Supplemental information

MicroRNA-133a-dependent inhibition of proximal tubule angiotensinogen by renal TNF

Shoujin Hao, Joseph Salzo, Hong Zhao, Mary Hao, Zbigniew Darzynkiewicz, and Nicholas R. Ferreri

Department of Pharmacology, New York Medical College, Valhalla, NY 10595

This work was supported by NIH grant R01HL133077
To whom correspondence should be addressed:
Dr. Nicholas R. Ferreri
Dept. of Pharmacology
New York Medical College
Valhalla, NY 10595
Phone: 914-594-4079
FAX: 914-347-4956
E-mail: nick_ferreri@nymc.edu
Key words: TNF, angiotensinogen, miR-133a, dietary salt

METHODS

A. Preparation, collection, and separation of samples

Preparation and collection of tissues: The collection, isolation, and preparation of renal cortex and liver were performed as in previous studies. Briefly, renal cortex and liver were excised from male C57BL/6J mice that were anesthetized with 100 mg/kg ketamine-20 mg/kg xylazine (ip). The kidneys were perfused with sterile 0.9% saline via retrograde perfusion of the aorta, removed, and cut along the corticopapillary axis. The renal cortical tissues were excised, then separated and further washed three times with fresh RPMI1640 medium. Liver pieces were cut, isolated, collected, and washed three times using fresh medium.

Preparation and isolation of murine proximal tubules (mPT): mPT were isolated in a manner similar to that previously described. Briefly, kidneys were immediately harvested and washed in sterile HBSS medium. The renal cortical regions were minced with a sterile blade, and incubated for 10 min at 37°C in a 0.05% collagenase solution gassed with 95% oxygen. The collagenase digestion step was repeated three times and filtered through a 210-µm mesh sieve (Fisher Scientific, Houston, TX, USA). The filtered solution was centrifuged at 500 rpm for 10 min; and the pellet was resuspended in Krebs-Henseleit saline (KHS, pH 7.4) solution. The KHS solution was bubbled with 95% O_2/5% CO_2 during incubation for 30 min at 37 °C. Then 5 mL of the renal tubule solution was slowly added to the top of the centrifuge tubes containing 30 mL of 45% Percoll (Sigma, USA) solution and 5 mL of 90% Percoll solution. The tubes were centrifuged at 20,000 g and 4 °C for 30 min, which produced 4 banded layers. The mPT within the 3rd layer was collected and centrifuged at 1500 rpm at room temperature for 2 min to remove the Percoll solution. The pelleted mPT tubules were used to establish primary cultures of mouse mPT cells.

Preparation and culture of primary mPT cells: Primary cultures of murine mPT cells were performed in a protocol similar to that previously described. Briefly, the fresh mPT tubules were placed in 6 well plates containing basal culture Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 5 × 10^8 mol/L hydrocortisone, 5 µg/mL transferrin, 2 mmol/L butyrate, 2 mmol/L alanine, and 2 mmol/L lactate. The bicarbonate concentration was adjusted to 24 mmol/L to maintain the medium at pH 7.4 to 7.5. After 48 h of the culture, the medium was changed, and every two days thereafter. After 6–7 days, monolayers of cells were 70–80% confluent, the medium was removed, and cells were placed in 1 ml of serum-free OPTI-MEM medium containing different plasmid DNA constructs and 10 µl Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, for 4 h at 37°C/5% CO_2. After the transfection period, 1 ml of culture medium containing 20% FBS was added, and the cells were incubated overnight at 37°C/5% CO2. The medium was then removed, and cells were cultured for an additional 12–48 h in the culture medium containing 10% FBS. After the primary culture of mPT cells were sub-confluent, the cells were harvested with trypsin-EDTA (Invitrogen, USA) and the purified mPT cells will be used for further analysis.

B. Preparation, amplification and purification of vectors

Plasmid and Lentivirus targeting vectors: The inhibitory construct for TNF (U6-TNF-ex4), which was cloned into pcDNA3.1, was designed by targeting exon 4 of the TNF gene; the TNF reverse primer for the 3′ -end was gcagctagcCTCGAGAAAAAACCCAGTGATAGAGGTTACCCTACACAAAGGTAACCTC TATCACTGGGAAACAAGGCTTTTCTCCAAGGGATA. Silencing of TNF mRNA also was accomplished using the lentiviral vector psiLv-U6 (GeneCopoeia). The target sequence of the inhibitory construct for TNF (U6-TNF-ex4) was GATGGGTTGTACCTTGTCT; and the constructs were designed by targeting exon 4 of the TNF gene; scrambled U6-shRNA (U6) was used as a negative control. PCRs for TNF silencing and nonsilencing shRNA controls employed a common primer for the 5′ -end, forward: gcagaattcGATCCGACGCCGCCATCTCT; the 3′ -end for scrambled nonsilencing shRNA was gcagctagcCTCGAGAAAAAAGAACGTTCGATAATGGATCCTACACAAAGATCCATTAT CGAACGTTCAAACAAGGCTTTTCTCCAAGGGATA. All constructs were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing as previously described. psPAX2 and pMD2.G plasmids were used for preparation of lentivirus (Addgene MIT, Cambridge, MA).

Lentivirus amplification and purification: HEK293-T cells were grown in 20-cm2 flasks to generate lentivirus. Briefly, the packaging cells were seeded at a density of 7 × $10e^5$ cells per flask in 5 ml media (DMEM/10% FBS/no antibiotics) 24 h before transfection and grown at 37°C/5% CO2. DNA for transfection was prepared by mixing 1.0 µg psPAX2 and 0.1 µg pMD2.G with 1.5 µg pLKO.1 or psiLV plasmids in each flask. A mixture of 150 µl OptiMEM (GIBCO) and 6 µl FUGENE (Roche) was then added to the DNA, and this mixture was incubated before adding to the packaging cells. Cells were incubated for 12 h, and the media were changed to remove remaining transfection reagent. The lentivirus encoding shTNF (U6-TNF-ex4) were collected, pooled, filter purified with Lenti-X Maxi purification kit, and then frozen at −80°C for long-term storage.

***miR-133a RNA and gene overexpression*:** All constructs and vectors for miR-133a were designed and generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing in a protocol similar to that previously described. The mouse specific miR-133a RNA mimic was designed and the pcDNA3.2/V5 mmu-miR-133a-1 (miR-133a) was purchased from Addgene. The sequence of the negative control (5’-3’) was sense for the 5′-end: 5′-UUCUCCGAACGUGUCACGUTT-3’ and antisense for the 5′-end: 5′-ACGUGACACGUUCGGAGAATT-3’; the sequence of the miR133a mimic (5’-3’) was sense for the 5′-end: 5’-UUUGGUCCCCUUCAACCAGCUG-3’; and antisense for the 5′-end: 5’-GCUGGUUGAAGGGGACCAAAUU-3’. Subcloning of pcDNA3.2/V5 mmu-miR-133a-1 into a pLKO.1 vector and cotransfecting HEK293-T cells with pLKO.1 was performed to generate lentivirus encoding miR-133a. DNA for transfection was prepared as described above. HEK293-T cells were transduced for 48 hr with synthetic miR-133a or a negative control miR-133a lentivirus and overnight

infection was performed in the presence of 8µg/ml polybrene. Cells were incubated and lentiviral supernatants were collected after transduction, then the supernatants were pooled, and filter purified with Lenti-X Maxi purification kits. The titer of filter-purified lentivirus was approximately 2×10^8 transducing units. Transduction efficiency was monitored by flow cytometry analysis described previously.

Administration of U6-TNF-ex4, miR-133a, or TNF in vivo: Mice were anesthetized with 100 mg/kg ketamine-20 mg/kg xylazine (i.p.) and a 31-G needle was inserted at the lower pole of the each kidney parallel to the long axis and was carefully pushed toward the upper pole. As the needle was slowly removed, 50µl filter-purified lentivirus (U6 or U6-TNF-ex4 ~5×10^7 transducing units) was injected. Previous studies showed that lentiviral-mediated EGFP DNA and protein expression in kidney parenchyma was robust after 72h, and no toxicity was observed in mice treated with the lentiviral vector as survival was not affected in lentivirus-injected or PBS-injected animals. Similarly, 50µl filter-purified miR-133a as well as murine recombinant TNF (5ng/g body weight), or saline control was injected into both kidneys.

Isolation of total RNA and amplification of cDNA fragments: Total RNA was isolated from mouse tissues or the cultures of primary mPT cells by addition of 1 ml Tri-Zol reagent. Chloroform, Isopropanol, and 75% EtOH were used for isolation, purification, and dehydration of total RNA. Ultimately, the RNA pellet was resuspended in 50 µl of RNase free dH_2O and stored at −80°C. After total RNA was treated with deoxyribonuclease I for 30 min, a 3-µg aliquot was used for cDNA synthesis using the Superscript Preamplification System (Life Technologies) in a 20 µl reaction mixture containing Superscript II reverse transcriptase (200 U/µl) and random hexamers (50 ng/µl). cDNA fragments were size fractionated on a 1% agarose gel and stained with ethidium bromide.

C. Experimental Analysis

Quantitative RT-PCR analysis for mRNA: A 0.5-µg aliquot of total RNA was converted to cDNA using random primers and PowerScript RT (Clontech). The cDNA from each RNA sample was placed in a 20 µl RT-PCR mixture using a FastStart DNA Master SYBR Green I kit (Roche) supplemented with 3 mM MgCl2 and Platinum Taqpolymerase (Invitrogen). The specific primer pairs for murine TNF and AGT are provided in Table S1. Input cDNAs were normalized using the housekeeping gene β-actin and the efficiency of primer pair amplification was determined from a standard curve generated using protocols described previously. The $2^{(-\Delta\Delta CT)}$ method was used to evaluate changes in mRNA accumulation.

qRT-PCR analysis for miR-133a: Total RNA was isolated as described above and cDNA was generated with Affinity Script RT enzyme from Stratagene (La Jolla, CA). Briefly, 1 µl random primer and 0.5 µg RNA or single tubule lysate was annealed at 95°C by adding 1 µl DTT, dNTP, and enzyme. The mixture was incubated for 1 h at 65°C. The primers for the pre-miR-133a were designed using BLAST software. Before conducting the experiments, we validated the RT-PCR with the mRNA purified as a template. The miR-

133a primers (2.5 µl, 12.5 nM) were mixed with 2 µl cDNA (200 ng), 12.5 µl 2× SYBR Green master or TaqMan probes. MxPro3000 (Stratagene) was used to carry out the experiments, and we used $2^{(-\Delta\Delta CT)}$ to analyze the comparative expression level of miR-133a. Four replicates were done for each miRNA, consisting of two replicate PCR reactions from each of the two replicate RT reactions, and the results were averaged.

Western blot analysis: Renal tissue or cells were homogenized and solubilized with RIPA buffer (cat no. 9806s, Cell Signaling Technology) for AGT after protease inhibitors (Roche Diagnostics) were added. The protein samples were heated in loading buffer, and protein concentration was determined with a Bio-Rad protein assay kit. Equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Following blocking at room temperature for 1 h with 5% skim milk, membranes were probed at 4°C overnight with the polyclonal antibody for AGT (Catalog# PA5-47888, ThermoFisher), monoclonal antibody for β-actin (Catalog# A1978, Sigma-Aldrich), and appropriate secondary antibodies followed by treatment with enhanced chemiluminescence substrate (Pierce). Bands on the membrane were visualized and analyzed using a UVP BioImaging System. Quantification of images of AGT protein levels was normalized to β-actin protein levels.

Laser-scanning cytometry analysis: The primary mPT cells were directly seeded onto two-chamber tissue culture-treated glass slides (BD Bioscience), washed several times with PBS, fixed with freshly prepared 4% paraformaldehyde in PBS for 1 h, and rinsed several times with fresh PBS and stored at 4°C. For staining, cells were permeabilized with 0.1% Triton X-100 in PBS for 1 h at room temperature. After each sequence with either anti-AGT primary (goat), or appropriate secondary antibody (donkey anti-sheep-conjugated with Alexa Fluor 647; Invitrogen), slides were washed five times with a high-salt solution containing 1% BSA and 2.3% sodium chloride in PBS, followed by a single wash with PBS. Cells were then washed three times and stained with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 min followed by a single wash with PBS. The slides were examined using a Nikon Microphot FXA microscope equipped for epifluorescence illumination. Laser-scanning cytometry (LSC; iCys; CompuCyte, Cambridge, MA) was used to measure expression of AGT. Briefly, nuclear and cytoplasmic fluorescence was measured by LSC using UV and 647-nm wavelength argon ion lasers to excite the fluorescence of DAPI and Alexa Fluor 647, respectively. Nuclear contouring was based on the blue fluorescence of DAPI. The intensity of blue (DAPI) and red (Alexa Fluor 647) fluorescence emission was measured by separate photomultipliers. The integrated value of red fluorescence representing AGT immunofluorescence was measured in the cytoplasm, which was defined by integration and peripheral contour settings, respectively, as described previously.

TNF ELISA: Determinations of TNF levels in renal tissues were performed in duplicate by ELISA (Pharmingen), according to the protocol provided by the manufacturer.

Radiotelemetry Mean Arterial Pressure measurements: The Dataquest IV telemetry system was used for direct measurement of systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), heart rate, and locomotor activity using a

radiotelemetry unit (PA-C10; Data Science International, St Paul, MN), which was subcutaneously secured in the neck-back area between the scapulae, with the catheter inserted into the aorta through the right common carotid artery as previously described. The mice were then allowed to recover for 1 week prior to assessment once baseline MAP was stable. The MAP was a mean for each animal and monitored once each 15 min for 3 hours at night (9:00-12:00pm). Each point represents the means ± SE of the mice in each group.

RESULTS/FIGURE LEGENDS

Fig.S1: The level of TNF mRNA or protein remaining after renal-specific silencing by shRNA-TNF lentivirus. The data indicate that the percent remaining of TNF mRNA (Fig. S1A) and protein expression (Fig. S1B) after administration of the U6-TNF-ex4 lentivirus is 18.3±4.2% and 19.2±3.9%, respectively (n=10). The percent remaining of mRNA expression was monitored by RT-qPCR (A), and percent remaining protein expression was evaluated by ELISA essay (B). Data are shown as mean ± SE (n=10).

Fig. S2: TNF downregulates AGT expressions in primary mPT cells under low-salt conditions. Addition of TNF (1nM) to primary mPT cells incubated in LS isosmotic medium for 4 hr significantly decreased AGT mRNA accumulation (A) and protein expression (B). Data are shown as mean ± SE (n=4).

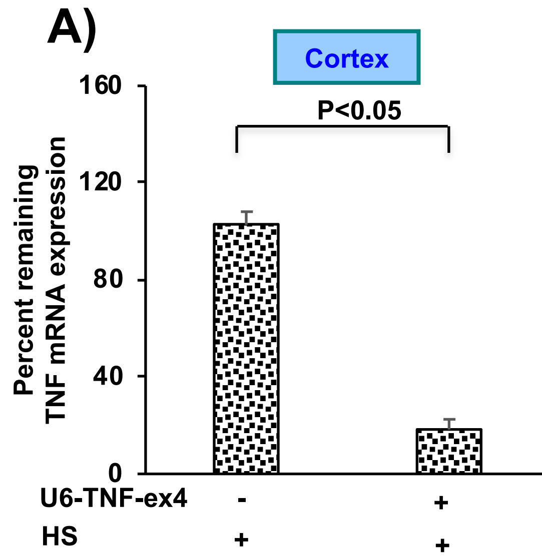
Fig. S3: miR-133a inhibits AGT expression in primary mPT cells subjected to HS conditions.
A): AGT mRNA expression was reduced in mPT cells exposed to high salt medium (400 mosmol/kg H2O) for 4 hr was measured by RT-PCR after treatment of lentivirus U6-TNF-ex4 and miR-133a or scramble for 12 hr. B): Western blotting using anti-AGT antibody (top) showed that the AGT protein expression was decreased in mPT cells expose to high salt medium for 4 hr after treatment of lentivirus U6-TNF-ex4 and miR-133a or scramble for 12 hr. Anti-β-actin antibody was used as a loading control (bottom). C) Positive control experiments showing that U6-TNF-ex4 inhibits TNF mRNA accumulation in mPT cells. Data are shown as mean ± SE (n=4). U6-TNF-ex4= short hairpin inhibitory construct for TNF.

Fig. S4: Renal-specific silencing of TNF using a lentivirus approach in vivo. Intrarenal injection of the shRNA-TNF lentivirus construct (U6-TNF-ex4) inhibits TNF mRNA levels in the renal cortex but not in the liver (A and B, respectively). Data are shown as mean ± SE (n=4).

Table S1. Oligonucleotide-specific primers for PCR

Primer	Sequence	PCR Size, bp	Position
F-AGT(m)	5-GAATTCTGGGTGGACAACAG-3		930-949
R-AGT(m)	5-GGGTTCTCTATCCAAGTCAG-3	220	1150-1131
F-TNF(m)	5-GAGAAGTTCCCAAATGGCCT-3		351-370
R-TNF(m)	5-GAGAACCTGGGAGTAGACAA-3	242	593-574

Figure S1



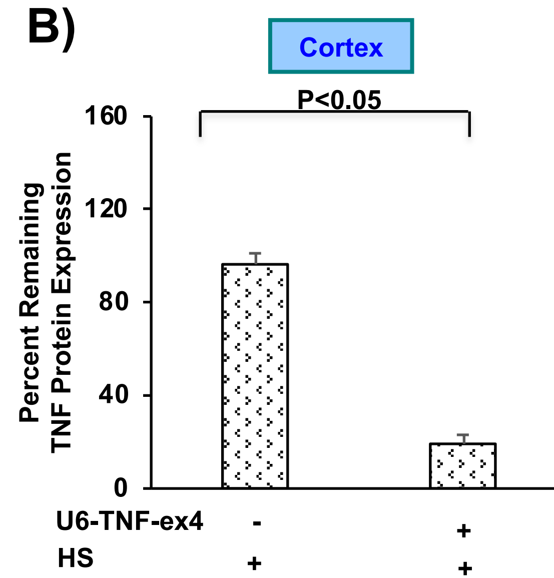
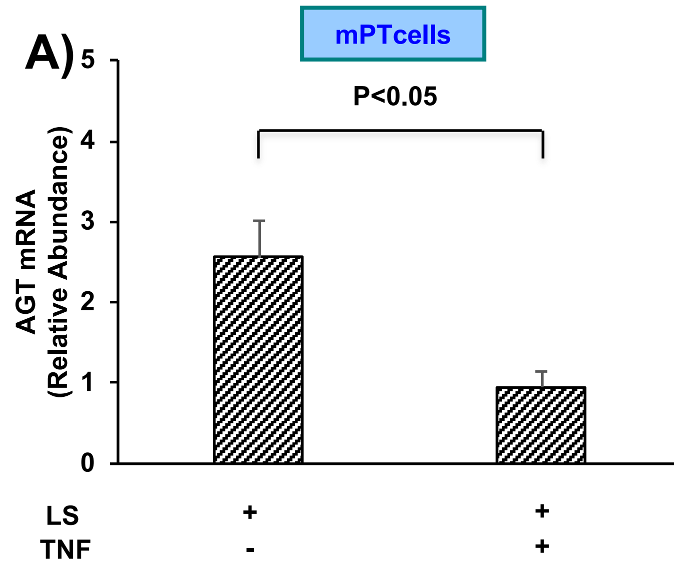


Figure S2



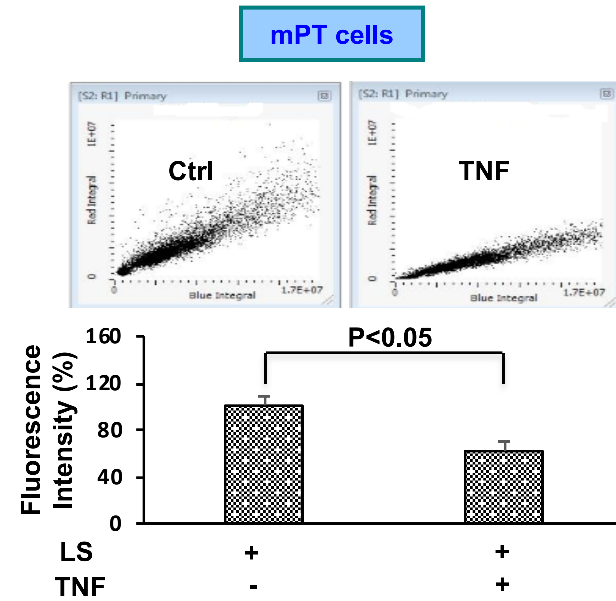


Figure S3

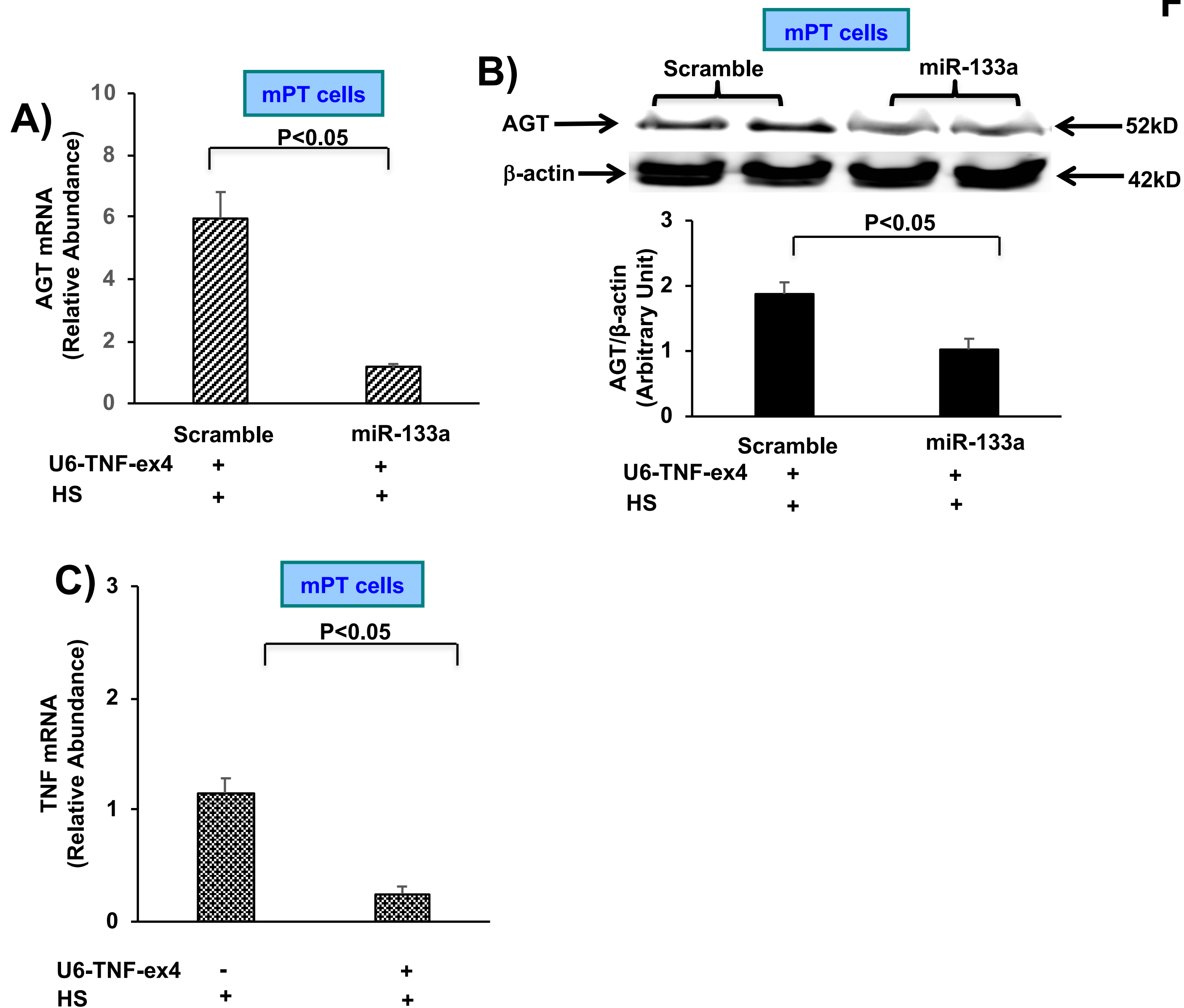


Figure S4