Activation of SIRT1 Promotes Renal Fibroblast Activation and Aggravates Renal Fibrogenesis

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Sirt1 activation aggravates renal fibrosis

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SIRT1, sirtuin 1; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; STAT3, signal transducer and activator of transcription 3; ERK1/2, extracellular signal regulated kinase 1/2; CKD, chronic kidney disease; HAT, histone acetyltransferase; HDAC, histone deacetylase, PCNA, proliferating cell nuclear antigen; UUO, unilateral ureteral obstruction; α-SMA, α-smooth muscle actin; ECM, extracellular matrix; TGFβ1; Transforming growth factor–β1; Ac-H3K, acetylation of histone H3 at lysine 9
Abstract

Although activation of sirtuin 1 (SIRT1) has been shown to protect the kidney from acute injury, its role in renal fibrosis remains controversial since both inhibition and activation of SIRT1 have been reported to attenuate renal fibrosis. To resolve this conflict, we further examined the effect of SIRT1 activators on the activation of renal interstitial fibroblasts and development of renal fibrosis in vivo and in vitro. In a murine model of renal fibrosis induced by unilateral ureteral obstruction, administration of SRT1720, a potent activator of SIRT1, accelerated deposition of collagen fibrils and increased expression of fibroblast activation markers (α-smooth muscle actin, collagen I and fibronectin) in the obstructive kidney of mice. In cultured rat renal interstitial fibroblasts (NRK-49F), exposure of cells to SRT1720 or YK-3-237, another SIRT1 activator, also resulted in enhanced expression of α-smooth muscle actin and fibronectin. Mechanistic studies showed that augmentation of renal fibrogenesis by SRT1720 is associated with elevated phosphorylation of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor β (PDGFRβ). SRT1720 treatment also increased the phosphorylation STAT3 and AKT in the fibrotic kidney and NRK-49F cells. However, SRT1720 treatment did not affect expression of proliferating cell nuclear protein, a proliferation marker and activation of ERK1/2 in vitro and in vivo. These results indicate that SIRT1 activating compounds can provoke renal fibrogenesis through a mechanism involved in the activation of EGFR and PDGFR signaling pathways and suggest that long-term use of SIRT1 activators risks the development and progression of chronic kidney disease.
Introduction

Tubulointerstitial fibrosis is a common event for the progression of chronic kidney disease (CKD) regardless of the primary causes of renal disease (Tampe and Zeisberg, 2014). Renal fibrogenesis is characterized by activation of renal interstitial fibroblasts and subsequent production of excessive amount of extracellular matrix proteins. The transformation of fibroblasts into myofibroblasts is a core event for the development of fibrotic lesions in the kidney (Zeisberg and Neilson, 2010). Transforming growth factor–β1 (TGFβ1) and other growth factors, like epidermal growth factor (EGF) and platelet derived growth factors (PDGF), are critically involved in the fibrotic process (Bonner, 2004; Liu et al., 2012; Liu et al., 2013). Those cytokines/growth factors trigger their cellular events through binding to their receptors and subsequent activation of multiple downstream signaling pathway such as signal transducer and activator of transcription 3 (STAT3), protein kinase B (AKT), and extracellular signal regulated kinase 1/2 (ERK1/2) (Qin and Han, 2010; Liu et al., 2012; Kitada et al., 2013; Liu et al., 2013; Ponnusamy et al., 2013). It is evident that activation of epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) contribute to activation of renal interstitial fibroblasts and development of renal fibrosis (Bonner, 2004; LeBleu and Kalluri, 2011; Liu et al., 2011; Liu et al., 2012).

Epigenetic modifications such as acetylation/deacetylation have also been linked to the pathogenesis of CKD (Liu et al., 2013; Van Beneden et al., 2013; Tampe and Zeisberg, 2014). Protein acetylation is regulated by a network of enzyme systems namely histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs can modulate both acetylation of both histone and non-histone proteins associated with fibrogenic process such as STAT3 (Rombouts et al., 2002; Pang and Zhuang, 2010; Qin and Han, 2010). Among the four classes of HDACs, Class I and II HDACs have been extensively studied for their role in tissue fibrogenesis (Pang et al., 2009; Pang and Zhuang, 2010; Pang et al., 2011). It is evident
that small molecular inhibitors acting on these two classes of HDACs were effective in attenuating fibrosis in multiple organs including kidney (Pang et al., 2009; Liu et al., 2013; Van Beneden et al., 2013). Apart from class I and II HDACs, the role of Sirtuins (SIRTs), a Class III HDAC, has also been intensively studied in animal models of kidney diseases. Among the seven members of SIRTs, SIRT1 is ubiquitously expressed in renal tissue, and has been shown to be implicated in renal protection under ischemic, hypoxic and calorie restricted conditions (Fan et al., 2013; Kitada et al., 2013; Dong et al., 2014). However, the role of SIRT1 in renal fibrogenesis remains controversial even though its activity increases in the fibrotic kidney (Ponnusamy et al., 2014). Earlier studies have indicated that activation of SIRT1 by resveratrol, a polyphenol found in red wine that is believed to be an activator of SIRT1, can alleviate renal fibrosis induced by unilateral ureteral obstruction in mice (Li et al., 2010). However, our recent studies showed that inhibition of SIRT1 and 2 with sirtinol attenuated renal fibrosis in the same model. In addition, specific inhibition of SIRT1 by EX527 or silencing of SIRT1 with siRNA suppressed activation of renal interstitial fibroblasts (Ponnusamy et al., 2014). These obviously conflicting results make it difficult to explain the functionality of SIRT1 in renal fibrogenesis and raise the issue whether the anti-fibrotic effect of resveratrol is due to its non-specific effects. It is well documented that this compound indeed has off-target effects (Pacholec et al., 2010). Indeed, a recent report from Ventruelli et al. (2013) showed that resveratrol has an inhibitory effect on class I, II and IV HDACs (Venturelli et al., 2013). In the view that blocking of class I/II HDACs can attenuate renal fibrosis (Pang et al., 2009; Liu et al., 2013), this suggests the possibility that resveratrol may alleviate renal fibrosis through a mechanism involved in the inhibition of class I/II HDACs rather than activation of SIRT1. Therefore, additional experiments are needed to characterize the role of SIRT1 activation in regulating renal fibrosis by using more specific SIRT1 pharmacological activators.
Efforts have been made to discover new molecules that are able to stimulate SIRT1 activities more specifically and potentially than resveratrol. SRT1720 is structurally unrelated to resveratrol and does not share “off-target” effects of resveratrol (Villalba and Alcain, 2012), therefore it is a useful tool for verifying putative SIRT1-dependent effects in vivo. In this study, we investigated that the influence of SRT1720 on the progression of renal fibrosis in a murine model of renal fibrosis induced by unilateral ureteral obstruction (UUO) and on the activation of cultured renal fibroblasts.

**Materials and Methods**

**Chemicals and Antibodies.** Antibodies to fibronectin, collagen I(A2), EGFR, glyceraldehyde-3 phosphate dehydrogenase (GAPDH), and proliferating cell nuclear antigen (PCNA) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other antibodies used in this study were purchased from Cell Signaling Technology (Danvers, MA, USA). SRT1720 was purchased from EMD millipore (USA). α-smooth muscle actin (α-SMA) and all other chemicals were purchased from Sigma (St Louis, MO).

**Cell Culture and Treatments.** Rat renal interstitial fibroblasts (NRK-49F) were cultured in Dulbecco’s modified eagle’s medium with F12 (DMEM-F12) containing 5% fetal bovine serum (FBS), 0.5% penicillin and streptomycin in an atmosphere of 5% CO2 and 95% air at 37°C. To reduce the interference of growth factors in serum with SRT1720 activity, NRK-49F cells were grown in DMEM-F12 with 2.5% FBS for 24 hours, and SRT1720 was directly added to subconfluent NRK-49F cells and incubated for 36 hours to determine the effects of SRT1720 on renal fibroblast activation.

**Animals and Experimental Design.** The unilateral ureteral obstruction (UO) model was established in 6-8 weeks old male C57 black mice that weighed 20-25g (Jackson Laboratory,
Bar Harbor, ME, USA) as described in our previous studies (Pang et al., 2009; Pang et al., 2010). Briefly, the abdominal cavity was exposed via a midline incision and the left ureter was isolated and ligated. The contralateral kidney was used as a control. To examine the effects of SRT1720 on renal fibrosis after UUO injury, SRT1720 at 200 mg/kg body weight (prepared in 50 μl of DMSO) was intraperitoneally (i.p) administered immediately after ureteral ligation and then given daily for 4 days. The dose of SRT1720 was selected based on a previous report (Imanishi et al., 2012). Control mice were injected with the equal volume of DMSO. The animals were sacrificed and the kidneys were collected at day 5 for protein analysis and histological examination. All experimental procedures were performed according to the United States guidelines to the care and use of laboratory animals. The protocol (#0135-13) was approved by Lifespan Animal Welfare Committee (IACUC) at Rhode Island hospital. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

**Masson Trichrome Staining.** For assessment of renal fibrosis, Masson trichrome staining was performed according to the protocol provided by the manufacture (Sigma, St. Louis, MO). The semi-quantitative analysis of collagen tissue area (blue colored area) was measured using ImageJ software developed at the national institute of health (NIH), USA. The positive staining area from each microscopic field (200X) was calculated and graphed.

**Immunoblot Analysis.** To prepare protein samples for western blotting, the kidney tissue samples were homogenized the cell lysis buffer (Cell Signaling Technology, Danvers, USA) with protease inhibitor cocktail (Roche Diagnostic Corporation, USA). After various treatments, cells were washed once with ice-cold PBS and harvested in a cell lysis buffer mixed with a protease inhibitor cocktail. The protein level was measured by the bicinchoninic acid (BCA) method and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After incubation with 5% non-fat milk for 1 hour at room
temperature, membranes were incubated with primary antibody overnight at 4°C and washed with Tris buffered saline with Tween 20 (TBS-T). Then membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour in room temperature. After washing the membrane with TBS-T, bound antibodies were visualized by chemiluminescence detection using Electrochemiluminescence (ECL) solution obtained from GE Healthcare Life Sciences, PA, USA.

**Densitometry Analysis.** The semiquantitative analysis of different proteins were carried out by using ImageJ software developed at NIH, USA. The quantification is based on the intensity (density) of band, which is calculated by area and pixel value of the band. The quantification data are given as the ratio between target protein and loading control (house keeping protein) and the ratio between phosphorylated and corresponding total protein.

**Statistical Analysis.** Data are presented as means ± SD and were subjected to one-way analysis of variance. Multiple means were compared using Tukey’s test, and differences between two groups were determined by Student’s t test. P < 0.01 was considered statistically significant.

**Results**

**SRT1720 enhances deposition of ECM components and activation of renal fibroblasts in the kidney after UUO injury.** Progressive interstitial fibrosis is the result of excessive production of extracellular matrix (ECM) components by activated fibroblasts (Zeisberg and Neilson, 2010). Our recent study have shown that SIRT1 and 2 activities are increased during renal fibrosis induced by UUO injury and treatment with SIRT1/2 selective inhibitors can attenuate deposition of ECM proteins and inhibited activation of renal interstitial fibroblasts in the mouse model of renal fibrosis induced by UUO (Ponnusamy et al., 2014),
suggesting that SIRT1/2 are critical regulators of renal fibrogenesis. If this conclusion is correct, SIRT1 activation would exert an opposite effect by enhancing renal fibrosis. To test this hypothesis, we collected the kidney after 4 days of UUO injury with/without SRT1720 treatment and then examined the effect of SRT1720 on renal fibrosis. Masson trichrome staining illustrates that the deposition and accumulation of ECM components were increased in the tubulointerstitial space as a consequence of myofibroblast activation, and administration of SRT1720 further increased the deposition of ECM components in the interstitial space (Fig. 1A). Semiquantitative analysis of Masson trichrome-positive areas revealed a 4-fold increase of ECM components in the obstructive kidney compared with sham kidney. SRT1720 treatment increased ECM deposition by more than 3-fold compared with UUO injury alone (Fig. 1B). Immunoblot analysis of whole kidney tissue lysate indicated that acetylation of histone H3 at lysine 9 (Ac-H3K9) was increased in the injured kidney and its level was significantly decreased in the kidney of mice treated with SRT1720, indicating that the dose of SRT1720 was effective in elevating renal SIRT1 deacetylase activity (Fig. 1, C and D). In addition, there was also an increase in the expression of total H3 in UUO injured kidney, but its level was not affected by SRT1720 treatment (Fig. 1, C and E). Immunoblot analysis showed that UUO injury increased the expression of SIRT1, but SRT1720 did not reduce its expression (Supplemental Fig. 1).

The expansion of renal interstitial fibrosis is classically manifested by an increase in the population of myofibroblasts, the phenotypically transformed fibroblasts that express α-SMA and produce ECM components (Meran and Steadman, 2011). By immunoblot analysis, we examined the expression of fibroblast activation and proliferation markers in UUO injured kidney of mice treated with or without SRT1720. As shown in Fig. 2, increased expression of fibroblast activation markers (α-SMA, collagen I and fibronectin) as well as proliferation markers (proliferating cell nuclear antigen, PCNA) were observed in the
obstructed kidney. Administration of SRT1720 profoundly increased (about 2-fold) expression level of α-SMA, collagen I and fibronectin (Fig. 2A-D), however, PCNA expression was not affected by this compound (Fig. 2, E and F). Thus, these data indicate that SRT1720 can induce aggressive activation of fibroblasts and accumulation of ECM, but not affect proliferation of interstitial fibroblast cells in the kidney after UUO injury.

**Treatment with SIRT1 activators potentiate cultured renal interstitial fibroblast activation.** To confirm the role of SIRT1 in mediating renal fibroblast activation in the mouse model, we examined the effect of SRT1720 on the expression of fibrogenic markers in cultured renal interstitial fibroblast cells (NRK-49F). NRK-49F grown in reduced level of serum (2.5% FBS) were exposed to various concentrations (0.5-2 μM) of SRT1720 for 36 hours, and expression level of fibroblast activation markers (α-SMA and fibronectin) were assessed. As shown in Fig. 3A, SRT1720 dose-dependently increased expression of those proteins. Densitometry analysis demonstrated that SRT1720 increased the expression of α-SMA (Fig. 3B) and fibronectin (Fig. 3C) by approximately 2-3 fold at a dose of 1 μM, and their levels were further enhanced in fibroblasts exposed to 2 μM of SRT1720. To demonstrate that this increase is due to the activation of SIRT1 deacetylase activity, we also examined the level of Ac-H3K9. As expected, SRT1720 treatment decreased the level of Ac-H3K9 in a dose dependent manner (0.5-2 μM). The level of Ac-H3K9 was significantly decreased (~70%) at a concentration of 1 μM SRT1720 treatment and further decreased more than 90% at a dose of 2 μM (Fig. 3, A and D). However, SRT1720 treatment did not increase the level of PCNA (Fig. 3, E and F), and cell proliferation even at the maximum concentration (2 μM) as indicated by cell counting (Fig. 3G) and MTT assay (Fig. 3H). Similarly, SRT1720 treatment did not increase PCNA level in cultured renal proximal tubular
cells (RPTC) (Data not shown). These data are consistent with our in vivo data, indicating that SRT1720 triggers activation, but not proliferation of renal interstitial fibroblasts.

We further examined the effect of YK-3-237, another potent activator of Sirt1 (Yi et al., 2013), on the activation of renal interstitial fibroblasts using NRK-49F cells. Our data demonstrated that exposure of cells to YK-3-237 also significantly reduced expression of α-SMA and fibronectin in a dose-dependent manner, with the maximum inhibition at 10 μM. This dose of the inhibitor completely blocked expression of Ac-H3K9, suggesting its effectiveness in the activation of Sirt1 (Supplemental 2).

Collectively, our data provide the strong evidence that SIRT1 plays a critical role in mediating activation of renal interstitial fibroblasts.

**SRT1720 enhances UUO-induced phosphorylation of EGFR and PDGFRβ in obstructed kidneys.** Activation of growth factor signaling pathways is involved in the regulation of fibrosis development. Our studies and those of others have shown that EGFR and PDGFRβ are two major contributors to renal fibroblast activation and renal fibrogenesis (Ludewig et al., 2000; Terzi et al., 2000; Bonner, 2004; Di Pascoli et al., 2013). To determine whether SRT1720 exerts its profibrotic effects through activation of these two receptors, we examined the effect of this agent on the phosphorylation of EGFR at Tyr1068 (Y1068) and PDGFRβ at Tyr751 (Y751). As shown in Fig. 4, the level of phospho-EGFR was increased by ~4 fold after 4 days in the kidney with UUO injury, and SRT1720 treatment enhanced phospho-EGFR level up to 4-fold when compared to UUO injured kidney treated with the vehicle. Interestingly, SRT1720 administration also increased phospho-EGFR levels more than 8-fold in the sham-operated kidney compared with the control kidney (Fig. 4, A and B). In addition, UUO injury resulted in increased expression of total EGFR, however SRT1720 treatment did
not alter its expression levels (Fig. 4, A and C). Similarly, SRT1720 was also effective in potentiating PDGFRβ phosphorylation in either sham-operated and UUO injured kidneys (Fig. 4, A and D) but not altering the level of total PDGFRβ (Fig. 4, A and E). Collectively, our data suggest that SRT1720 treatment enhances phosphorylation of both EGFR and PDGFRβ in the fibrotic kidney.

**SRT1720 enhances phosphorylation/activation of EGFR and PDGFRβ in cultured renal fibroblasts.** To specifically demonstrate that SRT1720 induced enhancement of EGFR and PDGFRβ activation occurs in renal fibroblasts during UUO injury, we further examined the effect of SRT1720 on their phosphorylation status in cultured renal fibroblasts. Consistent with our in vivo data, exposure to SRT1720 also dose-dependently increased the phosphorylation level of EGFR and PDGFRβ in cultured NRK-49F cells (Fig. 5, A and B). In addition, SRT1720 increased the level of total EGFR, with the maximum effect at 2 μM, but this agent did not affect the level of total PDGFRβ (Fig. 5, A and C). Thus, SRT1720 can enhance EGFR and PDGFRβ phosphorylation in renal fibroblasts, but with different effect on their total protein levels.

**SRT1720 enhances phosphorylation of STAT3, but not ERK1/2 in UUO injured kidney and cultured renal fibroblasts.** EGFR and PDGFRβ exert their biological functions through activation of several intracellular signaling pathways including STAT3, AKT and ERK1/2 (Ludewig et al., 2000; Liu et al., 2012; Tang et al., 2013), and these pathways are also associated with development of renal fibrosis (Pang et al., 2010; Lan and Du, 2015). Thus, we further examined the influence of SRT1720 on their phosphorylation in vivo and vitro. As shown in Fig. 6, A-C, the phosphorylation levels of STAT3 (Y705) and total STAT3 were
increased in the obstructed kidneys. Interestingly, SRT1720 administration further increased the level of both phosphorylated and total STAT3 in UUO injured and sham operated kidneys. Compared with sham-operated kidney, UUO injured kidney also showed an increase in the phosphorylation of AKT (S473) and SRT1720 treatment enhanced UUO-induced AKT phosphorylation as clearly indicated by the ratio of phospho-AKT/AKT. In sharp contrast to elevated phospho-AKT, administration of SRT1720 resulted in reduction in the level of AKT in both sham-operated and UUO injured kidney (Fig. 6, A, D and E). Although increased levels of phosphorylated and total ERK1/2 were detected in the obstructed kidney, treatment with SRT1720 did not affect these responses (Fig. 6, F and G).

We further examined the effect of SRT1720 on the phosphorylation and expression of STAT3, AKT, ERK1/2 in cultured renal fibroblasts. As shown in Fig. 7, SRT1720 treatment at 1 and 2 μM remarkably increased the phosphorylation level of STAT3 (Fig. 7, A and B). Similarly, SRT1720 at 2 μM also enhanced AKT phosphorylation (Fig. 7, A and C), however, phosphorylation of ERK1/2 was not affected by SRT1720 (Fig. 7, A and D). In addition, SRT1720 treatment resulted in decrease of total AKT (Fig. 7, A and C), but not total STAT3 (Fig. 7, A and B) or ERK1/2 (Fig. 7, A and D) levels in cultured renal fibroblasts.

Collectively, these data indicate that SIRT1 activation by SRT1720 regulates phosphorylation of STAT3 and AKT, but not ERK1/2, suggesting that SIRT1 has a diverse role in regulating activation of intracellular signaling pathways.

**Discussion**

Sirtuin-activating compounds such as resveratrol have been widely studied for their ability to treat aging-related disorders in metabolic, cardiovascular, and neurodegenerative diseases
However, due to the poor specify and bioavailability of resveratrol, several molecules that are structurally unrelated to resveratrol has been developed to stimulate sirtuin activities, which are more potent and specific than resveratrol. SRT1720 is a newly developed and effective SIRT1 activator that has been shown to have the potential to treat diabetes and other diseases (Dong, 2012). In this study, we examined the pharmacological effect of SRT1720 on the development of renal fibrosis and activation of renal fibroblasts in a mouse model of UUO and cultured renal interstitial fibroblasts. Our results showed that activation of SIRT1 with SRT1720 potentiated these pathological processes. Moreover, exposure of cultured renal interstitial fibroblasts to YK-3-237, another SIRT1 activator, also promoted their activation as demonstrated by increased expression of α-smooth muscle actin and fibronectin. These results provide the strong evidence that SIRT1 plays an essential role in mediating renal fibrogenesis after chronic kidney injury.

Our findings are contrary to a previous report in which activation of SIRT1 was demonstrated as an anti-fibrotic mechanism in the kidney by using resveratrol (Li et al., 2010). Currently, the molecular basis behind the striking inconsistencies generated by these two SIRT1 activators in term of fibrosis regulation remains unclear. One possible explanation is that resveratrol acts on other targets rather than on SIRT1. In this regard, it has been reported that resveratrol has a property to inhibit the activity of class I, II and IV HDACs (Venturelli et al., 2013). As blocking class I/II HDACs with their specific inhibitors or siRNA has been shown to suppress renal fibroblast activation (Pang et al., 2009; Pang et al., 2011; Liu et al., 2013) and those class I/II HDACs inhibitors are also effective in attenuating renal fibrosis (Pang et al., 2009; Liu et al., 2013), resveratrol induced inhibition of these enzymes may override its role as the activator of SIRT1, thereby exhibiting an anti-fibrotic
effects. Structural and chemical studies indicate that SRT1720 is a hundred-fold more potent in stimulating SIRT1 activity when compared with resveratrol (Villalba and Alcain, 2012).

SRT1720 induced enhancement of the fibrotic process may be involved in the activation of EGFR and PDGFRβ and their downstream signaling pathways. Previous studies from our laboratory and others groups have demonstrated that sustained activation of EGFR and PDGFRβ is associated with the activation of renal fibroblasts, expression of pro-fibrotic factors and uncontrolled accumulation of ECM in the interstitial space (Pang et al., 2010; LeBleu and Kalluri, 2011; Liu et al., 2011; Liu et al., 2012; Liu et al., 2013). Class I/II HDACs have been suggested to mediate activation of several growth factor receptor signaling pathways, including EGFR and STAT3 activity in the kidney after chronic injury (Bruzzese et al., 2011; Chou et al., 2011; Liu et al., 2013). Similar to this, inhibition of SIRT1 reduced the phosphorylation of EGFR and PDGFRβ in cultured renal fibroblasts and fibrotic kidneys (Ponnusamy et al., 2014). All these studies provide the strong evidence that HDACs including SIRT1 can function as a positive regulator of cellular membrane receptors associated with fibrotic process. Thus, stimulation of SIRT1 by SRT1720 would result in the activation of these profibrotic signaling molecules. The mechanism by which SRT1720 enhances the phosphorylation of receptor tyrosine kinases remains unknown. One possibility is that SRT1720 induced SIRT1 activation reduces the activity of tyrosine phosphatase. In supporting this hypothesis, SIRT1 is found to suppress the expression of protein tyrosine phosphatase 1B (Sun et al., 2007; Gagarina et al., 2010), a member of dephosphorylating protein family, which negatively regulates the activity of growth signaling molecules including EGFR and PDGFRβ (Gu et al., 2003; Ponnusamy et al., 2013). Nevertheless, we cannot rule out the possibility that SRT1720 can also stimulate renal fibrosis through stimulation of TGF-β signaling activation as it has been reported that activation of SIRT1 increased Smad reporter activity, enhanced transcription of TGF-β target genes and promoted
release of collagen, whereas knockdown of SIRT1 inhibited TGF-β/SMAD signaling, and reduced collagen release in skin fibroblasts (Zerr et al., 2014). Further experiments are needed to examine the role of SIRT1 in the regulation of this signaling pathway in renal interstitial fibroblasts.

Interestingly, SRT1720 administration increased the level of phosphorylated and total STAT3 in both sham operated and fibrotic kidney. The structural analysis of STAT3 revealed that both phosphorylation (Tyr705) and acetylation (Lys685) are located in SH2 domain of STAT3 protein. So acetylation and phosphorylation may work mutually to regulate STAT3 activity (O'Shea et al., 2005). As such, SRT1720 mediated increase of SIRT1 activity may promote the phosphorylation of STAT3 by inhibiting its acetylation in addition to its influence on protein tyrosine phosphatase system. On the other hand, SRT1720 mediated SIRT1 activation may regulate STAT3 through suppressing the activity/expression of suppressor of cytokine signaling (SOCS) family members such as SOCS1 and SOCS3, the negative regulators of STAT3. In fact, these two SOCS not only promote dephosphorylation but also ubiquitination and degradation of STAT family of proteins (Kile et al., 2002; Croker et al., 2008). Therefore, SIRT1 mediated inhibition of SOCS 1/3 may abolish STAT3 degradation process, thereby increasing the stability of STAT3. As a result, both phosphorylated and total STAT3 levels are increased. A further detailed study should be carried out to unveil the mechanism of SRT1720 induced up-regulation of phosphorylated and total STAT3 level.

It is quite surprising that SRT1720 treatment reduced total AKT level in both fibrotic and sham operated kidney without affecting the phosphorylation of AKT (ser473), which results an increase in the ratio of pAKT to AKT. In general, phosphorylation of ser473 at the hydrophobic motif site is necessary for full activation of AKT molecule, and it is also the major site engaged with many physiological and pathological functions of AKT (Liao and
Hung, 2010). Recent studies indicated that the AKT activity is controlled by negative feedback loop, which is triggered by hyperactivation of AKT and this hyperactivation has the capacity to regulate AKT stability and/or expression (Hart and Vogt, 2011; Wu et al., 2011). In supporting this, Wu et al. (2011) found that consecutive activation of AKT by ser473 phosphorylation promotes its rapid degradation by polyubiquitination dependent proteosomal pathway thereby it turns off AKT signaling (Wu et al., 2011). Given that SIRT1 promotes AKT phosphorylation through deacetylation (Horio, 2012; Li et al., 2013), SRT1720 induced SIRT1 activity might stimulate sustained activation of AKT, which in turn leads to degradation of AKT and reduction of total AKT levels.

Sirtuin activity has been linked to metabolic control, cell survival, DNA repair, development, neuroprotection and healthy aging (Stunkel and Campbell, 2011; Villalba and Alcain, 2012). Because sirtuin activation could have beneficial effects on human diseases, there is a growing interest in the discovery of small molecules able to stimulate sirtuin activity. Several small molecule activators of SIRT1 that are structurally unrelated to resveratrol have been developed. Among them, SRT1720 is one of the most effective SIRT1 activators. The role of SIRT1720 to treat insulin resistance, diabetes and other diseases has been tested in animals models and drugs similar to SRT1720 are currently in human clinical trials (Villalba and Alcain, 2012). However, due to the fibrosis promoting effect of SIRT activators, a large dose and long-term application of them should be avoided when they are used clinically in the future.

In summary, this is the first study to demonstrate that activation of SIRT1 by SRT1720 potentiates renal fibroblast activation and renal fibrogenesis. The profibrotic effects of SRT1720 are associated with activation of EGFR and PDGFRβ and their downstream signaling molecules STAT3 and AKT. On this basis, a caution should be put on
the application of SIRT1 activators to avoid induction of kidney fibrotic disorders or acceleration of pre-existing chronic kidney disease when they are used for renal diseases.

**Authorship contribution**

Participated in research design: Ponnusamy and Zhuang S

Conducted experiments: Ponnusamy, Zhou and Tolbert

Contributed new reagents or analytic tools: Ponnusamy, and Tolbert

Performed data analysis: Ponnusamy, Zhou

Contributed to the writing of the manuscript: Ponnusamy, Zhuang M, Bayliss, Zhuang S
References


**Footnotes**

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Figure Legends

Figure 1. SRT1720 enhances the deposition of ECM and development of fibrosis in obstructed kidneys. (A) Photomicrographs illustrating Masson trichrome staining of kidney tissue after treatment with or without SRT1720. (B) The Masson trichrome-positive tubulointerstitial area (blue) relative to the whole area from 10 random cortical fields (200 X) (mean ± SD) was analyzed. Data are represented as the mean ± SD. Means with different superscript letters are significantly different from one another (P< 0.01). (C) Kidney tissue lysates were subjected to immunoblot analysis with antibodies for acetyl-H3K9 (Ac-H3K9), total H3 or β-actin. The levels of Ac-H3K9 and total H3 were quantified by densitometry and normalized with β-actin (D and E). Values are means ± SD (n=6). Bars with different letters (a-c) are significantly different from one another (P< 0.01).

Figure 2. Administration of SRT1720 increases renal interstitial fibroblast activation in obstructed kidneys. Kidney tissue lysates were subjected to immunoblot analysis with antibodies against α-SMA, collagen I, fibronectin, proliferating cell nuclear antigen (PCNA) or β-actin (A and E). Representative immunoblots from 3 independent experiments are shown. The levels of α-SMA, collagen I, fibronectin and PCNA were quantified by densitometry and normalized with β-actin (B-D, and F). Values are means ± SD (n=6). Bars with different letters (a-c) are significantly different from one another (P< 0.01).

Figure 3. SRT1720 treatment enhances activation of cultured renal interstitial fibroblasts. NRK-49F cells were cultured in 2.5% FBS containing medium and incubated with different concentrations of SRT1720 (0-2 μM) for 36 hours. Then, cell lysates were prepared and subjected to immunoblot analysis with antibodies against α-SMA, fibronectin,
acetyl-H3K9 (Ac-H3K9), proliferating cell nuclear antigen (PCNA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A and E). Representative immunoblots from 3 independent experiments are shown. The levels of Ac-H3K9, α-SMA, fibronectin and PCNA were quantified by densitometry and normalized with GAPDH (B-D, and F). NRK-49F cells were treated with indicated concentration of SRT1720 for 36 hours and cells were randomly photographed in bright field (200×) and cell proliferation was measured by cell counting (G), or the MTT assay (H). Values are means ± SD of 3 independent experiments. Bars with different letters (a-d) are significantly different from one another (P< 0.01).

Figure 4. SRT1720 increases phosphorylation of EGFR and PDGFRβ in obstructed kidneys. Kidney tissue lysates were prepared and subjected to immunoblot analysis with antibodies against phospho-EGFR (Tyr1068), EGFR, phospho-PDGFRβ (Tyr751), PDGFRβ or β-actin (A). The phospho-EGFR, phospho-PDGFRβ, EGFR, PDGFRβ, and β-actin were quantified by densitometry. The phospho-EGFR level was normalized to total EGFR level (B) and phospho-PDGFRβ level was normalized with total PDGFRβ (D). The levels of EGFR and PDGFRβ were normalized with β-actin (C and E). Values are means ± SD (n=6). Bars with different letters (a-d) are significantly different from one another (P< 0.01).

Figure 5. SRT1720 enhances phosphorylation of EGFR and PDGFRβ in cultured renal interstitial fibroblasts. NRK-49F cells were cultured in 2.5% FBS containing medium and then incubated with different concentration of SRT1720 (0-2 μM) for 36 hours (A-C). Cell lysates were prepared and subjected to immunoblot analysis with antibodies for phospho-EGFR (Tyr1068), EGFR, phospho-PDGFRβ (Tyr751), PDGFRβ or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A). Representative immunoblots from 3 experiments
are shown. The phospho-EGFR, phospho-PDGFRβ, EGFR, PDGFRβ and GAPDH were quantified by densitometry. (B) The phosphorylated EGFR and PDGFRβ were normalized to total protein levels. (C) The levels of EGFR and PDGFRβ were normalized with GAPDH. Values are means ± SD of 3 independent experiments. Bars with different letters (a,b) are significantly different from one another (P< 0.01).

Figure 6. SRT1720 enhances STAT3 phosphorylation in obstructed kidneys. Kidney tissue lysates were prepared and subjected to immunoblot analysis with antibodies for phospho-STAT3 (Tyr705), STAT3, phospho-AKT (Ser473), AKT, phospho-ERK1/2, ERK1/2 or β-actin (A). The levels of phosphorylated and total proteins were quantified by densitometry. The level of phosphorylated proteins were normalized to its corresponding total protein (B, D, and F). The levels of STAT3, AKT and ERK1/2 were normalized with β-actin (C, E, and G). Values are means ± SD (n=6). Bars with different letters (a-d) are significantly different from one another (P< 0.01).

Figure 7. SRT1720 enhances STAT3 phosphorylation in cultured renal interstitial fibroblasts. NRK-49F cells were cultured in 2.5% FBS containing medium and then with different concentration of SRT1720 (0-2 μM) for 36 hours (A-D). Cell lysates were prepared and subjected to immunoblot analysis with antibodies for phospho-STAT3 (Tyr705), STAT3, phospho-AKT(Ser473), AKT, phospho-ERK1/2, ERK1/2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A). Representative immunoblots from 3 experiments are shown. The phosphorylated and total proteins were quantified by densitometry and phosphorylated protein levels were normalized to its corresponding total protein level. The levels of total STAT3, AKT and ERK1/2 were normalized with GAPDH (B-D). Values are means ± SD of
3 independent experiments. Bars with different letters (a,b) are significantly different from one another ($P < 0.01$).
Figure 3
Figure 4

A

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<tr>
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B

![Graph B: pEGFR/EGFR](image)

C

![Graph C: EGFR/β-actin](image)

D

![Graph D: pPDGFRβ/PDGFRβ](image)

E

![Graph E: PDGFRβ/β-actin](image)
Figure 5

A  SRT1720 (µM)  
0  0.5  1  2  
  pEGFR (Y1068)  
  EGFR  
  pPDGFRβ (Y751)  
  PDGFRβ  
  GAPDH  

B  
Relative abundance  
0  0.5  1  2  
SRT1720 (µM)  
  pEGFR/EGFR  
  pPDGFRβ/PDGFRβ  

C  
Relative abundance  
0  0.5  1  2  
SRT1720 (µM)  
  EGFR/GAPDH  
  PDGFRβ/GAPDH  

Legends:  
- a: significant difference from 0 µM  
- b: significant difference from 0.5 µM  

Figure 6

A

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</tbody>
</table>

B

\[
p\frac{\text{STAT3}}{\text{STAT3}}
\]

C

\[
\frac{\text{STAT3}}{\beta-\text{Actin}}
\]

D

\[
p\frac{\text{AKT}}{\text{AKT}}
\]

E

\[
\frac{\text{AKT}}{\beta-\text{Actin}}
\]

F

\[
p\frac{\text{ERK1/2}}{\text{ERK1/2}}
\]

G

\[
\frac{\text{ERK1/2}}{\beta-\text{Actin}}
\]
Figure 7

A

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<tbody>
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B

![Bar chart showing relative abundance of pSTAT3/STAT3 and STAT3/GAPDH with SRT1720 (μM) as the x-axis.](chartB.png)

C

![Bar chart showing relative abundance of pAKT/AKT and AKT/GAPDH with SRT1720 (μM) as the x-axis.](chartC.png)

D

![Bar chart showing relative abundance of pERK/ERK and ERK/GAPDH with SRT1720 (μM) as the x-axis.](chartD.png)