Research Paper

The program of renal fibrogenesis is controlled by microRNAs regulating oxidative metabolism

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\textbf{A B S T R A C T}

Excessive accumulation of extracellular matrix (ECM) is the hallmark of fibrotic diseases. In the kidney, it is the final common pathway of prevalent diseases, leading to chronic renal failure. While cytokines such as TGF-β play a fundamental role in myofibroblast transformation, recent work has shown that mitochondrial dysfunction and defective fatty acid oxidation (FAO), which compromise the main source of energy for renal tubular epithelial cells, have been proposed to be fundamental contributors to the development and progression of kidney fibrosis. MicroRNAs (miRNAs), which regulate gene expression post-transcriptionally, have been reported to control renal fibrogenesis. To identify miRNAs involved in the metabolic derangement of renal fibrosis, we performed a miRNA array screen in the mouse model of unilateral ureteral obstruction (UUO). MiR-150-5p and miR-495-3p were selected for their link to human pathology, their role in mitochondrial metabolism and their targeting of the fatty acid shunting enzyme CPT1A. We found a 2- and 4-fold upregulation of miR-150-5p and miR-495-5p, respectively, in both the UUO and the folic acid induced nephropathy (FAN) models, while TGF-β1 upregulated their expressions in the human renal tubular epithelial cell line HKC-8. These miRNAs synergized with TGF-β regarding its pro-fibrotic effect by enhancing the fibrosis-associated markers Acta2, Col1α1 and Fn1. Bioenergetics studies showed a reduction of FAO-associated oxygen consumption rate (OCR) in HKC-8 cells in the presence of both miRNAs. Consistently, expression levels of their mitochondrial-related target genes CPT1A, PGC1α and the mitochondrial transcription factor A (TFAM), were reduced by half in renal epithelial cells exposed to these miRNAs. By contrast, we did not detect changes in mitochondrial mass and transmembrane potential (ΔΨm) or mitochondrial superoxide radical anion production. Our data support that miR-150 and miR-495 may contribute to renal fibrogenesis by aggravating the metabolic failure critically involved in tubular epithelial cells, ultimately leading to fibrosis.

1. Introduction

Chronic kidney disease (CKD) is a clinical condition where the reduction of renal function is maintained. It is generally considered to be irreversible and progressive. It represents an important public health problem that can affect 12–14% of the general population \cite{1}. It may be present in about 30–40% of patients with highly prevalent pathologies such as diabetes mellitus and hypertension, where it contributes to dictate evolution and prognosis. Regardless of the disease etiology, progression of CKD leads to tubule-interstitial and glomerular fibrosis...
modified Eagle medium supplemented with 15 mM Hepes, 5% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin and streptomycin (Gibco, Rockville, MD), and 50 μg/mL streptomycin (Gibco, Carlsbad, CA). Cells were incubated at 37 °C and 5% CO2 in a humidified atmosphere.

miRNAs were transfected into HKC-8 cells using lipofectamine-pre-miRNA™ (Invitrogen, Carlsbad, CA). Cells were incubated with the lipofectamine-pre-miRNA™ for 6 h. Subsequently, 5 ml fresh medium containing 10% FBS were added to the culture dishes and the cells were maintained in culture for 6 h until used for subsequent experiments. In case of combination with TGF-β1 treatment, it was applied to the cells after miRNAs overexpression according to the previous section.

**Immunoblot.** Cells were washed in PBS, homogenized and lysed in 100 μL RIPA lysis buffer containing 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40 and 25 mM Tris–HCl pH 7.6, in the presence of protease (Complete, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO) and harvested by scraping. A quarter piece of each kidney sample (obtained after dissection in half both lengthwise and crosswise) was homogenized in 300 μl RIPA buffer with 5 mm stainless steel beads (Qiagen, Valencia, CA) using TissueLyser LT (Qiagen, Valencia, CA) vibrating at 50 Hz for 15 min at 4 °C. Samples were clarified by centrifugation at 10,000 g for 15 min at 4 °C. The pellet was then discarded, and the supernatant was kept as protein lysate. Protein concentrations were determined by the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and was measured in Glomax®-Multi Detection system (Promega, Madison, WI). Equal amounts of protein (10–50 μg) from the total extract were separated on 8–10% SDS–polyacrylamide gels and transferred onto nitrocellulose blotting membranes (GE Healthcare, Chicago, IL) at 12 V for 20 min in a semi-dry Trans-Blot Turbo system (Bio-Rad, Hercules, California). Membranes were blocked by incubation for 1 h with 5% non-fat milk in PBS containing 0.5% Tween-20 and blotted overnight with the specific antibodies: CPT1A (Ab128568, Abcam; 1:1000; 4 °C overnight), α-SMA (sc-32251, Santa Cruz Biotechnology; 1:1000; 4 °C overnight), GAPDH (MAB374, Millipore; 1:15000; 1 h, room temperature). After incubation with IRDye 800 goat anti-rabbit and IRDye 600 goat anti-mouse secondary antibodies, membranes were imaged in triplicates with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band densitometry was performed using the ImageJ 1.48 software (http://rsb.info.nih.gov/ij) and relative protein expression was determined by normalizing to GAPDH. Fold changes were normalized to values of control condition.

**RNA extraction.** Total RNA was extracted from HKC-8 cells or mouse kidneys using the miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA quantity and quality were determined at 260 nm by a Nanodrop-1000 spectrophotometer (Thermo Scientific, Rockford, IL).

**Analysis of mRNA expression.** Reverse transcription (RT) was carried out with 500 ng of total RNA using the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA). qRT–PCR was performed with the IQ™SYBR Green Supermix (Bio-Rad, Hercules, CA), using a 96-well Bio-Rad CFX96 RT–PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) according to the manufacturers’ instructions. A Ct value was obtained from each amplification curve using CFX96 analysis software provided by the manufacturer. Relative mRNA expression was determined using the 2–ΔΔCt method [17]. The 18S gene was used for normalization purposes. The primer sequences used for mRNA quantification were: CPT1A (FW: TGCTTACGGCGCCAAACTG, RV: TGGAATCTGGTATCCCAA), ACTA (FW: TTACATGTCCCCAGCAGTGA, RV: GAAGGAAATGACCGACGCAG), COL1A1 (FW: GGAGACCGAGGAGGAGGAGA, RV: TGTTTGCCGTCTAAATGGCC), FN1 (FW: GGCTGCTGGCTGGGGATG, RV: CCAATGCAGCCACATAGCAG), PGC1A (FW: TGCCCTGGATTTGTGACAG, RV: TTGGTACGGTGGGGATG), TF AM (FW: CATCAGTCCTGGGCAATGTCC, RV: CCACCTCGCCCTTAACGAT), 18S (FW: AGCTATACCTGCTACCTGCT, RV: GCTTAATTGGCCTACCAAGGGG). Fold changes were normalized to values of control condition.

**Quantification of miRNA expression.** Quantification of miRNAs expression was performed using the murCURY Locked Nucleic Acid
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mitochondria. Substrates/inhibitors were prepared in the same medium in which the experiment was conducted and were injected from the reagent ports automatically at the times indicated. Measurements were registered for 3-min periods of time (over a total period of 2 h) and values were normalized for total protein content. Protein was extracted from wells with 0.1% NP-40-PBS solution, and quantified with BCA protein assay (Thermo Scientific, Rockford, IL). Four wells were used for each experimental group. The seahorse XFp Cell Mito Stress Test was used to determine the key parameters of mitochondrial function: basal mitochondrial respiration, ATP-linked respiration, proton leak (non-ATP–linked oxygen consumption), maximal respiration, non-mitochondrial respiration and reserve respiratory capacity, as previously described [27].

Luciferase assay. To characterize the miRNA-150 and miRNA-495 candidate binding sites in the human CPT1A 3′-UTR, a luciferase reporter assay containing the CPT1A 3′-UTR (SwitchGear Genomics, Carlsbad, CA, Product ID SB13447) was used. Site-directed mutations in the seed regions of predicted miR-150 and miR-495 sites within the 3′-UTRs were generated by using the MultiSite-QuickChange directed mutagenesis kit (Strategene, La Jolla, CA) according to the manufacturer’s protocol. The primer sequences used were for miR-495 PM1 (FW: GCATTCATCGAGGGTGAATTTGTTCTAGAAAAAGAAAATGTTATTCATTTGCTGCC, RV: GGCACAccAACCACCACAAATCAGATGAATAACACATTCTTTCCTTTTTCACAGA- CAAAAGTTTACCTGCTGAGATGC) and PM3 (FW: GCTGTTACGACGCGCATTGTTGATCTACATT-GAATTCCGCTCAGCAGGGCAAGATCTTTTATTAGCATTTACCATGTGCTCGTAGTACACAAAGTCTTACGTCGTTGAAGACA) and for miR-150 PM2 (FW: CTCATGGTGATTAACCCGAGGATCTACAGAGGCGGAAAAGCGGCCGCG, RV: CCGCCCGTTGGCCTGCTGCTCTTAGAAGCTCTTCAAGTCTGCTGAGATACCCAGCTGAGG, All constructs were sequenced before use to confirm their proper structure. HKC-8 cells were seeded in a 24-well plate and were transiently co-transfected with 200 ng pLightSwitch_CPT1A_3′-UTR (intact or mutated constructions) and 4 ng pGL3-Promoter (a firefly luciferase reporter under the control of the SV40 promoter) (Promega Corporation, Madison, WI, USA) reporter plasmids and 40 nM of either mirVana™ miRNA mimic of miR-150, miR-495 or mirVana™ miRNA mimic negative control (Ambion company, USA) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) when they reached a confluence of 70% as described above. Four wells were used for each experimental group. Luciferase assays were performed 24 h later using the Dual-Luciferase reporter system (Promega, Madison, WI). The renilla and firefly luciferase signals were detected using a Glomax multidection system (Promega Corporation, Madison, WI, USA). The activity of renilla luciferase was normalized by the firefly luciferase activity.

Mitochondrial membrane potential (MMP). Changes in MMP were determined as differences in tetramethylrhodamine methyl ester (TMRM) fluorescence (Invitrogen, Carlsbad, CA, USA). It accumulates in negatively charged polarized mitochondria and fluoresces in orange. When mitochondrial membrane potential collapses in apoptotic or metabolically stressed cells, the TMRM reagent is dispersed throughout the cell cytosol and fluorescence levels drop dramatically. HKC-8 cells were cultured and transfected as described in the cell culture and transfection procedure sections. Next, growth medium was replaced by phenol-red free Hank’s Balanced Salt Solution (HBSS) with 10 mM Hepes. Treatments with oligomycin (5 μM) and FCCP (4 μM) for 5 min were used as positive and negative control conditions. Next, cells were stained with TMRM for 30 min at 37 °C. Cells were harvested with trypsin, centrifuged (3000 rpm, 5 min) and the pellet was resuspended in 200 μL HBSS with 1% Bovine Serum Albumin (BSA) and 5 mM ethylenediaminetetraacetic acid (EDTA). Fluorescence intensity was measured by flow cytometry using an emission wavelength of 570 nm for TMRM (FL2) [28] in a BD FacsCantoTM II system (BD Bioscience, San José, CA) and analyzed with the FlowJo 10.2 software (FlowJo, LLC, Ashland, OR). For each experimental condition, at least 20,000 singlets were analyzed in triplicates.

Mitochondrial superoxide radical anion production. Evaluation of superoxide radical anion production was performed by using MitoSOX™ Red mitochondrial superoxide radical anion indicator (Invitrogen, Carlsbad, CA, USA), a highly selective fluorogenic dye for mitochondrial superoxide radical anion in live cells, according to the manufacturer’s instructions. HKC-8 cells were cultured and transfected as described in the cell culture and transfection procedure sections. Next, growth medium was replaced by phenol-red free Hank’s Balanced Salt Solution (HBSS) with 10 mM Hepes. Treatment with antimycin A (150 μM) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (50 μM) for 5 min were used as positive and negative control conditions. Next, cells were stained with 5 μM MitoSOX™ Red for 30 min at 37 °C. Cells were harvested as described in the mitochondrial membrane potential section. Fluorescence intensity was measured by flow cytometry using an emission wavelength of 580 nm for MitoSOX™ Red (FL2) in a BD FacsCantoTM II system (BD Bioscience, San José, CA) and analyzed with the FlowJo 10.2 software (FlowJo, LLC, Ashland, OR). For each experimental condition, at least 20,000 singlets were analyzed in triplicates.

Mitochondrial labeling. Evaluation of mitochondrial content was performed using MitoTracker™ green FM (Invitrogen, Carlsbad, CA, USA), which passively diffuses across the plasma membrane and accumulates in active mitochondria regardless of mitochondrial membrane potential, according to the manufacturer’s instructions. HKC-8 cells were cultured and transfected as described in the cell culture and transfection procedure sections. Next, growth medium was replaced by phenol-red free Hank’s Balanced Salt Solution (HBSS) with 10 mM Hepes. Cells were stained with 150 nM with MitoTracker™™ green FM for 30 min at 37 °C. For flow cytometry analysis, cells were harvested as described in the mitochondrial membrane potential section. Fluorescence intensity was measured using an emission wavelength of 516 nm for with MitoTracker™™ green FM (FL1) in a BD FacsCantoTM II system (BD Bioscience, San José, CA) and analyzed with the FlowJo 10.2 software (FlowJo, LLC, Ashland, OR). For each experimental condition, at least 20,000 singlets were analyzed in triplicates. For fluorescence imaging, nuclei were also stained with DAPI (Sigma, St. Louis, MO) for 5 min at RT. Living cells were visualized by an inverted Zeiss LSM 710 confocal microscope with a Cell observed, a 63X/1.2 Water C-Apochromat Corr UV-VIS-IR M27 objective and analyzed with the Zeiss Zen2010b sp1 software (Zeiss, Oberkochen, Germany).

Mitochondrial copy number determination. Genomic DNA was extracted from HKC-8 cells using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Mitochondrial abundance was determined with the Human Mitochondrial DNA Copy Number Assay Kit (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions. MtDNA copy number was presented as the mtDNA-to-nuclear DNA ratio. Human CKD patient samples. A cohort of 100 CKD patients (stage 3–4) from Hospital Prinçipe de Asturias was selected for the analysis of miRNA plasma levels. It included two different subgroups classified according to the evolution of their renal function based on the GFR MDRD indicator [29] over a period of 24 months: 50 patients presented less than 10% of kidney function deterioration while the rest of them had experienced at least a 40% reduction in kidney function or had initiated renal replacement therapy (dialysis). We also quantified the degree of fibrosis in kidney biopsies from a different cohort of 26 patients with graft dysfunction following kidney transplantation from Hospital Ramón y Cajal. All biopsies were evaluated according to the Banff 2007 criteria [30].

Statistical analysis. Data were analyzed using nonparametric tests except where indicated. The difference between two independent groups was examined with Mann-Whitney test, while more than two groups were compared with Kruskall-Wallis test. A P-value of 0.05 or less was considered statistically significant (t-test; P < 0.05, *; P < 0.01, **; P < 0.001, ***). Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Data are reported as mean ± standard error of mean (SEM).
miRNA expression data in UUO-induced fibrotic kidneys. To decipher the contribution of miRNAs to the metabolic regulation of renal fibrogenesis, the 7days UUO model was performed in WT mice. RNA was isolated from control and fibrotic kidney samples and the miRNA expression profile was analyzed in a customized microRNA array. Selection of miRNAs was based on their potential targeting of key enzymes involved in mitochondrial metabolism, redox processes and circadian rhythm after “in silico” analysis (more details in the Methods section). The heat map revealed clearly distinct expression patterns in some miRNAs when comparing fibrotic and control kidney samples (Fig. 1A). The volcano plot analysis showed that 73 of 175 miRNAs were differentially expressed, 17 up- and 56 down-regulated, in the fibrotic kidney samples compared to the control ones (Fig. 1B, Supplementary Table 1). Among them, specific miRNAs that could regulate the fibrotic outcome were upregulated in fibrotic kidney samples compared to the control ones, with a fold change of 3.69 (P-value: 0.012) and 1.95 (P-value: 0.012), respectively.

**MiR-150 and miR-495 enhance the TGF-β profibrotic response in human renal tubular epithelial cells.** Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to confirm the change of miR-150-5p and miR-495-3p expression that had been identified by miRNA expression profiling in the UUO kidney samples (Fig. 2A and B). In addition, kinetics of miR-150-5p and miR-495-3p expression in the kidneys 3, 5, 7, 10 and 15 days after the UUO procedure was analyzed by qRT-PCR. The expression of these miRNAs was significantly enhanced by 2-fold at 5 days after UUO and remained elevated 15 days after UUO (Fig. 2C and D). The expression level of these miRNAs was also evaluated in the folic acid nephropathy (FAN) model. We found a 4- and 2-fold upregulation of miR-495-3p and miR-150-5p, respectively, in fibrotic kidney samples from the FAN model (Fig. 2E). TGF-β1, one of the master regulators of fibrogenesis, also induced miR-150 and miR-495 expression in HKC-8 cells more than 2-fold and 3-fold, respectively, after 48 h, supporting a potential role for these miRNAs in TGF-β signaling-related events (Fig. 3A and B). To assess whether miR-150 and miR-495 were involved in the pro-fibrotic transformation of tubular epithelial cells by TGF-β1, the human cell line HKC-8 was transfected with miR-150-5p or miR-495-3p and treated with TGF-β1 for different times. Increasing miR-150-5p and miR-495-3p levels significantly enhanced the TGF-β1-induced mRNA level expression of the fibrosis-associated markers Acta2, Col1α1 and Fn1 (Fig. 3C and D). Similarly, overexpression of miR-150-5p or miR-495-3p strongly reduced CPT1A and enhanced α-SMA protein abundance (Fig. 3E and F). These data suggest that these miRNAs may participate in the pathogenesis of renal fibrosis by promoting TGF-β1-dependent epithelial dedifferentiation of tubular cells, as well as by compromising the expression of a critical enzyme involved in FAO.

MiR-150 and miR-495 induce mitochondrial bioenergetics changes and reduce the expression of genes involved in crucial mitochondrial function. To gain insight into the metabolic consequences induced by the administration of miR-150-5p or miR-495-3p we studied the bioenergetics profile of human renal tubular epithelial cells. **Fig. 1. miRNA expression data in UUO-induced fibrosis.** (A) Heat map showing relative miRNA expression of contralateral and obstructed kidneys of C57/BL6-J mice subjected to the UUO procedure for 7 days (n = 6). The scale bar ranges from green to red (high to low expression) and numbers represent ΔCt values. On the left side are indicated the miRNAs selected for study. (B) Volcano plot analysis of UUO-modulated miRNAs. Log_{10} relative quantification (RQ) and negative (-) log_{10} adjusted (adj.) P-values are plotted on the x- and y-axis, respectively. Each miRNA is represented by a dot. 73 of 175 miRNAs showed an altered expression in the fibrotic samples (adj. P-value > 0.05). Selected miRNAs for further analysis are highlighted. (C) miR-150-5p and miR-495-3p were selected on the basis of their power for in silico targeting of the genes CPT1A, TFAM and PGC1α. For each selected microRNA, conserved seed target sites in the 3′ UTR of these genes, their fold change and significance (adj. P-value) are indicated.
TGF-β1 was used as the model cytokine involved in pro-fibrotic associated changes. To determine the bioenergetics status of these cells, FAO-associated oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of HKC-8 cells were measured during sequential treatment with compounds that modulate mitochondrial activity in the presence of palmitate, using a Seahorse XF24 Extracellular Flux Analyzer (more details in the Methods section). After 48h with TGF-β1, HKC-8 cells in the presence of miR-150-5p showed a consistent decrease in basal, OCR coupled to ATP generation, ATP-linked respiration and maximal respiration, which relates to FCCP-sensitive OCR (Fig. 4A). The impairment in the OXPHOS was mirrored by a decrease in ATP content in HKC-8 cells over-expressing miR-150-5p (Fig. 4C). We did not observe that these miRNAs induced variations in ECAR (data not shown). Consistent with this, mRNA expression levels of their mitochondrial-related target genes CPT1A, PGC1α and the mitochondrial transcription factor A (TFAM), were reduced by half in cells treated with miR-150 mimic in comparison to miRNA mimic NC-treated cells (Fig. 4E). Studies using the same experimental approach with miR-495-3p yielded similar results (Fig. 4B, D, F). However, mitochondrial mass, determined by the mtDNA copy number and mitochondrial staining with MitoTrackerTM green FM, was not significantly modulated by miRNA-150 and miRNA-495 both in basal conditions and under TGF-β treatment. A decreasing trend in this parameter was observed basally (Supplementary Fig. 2A, B, C). Overall, these data support that miR-150 and miR-495 promote a pro-fibrotic action in tubular epithelial cells through the impairment of mitochondrial function.

MiR-150 and miR-495 do not alter mitochondrial transmembrane potential (∆Ψm) and mitochondrial superoxide radical
Fig. 3. MiR-150 and miR-495 enhance TGF-β1-dependent pro-fibrotic response in human renal tubular epithelial cells. (A, B) RT-PCR analysis of miR-150-5p (A) and miR-495-3p (B) expression in HKC-8 cells treated with 10 ng/ml TGF-β1 for the indicated times. (C, D) mRNA levels of alpha-smooth muscle actin (α-SMA), alpha 1 type-1 collagen (Col1α1), fibronectin (FN) from cells transfected with miR-NC and mimic miR-150-5p (C) or miR-495-3p (D) were determined by qRT-PCR using Sybr green. Cells were treated with TGF-β1 (10 ng/ml) after miRNA overexpression where indicated (see also methods section). (E, F) Immunoblots depicting CPT1A and α-SMA levels protein levels in cells transfected with miR-NC and mimic miR-150-5p (E) or miR-495-3p (F) for the indicated time points. GAPDH was used for normalization purposes. Bar graphs (right panels) represent the mean of the fold change expression ± s.e.m. from 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared to their corresponding control conditions; #P < 0.05, ##P < 0.01, ###P < 0.001 compared to cells treated with miR-NC with the same experimental condition.
Fig. 4. MiR-150 and miR-495 enhance TGFβ1-induced FAO repression in HKC-8 cells. (A, B) Oxygen consumption rate (OCR) of HKC-8 cells transfected with 40 nM miR-NC and mimic miR-150-5p (A) or miR-495-3p (B) and exposed to 10 ng/ml TGF-β1 after miRNA overexpression (see also methods section). Bar graphs (right panels) show the rates of OCR associated to basal, proton-leak, ATP-linked, maximum and reserve capacities and non-mitochondrial respiratory statuses. Data are represented after normalization by protein amount. (C, E) ATP levels in HKC-8 cells transfected with miR-NC and mimic miR-150-5p (C) or miR-495-3p (E). (D, F) mRNA levels of CPT1A, PGC1A and TFAM in HKC-8 cells transfected with miR-NC and mimic miR-150-5p (D) or miR-495-3p (F) were determined by qRT-PCR using Sybr green. Bar graphs show the mean ± s.e.m. of 4 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared to their corresponding control conditions; #P < 0.05, ##P < 0.01, ###P < 0.001 compared to cells treated with miR-NC with the same experimental condition.
anion production. Mitochondrial membrane potential (ΔΨm) generated by proton pumps (Complexes I, III and IV) is an essential component in the process of energy storage during oxidative phosphorylation. Increased ΔΨm can lead to mitochondrial redox perturbation, while its decrease causes a reduction of ATP production, compromising cell viability [254]. Superoxide radical anion (O₂•⁻) is a free radical that can be generated in the mitochondria as a consequence of electron leakage that may occur within several steps of the ETC. Generation of O₂•⁻ within the mitochondrial matrix also critically depends on the NADH/NAD⁺ and CoQH²/CoQ ratios and the local O₂ concentration [255]. Its overproduction may lead to the loss of mitochondrial redox homeostasis through different mechanisms, a condition that has been related to many pathological states including AKI and CKD [201]. ΔΨm was examined with the TMRM dye and the superoxide radical anion production with MitoSOX dye. First, we validated these assays by the treatment of cells with FCCP and CCCP as negative controls, for TMRM and MitoSOX respectively, and Oligomycin and Antimycin A as positive controls, for TMRM and MitoSOX respectively, in the human cell line HKC-8. In the case of the TMRM assay, oligomycin treatment induced a 3-fold increase in fluorescence mean of the TMRM compared to the control dye, while FCCP treatment resulted in a 9-fold decrease. Antimycin A and CCCP produced a 35-fold increase and 2-fold decrease, respectively, in the MitoSOX assay (Supplementary Fig. 3A and B). To determine if miR-150 and miR-495 effects on mitochondrial metabolism triggered alterations in these parameters, HKC-8 cells were transfected with a miR-150-5p or miR-495-3p mimic or the corresponding miR-NC. No statistically significant differences in the fluorescence quantification of the TMRM or MitoSOX probes were found between the treatment conditions with the selected miRs and miR-NC (Supplementary Fig. 3C).

miR-150 and miR-495 levels are not affected in plasma and kidney samples from CKD patients. We assessed the expression of the selected miRNAs in plasma and kidney biopsies from patients with CKD. Analysis of kidney biopsies from 26 patients (Hospital Ramón y Cajal) did not show correlation between the degree of fibrosis and miR-150-5p and miR-495-3p levels, respectively (Supplementary Fig. 4A and B). Circulating plasma levels of miR-150-5p, the miRNA with the highest kidney expression of the two, were also determined in a cohort of 100 CKD patients (Hospital Príncipe de Asturias). Due to limitations in sample availability, we were unable to determine serum values of miR-495-3p in this cohort. These patients were previously divided in two subgroups based on the evolution of their renal function over a period of 24 months, so that 50 patients presented less than 10% of kidney function deterioration while the rest of them had experienced at least a 40% reduction in kidney function. The qRT-PCR quantification of plasma levels of miR-150-5p did not show a clear trend towards a differential expression in the two subgroups of patients (Supplementary Fig. 4C).

4. Discussion

The discovery of miRNAs as key regulators of pathophysiological processes has fostered research on their use as therapeutic agents in almost every clinical setting, including kidney disease [31]. Renal fibrosis is a highly prevalent outcome of chronic kidney disease (CKD) with independence of the etiology, and its presence is a clear predictor of evolution. Hence, a significant effort has been devoted to identify miRNAs linked to kidney fibrosis [32,33]. In this study we focused on the increasingly important role of mitochondrial derangement in renal damage and we identified two miRNAs, miR-150 and miR-495, which were previously unknown actors in the scene. Moreover, we found that they exert a profound effect on cellular bioenergetics by synergizing with TGF-β and modulating key metabolic routes related to fatty acid oxidation.

Epithelial injury co-exists with a defect in fatty acid oxidation, the main source of energy for tubular epithelial cells [5]. It leads to their dedifferentiation inducing tubular function impairment, triggering cell cycle arrest and promoting the release of critical pro-fibrotic cytokines that may contribute to activate interstitial myofibroblasts [5,34]. MiR-150 and miR-495 expression was increased in mouse fibrotic kidneys and in human kidney epithelial cells (HKC-8) after treatment with TGF-β, suggesting their involvement in the pro-fibrotic response. Of interest, in silico analysis of the genomic regions coding for miR-150 and miR-495 permits to identify binding sites for transcription factors related to TGF-β signaling and other pathways involved in UDOM damage (searches in genecards.org for miR-150 and miR-495). Both miR-150 and miR-495 decreased the expression level of their target genes PPT1A, PGC1α and TFAM, which was reflected in a decreased basal, ATP-linked and maximal OCR and ATP content after treatment with miR-150 and miR-495. This effect was accompanied by an enhanced TGF-β pro-fibrotic response in HKC-8 cells. We found that TGF-β decreased mitochondrial mass and mitochondrial DNA. After treatment with miRNA-150 and miRNA-495 a decreasing trend was observed in mitochondrial content in basal conditions, even though not reaching significance. This points to a mechanism independent from the role of PGC1α and TFAM and most likely related to a metabolic effect linked to PPT1A downregulation as the main variable affected by the presence of the miRNAs. Alterations in mitochondrial bioenergetics are critical in kidney disease [35]. In keeping, we have recently shown that PPT1A is a critical enzyme for the preservation of a healthy tubular compartment, as its overexpression protects from kidney fibrosis [6]. However, PGC1α and TFAM genes are also closely related to mitochondrial function and biogenesis [36]. The impaired expression of these genes is associated with CKD, while its preservation contributes to kidney integrity as shown in several models of damage. Thus, Han et al. found that tubular gain-of-function of PGC1α protected mice against Notch-induced kidney fibrosis and reversed the mitochondrial dysfunction associated with this model [37]. Similarly, re-expression of Tfam in tubule cells prevented Notch-induced metabolic and pro-fibrotic reprogramming [38]. Thus, it is conceivable that the decreased mRNA expression of these mitochondrial genes, induced by miR-150 and miR-495, also contributes to a damaged epithelial phenotype.

The decline we observed in OCR associated with epithelial cell damage is in accordance with previous reports, including our studies with miR-33 and miR-9 [15,16]. Whereas Kang et al. also reported that glucose oxidation was lower in human and mouse models of kidney fibrosis [5], our data show that the microRNA-mediated decrease in OCR does not induce per se variations in ECAR, suggesting that the glycolytic shutdown in fibrotic conditions most likely depends on fibrotic stimuli rather than on impaired oxidative phosphorylation. Several miRNAs have been identified as major modifiers of mitochondrial function, including the archetypal pro-fibrotic miRNA, miR-21 [39]. The suppression of PPAR-α expression by miR-21 has been invoked as a mechanism by which this miRNA exerts its pro-fibrotic action. In addition to inhibiting FAO, the target genes for miR-21 indicated that this miRNA silences a wide range of mitochondrial processes. Other examples include miR-9 [16], miR-33 [15] and miR-30e [40,41]. However, to our knowledge there are few reports addressing the microRNA regulation of PPT1A, PGC1α and TFAM genes in the context of kidney fibrosis. Interestingly, microRNA-214 which promotes epithelial-to-mesenchymal transition (EMT) during renal fibrosis, directly targets TFAM in colon cancer cells [42]. This repression was also reported for miR-590-3p [43]. The miR-29 family, one of the best characterized regulators of ECM production in organ fibrosis, directly targets TFAM and promotes mitochondrial biogenesis. Thus, pathological silencing of miR-29 leads to PGC1α upregulation, generating profound alterations in mitochondrial biogenesis that contribute to cardiac disease [44]. While our data are clearly supportive of a role for miR-150 and miR-495 in kidney fibrosis, further in vivo studies would be necessary to confirm this contention.

To evaluate the integrity of mitochondrial electron transport chain (ETC), we focused on two features: ΔΨm and superoxide radical anion production. We observed that miR-150 and miR-495 did not alter these
parameters. A stable ΔΨm is required for cell viability and its magnitude varies among cell types [45]. Its maintenance is strictly fine-tuned as an increase in ΔΨm may lead to perturbations in the redox state, while its decrease causes a reduction of ATP production and may trigger apoptosis. In our study, changes in ΔΨm were quantified with the cationic fluorescent dye TMRM, while mitochondrial superoxide radical anion production was measured with the fluorogenic dye MitoSOX Red. The employment of these tools in conditions affecting renal function is not conspicuous in the literature. Nevertheless, in the face of the absence of changes in our study, we did not pursue in depth other strategies to evaluate redox hormesis and hence, we cannot exclude that these miRNAs may alter the nucleophilic tone or the function of the several components of the ETC.

In the clinical setting miR-150 has been suggested as a pro-fibrotic miRNA in lupus nephritis by modulating TGF-β signaling [46,47], while miR-495 was associated with a protective role in diabetic cardiac fibrosis [48]. To find out their potential involvement in CKD, we evaluated their serum and parenchymal levels in two different patient cohorts. Serum levels of miRNAs have been proposed as potential biomarkers for CKD progression [49,50]. Thus, serum circulating miR-21 levels and abundance of miR-29c in urinary exosomes correlate with kidney fibrosis, suggesting their potential role as biomarkers [51,52]. However, miR-150 and miR-495 levels were not different either in plasma or kidney samples from CKD patients. The different microRNA expression patterns among species and cell types may explain this absence of difference in miR-150 and miR-495 levels in fibrotic tissue [53]. While in some cases reported microRNA plasma levels could be reflecting those in the kidney tissue, other mechanisms related to the secretion of miRNAs to the circulatory system or a counterbalance of this secretion by other organs should also be considered [13,54].

Overall, our results support that miR-150 and miR-495 promote renal epithelial cell dedifferentiation most likely through energy deprivation, conveyed by the repression of CPT1A, PGC1α and TFAM mitochondrial function-related genes. They also lend a basis to pursue their role in vivo, as well as their potential usefulness as diagnostic or prognostic biomarkers.

Author contributions
SL conceived and directed research. VM designed, performed and analyzed the majority of experiments. RR assisted with the microRNA array analysis. DRP and LGB performed studies in two different cohorts of CKD patients. All authors helped with the discussion of the results and SL and VM wrote the manuscript.

Declaration of competing interest
The authors have no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101851.
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