Exosomal lncRNA TUG1 Derived From Human Urine-derived Stem Cells Attenuates Renal Ischemia/reperfusion Injury by Interacting With SRSF1 to Regulate ASCL4 Mediated Ferroptosis

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Research Article

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Abstract

Background: Human urine-derived stem cells (USCs) derived exosomes protect against kidney ischemia/reperfusion injury (IRI), while the underlying mechanisms of this protective effect remain unclear.

Methods: Human USCs and USCs derived exosomes (USC-Exo) were isolated and verified by morphology and specific biomarkers. The effects of USC-Exo on ferroptosis and kidney injury were detected in the IRI-induced acute kidney injury (AKI) model in C57BL/6 mice. The effects of USC-Exo on ferroptosis and lncRNA taurine upregulated gene 1 (TUG1) were detected in hypoxia/reoxygenation (H/R) treated human proximal tubular epithelial cells (HK-2). The interaction of SRSF1 and TUG1, ACSL4 were checked via RNA pull down/RIP and RNA stability assays. The effects of lncRNA TUG1 on SRSF1/ACSL4 mediated ferroptosis were verified in H/R treated HK-2 cells and the IRI-induced AKI mouse models.

Results: USC-Exo treatment improved kidney injury and ameliorated ferroptosis in IRI-induced AKI mouse models. USC-Exo were rich in lncRNA TUG1, which regulated ferroptosis in HK-2 cells exposed to H/R. Mechanistically, lncRNA TUG1 regulates the stability of ACSL4 mRNA by interacting with RNA binding protein SRSF1. In addition, SRSF1 up-regulation or ACSL4 down-regulation partially reversed this protective effect of lncRNA TUG1 on ferroptosis in H/R treated HK-2 cells. Further, ACSL4 up-regulation partially reversed the protective effect on kidney injury and cell ferroptosis by lncRNA TUG1 in IRI-induced AKI mice.

Conclusion: Collectively, lncRNA TUG1 carried by USC-Exo regulated ACSL4 mediated ferroptosis by interacting with SRSF1, then protected IRI-induced AKI. Potentially, USC-Exo rich in lncRNA TUG1 can serve as a promising therapeutic method for IRI-induced AKI.

Highlights

1. lncRNA TUG1 carried by USC-Exo regulates cell ferroptosis.
2. lncRNA TUG1 regulates the stability of ACSL4 mRNA by interacting with SRSF1.
3. lncRNA TUG1 alleviates IRI-induced AKI by suppressing ACSL4 mediated ferroptosis.

Introduction

Ischemic/reperfusion injury (IRI) is one of the common reasons for acute kidney injury (AKI), which is related to high morbidity and mortality [1, 2]. Addition to the high mortality rates, patients with AKI has a high risk for progression to chronic kidney disease developing and hastened the development of end-stage renal disease [3]. Unfortunately, despite recent advances in our understanding of the pathogenesis of IRI-induced AKI, largely therapeutic strategies are often ineffective [3, 4]. Therefore, efforts focused on

clarifying pathophysiological mechanisms will hopefully find a new therapy to prevent and treat IRI-induced AKI.

Increasing evidence has been demonstrated that stem cell therapy is a new promising therapy for kidney diseases [5–8]. Different types of stem cells, such as mesenchymal stem cells and spermatogonia stem cells, have been shown to either attenuate kidney dysfunction or pathological morphology improvement in AKI [6, 9]. Human urine-derived stem cells (USCs) were recently identified as a new therapeutic strategy for kidney diseases [10, 11]. Moreover, the patient-derived USCs were identified as a novel biomarker to predict the outcome of the kidney disease [12]. Some studies have revealed that USCs alleviated IRI-induced AKI in a rat model [11]. However, the specific mechanism of USCs' protective effect on IRI-induced AKI is complicated and has not been fully elucidated.

The paracrine mechanism of exosomes is one of the main mechanisms of stem cell therapy for AKI [13, 14]. Exosomes are vesicles with a bilayer membrane structure rich in proteins, lipids, miRNAs, lncRNA, and other RNA species [15]. Exosomes are intracellular components carrying biologic signals from one cell type or tissue to another, which are taken up by other cells and participate in regulating the pathological process of diseases [16]. Exosomes derived from different stem cells have been proved to effectively protect against AKI *in vivo* and *in vitro* [6, 10]. Some studies suggest that lncRNA carried by exosomes may have an essential role in the immune response of diseases' pathology and represent a novel target for AKI. lncRNA taurine upregulated gene 1 (TUG1) was downregulated in AKI, while upregulated TUG1 not only presented a protective effect on kidney injury but also reduced IRI-induced AKI [17, 18]. However, the mechanism of the protective effect of lncRNA TUG1 on IRI-induced AKI is unclear.

Recently ferroptosis was discovered as a new regulated cell death which is dependent on iron and reactive oxygen species [19]. Mis-regulated ferroptosis has been demonstrated in multiple physiological and pathological processes like cancer, tissue injury, T-cell immunity and so on [20]. Acyl-CoA synthetase long-chain family member 4 (ACSL4) is a sensitive monitor of ferroptosis, and an important contributor of ferroptosis [21]. ACSL4 downregulation reduces cell ferroptosis and protects the brain and gut IRI [22, 23]. However, the role of ACSL4 mediated ferroptosis in IRI-induced AKI is not clear.

lncRNA has been shown to regulate the stability and expression of downstream target mRNA and participate in the disease process by interacting with RNA binding proteins [24]. Serine/Arginine Splicing Factor 1 (SRSF1) is an RNA binding protein from the SR protein family of splicing regulators, which has been proved involved in mRNA metabolism, such as mRNA splicing, stability, and translation, as well as other mRNA-independent processes [25]. Studies have shown that SRSF1 expression is down-regulated in myocardial IRI, and its overexpression can inhibit myocardial cell apoptosis [26]. In addition, starBase, an online bioinformatics tool, predicted that RNA binding protein SRSF1 had a potential binding relationship with lncRNA TUG1 and ACSL4. However, whether lncRNA TUG1 regulates ACSL4 expression by interacting with SRSF1 is unknown, and the role of this interaction in IRI-induced AKI is not elucidated.

In this study, we investigated the potential protective role of lncRNA TUG1 carried in USCs-exosomes (USC-Exo) by using *in vivo* and *in vitro* models of IRI-induced AKI. Based on the above findings, we

hypothesized that lncRNA TUG1 carried by USC-Exo may regulate ACSL4-mediated cell ferroptosis by interacting with SRSF1, then alleviate IRI-AKI.

Materials And Methods

Isolation and identification of USCs

Human USCs were isolated from urine samples were collected from healthy male donors (22-28 years old) as previously described [27]. Urine samples were centrifuged at 400 g for 10 minutes at room temperature (RT). After discard the supernatants, the sediments were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS, Gibco), human epidermal growth factor (hEGF, 10 ng/mL, Gibco), transforming growth factor β (TGF- β , 1 ng/mL, Gibco), basic fibroblast growth factor (bFGF, 2 ng/mL, Gibco), platelet-derived growth factor (PDGF, 2 ng/mL, R&D systems), hydrocortisone (0.5 mM, Sigma), insulin (25 mg/mL, Invitrogen), transferrin (20 mg/mL, Sigma), epinephrine (549 ng/mL, Sigma), triiodothyronine (50 ng/mL), L-glu, and 20 IU/mL of antibiotics. After counting by a hemocytometer, cells were seeded into cellular culture flasks and cultured in a cell incubator at 37 °C for 7 days to allow them to adhere to the substrate. The nonadherent cells were removed by washing with PBS.

USCs were identified by flow cytometry, western blot, and qPCR. USCs were stained with the positive surface antigens antibodies PE-CD29 (#303004), APC-CD90 (#328114), and FITC-CD44 (#397518) and negative surface antigens antibodies FITC-CD45 (#304054), APC-CD34 (#343510), and PE-HLA-DR (#327008) for 30 min at RT. All antibodies from experiments were purchased from Biolegend (San Diego, CA, United States). Isolated cells were analyzed by flow cytometry using a FACSCanto II (BD Biosciences, San Jose, CA, United States). Isotype antibodies were used as controls. The specific kidney markers NEPHRIN and WT-1 were used to confirm USCs by western blotting. Stem cell pluripotency markers OCT4 and NANOG were detected by qPCR.

Isolation and identification of USC-Exo

Human USC-Exo were separated from USCs conditioned medium using total exosome isolation kits according to the company instruction (#4478359, Invitrogen). Briefly, USCs cell culture media was harvested and centrifuged. Then, the culture media was mixed with exosome isolation reagent and incubated at 4°C overnight. After incubation, the mixtures were centrifuged at 10,000 g for 1 h at 4°C. After discarding the supernatants, exosomes were contained in the pellet of the tube.

For identification, the exosome pellets were fixed with 2% glutaraldehyde for 2 h. After washing, the grids were contrasted in 2% uranyl acetate, dried, and then examined by TEM (Morgagni 268D, Philips, Holland). Exosome's markers and other different specific cell particles surface markers: CD9, CD63, CD81, TSG101, Cytochrome C, Calnexin and Syntaxin 6 were analyzed by flow cytometry and western blotting to identify USC-Exo.

Mouse renal ischemia/reperfusion (I/R) model

Mouse renal I/R model was performed in male C57BL/6 mice (8-12 weeks old) as previously described [28]. Briefly, the mice were anesthetized with pentobarbital sodium by intraperitoneal injection and lay on the right side. Dorsal incisions of both left and right sides were made to expose kidneys. The right kidney artery was gently separated with cotton swabs and occluded with a microvascular clamp to induce renal ischemia for 45 min. The left renal pedicle clamping and ischemia were the same as right. After ischemia, the micro-aneurysm clips were removed to start the reperfusion. The wounds were sutured and resuscitated with warm sterile saline intraperitoneally. All operations were the same in the sham group except for clamping and ischemia.

Animal treatment

Experimental mice were randomly included into following groups: sham (n = 6); I/R (n = 6); I/R+PBS (n = 6), mice underwent renal I/R and injected the same volume of PBS 15 min before the renal artery clamp was placed; renal I/R+USCs-Exo (n = 6), mice underwent renal I/R and pre-treated with USCs-Exos (20 µg/mL) intravenously 15 min before the renal artery clamp was added; I/R + ferrostatin-1 (Fer-1, ferroptosis inhibitor, #S7243, selleckchem, German) (n=6), mice underwent renal I/R and pre-treated with Fer-1 (5 mg/kg) intraperitoneally 15 min before the renal artery clamp was placed. For I/R + negative control lncRNA (NC) group (n = 6), mice were pre-treated with the same volume of NC 1 h before underwent renal I/R; renal I/R + lncRNA TUG1 (n=6), mice were pre-treated lncRNA TUG1 (*i.p.*, 60 mg/kg) 1 h before underwent renal I/R; I/R + lncRNA TUG1 + ACSL4 (n=6), mice were pre-treated lncRNA TUG1 and ACSL4 (*i.p.*, 60 mg/kg) 1 h before underwent renal I/R. Blood and tissue samples were collected at 24 h post I/R.

Biochemical assays

Commercially available blood urea nitrogen (BUN, #EIABUN, Invitrogen) and serum creatinine (Scr) detection kits (#C011-2-1, Jiancheng Bioengineering Institute, Nanjing, China) were used to measure the levels of biochemical kidney parameters by following the manufacturer's instructions, using an Olympus AU5400 Automatic Biochemical Analyzer (Olympus, Tokyo, Japan).

Malondialdehyde (MDA) and glutathione (GSH) Assay

The levels of MDA and GSH in kidney tissues or cell lysates was assessed using a Lipid Peroxidation (MDA) Assay Kit (#S0131M, Beyotime Biotechnology, Shanghai, China) and GSH Assay Kit (#S0053, Beyotime Biotechnology) according to the manufacturer's instructions with a microplate fluorometer at 450 and 405 nm, respectively.

Hematoxylin and eosin (H&E) dye and transferase terminal UTP nick-end labeling (TUNEL) staining

Kidney sections were fixed in 10% buffered formalin and embedded in paraffin, then 3 µm sections were stained with H&E for 3 min, and visualized under an optical microscope (Olympus Optical, Tokyo, Japan). Cell apoptosis of kidney tissue or HK-2 cells was detected by using TUNEL assay (Roche, Basel, Switzerland) according to the manufacturer's instructions. The kidney tissue slides or HK-2 cells were

fixed, paraffin-embedded and labeled with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides including tetramethyl rhodamine-labeled (TMR-labeled) dUTP at RT for 1 h. After staining the nucleus with DAPI, tissues and cells were covered by a fluorescent mounting medium. The TUNEL-positive cells were counted in each sample, and the percentage of TUNEL-positive cells was calculated from four different samples.

Iron assay

For iron concentration (Fe^{2+} level) detection, cell lysates or kidney tissue was homogenized using PBS. After centrifugation, iron concentration in the supernatant was assessed by an Iron Assay Kit (#ab83366, Abcam) according to the manufacturer's instructions.

ROS level measurement

ROS level detection was performed using the ROS Assay Kit (#S0033M, Beyotime Biotechnology) as described in the manufacturer's instructions. In brief, cells were collected and suspended in diluted DCFH-DA, followed by incubation for 20 min at 37°C. After washing the cells three times with a serum-free cell culture medium, fluorescence was examined at the excitation/emission 488/525 nm on a fluorescence microplate reader.

qPCR analysis

Total RNA was extracted from cells or tissues with TRIzol Reagent (#15596026, Thermo Fisher Scientific). A PrimeScript™ RT Reagent Kit (#RR600A, TaKaRa Bio) was used for RNA reverse transcription. Then cDNAs were subjected to quantitative real-time PCR using gene-specific primers according to TB Green™ Premix Ex Taq™ (TaKaRa Bio, Inc.) protocols. Human-18S ribosomal mRNA levels or GAPDH was used as internal controls. Data were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [29]. The primers used in this study are presented in Table 1.

Table 1
Sequence of primers for qPCR analysis.

Gene	Primer sequence
OCT4	Forward: 5'-CTTGAATCCCGAATGGAAAGGG-3'
	Reverse: 5'-GTGTATATCCCAGGGTGATCCTC-3'
NANOG	Forward: 5'-GTCCCAAAGGCAAACAACCC-3'
	Reverse: 5'-ATCCCTGCGTCACACCATTG-3'
TUG1	Forward: 5'-ACGACTGAGCAAGCACTACC-3'
	Reverse: 5'-CTCAGCAATCAGGAGGCACA-3'
SRSF1	Forward: 5'-CCGCAGGGAACAACGATTG-3'
	Reverse: 5'-GCCGTATTTGTAGAACACGTCCT-3'
ACSL4	Forward: 5'-TTGGGCATTCTCCAAGTAG-3'
	Reverse: 5'-CCTGCAGCCATAGGTAAAGC-3'
GAPDH	Forward: 5'-AGGTCGGAGTCAACGGATTT-3'
	Reverse: 5'-TGACGGTGCCATGGAATTTG-3'

Western blotting

After protein extraction, the concentration was determined using bicinchoninic acid (BCA) protein assay. Then, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore). After blocking, the membrane was incubated with primary antibodies (Table 2) at 4°C overnight and HRP-conjugated secondary antibody HRP-conjugated secondary antibody (goat anti-rabbit, #A1055, BOSTER Biological Technology, Wuhan, China) at RT for 1 h. The signals were visualized with ECL substrate (Bio-Rad Laboratories, Inc.).

Table 2
Primary antibodies used in western blotting assay.

Antibody	Company (Cat. No.)	Working dilution
NEPHRIN	Abcam (ab216341)	1:1000
WT-1	Abcam (ab89901)	1:500
CD9	Abcam (ab92726)	1:2000
CD63	Abcam (ab134045)	1:5000
TSG101	Abcam (ab125011)	1:5000
Cytochrome C	Abcam (ab133504)	1:5000
Calnexin	Cell Signaling (#2679)	1:1000
Syntaxin 6	Cell Signaling (#2869)	1:2000
ACSL4	Abcam (ab155282)	1:20000
GPX4	Bioss (bs-3884R)	1:1000
FTH1	Cell Signaling (#4393)	1:1000
COX2	Cell Signaling (#12282)	1:1000
SRSF1	Cell Signaling (#14902)	1:1000
GAPDH	Bioss (bs-10900R)	1:1000

Uptake of Exosomes

The PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) was used to label exosomes. Briefly, HK-2 cells were stained with PKH67 dye for 3 min. After centrifugation, the labeled exosomes were resuspended in PBS. The labeled exosomes were added into the culture medium of HK-2 cells. After incubation for 0, 1, 3 h in the dark, the cells were fixed in 4% paraformaldehyde for 20 min and stained with DAPI. Observing exosomes' uptake was visualized using Zeiss LSM900 confocal microscopy system. The images obtained by confocal microscope were merged and combined by FIJI Image J.

Cell culture and transfection

HK-2 cells (from ATCC) were cultured in DMEM/F12 medium supplemented with 10% FBS at 37°C in an incubator. shRNA TUG1 (sh-TUG1, GACTACCTTCCCTGTGCTATT), shRNA ACSL4 (sh-ACSL4, CCAGTGTTGAACTTCTGGAAA) and shRNA negative control (sh-NC) were designed and synthesized by GenePharma (Shanghai, China). For overexpression, TUG1, SRSF1, ACSL4 cDNA were PCR-amplified and then cloned into the expression vector pcDNA3.1 (RiboBio, Guangzhou, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection of plasmids into HK2 cells by following the

protocol from the manufacturer. For experiments *in vivo*, TUG1 and ACSL4 expression plasmids were packaged with pPACKH1 Lentivector Packaging Plasmid Mix (System Biosciences, Palo Alto, CA, USA).

Cell Counting Kit-8 (CCK8) assay

A CCK8 assay kit (#ab228554, abcam) was used for the cell viability assay. HK2 cells were seeded in a 96-well plate and co-cultured with PBS, USCs-derived conditioned medium and exosomes followed exposed to H/R. Then, 10 μ L of CCK8 solution was added into each well and incubated for 2 h at RT. The OD value at 450 nm wavelength was determined using by BioTek Synergy Neo2 multi-mode reader (BioTek Instruments, Inc.).

Subcellular fractionation and fluorescence in situ hybridization (FISH) assay

For subcellular fractionation, cytoplasmic and nuclear RNAs from HK-2 cells were isolated by using NE-PER Reagent (Thermo Scientific). Then, qPCR measurement was performed to assess TUG1 distribution and normalized to GAPDH (cytoplasm) and U1 (nucleus). For FISH assay, End-labeled 6-carboxyfluorescein (FAM) probes were synthesized for TUG1 (Invitrogen). Cells were seeded on coverslips and fixed using 4% formalin. The cells were stained in hybridization buffer (0.7 M NaCl, 0.1 M Tris (pH 8.0), 0.1% SDS, and 10 mM EDTA) containing the probes. Cells were heated at 55°C for 30 min, mounted using a mounting medium containing DAPI and were analyzed by fluorescence microscopy (Olympus Optical, Tokyo, Japan).

RNA immunoprecipitation (RIP)

RIP experiments were performed using a EZMagna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, HK-2 cells were harvested and lysed in RIP lysis buffer on ice for 30 min. Subsequently, cell extracts were incubated with RIP buffer (containing magnetic beads conjugated to antibodies against SRSF1 or normal anti-rabbit IgG (Millipore)) for 2 h at RT. After washing, beads were resuspended in Trizol and co-precipitated RNAs were isolated for qPCR examination.

RNA pull-down assay

Biotin-labeled full length TUG1 RNA probe was prepared with the Biotin RNA Labeling Mix (#11685597910, Roche, Basel, Switzerland). Biotinylated RNAs were mixed with lysates prepared from HK-2 cells and incubated at 4°C for 1 h. The mixtures were incubated with washed Streptavidin agarose beads (#S1638, sigma) at 4°C for 1 h in binding buffer. RNA bound to the probe was eluted and the retrieved protein was visualized by western blot.

mRNA Stability Assay

HK-2 cells were seeded into 6 well plates and transfected TUG1 and/or SRSF1 overexpression plasmid for 48 h, followed by treatment with Actinomycin D (5 μ g/mL). At 0, 3, 6 h time points, Trizol reagent was added to extract RNA from the cells and ACSL4 mRNA remaining was analyzed via qPCR assay.

Statistical Analysis

GraphPad Prism version 8.0 software (GraphPad Software, Inc.) was applied for statistical analyses in all experimental data. Student's *t*-test was used for the comparisons of two groups. Comparisons of multiple groups were performed by using one-way ANOVA and Tukey's multiple comparison test. A P-value <0.05 was considered to indicate a statistically significant difference between study groups.

Results

Human USCs were isolated and identified

After 3-5 days of primary culture, adherent cells and spindle-shaped colonies were observed. At passage 3, isolated cells showed a spindle-shape and fibroblast-like morphology and rapid proliferation rate (Figure 1A). Human urine-derived cells were analyzed by flow cytometry, which showed that urine-derived cells positively expressed MSCs markers CD44, CD29 and CD90, while negatively expressed hematopoietic antigens CD45, CD34 and HLA-DR (Figure 1B). Western blot analysis demonstrated that urine-derived cells were highly expressed specific kidney markers NEPHRIN and WT-1 (Figure 1C). In addition, stem cell pluripotency markers OCT4 and NANOG were detected in urine-derived cells by qPCR (Figure 1D). Collectively, we successfully isolated human USCs.

USC-Exo were isolated and identified

TEM, nanoparticle tracking analysis (NTA), flow cytometry, and western blotting were used to identify the USCs derived cell particles. These particles showed bilayer membrane vesicles the same as exosomes with a diameter of 60-80 nm (Figure 2A-B). Flow cytometry showed that exosomes markers CD9, CD63 and CD81 were expressed in USCs derived cell particles (Figure 2C). Western blot also confirmed that CD9 and CD63 were positive in particles from human USCs, while TSG101, Cytochrome C, Calnexin and Syntaxin 6 were negative in USCs-particles (Figure 2D). These results suggested that we successfully isolated USC-Exo.

USC-Exo ameliorates ferroptosis in the kidney after IRI-induced AKI

To investigate the effect of USC-Exo on the IRI induced kidney injury and ferroptosis, IRI-AKI mice were treated with USC-Exo or ferroptosis inhibitor Fer-1 or vehicle before IRI 15 min (Figure 3A). In this work, the serum levels of BUN and creatinine were increased in I/R mice compared to sham group, and this increase was significantly decreased by USC-Exo or Fer-1 treatment (Figure 3B). The morphological tubular HE and TUNEL dye showed that the kidney tissues of I/R mice were edematous with larger cellular volume, vacuolar degeneration, tubular epithelial cell death and glomerular structure was disordered accompanied by narrowed lumen of the renal tubules. These pathological lesions were alleviated by USC-Exo or Fer-1 treatment (Figure 3C). The tissue levels of MDA and iron were increased, GSH was decreased in I/R mice compared with sham group, and USC-Exo or Fer-1 treatment could partly reverse this trend (Figure 3D-E). In addition, I/R induced upregulated expression of ACSL4 and COX2, and down-regulated expression of GPX4 and FTH1 in kidney tissues, while those effects were inhibited by USC-

Exo or Fer-1 treatment (Figure 3F). Taken together, USC-Exo suppresses tubular epithelial cell ferroptosis and has a protective effect on IRI-induced AKI.

LncRNA TUG1 carried by USC-Exo suppresses H/R induced ferroptosis in HK-2 cells

Next, the expression of lncRNA TUG1 was significantly increased in the isolated USC-Exo compared with whole cell lysis (Figure 4A). PKH67 and DAPI dye was used to label exosomes and fluorescence microscopy observed that exosomes can be uptaken by HK-2 cells (Figure 4B). To verify the role of lncRNA TUG1 carried by USC-Exo in ferroptotic protection, HK-2 cells exposed to H/R were treated with or without USC-Exo, and we used shRNA tool to knockdown TUG1 expression. H/R decreased lncRNA TUG1 expression in HK-2 cells compared with control group, while USC-Exo reversed TUG1 expression in HK-2 cells after H/R (Figure 4C). The expression of TUG1 in sh-TUG1 USC-Exo was lower than that in USC-Exo and sh-NC treated USC-Exo (Figure 4D). Moreover, H/R significantly decreased cell viability and increased cell apoptotic rate in HK-2 cells, these effects were partially reversed by USC-Exo. While Exo isolated from USCs transfected with TUG1 shRNA reduced the increase in cell viability and decrease in apoptosis caused by USC-Exo (Figure 4E-F). USC-Exo inhibited the increased effect on the levels of MDA, iron, ROS and decreased effect on GSH level by H/R in HK-2 cells, while TUG1 silenced USC-Exo could suppress these changes caused by USC-Exo (Figure 4G-I). In addition, USC-Exo suppressed the increased ACSL4 and COX2 expression and the decreased GPX4 and FTH1 expression by H/R in HK-2 cells, while this trend was reversed by Exo isolated from USCs transfected with sh-TUG1 (Figure 4J). These results suggested that lncRNA TUG1 carried by USC-Exo suppresses H/R induced ferroptosis in HK-2 cells.

LncRNA TUG1 regulates ACSL4 mRNA stability by interacting with SRSF1

Subcellular fractionation analysis and FISH assay showed TUG1 was expressed in both cytoplasm and nuclear (Figure 5A-B). RNA-pull down assay was proceed as shown in Figure 5C and the results demonstrated that SRSF1 was existed in the samples of pull down, which suggested that TUG1 was interacted with SRSF1 (Figure 5D). In addition, SRSF1 antibody caught more TUG1 compared with IgG (Figure 5E). To determine which location of SRSF1 is interacted with lncRNA TUG1, we designed and purified SRSF1 full length protein and some protein fragments, like RRM1, RRM2 and C terminal structural domain. We found that all three protein domains were involved into the interaction between TUG1 and SRSF1 (Figure 5F-G). Additionally, USC-Exo significantly suppressed H/R induced ACSL4 mRNA and protein expression (Figure 5H). Furthermore, TUG1 and SRSF1 treatment significantly down-regulated ACSL4 mRNA and protein expression (Figure 5I). Next, RIP assay showed ACSL4 mRNA was enriched by SRSF1 vs control IgG antibody, moreover, this enrichment was significantly increased by TUG1 overexpression (Figure 5J). Furthermore, TUG1 and SRSF1 could promote the stability of ACSL4 mRNA (Figure 5K). Therefore, lncRNA TUG1 regulates the stability of ACSL4 mRNA by interacting with SRSF1.

LncRNA TUG1 suppresses H/R induced ferroptosis by regulating SRSF1/ACSL4 in HK-2 cells

Then, we determined whether the inhibition of ferroptosis by lncRNA TUG1 through SRSF1/ACSL4 in HK-2 cells. We found that downregulation of TUG1 by shRNA transfection reversed the protective effects of USC-Exo on the cell viability and death in H/R treated HK2 cells. while SRSF1 up-regulation or ACSL4 down-regulation partially reversed these effects of sh-TUG1 on cell viability and death in USC-Exo treated HK2 cells stimulated by H/R (Figure 6A-B). The levels of MDA, iron level and ROS were increased and GSH was decreased by TUG1 inhibited USC-Exo in USC-Exo treated HK2 cells stimulated by H/R (Figure 6C-E). While SRSF1 upregulation or ACSL4 downregulation partially reversed this reduced effect by sh-TUG1 treated USC-Exo. Moreover, the protein expression of ACSL4 and COX2 were decreased and GPX4 and FTH1 were increased in USC-Exo treated HK2 cells stimulated by H/R, while TUG1 downregulated USC-Exo markedly reversed those effect by USC-Exo in H/R treated HK-2 cells (Figure 6F). And, SRSF1 upregulation or ACSL4 downregulation partially reversed the trend of sh-TUG1 transfected USC-Exo on ferroptosis (Figure 6F). These data suggest that lncRNA TUG1 ameliorates H/R-induced ferroptosis by regulating SRSF1/ACSL4 in HK-2 cell.

lncRNA TUG1 ameliorates IRI-induced AKI by suppressing ACSL4 mediated ferroptosis

Finally, the *in vivo* impact of lncRNA TUG1 on ferroptosis and kidney injury were assessed. We found that lncRNA TUG1 treatment significantly reduced levels of serum creatine and BUN induced by IRI-induced AKI mice (Figure 7A). The morphological tubular HE and TUNEL dye also showed that lncRNA TUG1 treatment significantly alleviated IRI-induced kidney injury evidenced by decreased kidney injury score and inhibited tubular epithelial cell death (Figure 7B). However, ACSL4 overexpression partially reversed this protective effect on kidney injury by lncRNA TUG1 (Figure 7A-B). We also found that the levels of MDA, iron were significantly increased and GSH level was remarkably decreased in IRI-induced AKI mice compared to the sham mice, while lncRNA TUG1 markedly decreased those in IRI-induced AKI mice (Figure 7C-E). Moreover, ACSL4 partially reversed this reduced effect by lncRNA TUG1. Additionally, the protein expression of ACSL4 and COX2 were increased, and GPX4 and FTH1 were decreased in IRI-induced AKI mice compared to the sham mice, while lncRNA TUG1 markedly reversed those in IRI-induced AKI mice (Figure 7F). And, ACSL4 up-regulation partially reversed the inhibition effect on ferroptosis by lncRNA TUG1 (Figure 7F). Taken together, lncRNA TUG1 ameliorates IRI-induced kidney injury by suppressing ACSL4 mediated ferroptosis (Figure 8).

Discussion

Despite significant advances in the treatment, the mortality of acute renal failure remains high. Therefore, efforts to clarify the specific pathogenesis are essential for finding a new therapy for IRI-induced AKI. Several stem cells have been identified as the most promising therapy for structural and functional repair during AKI [30]. For instance, some studies have demonstrated that human USC treatment improves kidney injury in a rat ischemic AKI model [10]. As reported, extracellular vesicles, such as exosomes, have been described as a different cell-to-cell communication way of transferring microRNAs, mRNAs, and proteins to target cells [31]. Convincing evidence indicates that extracellular vesicles like exosomes secreted by stem cells present protective effects on AKI [32]. In an IRI-induced AKI rat model, USC-Exo can

reduce tubular cell apoptosis and protect against kidney injury by transferring miR-216a-5p [33]. In line with the previous studies, USC-Exo treatment showed a protective effect on IRI-induced AKI with evidence of decreased BUN and serum creatine levels and reduced cell death of tubular epithelial cells. However, the mechanism of the protective effect of USC-Exo on IRI-induced AKI is still unclear.

Ferroptosis was recently discovered as a new iron- and reactive oxygen species (ROS)-dependent form of regulated cell death [19]. Studies revealed that dysregulation of iron metabolism plays an essential role in AKI. In the present study, we found that IRI increased the expressions of MDA, iron, ROS, ACSL4, and COX2, and decreased GSH, GPX4, and FTH1 in the kidney tissues. These data suggested that ferroptosis was upregulated in IRI-induced AKI. Convincing evidence proved that strategies like iron chelation therapy, targeting iron metabolism-related proteins, and direct inhibitors of ferroptosis may provide new therapeutic methods for AKI [34, 35]. In our experiments, we observed that USC-Exo treatment had the same protective effect as ferroptosis inhibitor Fer-1 in IRI-induced kidney injury. USC-Exo treatment suppresses tubular epithelial cell ferroptosis *in vitro* and *in vivo* and has a protective effect on IRI-induced kidney injury.

Exosomes are vesicles with a bilayer membrane structure, which are rich in proteins, lipids, miRNAs, lncRNA, and other RNA species [15]. Increasing evidence suggests that lncRNA carried in exosomes may have a vital role in the immune response of pathology of diseases and represent a novel target for AKI. As reported, lncRNA TUG1 was downregulated in LPS-induced and IRI-induced AKI. At the same time, upregulating TUG1 presented a protective effect on IRI-induced kidney injury [17, 18]. During the experiments, we observed that the levels of lncRNA TUG1 were significantly increased in USC-Exo. Thus, we speculated that lncRNA TUG1 might participate in the protective effect of USC-Exo on IRI-induced AKI by suppressing ferroptosis for further investigation. Consistent with our speculation, further experiments confirmed that TUG1 silenced USC-Exo showed lower expression of TUG1. Furthermore, it reversed the ferroptosis protection effects of USC-Exo in H/R treated HK-2 cells. Therefore, these results suggested that USC-Exo suppresses H/R induced ferroptosis by transferring lncRNA TUG1 in HK-2 cells. However, the molecular mechanism for suppressing ferroptosis by lncRNA TUG1 carried by USC-Exo remains unclear.

Abundant evidence has shown that lncRNA regulates the stability and expression of downstream target mRNA and participates in the process of disease by interacting with RNA-binding proteins [24]. SRSF1 is the archetype member of the SR protein family of splicing regulators, which has been involved in several key aspects of mRNA metabolism, such as mRNA splicing, stability, and translation, as well as other mRNA-independent processes [25]. Studies have shown that SRSF1 expression is down-regulated in myocardial IRI, and its overexpression can inhibit myocardial cell apoptosis [26]. In addition, ACSL4 is a sensitive monitor of ferroptosis and an important contributor of ferroptosis [21]. Silent ACSL4 gene reduces cell ferroptosis and protects the brain and gut IRI [22, 23]. In present study, we found that lncRNA TUG1 was interacted with SRSF1 evidenced by RNA pull down assay and RIP assay. We also proved that lncRNA TUG1 regulates the stability of mRNA ACSL4 by interacting with SRSF1. Therefore, we found that lncRNA TUG1 suppressed ACSL4 mediated ferroptosis by interacting with SRSF1 in HK-2 cells.

Conclusion

In conclusion, the present study demonstrated a potential mechanism underlying the protective role of USC-Exo rich in lncRNA TUG1 against IRI-induced AKI, which was involved in inhibiting ACSL4 mediated ferroptosis through interacting with SRSF1. These findings suggest that the reprogramming of iron homeostasis is essential in renal injury, and USC-Exo and TUG1 overexpression are a promising therapeutic method for the treatment of IRI-AKI.

List Of Abbreviations

ACSL4, acyl-CoA synthetase long-chain family member 4; AKI, acute kidney injury; BUN, blood urea nitrogen; DMEM, Dulbecco's modified eagle medium; FISH, fluorescence in situ hybridization; GSH, glutathione; HK-2, human proximal tubular epithelial cells; IRI, ischemic/reperfusion injury; MDA, malondialdehyde; RIP, RNA immunoprecipitation; ROS, reactive oxygen species; Scr, serum creatinine; SRSF1, Serine/Arginine Splicing Factor 1; TEM, transmission electron microscopy; TUG1, taurine upregulated gene 1; TUNEL, transferase terminal UTP nick-end labeling; USCs, urine-derived stem cells.

Declarations

Acknowledgments

Not applicable

Ethics approval and consent to participate

The animal experiment protocols were approved by the MDKN Biotech Co., Ltd. (Beijing) and ethics committee of Capital Medical University Beijing Chaoyang Hospital.

Consent for publication

All authors have signed the consent for publication.

Availability of data and material

All data are available from the first author (Zejia Sun) on reasonable request.

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Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

This study was designed by ZJS. QB and ZJS made great contribution to the data collection of this study, QB and JYW were responsible for carrying out the experiments, data analysis and ascertained the integrity as well as accuracy of the data. ZJS prepared and revised this manuscript. WW funded the research. ZJS, JYW and QB contributed equally to this work and should be listed as first co-author. All authors have read and approved the manuscript.

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Figures

Figure 1

Isolation and Identification of human USC. (A) The morphology of isolated USCs was shown by microscope. (B) USCs were stained with surface antigens antibodies and analyzed by flow cytometry. (C) The expression of NEPHRIN and WT-1 in USCs were assessed by western blot analysis. (D) The gene expression of OCT4 and NANOG in HUSCs were assessed by qPCR analysis.

Figure 2

Isolation and identification of USC-Exo. (A) The morphology and size of isolated USC-derived particles were checked by TEM and nanoparticle tracking analysis. (C) USC-Exo were stained with surface antigens antibodies CD9, CD63 and CD81, and analyzed by flow cytometry. (D) The protein expressions of CD9, CD63, TSG101, Cytochrome C, Calnexin and Syntaxin 6 in USC-Exo were assessed by western blot analysis.

Figure 3

USC-Exo ameliorates ferroptosis in IRI-induced AKI. (A) Diagram of IRI-AKI model and treatment. (B) Serum levels of creatinine and BUN were assessed by colorimetric method. (C) The injury and cell death of kidney tissues showed by HE dyes and TUNEL assay. (D) The levels of MDA and GSH in the kidney tissues were measured by commercial kits. (E) The levels of Fe^{2+} in the kidney tissues of mice were

assessed by commercial kits. **(F)** The protein levels of ACSL4, GPX4, FTH1 and COX2 in the kidney tissues were assessed by western blot. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. I/R group.

Figure 4

LncRNA TUG1 carried by USC-Exo suppressed H/R induced ferroptosis in HK-2 cells. **(A)** The expression of TUG1 in isolated USC-Exo was measured by qPCR. **(B)** The uptake of USC-Exo by HK-2 cells was checked by PKH67 immunofluorescence dyes. **(C)** The effect of USC-Exo on the expression of TUG1 in HK-2 cells was detected by qPCR. **(D)** The expression of TUG1 in USC-Exo, sh-NC treated USC-Exo and TUG1 silenced USC-Exo was checked by qPCR. **(E)** Cell viability in HK-2 cells after treated with H/R and sh-NC treated USC-Exo and TUG1 silenced USC-Exo was measured by CCK-8 kits. **(F)** Cell death in HK-2 cells was checked by TUNEL assay. **(G)** The levels of MDA and GSH in HK-2 cells were assessed by using commercial kits. **(H)** The levels of Fe^{2+} in HK-2 cells were assessed by using commercial kits. **(I)** The

levels of ROS in HK-2 cells were detected using DCFH-DA probe. **(J)** The protein levels of ACSL4, GPX4, FTH1 and COX2 in HK-2 cells were detected by western blot. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5

LncRNA TUG1 regulates the stability of mRNA by interacting with SRSF1. **(A-B)** The localization of TUG1 in HK-2 cells was determined by subcellular fractionation assay and RNA FISH assay. **(C)** The schematic illustration of RNA pull down assay. Interaction between lncRNA TUG1 and SRSF1 was confirmed by **(D)** RNA pull-down and **(E)** RIP assay. **(F)** Schematic illustration of SRSF1 full-length protein and truncations; their expression in E. coli was shown in the SDS-PAGE gel below. **(G)** The binding of TUG1 to SRSF1 full-length protein and truncated proteins was assessed by RIP. **(H)** The effect of USC-Exo on the expression of ACSL4 was checked by qPCR and western blot. **(I)** The effect of TUG1 and SRSF1 on the ACSL4 expression were assessed by qPCR and western blot. **(J)** RIP to determine the interaction between ACSL4 mRNA and SRSF1 protein using anti-SRSF1 or IgG (negative control) in HK2 cells in the presence or absence of TUG1 overexpression. **(K)** The effect of TUG1 and SRSF1 on the stability of ACSL4 mRNA was measured by qPCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

LncRNA TUG1 suppressed H/R induced ferroptosis in HK-2 cells by regulating ARSF1/ACSL4. (A) Cell viability was assessed by CCK-8 kits. (B) Cell death of HK-2 cells was measured by TUNEL assay. (C) The levels of MDA and GSH in the supernatants of HK-2 cells were detected by commercial kits. (D) The levels of Fe^{2+} in HK-2 cells were checked by commercial kits. (E) The levels of ROS in HK-2 cells were detected

using DCFH-DA probe. **(F)** The protein levels of ACSL4, GPX4, FTH1 and COX2 in HK-2 cells were assessed by western blot. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7

LncRNA TUG1 ameliorates IRI-induced kidney injury by suppressing ACSL4 mediated ferroptosis. (A) Serum levels of creatinine and BUN were assessed by colorimetric method. **(B)** The injury and cell death

of kidney tissues showed by HE dyes and TUNEL assay. **(C)** The levels of MDA and GSH in the mice were assessed by using commercial kits. **(D)** The levels of Fe^{2+} in the mice were checked by using commercial kits. **(E)** The protein levels of ACSL4, GPX4, FTH1 and COX2 in the kidney tissues were assessed by western blot. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8

USC-Exo ingested by kidney cells upregulates lncRNA TUG1 expression, which promotes ACSL4 mRNA decay by interacting with SRSF1, then suppresses ACSL4 mediated cell ferroptosis, furtherly improves IRI induced AKI.