyde for 10 min. Tissue was permeabilized in 0.05% Triton X-100 (in PBS) for 10 min, and quench-fixed in 50 mM NH_4Cl for another 10 min. Samples were blocked with 5% bovine serum albumin for 1 h, followed by incubation overnight with primary antibody. Sections were subsequently washed with PBS and incubated for 60 min with FITC-labeled anti-rabbit IgG (111-095-144; diluted 1:100; Jackson Immuno Research Lab, West Grove, PA). Nuclei were stained by DAPI. Images were visualized with an Olympus 1X51 inverted fluorescence microscope and captured by DP73-1-51-17MP color camera. Muscle fiber cross-sectional area was determined in TA using an anti-laminin antibody (1:50 dilution; Sigma-Aldrich) and at least 500 individual myofibers per muscle were measured. Kidneys dehydrated with increasing concentrations of sucrose, then embedded in tissue freezing medium and frozen in liquid N_2. Sections (10 micron) were cut, mounted on slides and stained with primary antibodies as described for muscle above.

Luciferase reporter assay and transfection: Effectene transfection reagent was used for transfection (Qiagen, Valencia, CA). Firefly and Renilla luciferase activities were measured by dual-luciferase assays (Promega) using TD-20/20 Luminometer (Turner designs, Sunnyvale, CA) [7]. The luciferase report vectors (pMIR-REPORT Luciferase) were purchase from Applied BIOSYSTEMS (Waltham, MA) and constructs were made by Emory Integrated Genomics Core.

Statistical analysis: Data were presented as mean ± se. To identify significant differences between two groups, comparisons were made by using the Student's t-test. When multiple

treatments were compared, ANOVA was performed with a post hoc analysis by the Student-Newman-Keuls test. The relationship between muscle and kidney fluorescence intensity was calculated by linear regression modeling. Differences with P values < 0.05 were considered significant.

Reference:

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