

Resveratrol attenuates renal injury and fibrosis by inhibiting transforming growth factor- β pathway on matrix metalloproteinase 7

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Abstract

Renal injury has a strong relationship to the subsequent development of renal fibrosis. In developing renal fibrosis, tubular epithelial cells in the kidney underwent epithelial–mesenchymal transition (EMT). Matrix metalloproteinase 7 (MMP7) was reported to reduce E-cadherin and induce EMT by up-regulation of β -catenin/lymphoid enhancer-binding factor 1 (LEF1) signaling. In this research, we tried to evaluate the role of resveratrol (RSV) on EMT process in renal injury and fibrosis. Human tubular epithelial cell HK-2 cells were treated with aristolochic acid (AAs) and transforming growth factor- β (TGF- β) to induce EMT with or without the administration of RSV. The inhibitory role of RSV on EMT in renal injury and fibrosis was determined by Western blotting, real-time PCR, and immunofluorescence staining. The EMT repressing role of RSV was also evaluated *in vivo* by renal ischemia-reperfusion (I/R) injury and unilateral ureteral obstruction (UUO) models. The underlying mechanism was investigated by shRNA interfering MMP7 and sirtuin 1 (SIRT1) expression. The results indicated that RSV reversed human kidney 2 (HK-2) cell EMT, renal I/R injury, and renal fibrosis. MMP7 inhibition was responsible for RSV-induced EMT repression. SIRT1 was up-regulated by RSV inhibited TGF- β pathway on MMP7 via deacetylating Smad4. In conclusion, RSV attenuated renal injury and fibrosis by inhibiting EMT process which was attributed to the fact that the up-regulated SIRT1 by RSV deacetylated Smad4 and inhibited MMP7 expression.

Keywords: Resveratrol, epithelial–mesenchymal, transition, renal fibrosis, matrix metalloproteinase 7

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Introduction

Renal injury is a complex disorder that occurs in a variety of settings with clinical manifestations. It has a strong relationship to the subsequent development of chronic kidney diseases, like renal fibrosis. In developing renal fibrosis, tubular epithelial cells in the kidney underwent epithelial–mesenchymal transition (EMT). The blockage of EMT reduced fibrosis after renal obstructive injury.¹ In injury and fibrosis, matrix metalloproteinases (MMPs) participated in regulating abnormal epithelial response to injury, fibroblast proliferation, extracellular matrix accumulation, and aberrant tissue remodeling.² It has been reported that MMP7 expression dramatically increased during several renal disease states and acute lung injury.^{3–5} Chen *et al.*⁶ found that MMP7 was over-expressed in the aging kidney. The role of MMP7 on the induction EMT of human squamous carcinoma cell line was reported by Shibata *et al.*⁷

They found that MMP7 could reduce E-cadherin and induce EMT by up-regulation of β -catenin/LEF1 signaling.

Resveratrol (RSV; trans-3,5,4'-trihydroxystilbene), a stilbene polyphenol from grapes, wine, mulberries, and peanuts, has been reported to have various pharmacological effects, including protection against coronary heart disease, anti-inflammatory properties, and chemo-preventive of cancer, etc.^{8–10} In basic research, RSV is also a star natural compound in attenuating renal injury and fibrosis, including glycerol-induced renal injury,¹¹ sepsis-induced renal injury,¹² cisplatin-induced renal injury,¹³ ischemia-reperfusion (I/R) injury,¹⁴ and ureteral obstruction (UUO)-induced renal fibrosis,¹⁵ etc. Most of the researches focused on the anti-inflammatory and anti-oxidation effects of RSV. RSV ameliorated renal injury and renal fibrosis by suppressing the inflammatory process and by inhibiting lipid peroxidation.¹¹ The inhibitory effect of RSV on EMT has been revealed in prostate cancer,¹⁶ ovarian cancer,¹⁷ breast

cancer,¹⁸ and pancreatic cancer.¹⁹ In a recent research by Bai et al.,²⁰ RSV inhibited EMT in renal tubular cells by antagonizing the hedgehog signaling pathway. Based on these understandings, we speculated that as the activator of sirtuin 1 (SIRT1),¹² whose loss in tubular epithelial cells exacerbated injury induced kidney fibrosis,²¹ RSV could inhibit EMT in renal injury and renal fibrosis by activating SIRT1.

In this study, we investigated the role of RSV in EMT process which is a key step in renal injury and fibrosis and try to uncover the underlying mechanism beneath the protective role of RSV from a novel aspect.

Materials and methods

Cell and reagents

Human tubular epithelial cell human kidney 2 (HK-2) cells were from ATCC. HK-2 cells were supported in RPMI-1640 medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (Gibco) at 37 °C in an air supplemented with 5% CO₂ under humidified condition. RSV and aristolochic acids (AAs) were purchased from Sigma Aldrich (St. Louis, MO). Recombinant human transforming growth factor- β (TGF- β) was purchased from PeproTech (Rocky Hill City, NJ). Primary antibodies against E-cadherin, α -smooth muscle actin (α -SMA), type I collagen (COL1A1), β -catenin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MMP7, and SIRT1 were obtained from Cell Signaling Technology (Beverly, MA). The antibody against acetyl-lysine was purchased from Santa Cruz Biotechnology.

Renal I/R injury and UO models

Renal I/R injury and UO models were generated in eight-week-old male C57BL/6 mice ($n = 8$ per group). I/R injury was performed by clamping both renal pedicles for 30 min with vascular clamps. Blood sample from I/R injury model was collected every day after surgery. Mice were sacrificed four days following the experiment and kidneys were collected for further analysis. For UO model, 4-0 silk was used to ligate the left ureter of the anesthetized mice.¹⁵ The mice in the RSV treatment group received a daily dose of 20 mg/kg (body weight) of RSV by gavage. Mice in control group, I/R injury, and UO group were treated with vehicle. The mice in UO model were sacrificed six weeks after left ureter ligation and kidneys were collected.

Western blotting

Protein levels were detected by Western blotting following published protocols.²¹ The protein levels were determined by incubating with primary antibodies followed by horseradish peroxidase (HRP)-conjugated second antibody. The signal was developed with enhanced chemiluminescence (ECL) (Millipore, Switzerland).

qRT-PCR

Total RNA was extracted by TRIzol reagent (TaKaRa, Japan) and reverse transcribed to cDNA using Primescript RT master mix (TaKaRa). The relative mRNA levels were

determined by quantitative real-time PCR using the SYBR Green PCR Kit (TaKaRa) on ABI PRISM 7300Sequence Detector. GAPDH was set as internal control. The mRNA relative expression level was calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequences were as the following: for *hE-cadherin* gene, forward primer 5'-CGAGAGCTACACGTTACGG-3', reverse primer 5'-GGGTGTCGAGGGAAAAATAGG-3'; for *ha-SMA* gene, forward primer 5'-TCTG GAGGCACAA CTGGCATCGT-3', reverse primer 5'-TACATATGTT GTCCCC TGATAG-3'; for *hCOL1A1* gene, forward primer 5'-GAGGGCCAAGACGAAG ACATC-3', reverse primer 5'-CAGATCACGTCATCGCACAAC-3'; for *hMMP7* gene, forward primer 5'-GAGTGAGCTACAGT GGGAAACA-3', reverse primer 5'-CTATGACGCGGGA GTTTAACAT-3'; for *hSIRT1* gene, forward primer 5'-TAG CCTT GTCAGATAAGGAAGGA-3', reverse primer 5'-ACAGCTTCACAGTCAACTTT GT-3'; for *mE-cadherin* gene, forward primer 5'-CAGGTCTCCTCATGGCTTT GC-3', reverse primer 5'-CTTCCGAAAAGAAGGCTGTCC-3'; for *ma-SMA* gene, forward primer 5'-GTCCCAGACAT CAGGGAGTAA-3', reverse primer 5'-GTC CCAGACAT CAGGGAGTAA-3', reverse primer 5'-TCGGATACTTCA GCGTC AGGA-3'; for *mCOL1A1* gene, forward primer 5'-GCTCCTCTTAGGGGCCACT-3', reverse primer 5'-CCA CGTCTCACCATGTTGGG-3'; for *hGAPDH* gene, forward primer 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse primer 5'-GGCTGTTGTC ATACTTCTCATGG-3'; for *mGAPDH* gene, forward primer 5'-AGGTCCGGTGTG AACGGATTG-3', reverse primer 5'-TGTAGACCATGTG GTTGAGGTCA-3'.

Immunofluorescence staining

HK-2 cells were labeled with antibodies against β -catenin and MMP7. The slides were exposed to AlexFluor594 anti-rabbit secondary antibodies (Invitrogen, CA). The nucleus was stained with DAPI (Invitrogen). The staining was visualized by fluorescence microscope.

Renal function

The serum creatinine was detected by S-Cr ELISA kit (ExCell Bio, China). The blood urea nitrogen (BUN) level was determined by a commercial ELISA kit (Neogen, Shanghai) following the manufacturer's instructions.

Immunoprecipitation

The immunoprecipitations were carried out by using Pierce Direct IP Kit (Thermo Scientific). HK-2 cells were incubated with agarose beads coupled with Smad4 antibody. The Smad4 acetylation was determined by incubation with acetyl-lysine antibody.

Statistical analysis

All the data were expressed as mean \pm SD from three individual experiments and were analyzed by the Student's *t*-test and two-way ANOVA with Bonferroni correction. $P < 0.05$ was considered significant.

Results

RSV reversed HK-2 cell EMT induced by AAs and TGF- β

To induce EMT in HK-2 cells, we used AAs and TGF- β in the experiments. HK-2 cells were incubated with AAs or TGF- β for 48h, and were harvested for protein and mRNA analysis. As shown in Figure 1(a) and (b), AAs inhibited E-cadherin and increased α -SMA and COL1A1 protein and mRNA levels. A similar result was observed in TGF- β treated HK-2 cells (Figure 1(a) and (c)). This confirmed that HK-2 cell underwent EMT. However, the

administration of RSV inhibited E-cadherin decrease and α -SMA and COL1A1 increase. RSV blocked the EMT process induced by AAs and TGF- β . Because nuclear re-localization of β -catenin/LEF was one of the major criteria for EMT, we further detected the location of β -catenin in the cells by separating the cytoplasm and nuclear protein. We found a translocation of β -catenin from the cytoplasm to the nuclear under AAs and TGF- β treatment which could be inhibited by RSV administration (Figure 1(d)). The immunofluorescence staining of β -catenin was in consistency with the Western blotting results (Figure 1(e)).

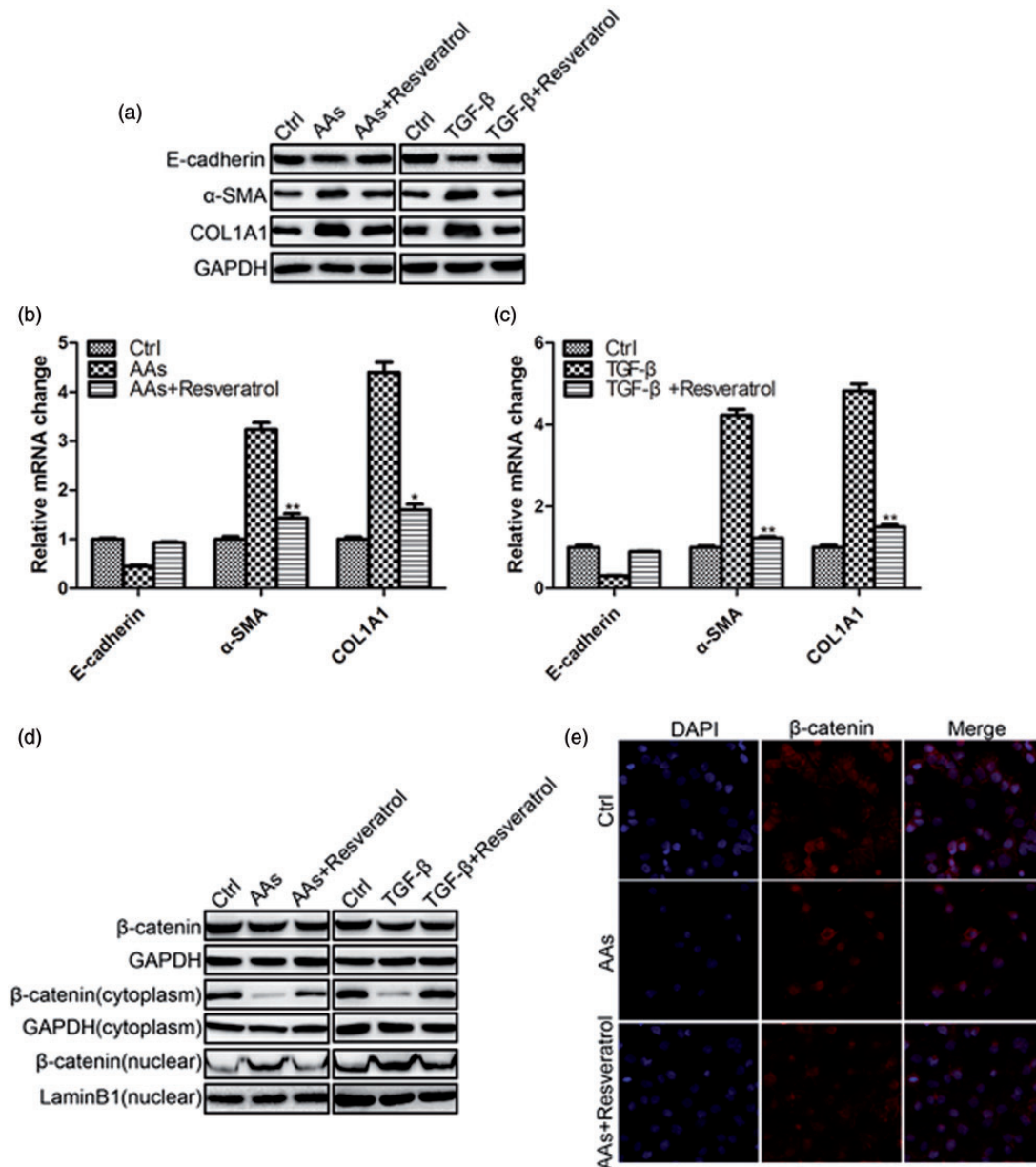


Figure 1 RSV reversed HK-2 cell EMT induced by AAs and TGF- β . a. Western blotting results of E-cadherin, α -SMA, and COL1A1 in HK-2 cells 48h following the treatment of AAs (10 μ mol/L)/TGF- β (10 ng/mL) with or without the presence of RSV (20 μ mol/L). b. Real-time PCR results of E-cadherin, α -SMA, and COL1A1 in HK-2 cells 48h following the treatment of AAs (10 μ mol/L) with or without the presence of RSV (20 μ mol/L). c. Real-time PCR results of E-cadherin, α -SMA, and COL1A1 in HK-2 cells 48h following the treatment of TGF- β (10 ng/mL) with or without the presence of RSV (20 μ mol/L). d. RSV reversed HK-2 cell EMT by inhibiting the translocation of β -catenin into the nuclear. e. Immunofluorescence staining of β -catenin in HK-2 cells 48h following the treatment of AAs with or without the presence of RSV (* P < 0.05 AAs vs. AAs + RSV, ** P < 0.01 AAs vs. AAs + RSV). (A color version of this figure is available in the online journal.)

RSV reversed renal I/R injury and renal fibrosis induced by UO

To verify the inhibitory effect on EMT by RSV *in vivo*, we adopted acute renal injury and renal fibrosis model in mice. After I/R, the serum creatinine and BUN were detected. In I/R injury group, the creatinine elevated sharply and peaked at the second day, and then slowly declined. The creatinine in RSV-treated group was lower than AAs group (Figure 2(a)). The BUN changes were similar to creatinine changes. RSV decreased the BUN elevation after I/R injury (Figure 2(b)). The EMT markers E-cadherin, α -SMA and COL1A1 after renal injury and fibrosis were detected by Western blotting. After I/R injury and renal fibrosis, E-cadherin decreased in the kidney, while α -SMA and COL1A1 increased, which proved that EMT existed in renal injury and fibrosis. A daily dose of 20 mg/(kg body weight) of RSV partially inhibited the changes of the EMT markers (Figure 2(c)).

The above results proved that RSV attenuated renal injury and fibrosis by inhibiting EMT process *in vitro* and *in vivo*.

MMP7 inhibition was responsible for RSV-induced EMT repression

Because MMP7 was reported to increase in renal injury and it could reduce E-cadherin and induce EMT,^{6,7} we further detected the level of MMP7 in HK-2 cells. We found the increase of MMP7 in HK-2 cells after AAS and TGF- β treatment and RSV inhibited MMP7 elevation (Figure 3(a) to (c)). As MMP7 could induce EMT through nuclear β -catenin

translocation, we speculated that MMP7 might be a key protein in AAs and TGF- β induced EMT. Therefore, we further studied MMP7 expression in HK-2 by transfecting MMP7 shRNA. The interfering efficiency was determined by real-time PCR method which showed an interfering efficiency of about 70% (Figure 3(d)). After MMP7 shRNA transfection for 48 h, HK-2 cells were incubated with AAs/TGF- β for another 48 h. The Western blotting results showed that MMP7 shRNA transfection inhibited AAs/TGF- β induced E-cadherin decrease and α -SMA and COL1A1 increase.

SIRT1 up-regulation by RSV inhibited TGF- β pathway on MMP7 by deacetylating Smad4

Because SIRT1 could be activated by RSV²² and the loss of SIRT1 in tubular epithelial cells exacerbated injury induced kidney fibrosis,²¹ we further detected the SIRT1 level after RSV treatment. RSV up-regulated the protein level of SIRT1 dramatically (Figure 4(a)). To confirm the necessary role of SIRT1 in RSV-induced EMT repression, SIRT1 shRNA was transfected into HK-2 cells. Figure 4(b) showed the interfering efficiency of SIRT1 shRNA to be about 80%. SIRT1 interfering induced EMT in HK-2 cells as manifested by the increase of α -SMA and COL1A1 protein level which could not be repressed by RSV (Figure 4(c)). The increased α -SMA and COL1A1 level might be the result of increased MMP7, because SIRT1 interfering also promoted the increase of MMP7 protein level (Figure 4(d) and (e)). Because SIRT1 deacetylated the positive regulator of TGF- β signaling Smad4 to inhibit the expression of

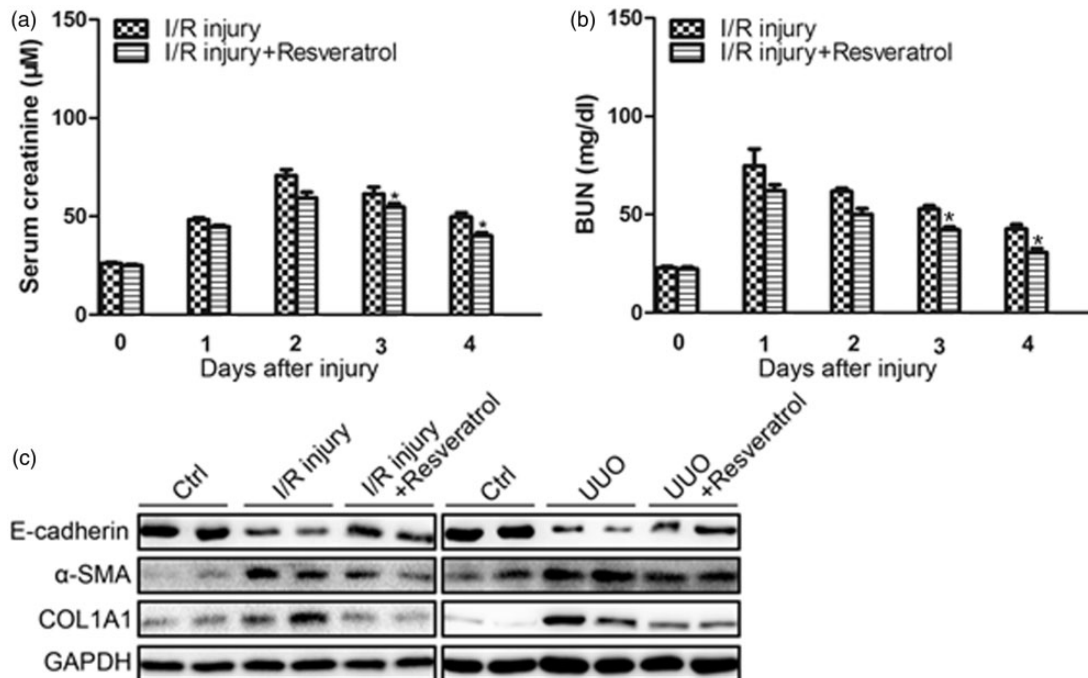


Figure 2 RSV reversed renal I/R injury and renal fibrosis induced by UO. a. Serum creatinine changes over time following I/R injury ($n = 6$ in each group). b. BUN levels over time following I/R injury ($n = 6$ in each group). c. Western blotting results of E-cadherin, α -SMA, and COL1A1 in the kidney of mice after I/R injury for 48 h with or without the presence of RSV (left panel). Western blotting results of E-cadherin, α -SMA, and COL1A1 in the kidney of mice after UO for six weeks with or without the presence of RSV (right panel). (* $P < 0.05$ I/R injury vs. I/R injury + RSV)

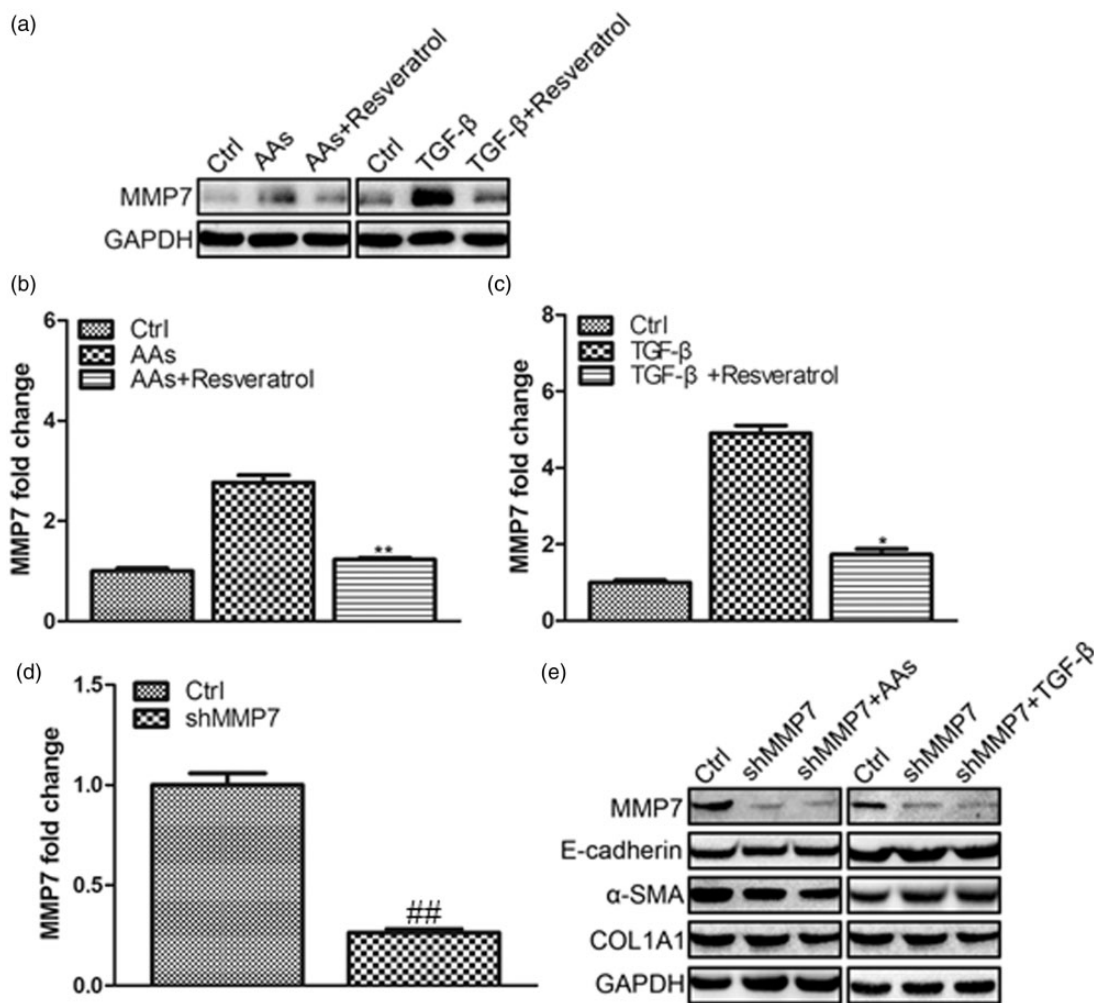


Figure 3 MMP7 inhibition was responsible for RSV-induced EMT repression. a. Western blotting results of MMP7 in HK-2 cells 48 h following the treatment of AAs (10 μ mol/L)/TGF- β (10 ng/mL) with or without the presence of RSV (20 μ mol/L). b. Real-time PCR results of MMP7 in HK-2 cells 48 h following the treatment of AAs (10 μ mol/L) with or without the presence of RSV (20 μ mol/L). c. Real-time PCR results of MMP7 in HK-2 cells 48 h following the treatment of TGF- β (10 ng/mL) with or without the presence of RSV (20 μ mol/L). d. Interfering efficiency of MMP7 shRNA verified by real-time PCR. e. Inhibition of MMP7 reversed AAs (10 μ mol/L)/TGF- β (10 ng/mL) induced EMT. (* P < 0.01 AAs vs. AAs + RSV, * P < 0.05 TGF- β vs. TGF- β + RSV, ## P < 0.01 Ctrl vs. shMMP7)

MMP7,²¹ we immunoprecipitated Smad4 from HK-2 cells after RSV incubation/ SIRT1 shRNA transfection and probed Western blotting with antibody against acetyllysine. We found SIRT1 interfering increased the acetylation of Smad4 (Figure 4(f)).

The above results proved that RSV up-regulated SIRT1 which deacetylated Smad4 and inhibited MMP7 expression, thus leading to EMT repression.

Discussion

In the present study, we evaluated the inhibitory role of RSV on EMT process in renal injury and fibrosis. RSV reversed the reduction of E-cadherin and the increase of α -SMA and COL1A1 in both protein and mRNA levels. RSV inhibited the profibrotic protein MMP7 level, therefore, attenuated UO-induced renal fibrosis. The underlying mechanism was also investigated. We found that SIRT1 up-regulation by RSV was responsible for the downstream blockage of EMT process. SIRT1 inhibited MMP7 protein level by deacetylating Smad4 which was a transcription factor for

MMP7 expression, and the interfering of SIRT1 induced the up-regulation of MMP7 and EMT.

RSV is a polyphenol found in red wine that is associated with a number of health benefits.²²⁻²⁴ The renal protective effects of RSV have been reported previously. RSV can ameliorate several types of renal injury through its antioxidant effect and SIRT1 activation.²³ SIRT1 is an NAD⁺-dependent deacetylase and functions in multiple cellular processes like apoptosis, mitochondrial biogenesis, inflammation, etc. through the deacetylation of target proteins.²⁵ He *et al.*²⁶ found that SIRT1 is an important protective factor for mouse renal medullary interstitial cells following oxidative stress. Jung *et al.*¹³ found that SIRT1 up-regulation by RSV decreased acetylation of NF- κ B p65 subunit and attenuate cisplatin-induced renal cell damage.

In renal injury and fibrosis, renal tubular epithelial cells undergo EMT in which they lose the epithelial phenotype and acquire the new features of mesenchyme.²⁷ When renal fibrogenesis sets in, more than one-third of all disease-related fibroblasts originate from tubular epithelial cells at the site of injury.²⁸ This reinforces the notion that EMT exists

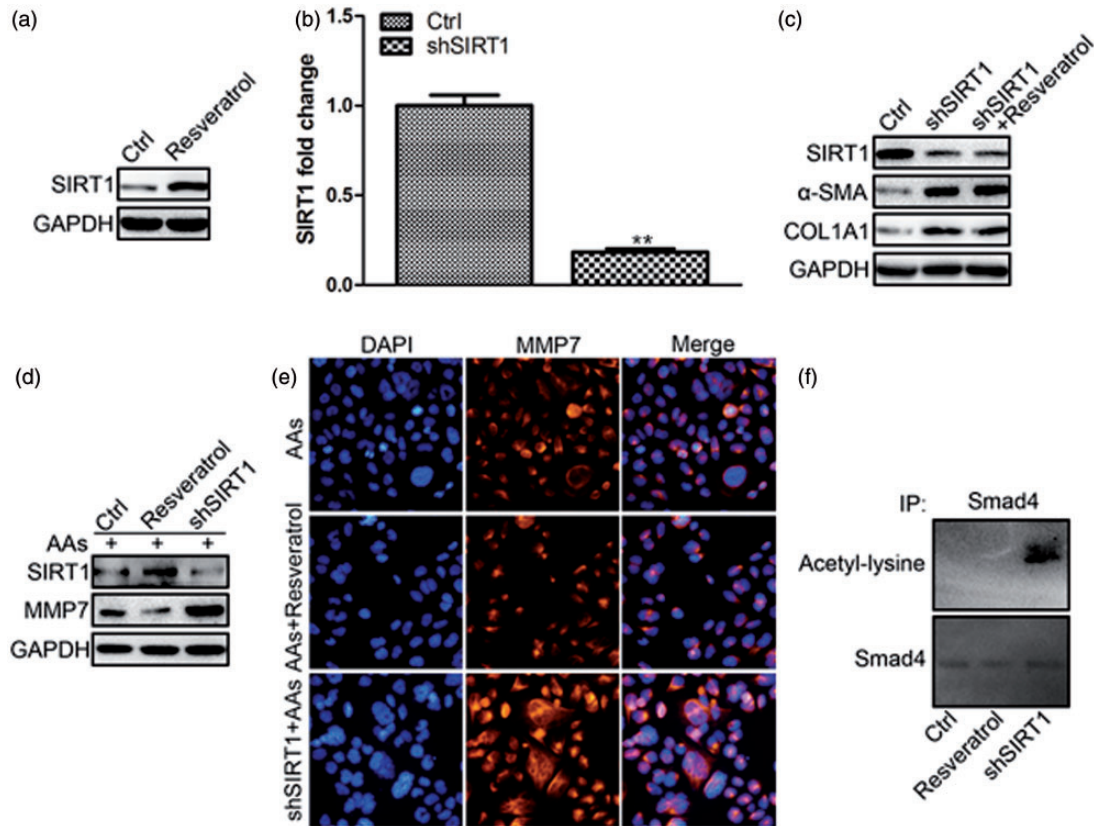


Figure 4 SIRT1 up-regulation by RSV inhibited TGF- β pathway on MMP7 by deacetylating Smad4. a. RSV up-regulated SIRT1 protein level. b. Interfering efficiency of SIRT1 shRNA verified by real-time PCR. c. Inhibition of SIRT1 induced HK-2 cell EMT. d. SIRT1 up-regulation inhibited MMP7 protein level. e. SIRT1 up-regulation by RSV inhibited MMP7 protein level visualized by immunofluorescence staining of MMP7 in HK-2 cells. f. SIRT1 up-regulation by RSV deacetylated Smad4 which inhibited MMP7 activity (** $P < 0.01$ Ctrl vs. shSIRT1). (A color version of this figure is available in the online journal.)

in renal injury and fibrosis. In the EMT process of renal tubular cells, loss of epithelial adhesion properties, de novo α -SMA expression and actin reorganization, disruption of tubular basement membrane, and enhanced cell migration happen. In this research, human tubular cell HK-2 cells were treated with AAs and TGF- β to induce EMT *in vitro*, while renal I/R injury and UUO models in mice were used to induce EMT *in vivo*. The renal I/R injury and longtime UUO models can mimic renal epithelial injury and renal fibrosis, respectively. We found that AAs and TGF- β could induce EMT in HK-2 cells. Renal I/R injury and longtime UUO also induced EMT in the kidney. The induced EMT could be reversed by RSV administration. RSV attenuated renal injury and fibrosis by inhibiting EMT process.

The growth factor TGF- β can initiate and maintain EMT in a variety of biological system and pathophysiological context by activating major signaling pathways and transcriptional regulators integrated in extensive signaling networks.²⁹ Boutet *et al.*³⁰ found that aberrant Snail1 activation in the adult kidney is sufficient to induce renal fibrosis in transgenic mice. TGF- β signaling regulates Snail activation, which triggers EMT and the conversion of epithelial cells into myofibroblasts.³¹ Thiery *et al.*,³¹ found that in renal proximal tubular cells, TGF- β -induced β -catenin was required for synthesis of α -SMA as a

marker of EMT. In this research, we found the translocation of β -catenin into the nucleus which could be inhibited by RSV.

It has been reported that MMP7 expression increases during several renal diseases and in aging kidneys.^{3,6} MMP7 can reduce E-cadherin and induce EMT by up-regulation of β -catenin/LEF1 signaling.⁷ In TGF- β signaling, there is a strong relationship between MMP7 and Smad4. Kitamura *et al.* found that MMP7 was not required for the invasion or fibrosis of the colon cancer whose Smad4-dependent TGF- β family signaling is blocked.³² Simic *et al.*²¹ found that TGF- β regulated MMP7 expression, while SIRT1 deacetylated Smad4 which re-pressed the activity of MMP7. Some scientists^{33,34} also found that by associating and deacetylating Smad4, SIRT1 enzyme could influence MMP7 expression and enzyme activity. In this research, we found that the up-regulated SIRT1 by RSV deacetylated Smad4 and inhibited MMP7 expression.

In summary, we found that RSV attenuated renal injury and fibrosis by inhibiting EMT process. The up-regulated SIRT1 by RSV inhibited MMP7 expression by deacetylating Smad4.

Author contributions: ZX and QZ conceived and designed the study; ZX, CC, and WZ performed laboratory experiments; ZX, CC, and TM analyzed data and interpreted the

results; ZX, CC, and QZ created the figures and drafted the manuscript; ZX, CC, WZ, and QZ edited and revised manuscript; all authors approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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