

Meclofenamic acid promotes cisplatin-induced acute kidney injury by inhibiting fat mass and obesity-associated protein-mediated m⁶A abrogation in RNA

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Running Title: m⁶A in AKI

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ABSTRACT

The role of RNA methylation on the sixth N atom of adenylyate (m⁶A) in acute kidney injury (AKI) is unknown. Fat mass and obesity-associated protein (FTO) reverses the m⁶A modification in cisplatin-induced AKI. Here, we aimed to determine FTO's role in AKI. We induced AKI in c57BL/6 mice by intraperitoneal cisplatin injection and treated the animal with vehicle or an FTO inhibitor meclfenamic acid (MA) for 3 days. Moreover, as an *in vitro* model, human kidney proximal tubular cells (HK2 cells) were treated with cisplatin. We found that the cisplatin treatment reduces FTO expression and increases m⁶A levels *in vivo* and *in vitro*. MA aggravated renal damage and increased apoptosis in cisplatin-treated kidneys, phenotypes

that were correlated with reduced FTO expression and increased m⁶A levels. Moreover, MA promoted apoptosis in cisplatin-treated HK2 cells, which was correlated with the reduced FTO expression and increased m⁶A in HK2 cells. FTO protein overexpression reduced m⁶A levels and inhibited apoptosis in cisplatin-treated HK2 cells and also blocked the MA-induced increase in m⁶A levels and apoptosis rates. In agreement, overexpression of the m⁶A-generating methyltransferase-like 3 and 14 (METTL3 and METTL14) or siRNA-mediated FTO knockdown promoted apoptosis and enhanced m⁶A levels in cisplatin-treated HK2 cells. MA increased p53 mRNA and protein levels in AKI both *in vitro* and *in vivo*, and FTO overexpression reduced p53 expression and reversed the MA-

induced p53 increase in AKI. In conclusion, reduced renal FTO expression in cisplatin-induced AKI increases RNA m⁶A levels and aggravates renal damages.

Cisplatin is a potent chemotherapy agent and widely used to treat malignant solid tumors. Since 90% cisplatin is excreted through kidney, about 30% patients are suffered from renal damages after one high-dose of cisplatin treatment, which hampered its clinical application (1). However, the mechanism of cisplatin-induced acute kidney injury (AKI) remains largely elusive.

Increasing evidences indicate that apoptosis plays a vital role in the progress of AKI (2). The apoptosis of renal tubular epithelial cells occurs several hours after the injection of cisplatin and reaches its highest level during 48-72 hours in cisplatin induced AKI. The p53 protein is an important regulator of apoptosis by regulating the homeostasis of Caspase and Bcl family. *In vitro* and *in vivo* studies showed that the expression of p53 is significantly increased in cisplatin-induced AKI (3,4). Inhibition of p53 attenuated cisplatin induced acute kidney injuries (5).

m⁶A, defined as the RNA methylation on the sixth N atom of adenylate, is the most common internal modification of RNA (6). Recent studies showed that the levels of m⁶A determine the stability and translation efficiency of mRNA, and thus m⁶A modification is involved in a large number of biological processes including apoptosis (7). m⁶A is a reversible modification, which can be catalyzed by several enzymes, such as methyltransferase-like 3 and 14 (METTL3 and METTL14) and can also be erased by enzymes such as Fat mass and obesity-associated protein (FTO) (8). Meclofenamic acid (MA) is a pharmaceutical compound, which has been reported to modify

cellular m⁶A levels by selectively targeting FTO (9).

The role of m⁶A modification in kidney disease is rarely studied in the mouse model of unilateral ureteral obstruction, the expression of FTO is increased and ablation of the FTO gene attenuates fibrogenic responses in this model, implying that m⁶A modification may play a role in renal fibrosis (10).

In this study, we aimed to explore the role of m⁶A modification in cisplatin-induced AKI and its downstream working mechanism.

Results

Cisplatin reduced FTO expression and enhanced m⁶A levels in mouse kidneys and HK2 cells

A mouse model of AKI was established by intraperitoneal injection of 17 mg/kg cisplatin, and kidneys were collected at day 3. As shown in Figure 1A, cisplatin treatment significantly decreased FTO expression by 79% in mouse kidneys. The m⁶A levels were further analyzed in mouse kidneys, and Dot Blot analysis showed that the m⁶A levels were significantly elevated by 14.7 folds in cisplatin treated mouse kidneys as compared with that in vehicle treated control kidneys (Figure 1B).

Human proximal tubular epithelial line (HK2 cells) were exposed to 10 μmol/L cisplatin for 24 h, and Western Blot analysis showed that FTO was significantly down-regulated by 67% in cisplatin treated cells as compared with that in control cells (Figure 1C). Figure 1D showed that the m⁶A levels were increased by 17.5 times in cisplatin treated cells.

Meclofenamic acid (MA) inhibited FTO expression, enhanced m⁶A levels and promoted apoptosis in cisplatin-treated kidneys

The effect of MA was tested in cisplatin-induced AKI. Treatment with cisplatin increased Blood urea

nitrogen (BUN) levels by 134% and serum creatinine (SCr) levels by 57% in mice, which were further elevated by 49% and 25% respectively after administration of MA (Figure 2A). Pathological analysis by hematoxylin-eosin (HE) staining revealed a mild histology injury, such as loss of brush border membranes, tubular casts and cast formation in cisplatin treated kidneys, and these changes were much more prominent in MA treated mice (Figure 2B). Further quantification showed that the kidney injury score was significantly increased in cisplatin treated kidneys, which was further aggravated by 44% by MA treatment (Figure 2B). We further examined the effect of MA on apoptosis in cisplatin-treated kidneys. As shown in Figure 2C and 2D, the ratio of Bax/Bcl-2 and Cleaved Caspase3 expression were increased by 109% and 147% respectively in mouse kidneys during cisplatin injury, which were further enhanced by 82% and 42% respectively by MA treatment. Immunofluorescent staining revealed that TUNEL positive cells in the cisplatin treated kidneys were 3 times more than that in control kidneys, and administration of MA further increased TUNEL positive signals by 118% in injured kidneys.

The effect of MA on FTO expression and m⁶A levels was assessed. As shown in Figure 3A, treatment with MA did not inhibit FTO expression in control kidneys but reduced its expression by 80% in cisplatin-treated kidneys. Besides, MA did not increase m⁶A levels in control kidneys, but significantly increased m⁶A levels in cisplatin-treated kidneys by 72% (Figure 3B).

MA inhibited FTO expression, enhanced m⁶A levels and promoted apoptosis in cisplatin treated HK2 cells

As an *in vitro* model, HK2 cells, a human proximal tubular epithelial cell line, were

stimulated with normal saline or 10 μmol/L cisplatin for 24 h with or without the addition of 100 mM MA. As shown in Figure 4 A, cisplatin increased the ratio of Bax and Bcl-2 by 184% and the expression of Cleaved Caspase3 by 331% in HK2 cells, which were further increased by 287% and 52% respectively after the addition of 100 mM MA for 24 h. Next, TUNEL staining showed that there was no TUNEL positive cells in control cells and there was 4% TUNEL positive cells upon cisplatin exposure, which were further increased by 64% after the treatment with MA (Figure 4B). Western Blot showed that the expression of FTO was decreased by 47% in cisplatin treated HK2 cells and administration of MA further reduced FTO expression by 67% (Figure 4 C). Whilst, m⁶A levels were enhanced by 360% upon cisplatin exposure and were further increased by 82% by MA treatment (Figure 4D).

Over-expression of FTO blocked the pro-apoptotic effect of MA *in vitro*

FTO plasmids were transfected 24 h prior to the stimulation of 10 μmol/L cisplatin and the treatment of 100 mM MA. As shown in Figure 5A, transfection of the FTO plasmids reduced the ratio of Bax/Bcl-2 by 46% and the expression of Cleaved Caspase-3 by 40% in cisplatin treated HK2. MA increased the ratio of Bax/Bcl-2 by 401% and the expression of Cleaved Caspase-3 by 344% in cisplatin treated HK2 when transfected with the empty vector, but it did not increase the ratio of Bax/Bcl-2 and the expression of Cleaved Caspase-3 in cisplatin treated HK2 when transfected with the FTO plasmid (Figure 5A). Moreover, TUNEL staining showed that over-expression of FTO significantly reduced apoptosis by 82% in cisplatin treated HK2 cells. MA increased TUNEL positive cells by 87% in cisplatin treated HK2 when transfected with the empty vector, but it did not

increase TUNEL positive cells in cisplatin treated HK2 when transfected with the FTO plasmid (Figure 5B). Western Blot analysis showed that the expression of FTO was increased in cisplatin treated HK2 cells by 166 times without the addition of MA or by 152 times with the addition of MA when transfected with the FTO plasmid (Figure 5C). Transfection of the FTO plasmid reduced m⁶A levels by 95% in cisplatin treated HK2 (Figure 5D). MA increased m⁶A levels by 135% in cisplatin treated HK2 when transfected with the empty vector, but it did not increase m⁶A levels in cisplatin treated HK2 when transfected with the FTO plasmid (Figure 5D).

Knockdown of FTO promoted apoptosis in cisplatin treated HK2 cells and enhanced m⁶A levels.

FTO si-RNA(Si-FTO) were transfected 24 h prior to the stimulation of 10 μmol/L cisplatin, which treated HK2 cells for another 24 h. As shown in Figure 6A, the expression of FTO protein in cisplatin treated HK2 cells was significantly reduced by 66% after Si-FTO transfection. And knockdown of FTO expression by si-RNA increased the ratio of Bax/Bcl-2 by 167% and the expression of Cleaved Caspase-3 by 85% in cisplatin treated HK2. In addition, TUNEL staining indicated that knockdown of FTO expression significantly promoted apoptosis by 27% in cisplatin treated HK2 cells. Moreover, transfection of the Si-FTO enhanced m⁶A levels by 34% in cisplatin treated HK2 (Figure 6C).

METTL3 promoted apoptosis and enhanced m⁶A levels in cisplatin treated HK2 cells.

As shown in Figure 7A, cisplatin treatment significantly increased METTL3 expression by 4.7 folds in mouse kidneys. METTL3 plasmid (pMETTL3) were transfected 24 h before the

stimulation of 10 μmol/L cisplatin, cells were harvested at 24 h after cisplatin treatment. As shown in Figure 7B, cisplatin treatment increased the expression of METTL3 protein in HK2 by 4.1 folds, and transfection of METTL3 plasmids increased METTL3 expression by 25.1 folds in cisplatin treated cells. Furthermore, overexpression of METTL3 significantly increased the ratio of Bax/Bcl-2 by 82.7 folds and the expression of Cleaved Caspase-3 by 87.9 folds in cisplatin treated HK2 cells. Moreover, overexpression of METTL3 significantly increased TUNEL positive cells by 64% in cisplatin treated HK2 cells (Figure 7C). In addition, transfection of METTL3 plasmids enhanced m⁶A levels by 138% in cisplatin treated HK2 cells (Figure 7D).

METTL14 promoted apoptosis and enhanced m⁶A levels *in vitro*.

As shown in Figure 8A, cisplatin treatment significantly increased METTL14 expression by 5.5 folds in mouse kidneys. METTL14 plasmids (pMETTL14) were transfected in HK2 cells for 24 h and then stimulated with 10 μmol/L cisplatin for another 24 h. As shown in Figure 8B, cisplatin treatment increased the expression of METTL14 protein in HK2 by 14.8 folds, and transfection of pMETTL14 increased METTL14 expression by 58.9 folds in cisplatin treated cells. Overexpression of METTL14 significantly increased the ratio of Bax/Bcl-2 by 299% and the expression of Cleaved Caspase-3 by 477% in HK2 cells with cisplatin treatment. Moreover, TUNEL staining revealed that transfection of METTL14 plasmids significantly promoted apoptosis by 81% in cisplatin treated HK2 cells (Figure 8C). In addition, the m⁶A levels were increased by 9 folds after transfection of METTL14 plasmids in cisplatin treated HK2 (Figure 8D).

FTO inhibited p53 expression

The mRNA expression of p53 was measured. As shown in Figure 9A, the expression of p53 mRNA increased by 67% in HK2 cells after cisplatin treatment for 12 h, which were further enhanced by 50% by MA treatment. Transfection of FTO plasmids reduced p53 mRNA by 36% at 12 h. FTO knockdown by si-RNA promoted p53 mRNA by 20.4 folds at 24 h after cisplatin administration in HK2 cells (Figure 9A). In kidney tissues, the expression of p53 mRNA was increased by 90% after cisplatin administration, which were further enhanced by 80% by MA treatment (Figure 9B).

We further analysis the expression of p53 in protein levels. As shown in Figure 9C, transfection of FTO plasmids reduced p53 expression by 74% in cisplatin treated HK2 cells. Treatment with MA increased the expression of p53 by 58% in cisplatin treated HK2 cells when transfected with the empty vector, but it did not increase the expression of p53 in cisplatin treated HK2 cells when transfected with the FTO plasmid (Figure 9C). And transfection of si-FTO increased the p53 protein expression by 44 % in HK2 cells treated with cisplatin (Figure 9C). In kidney tissues, the expression of p53 protein increased by 192% after cisplatin administration, which were further enhanced by 60% by MA treatment (Figure 9D).

Discussion

The role of m⁶A modification in AKI is unknown. In the current study, we showed that FTO is down-regulated in cisplatin-induced AKI *in vivo* and *in vitro*, and METTL3 and METTL14 are up-regulated in cisplatin-induced AKI *in vivo* and *in vitro* which are correlated with increased m⁶A modification. Moreover, inhibition of FTO by meclofenamic acid (MA) or FTO si-RNA further increased m⁶A levels and promoted cisplatin-induced AKI. Furthermore, METTL3 and

METTL14 overexpression increased m⁶A levels and promoted cisplatin-induced AKI. Our data suggest that increased m⁶A modification is detrimental in cisplatin-induced AKI.

AKI is characterized by increased apoptosis, and excessive apoptosis in AKI causes the deterioration of renal function (11). The effect of m⁶A modification on apoptosis has been studied (12, 13). Over-expression of FTO promoted cell proliferation and inhibited apoptosis in acute myeloid leukemia cell lines, which was associated with reduced global mRNA m⁶A levels (12). In contrast, knockdown of FTO increased m⁶A levels and promoted apoptosis of acute myeloid leukemia cells (12). In an animal model of lipopolysaccharide-induced liver injury, dietary curcumin increased the abundance of m⁶A, which was correlated with increased expression of pro-apoptotic genes (13). Thus, we hypothesized that m⁶A demethylation by FTO in cisplatin-induced kidney injury inhibits apoptosis. Indeed, we showed that increased m⁶A modification by the FTO inhibitor MA was paralleled with enhanced apoptosis in cisplatin-treated kidneys, which was further confirmed *in vitro*. We also showed that over-expression of FTO in cisplatin-treated HK2 cells reduced m⁶A levels and inhibited apoptosis. Moreover, over-expression of FTO reversed the enhanced m⁶A modification by MA and blocked the pro-apoptotic effect of MA. Furthermore, we confirmed the pro-apoptotic effect of m⁶A in cisplatin induced AKI by knockdown of FTO or transfection of two methyltransferase METTL3 or METTL14.

P53, a central player in several types of programmed cell deaths, influences transcription of genes that are related to classical mitochondria apoptosis and non-classical apoptosis (14). Thus, we postulated that FTO inhibited apoptosis through p53. We showed that over-expression of FTO

inhibited the expression and methylation of p53 mRNA in cisplatin-treated HK2 cells. The FTO inhibitor MA and FTO si-RNA increased the expression of p53 in mRNA and protein levels in cisplatin treated cells and kidneys. Moreover, over-expression of FTO reversed the increased p53 expression by MA treatment in cisplatin treated cells.

In summary, treatment with cisplatin reduced FTO expression and increased the m⁶A levels in kidneys, which leads to the up-regulation of p53. MA aggravated the renal damages in cisplatin treated kidneys through p53 mediated apoptosis pathways (the Bax/Bcl-2 and Caspase3 pathways) (Figure 10). We conclude that FTO is protective in cisplatin-induced AKI through inhibiting m⁶A RNA methylation.

Experimental procedures

Animal

Male c57bl/6 mice were housed in the animal facilities of the Shanghai Jiao Tong University and fed with a standard diet. Mice were randomly divided into four groups: vehicle + NS group, vehicle + MA group, cisplatin + NS group, and cisplatin + MA group (n=8 per group). The mouse model of AKI was established by intraperitoneal injection of 17 mg/kg cisplatin (P4394, Sigma, St Louis, MO). MA (S5517, Selleck, USA) was given intraperitoneally for 3 times: 1 h prior to cisplatin injection, 24 h and 48 h after cisplatin injection at the dose of 10 mg/kg. At day 3 after the cisplatin injection, all mice were sacrificed to collect blood and kidney samples. Blood urea nitrogen (BUN) and serum creatinine (Scr) were assessed by automatic analyzer. The animal protocols were performed in accordance with the guide of the Care and Use of Laboratory Animals and were approved by Ethics Review Committee for Animal Experimentation of Shanghai Ninth People's

Hospital.

Cell culture and treatment

Human proximal tubular epithelial cells (HK2) were bought from the cell bank of Chinese Academy of Sciences, which were cultured in DMEM-F12 medium (C11330500BT, Gibco, Thermo Fisher Scientific) with 10% fetal bovine serum (10270-106, Gibco, Thermo Fisher Scientific). To establish *in vitro* model of AKI, HK2 cells were stimulated with normal saline or 10 μmol/L cisplatin for 24 h. In some experiments, HK2 cells were treated with normal saline or 100 mM MA. FTO plasmids were transfected by Lipofectamine 2000 (11668-027, Invitrogen, CA) in HK2 cells according to the manufacture's instruction. The FTO plasmids were constructed by subcloning the human FTO coding sequence into mammalian vector GV358. The empty GV358 vector was used as control. The human FTO si-RNA sequences were as follows: Forward, 5'GGACAAUGAUGAUGUCUCU-3'; Reverse, 5'AGAGACAUCAUCAUUGUCC-3'. The METTL3 plasmids were constructed by subcloning the human METTL3 coding sequence into mammalian vector pCMV3. The empty pCMV3 vector was used as control. The METTL14 plasmids were constructed by subcloning the human METTL14 coding sequence into mammalian vector GTP-C-3FLAG. The empty GTP-C-3FLAG vector was used as control.

HE staining and renal pathology

Kidney tissues were fixed in 4 % formaldehyde solution and then embedded in paraffin. Sliced tissue sections were stained with hematoxylin and eosin. Tubular injuries were semi-quantitatively scored as described previously (15): normal (score = 0), below 10% (score = 1), 10-25% (score = 2), 25%–75% (score = 3) and over 75% (score = 4) of

damaged areas.

Western Blot

Western Blot was performed as described previously (16). In brief, total protein were extracted from kidney tissues or HK2 cells using RIPA lysis buffer (P0013B, Beyotime, Shanghai, China). Protein samples were separated by SDS-PAGE electrophoresis and transferred to PVDF membrane. The membrane was incubated with primary antibodies overnight, which was followed by the incubation of the secondary antibody (A0216 or A0208, Beyotime, Shanghai, China). Primary antibodies for FTO (45980) and Cleaved Caspase-3 (9661) were purchased from Cell Signaling Technology (Beverly, MA). The antibodies for Bax (Ab32503) and Bcl-2 (Ab182858) were obtained from Abcam (Cambridge, UK), and the β -actin (60008-1-Ig) antibody was bought from Proteintech (PA, USA).

RNA extraction and PCR

Total RNA was extracted using Trizol (15596-026, Sigma, St Louis, MO) according to the manufacture's instruction, which was reverse transcribed to cDNA by TaKaRa PrimeScript RT reagent kit (RR0036A, Kyoto, Japan). The primer sequences listed in Table 1 were used for quantitative Polymerase Chain Reaction (RR820A, Q-PCR).

Dot Blot

Total RNA was preheated to 95 °C for 3 minutes, and then was spotted on positively charged Nylon membrane (RPN303B, Amersham, USA). After blocking with nonfat milk, the m⁶A was probed

with a specific antibody for m⁶A (ABE572, Millipore, USA) and followed by the second antibody (A0208, Beyotime, Shanghai, China). The signals were detected using ECL detection reagents (P36599, Millipore, MA, USA). The same membrane was further stained with methylene blue (319112, Sigma, St Louis, MO) to measure the quantity of RNA in membrane Blots. ImageJ (National Institutes of Health, USA) was used to quantify the signals of dot Blot.

TUNEL staining

Transferase-mediated dUTP nick-end labeling (TUNEL) kit (12156792910, Roche, Switzerland) and Hoechst (C1011, Beyotime, Shanghai, China) reagent were used to identify apoptotic cells in kidney sections and cultured cells according the manufacture's instructions. In brief, deparaffinized tissue sections were hydrated and then digested with Proteinase K (P6556, Sigma, St Louis, MO) for 30 minutes at room temperature. HK2 cells were fixed with 4% paraformaldehyde for 1 h at room temperature. Subsequently, kidney tissue sections or cells were washed with PBS, and then incubated with TUNEL reagent for 1 h at 37 °C. A fluorescence microscope was used to measure TUNEL-positive cells.

Statistical analysis

All data were analyzed using GraphPad Prism version 5.0 software (GraphPad Inc.CA, USA). The unpaired t-test or one-way ANOVA was used for statistical analysis. $P < 0.05$ was considered as statistical significance.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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FOOTNOTES

The abbreviations used are: acute kidney injury, AKI; fat mass and obesity-associated protein, FTO; meclfemomic acid, MA; human kidney proximal tubular cells, HK2 cells; RNA modification N6-methyladenosine, m⁶A; blood urea nitrogen, BUN; serum creatinine, SCr; hematoxylin-eosin, HE.

Tables

TABLE 1. Specific primer sequences for PCR

Gene name	Primer sequence
Mouse p53	Forward, 5'- CCCCTGTCATCTTTTGTCCCT-3' Reverse, 5'- AGCTGGCAGAATAGCTTATTGAG-3'
Mouse β -actin	Forward, 5'-GGCTGTATCCCCCTCCATCG-3' Reverse, 5'-CCAGTTGGTAACAATGCCATGT-3'
Human p53	Forward, 5'- CAGCACATGACGGAGGTTGT -3' Reverse, 5'- TCATCCAAATACTCCACACGC -3'
Human β -actin	Forward, 5'-AAGGAGCCCCACGAGAAAAAT-3' Reverse, 5'-ACCGAACTTGCATTGATTCCAG-3'

Figures

Fig 1

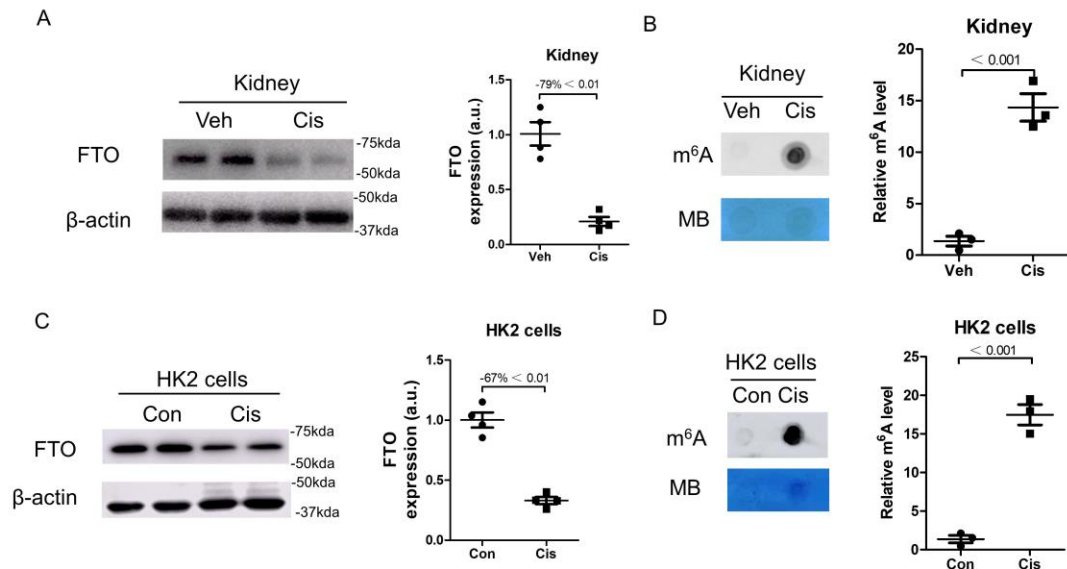


Figure 1. The expression of Fat mass and obesity-associated protein (FTO) was reduced and the m⁶A levels were increased in Cisplatin induced kidney injury

Kidney samples were collected 3 days after cisplatin administration. A. The expression of FTO in kidneys was assessed by Western Blot. B. Dot Blot was performed to assess the level of total RNA m⁶A in kidneys. C. Western Blot was performed to measure FTO expression in Human proximal tubular epithelial cells (HK2 cells). Protein samples were extracted from HK2 cells 24 h after cisplatin treatment. D. The m⁶A level in HK2 cells was assessed by Dot Blot. Data represent mean \pm SD and results are representative of three independent experiments. Veh, vehicle; Cis, cisplatin; Con, control.

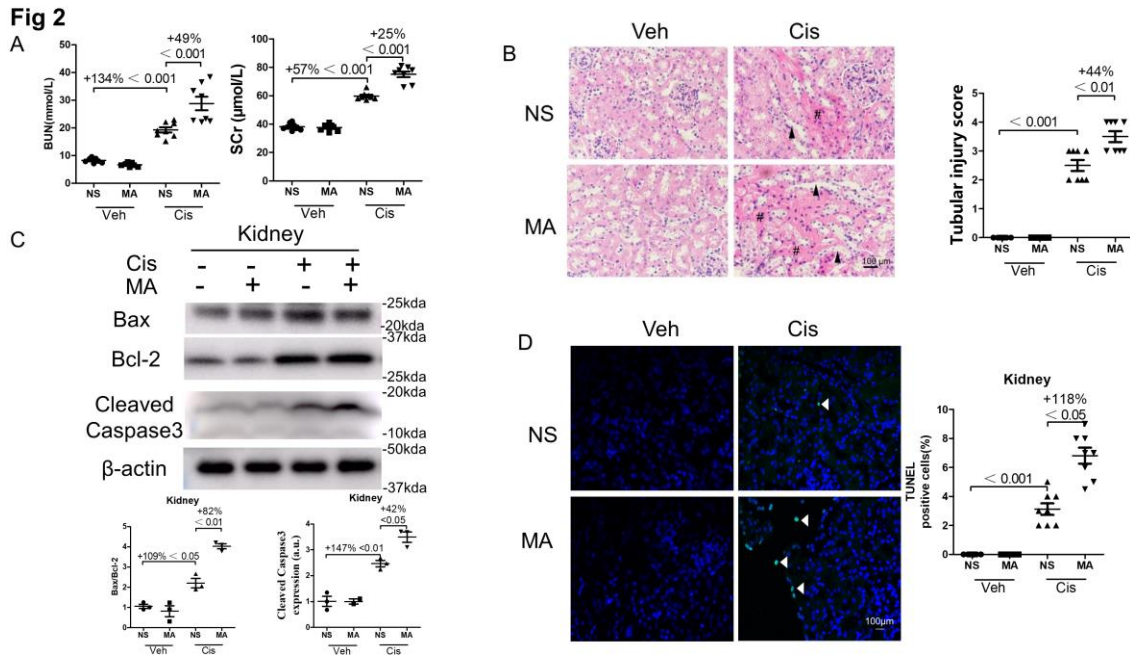


Figure 2. Meclofenamic acid (MA) deteriorated renal function and enhanced apoptosis in cisplatin-treated kidneys.

Blood and kidney samples were collected 3 days after cisplatin administration. A. Blood urea nitrogen (BUN) and serum creatinine (SCr) levels. B. Hematoxylin-eosin staining (HE) staining was performed and representative pathological images (original magnification $\times 400$, bar=100 μm) of kidneys were shown. Renal tubular casts (#); dilated renal tubules (arrow head). Scale bar = 100 μm . Tubular injury was semi-quantitatively scored. C. The expression of Bcl-2, Bax and Cleaved Caspase-3 in kidneys was assessed by Western Blot and then quantitatively analyzed. D. TUNEL staining was performed to measure apoptosis in the kidney tissue sections (original magnification $\times 400$, bar=100 μm). Data represent mean \pm SD and results are representative of three independent experiments. Veh, vehicle; Cis, cisplatin; NS, normal saline.

Fig 3

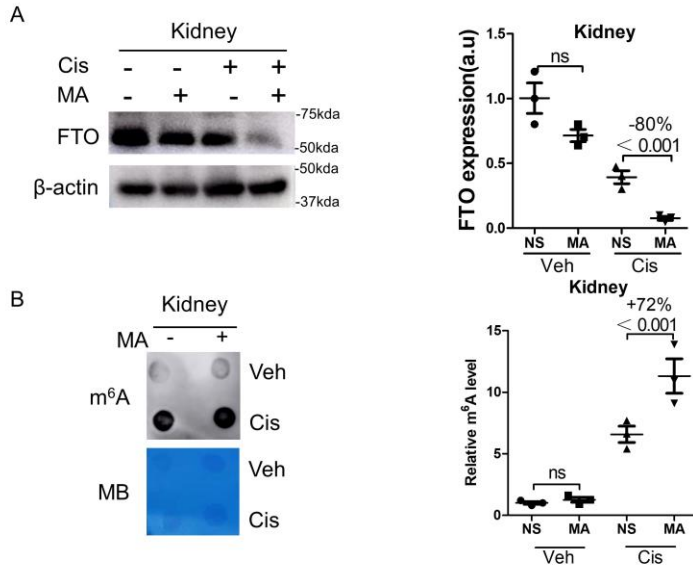


Figure 3. Meclofenamic acid (MA) inhibited FTO expression and enhanced m⁶A levels.

Kidney samples were collected 3 days after cisplatin administration. A The expression of FTO in kidneys was assessed by Western Blot, which was further quantified. B. Dot Blot was performed to measure the total RNA m⁶A level in kidneys, and then was quantified. Methylene blue (MB) staining was used to measure the quantity of RNA in membrane blots. Data represent mean \pm SD and results are representative of three independent experiments. Veh, vehicle; Cis, cisplatin; NS, normal saline.

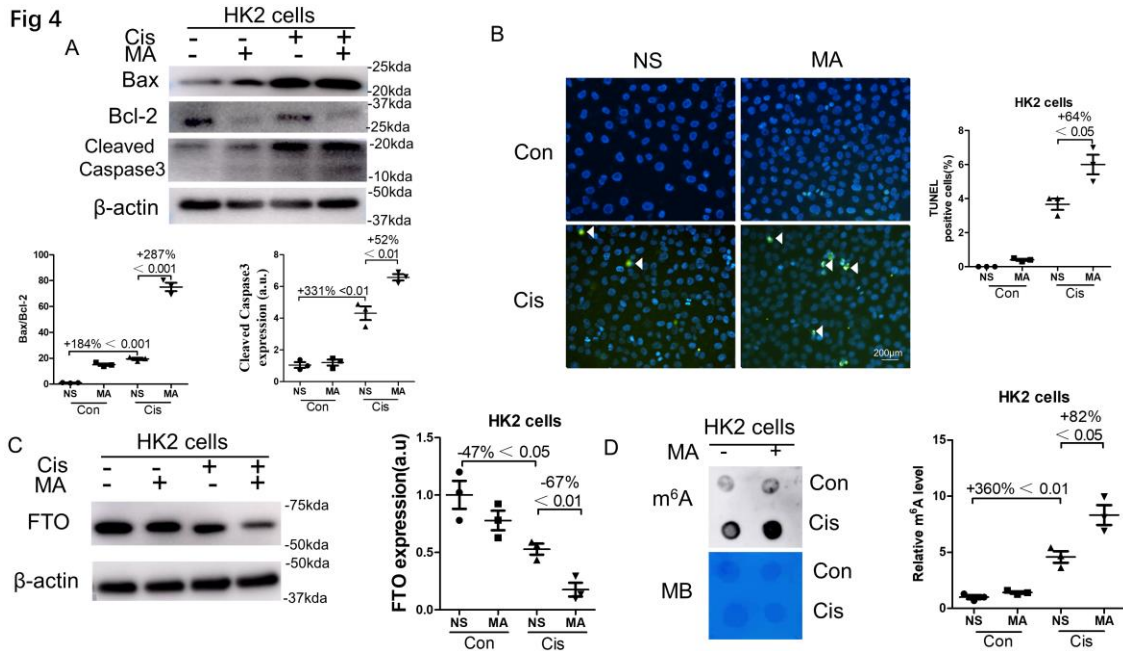


Figure 4. Meclofenamic acid (MA) inhibited FTO expression, enhanced m⁶A levels and promoted apoptosis in cisplatin treated HK2 cells.

HK2 cells, a human proximal tubular epithelial cell line, were stimulated with 10 μ mol/L cisplatin for 24 h in the presence or absence of 100 mM MA. A. The expression of Bcl-2, Bax and Cleaved Caspase-3 in HK2 cells was assessed by Western Blot and quantitative analyzed. B. TUNEL staining was performed to detect cell apoptosis (original magnification \times 400, bar=200 μ m). C. Western Blot was performed to measure FTO expression in HK2 cells, which was further quantified. D. Dot Blot was used to assess the m⁶A levels in HK2 cell's total RNA, and then was quantified. Methylene blue (MB) staining was used to measure the quantity of RNA in membrane Blots. Data represent mean \pm SD and results are representative of three independent experiments. Con, control; Cis, cisplatin; NS, normal saline.

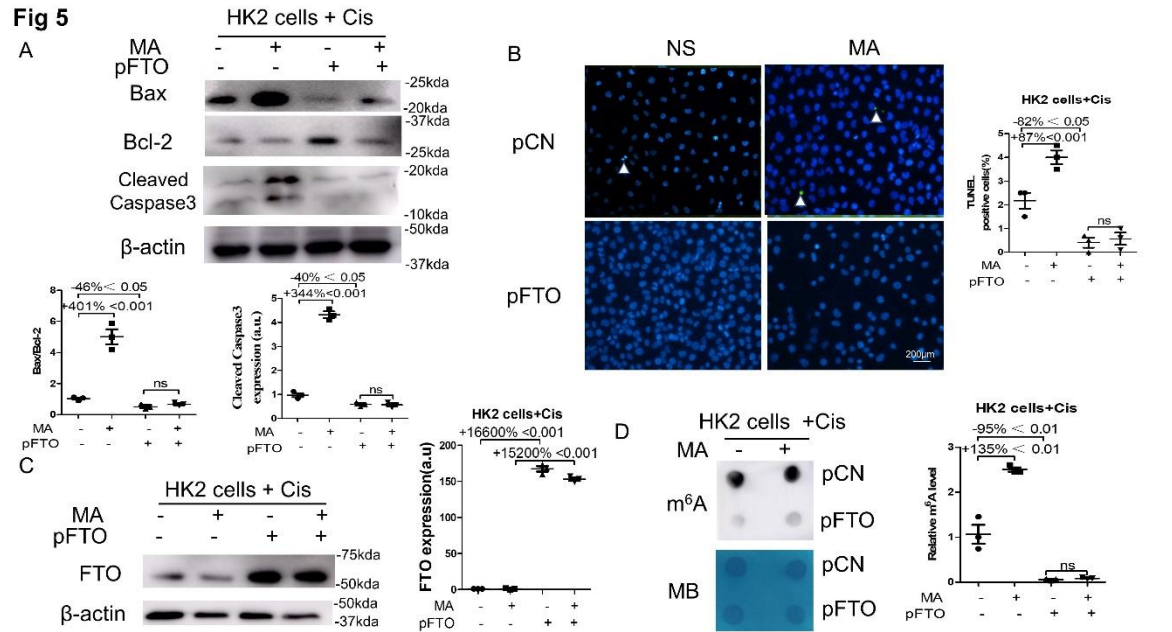


Figure 5. Over-expression of FTO blocked the pro-apoptotic effect of Meclofenamic acid (MA) *in vitro*.

HK2 cells were transfected with empty vector (pCN) or the FTO (pFTO) plasmid. 24 h after the transfection, HK2 cells were stimulated with 10 μ mol/L cisplatin for another 24 h in the presence or absence of 100 mM MA. A. The expression of Bcl-2, Bax and Cleaved Caspase-3 in HK2 cells was assessed by Western Blot and then were quantified. B. TUNEL staining was performed to measure cell apoptosis (original magnification \times 400, bar=200 μ m), and then was quantified. C. Western Blot was performed to measure FTO expression in HK2 cells, and then was quantified. D. Dot Blot was used to assess the m⁶A level in HK2 cells, which was further quantified. Methylene blue (MB) staining was used to measure the quantity of RNA in membrane Blots. Data represent mean \pm SD and results are representative of three independent experiments.

Fig 6

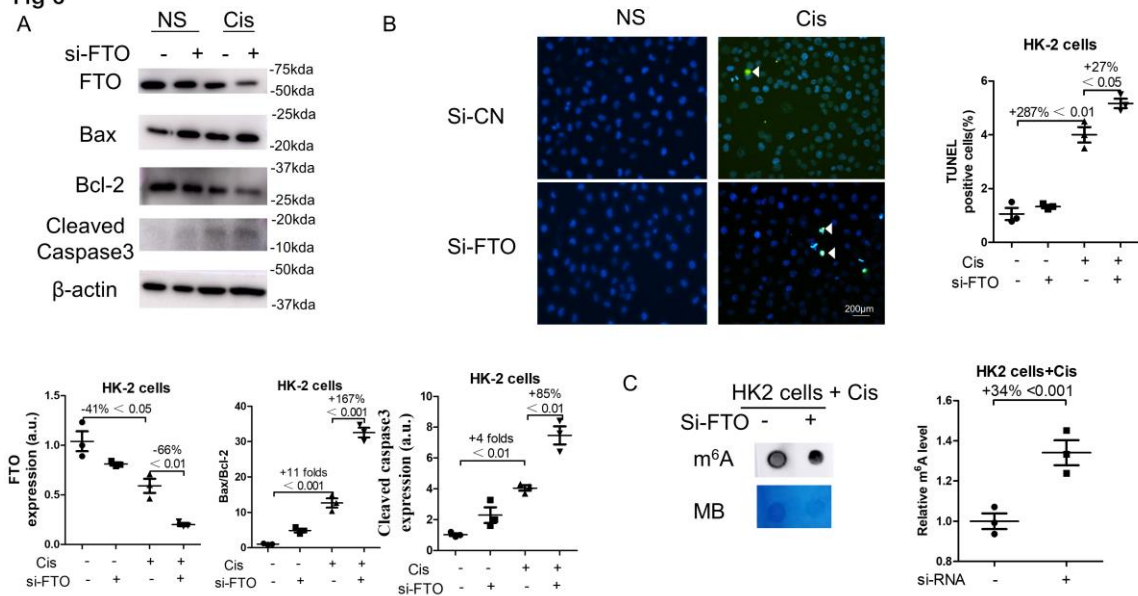


Figure 6. Knockdown of FTO promoted apoptosis in cisplatin treated HK2 cells and enhanced m6A levels.

HK2 cells were transfected with non-sense si-RNA (siNC) or the FTO si-RNA (Si-FTO). 24 h after the transfection, HK2 cells were stimulated with 10 μ mol/L cisplatin for another 24 h. A. The expression of FTO, Bcl-2, Bax and Cleaved Caspase-3 in HK2 cells was assessed by Western Blot and then were quantified. B. TUNEL staining was performed to measure cell apoptosis (original magnification $\times 400$, bar = 200 μ m), and then was quantified. C. Dot Blot was used to assess the m⁶A level in HK2 cells, which was further quantified. Methylene blue (MB) staining was used to measure the quantity of RNA in membrane Blots. Data represent mean \pm SD and results are representative of three independent experiments.

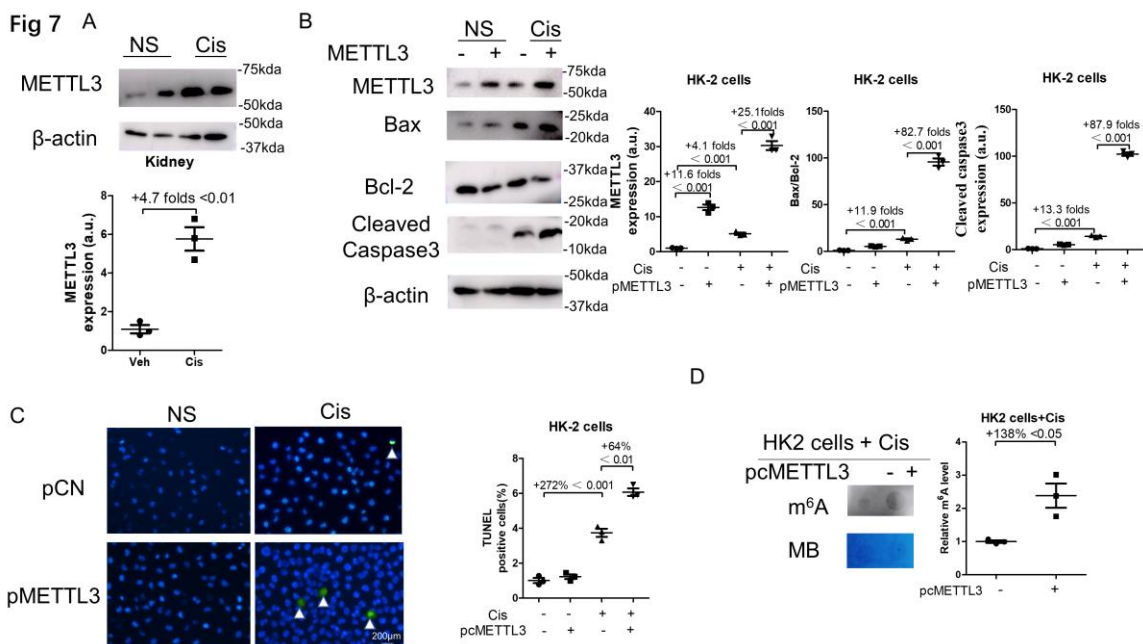


Figure 7. Over-expression of METTL3 promoted apoptosis and enhanced m⁶A levels in cisplatin treated HK2 cells.

A. Kidney samples were collected 3 days after cisplatin administration. The expression of METTL3 in kidneys was assessed by Western Blot. B. HK2 cells were transfected with empty vectors (pCN) or METTL3 (pMETTL3) plasmids, 24 h after the transfection, HK2 cells were stimulated with 10 μ mol/L cisplatin for another 24 h. The expression of METTL3, Bcl-2, Bax and Cleaved Caspase-3 in HK2 cells was assessed by Western Blot and then were quantified. C. TUNEL staining was performed to measure cell apoptosis (original magnification \times 400, bar=200 μ m), and then was quantified. D. Dot Blot was used to assess the m⁶A level in HK2 cells, which was further quantified. Methylene blue (MB) staining was used to measure the quantity of RNA in membrane Blots. Data represent mean \pm SD and results are representative of three independent experiments.

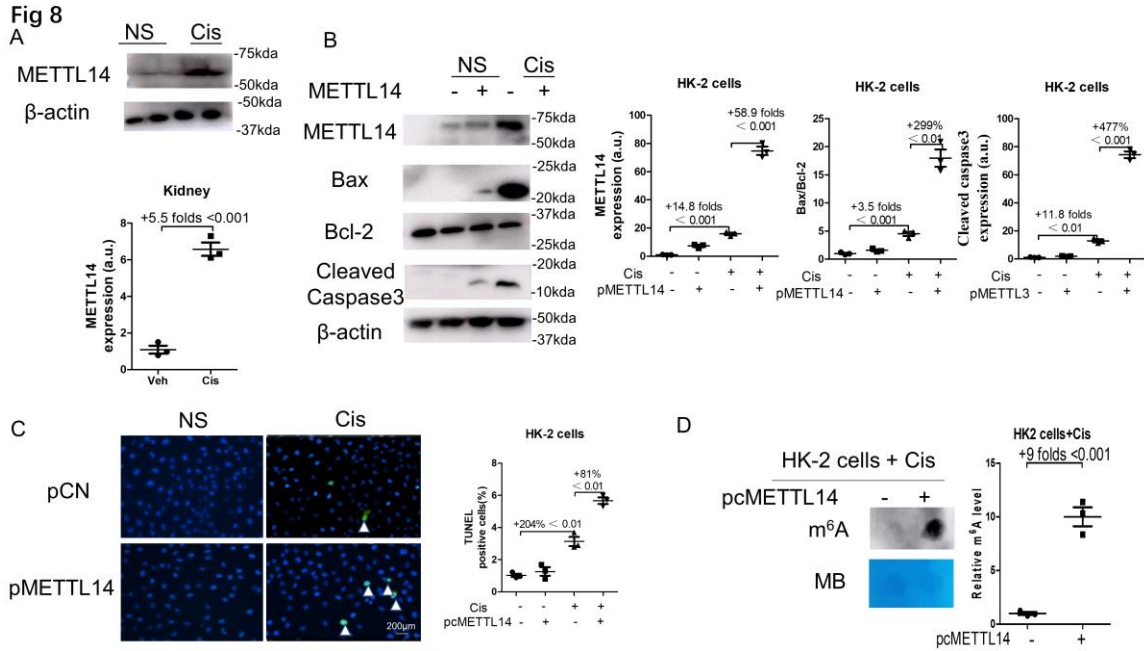


Figure 8. Over-expression of METTL14 promoted apoptosis and enhanced m⁶A levels *in vitro*.

A. Kidney samples were collected 3 days after cisplatin administration. The expression of METTL14 in kidneys was assessed by Western Blot. B. HK2 cells were transfected with empty vectors (pCN) or METTL14 (pMETTL14) plasmids, 24 h after the transfection, HK2 cells were stimulated with 10 μ mol/L cisplatin for another 24 h. The expression of METTL14, Bcl-2, Bax and Cleaved Caspase-3 in HK2 cells was assessed by Western Blot and then were quantified. C. TUNEL staining was performed to measure cell apoptosis (original magnification $\times 400$, bar = 200 μ m), and then was quantified. D. Dot Blot was used to assess the m⁶A level in HK2 cells, which was further quantified. Methylene blue (MB) staining was used to measure the quantity of RNA in membrane Blots. Data represent mean \pm SD and results are representative of three independent experiments.

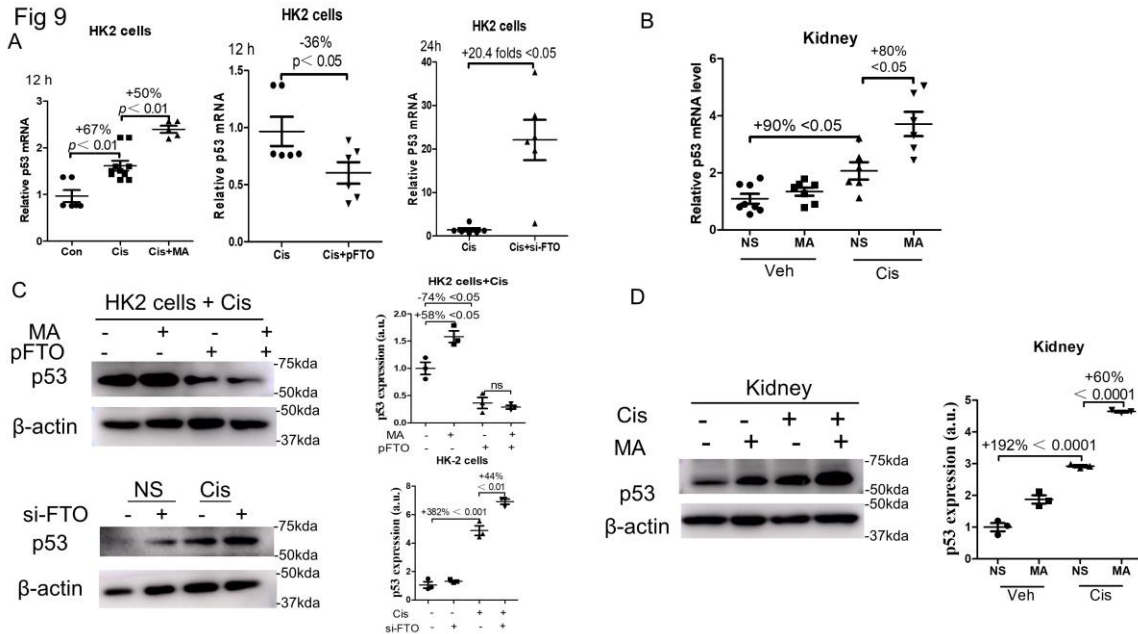


Figure 9. FTO inhibited p53 mRNA and protein expression *in vitro* and *in vivo*.

A. The left panel: HK2 cells were treated with 10 $\mu\text{mol/L}$ cisplatin for 12 h in the presence or absence of 100 mM meclufenamic acid (MA), and p53 mRNA was measured. The middle panel: HK2 cells were transfected with the FTO plasmid at 24 h prior to the stimulation of 10 $\mu\text{mol/L}$ cisplatin. The expression of p53 mRNA was measured 12 h after cisplatin stimulation. The right panel: HK2 cells were transfected with the si-FTO 24 h prior to the stimulation of 10 $\mu\text{mol/L}$ cisplatin. The expression of p53 mRNA was measured 24 h after cisplatin stimulation. B. The expression of p53 mRNA levels in cisplatin induced AKI kidneys treated with NS or MA. C. The upper panel: HK2 cells were transfected with empty vector or the FTO (pFTO) plasmid. 24 h after the transfection, HK2 cells were stimulated with 10 $\mu\text{mol/L}$ cisplatin for another 24 h in the presence or absence of 100 mM MA. Western Blot was performed to measure the expression of p53 in HK2 cells. The lower panel: HK2 cells were transfected with non-sense si-RNA (siNC) or the FTO si-RNA 24 h after the transfection, HK2 cells were stimulated with 10 $\mu\text{mol/L}$ cisplatin for another 24 h. Western Blot was performed to measure the expression of p53 in HK2 cells. D. Western Blot was performed to measure p53 expression in in cisplatin induced AKI kidneys treated with NS or MA. Data represent mean \pm SD and results are representative of three independent experiments. Veh, vehicle; Con, control; Cis, cisplatin; NS, normal saline.

Fig 10

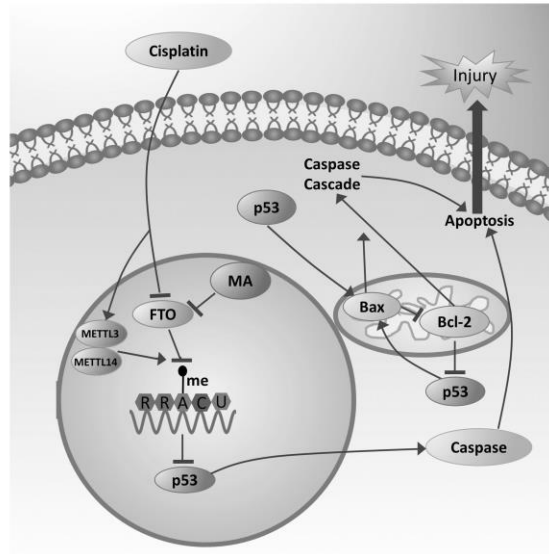


Figure 10. Meclofenamic acid (MA) promotes cisplatin-induced acute kidney injury through inhibiting FTO mediated m⁶A modification

Treatment with cisplatin reduced FTO expression and increased the m⁶A levels in kidneys, which leads to the up-regulation of p53. MA aggravated the renal damages in cisplatin treated kidneys through p53 mediated apoptosis pathways (the Bax/Bcl-2 and Caspase3 pathways).

Meclofenamic acid promotes cisplatin-induced acute kidney injury by inhibiting fat mass and obesity-associated protein-mediated m6A abrogation in RNA

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