MicroRNA-19a targets fibroblast growth factor-inducible molecule 14 and prevents tubular damage in septic AKI

Jun Hong[1], Bang-Chuan Hu[1], Liang Xu[1], Yang Zheng[1], Zi-Qiang Shao[1], Run Zhang[1], Xiang-Hong Yang[a], Ren-Hua Sun[1], Shi-Jing Mo[1]*

[1]*Department of Intensive Care Unit, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou 310014, Zhejiang, P.R.China*

Corresponding author:

*Shi-Jing Mo, MD, PhD

Department of Intensive Care Unit

Zhejiang Provincial People's Hospital

People's Hospital of Hangzhou Medical College

Hangzhou, Zhejiang 310014

P.R. China

Email: moshijing@hmc.edu.cn

Supplemental Information

Supplemental Materials and Methods

2.5. TUNEL assay

In brief, cells grown in 6-well plates were undergone the indicated treatments, incubated with the TUNEL reaction mixture for 60 min at 37˚C and stained with 5 mg/mL 4',6-diamidino-2-phenylindole (DAPI) in the dark. The reactions were terminated by rinsing three times with PBS and the number of apoptotic cells were observed and quantified using a fluorescent microscope (IX71; Olympus, Japan).

2.6. Dual-luciferase reporter (DLR) assay

Cells were seeded in triplicate in 24-well plates and allowed to settle for 24 hours. Indicated plasmids plus 1 ng pRL-TK *Renilla* plasmid were transfected into the cells using Lipofectamine 2000 Reagent (Life Technologies). Forty-eight hours after transfection, DualLuciferase Reporter Assay (Promega) was performed according to the manufacturer's instructions, as previously described[14,15]. The 3′ UTR regions of wild type and mutant Fn14 were amplified by PCR using the following primers: Fn14-WT: 5′-CGAGCTCGAAGCCTCAATCTGGGTCACAA-3′ (forward) and 5′-CCCGGGG GCATTATAGCCCCTCCGAGT-3′ (reverse). Fn14-Mut: 5′-CGAGCTCGAACTCGGAGGGGCTATAATGC-3′ (forward) and 5′-CCCGGGGGGAGATGGTTGTTTCCGTGT-3′ (reverse).

2.7. Real-time quantitative PCR (RT-qPCR)

Briefly, total RNA was isolated by Trizol (Invitrogen) and complementary DNA was synthesized with PrimeScript®RT Reagent Kit (Takana, Dalian, China) using Super Array PCR master mix (SuperArray Bioscience, USA). The sequences of the primers were as follows: Fn14 sense, 5′-GTGTTGGGATTCGGCTTGGT-3′; Fn14 antisense, 5′-GTCCATGCACTTGTCGAGGTC-3′. GAPDH sense, 5′-AGGTCGGTGTGAACGGATTTG-3′; GAPDH antisense, 5′-GGGGTCGTTGATGGCAACA-3′. Real-time PCR was then performed on an Applied Biosystems 7900HT cycler using Takana SYBR®
Primix Ex TaqTMKit (Takana, China).

2.8. Cellular ubiquitination assay

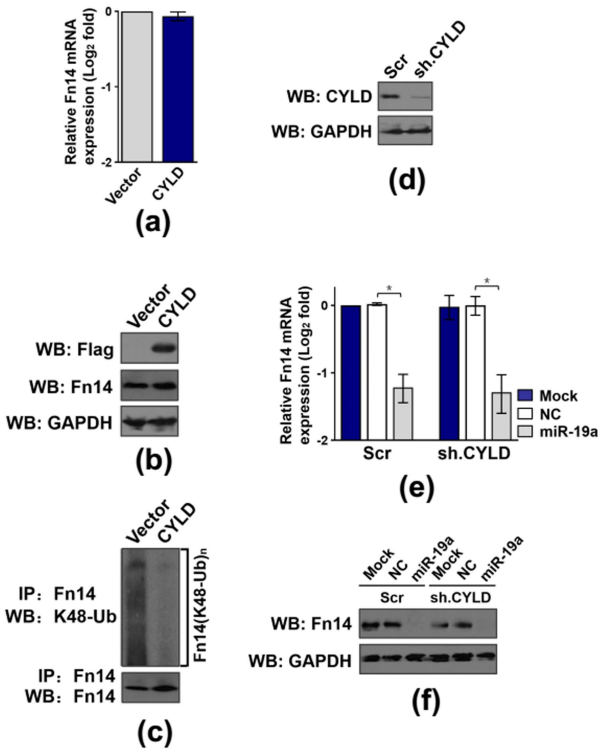
The indicated cells were lysed in radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors and phosphatase inhibitor cocktail (KeyGene, China), followed by immunoprecipitated using protein A/G-sepharose beads (Cwbiotech, China) overnight at 4°C. Polyubiquitinated Fn14 was detected with an anti-K48 Ub antibody.

2.9. Western-blotting

Briefly, total protein from each sample was fractionated in 10% sodium dodecyl sulfate (SDS)-polyacrylimide gel electrophoresis and transferred to the Immobilon™ PVDF Transfer Membranes (Millipore Corporation, Billerica, MA). The membrane was then blocked in 5% bovine serum albumin (BSA) and incubated with the primary antibodies as indicated overnight, followed by incubation with HRP-linked secondary antibodies and visualization using western chemiluminscent HRP Substrate Kit (PPLYGEN, Beijing, China).

2.11. Immunohistochemical staining

In brief, the rehydrated sections or coverslips were incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase conjugated secondary antibodies for 1h. Images were imaged with a AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss) and obtained using standard methods. At least three repeats were conducted for each calculation.



Supplemental Figure 1: Repression of Fn14 and CYLD by miR-19a is independent of each other. (a): RT-qPCR determining levels of Fn14 mRNA expression in murine MCT cells transfected with empty vector (Vector) and Flag-tagged wild-type CYLD. (b): Western-blotting analyses examining amount of Fn14 protein expression in murine MCT cells transfected with empty vector (Vector) and Flag-tagged wild-type CYLD. (c): Cellular ubiquitination assays detecting the levels of K48-linked polyubiquitination of Fn14 in murine MCT cells transfected with empty vector (Vector) and Flag-tagged wild-type CYLD. (d): Western-blotting analyses comparing levels of CYLD protein expression in murine MCT cells with or without CYLD shRNA (sh.CYLD) transfection. (e): RT-qPCR evaluating Fn14 mRNA expression in murine MCT cells transfected with mock (Mock), negative control (NC) as well as miR-19a mimics (miR-19a) in the presence or absence of CYLD shRNA (sh.CYLD) cotransfection. Scr, scrambled shRNA. *$P < 0.05$, one-way ANOVA, post hoc comparisons, Tukey's test. (f): Western-blotting analyses assessing levels of Fn14 protein expression in murine MCT cells transfected with mock (Mock), negative control (NC) as well as miR-19a mimics (miR-19a) in the presence or absence of CYLD shRNA (sh.CYLD) cotransfection.