Sirtuin 6 deficiency transcriptionally up-regulates TGF-β signaling and induces fibrosis in mice

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Running title: SIRT6 represses SMAD3 transcription factor

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Keywords: aging, aging-associated fibrosis, SIRT6 deacetylase, TGF-β signaling, SMAD3, caloric restriction, cardiac disease, extracellular matrix (ECM)

ABSTRACT

Caloric restriction has been associated with increased life span and reduced aging-related disorders and reduces fibrosis in several diseases. Fibrosis is characterized by deposition of excess fibrous material in tissues and organs and is caused by aging, chronic stress, injury, or disease. Myofibroblasts (myoFBs) are fibroblast-like cells that secrete high levels of extracellular matrix proteins, resulting in fibrosis. Histological studies have identified many-fold increases of myoFBs in aged organs where myoFBs are constantly generated from resident tissue fibroblasts and other cell types. However, it remains unclear how aging increases the generation of myoFBs. Here, using mice models, and biochemical assays, we show that sirtuin 6 (SIRT6) deficiency plays a major role in aging-associated transformation of fibroblasts to myoFBs, resulting in tissue fibrosis. Our findings suggest that SIRT6-deficient fibroblasts transform spontaneously to myoFB through hyperactivation of transforming growth factor β (TGF-β) signaling in a cell-autonomous manner. Importantly, we noted that SIRT6 haploinsufficiency is sufficient for enhancing myoFb generation, leading to multiorgan fibrosis and cardiac dysfunction in mice during aging. Mechanistically, SIRT6 bound to and repressed the expression of key TGF-β signaling genes by deacetylating SMAD family member 3 (SMAD3) and Lys-9 and Lys-56 in histone 3 (H3K9 and H3K56, respectively). SIRT6 binding to the promoters of genes in the TGF-β signaling pathway decreased significantly with age and was accompanied by increased binding of SMAD3 to these promoters. Our findings
reveal that SIRT6 may be a potential candidate for modulating TGF-β signaling to reduce multiorgan fibrosis during aging and fibrosis-associated diseases.

INTRODUCTION

Calorie restriction has been suggested as a strategy to increase life span and reduce aging-related disorders, including heart failure (1). Interestingly, calorie restriction reduces fibrosis in several disease models (2-4). Calorie restriction is believed to activate sirtuins, a family of class III histone deacetylase proteins (5). Sirtuins remove acetyl moieties from target lysine (Lys) residues thereby regulating the function of targets including histone and non-histone proteins. There are seven sirtuin isoforms in humans which are distributed in different subcellular compartments (5). Among the seven isoforms, SIRT6 is chromatin-associated that deacetylates histone 3 at Lys9, Lys18 and Lys 56 (5-7). In addition, SIRT6 regulates the activity of proteins by post-translational modifications such as deacetylation, ADP-ribosylation and demyristoylation. SIRT6 is also known to repress transcription factors HIF-1α, NF-kB, c-Myc, and c-Jun (5,8). Interestingly, SIRT6 deficiency induces accelerated aging in mice, while overexpression extends lifespan in male mice (9,10). Recent reports indicate that SIRT6 activation prevents several aging-related diseases including metabolic diseases, cancer, and inflammation (5,11).

One of the major contributors of tissue fibrosis is activation of TGF-β signaling (12). TGF-β superfamily consists of three ligands TGF-β1, -β2 and -β3, which are synthesized as latent precursors. Activated TGF-β1 binds to membrane receptors and initiates a series of phosphorylation-dependent signaling cascades which finally culminate into activation of Smad family of transcription factors (13). Activation of TGF-β is essential for proper embryogenesis, organ morphogenesis, cell polarity and wound healing (14-16). However, chronic hyperactivation of TGF-β signaling causes aging-related fibrotic diseases (17,18). Studies indicate that aging results in activation of TGF-β signaling in the brain (19), corneal epithelium (20), heart (21), lung (22), kidney (23) and liver (24). Moreover, the mRNA levels of different regulators of the TGF-β pathway, including TGFβRI, TGFβRII, SMAD3 and SMAD4 are significantly higher in senescent fibroblasts derived from old human donors compared to those from young donors (25). Based on several experimental studies, inhibition of TGF-β signaling was suggested to be a therapeutic target for the treatment of pathologic fibrosis (26). It is worth mentioning that neutralization of TGF-β by specific antibodies was considered to be one of the strategies for curing fibrotic diseases (27-30). However, TGF-β signaling inhibitors failed in clinical trials as anti-fibrotic drugs owing to severe adverse effects probably because of crosstalk between multiple signaling pathways (18,26). Till date, there is no definite therapy available for treatment of fibrotic diseases.

Our previous findings indicate that activation of SIRT6 specifically in cardiomyocytes prevents the development of pressure overload-induced cardiac hypertrophy by repressing IGF/Akt signaling (8). Recently, we found that SIRT6 is a critical regulator of global protein synthesis (31). In this work, we investigated the role of SIRT6 in regulating TGF-β signaling and development of multi-organ fibrosis during aging using in vitro cell culture and mice models. Our data suggest that SIRT6 transcriptionally regulates TGF-β signaling pathway genes, thus functioning as
SIRT6 represses SMAD3 transcription factor
guardian to protect against adverse tissue remodeling during aging.

RESULTS
SIRT6 deficiency activates TGF-β signaling in fibroblasts
SIRT6 deficient mice develop accelerated aging and aging-associated diseases such as heart failure, metabolic syndrome, and inflammation (7). In our previous work, we observed increased fibrosis in heart of SIRT6 deficient mice (8). Since TGF-β signaling is a well-established driver of fibrosis (17,18), we tested TGF-β signaling in various organs of SIRT6−/− mice and cardiac fibroblasts isolated from SIRT6−/−mice. Western blot analysis of kidney, heart, lung and the liver tissues of SIRT6 deficient mice revealed increased phosphorylation of SMAD3, suggesting activated TGF-β signaling (Figure 1A, Supplementary Figure 1A). Further, we observed a marked upregulation of TGF-β1 and TGFβRI protein levels in these tissues (Figure 1A, Supplementary Figure 1A). To further validate our findings, we assessed the TGF-β signaling in SIRT6 deficient cardiac fibroblasts. Consistent with in vivo findings, we observed increased phosphorylation of SMAD3 and enhanced levels of TGF-β1 in SIRT6 deficient cardiac fibroblasts, indicating activation of TGF-β signaling as shown in Figure 1B. Further, to rule out any confounding effects due to genomic instability and age-related degeneration observed in SIRT6−/−mice, we transiently depleted SIRT6 using SIRT6 specific siRNA in cardiac fibroblasts isolated from wild-type mice and assessed the activation of TGF-β signaling. We observed that depletion of SIRT6 spontaneously activated TGF-β signaling, as evidenced by increased phosphorylation of SMAD3 and the enhanced expression levels of TGF-β1 and TGFβRI (Figure 1C). However, we did not see any marked changes in the expression of SMAD1 in SIRT6 depleted cardiac fibroblasts (Figure 1C).

Next, we checked whether SB-505124, a TGF-β signaling pathway inhibitor treatment reduces spontaneous activation of TGF-β signaling in SIRT6-depleted fibroblasts. SB-505124 treatment indeed significantly reduced the activity of TGF-β signaling in control and SIRT6-depleted fibroblasts as measured by the TGF-β/Activin response element (TARE) containing luciferase reporter plasmid (Figure 1D). In addition, we performed luciferase reporter assays to test the transcriptional activity of α-SMA promoter, a well-characterized fibrosis-associated gene activated by TGF-β signaling. Notably, SIRT6 depleted cells showed increased luciferase reporter activity for the α-SMA promoter, which was attenuated by SB-505124 treatment (Figure 1E). In line with this, inhibition of TGF-β signaling markedly reduced the phosphorylation of SMAD3 and the protein levels of TGF-β1 and TGFβRI levels in SIRT6-depleted fibroblasts (Figure 1F, 1G, Supplementary Figure 1B). These findings suggest that hyperactivation of TGF-β signaling might be the contributing factor for the increased protein levels of TGF-β1 and TGFβRI levels in SIRT6-deficient cells. Since SIRT6 deficient mice show enhanced TGF-β signaling due to increased TGF-β1 and TGFβRI levels, we tested serum TGF-β1 levels by Sandwich ELISA. Surprisingly, our results suggest that TGF-β1 levels were not increased in the serum of SIRT6−/−mice (Figure 1H), which could possibly be due to failure in detectable
levels of serum TGF-β1, since TGF-β1 works mostly as paracrine growth factor (32).

**SIRT6 overexpression attenuates the activation of TGF-β signaling**

Since SIRT6 deficiency spontaneously activates TGF-β signaling, we tested the effect of transient overexpression of SIRT6 on TGF-β signaling. Overexpression of SIRT6-WT, but not SIRT6-H133Y or SIRT6-S56Y, the catalytic mutants of SIRT6, attenuated TGF-β signaling (Figure 2A). Further immunostaining results suggest that SIRT6 overexpression attenuates the basal and TGF-β1-induced phosphorylation of SMAD3 in cardiac fibroblasts (Figure 2B, 2C, Supplementary Figure 1C). Consistent with our observations, TARE reporter activity was inhibited by overexpression of SIRT6. Moreover, expression of SIRT6-H133Y or SIRT6-S56Y markedly enhanced the TARE reporter activity over basal levels, indicating a dominant negative effect of these mutants. Interestingly, SB-505124 treatment reduced the enhanced TGF-β signaling in SIRT6-H133Y and SIRT6-S56Y overexpressed cells (Figure 2D). Besides, we observed similar results with the promoter activity of α-SMA (Figure 2E). These findings suggest that the catalytic activity of SIRT6 is required for the inhibition of TGF-β signaling and the hyperactivation of TGF-β signaling might be the contributing factor for the enhanced SMAD3 activity.

**Deficiency of SIRT6 transcriptionally activates TGF-β signaling and induces fibrosis in mice**

Recent studies have shown association of H3K9 acetylation with myofibroblasts differentiation and extracellular matrix accumulation in nasal polyposis (33). SIRT6 is a chromatin associated histone deacetylase known to transcriptionally regulate IGF/Akt signaling by regulating acetylation of Histone 3 at lysine 9 (8). Therefore, we hypothesized that SIRT6 could transcriptionally modulate the expression of TGF-β signaling genes. To test this hypothesis, we quantified mRNA levels of key TGF-β signaling genes by real-time qPCR in SIRT6−/− mice hearts. The mRNA levels of TGF-β1, TGF-β2, TGFβRI and TGFβRII were significantly upregulated (Figure 3A). However, we did not observe significant changes in mRNA levels of TGF-β3 and SMAD3 in SIRT6−/− mice hearts (Figure 3A). These results are consistent with increase in protein levels of TGF-β1 and TGFβRI (Figure 1A, 1B and 1C). On the other hand, mRNA levels of TGF-β1, TGFβRI and TGFβRII were significantly downregulated in SIRT6 overexpressing cardiomyocytes and fibroblasts (Figure 3B, 3C). To test whether increased protein and mRNA levels of TGF-β1 in SIRT6-depleted cells are associated with increased transcriptional activation, we performed luciferase reporter assays with the construct containing the promoter region of TGF-β1. Our results suggest that SIRT6 deficiency increased TGF-β1 promoter activity, which was significantly inhibited following treatment with SB-505124 (Figure 3D). On the other hand, transient overexpression of SIRT6, but not SIRT6-H133Y or SIRT6-S56Y repressed the activity of TGF-β1 promoter (Figure 3E). Further, overexpression of SIRT6-H133Y and SIRT6-S56Y markedly enhanced the TGF-β1 promoter activity reporter activity over basal levels, suggesting that inhibition of SIRT6 by dominant negative SIRT6-H133Y or SIRT6-S56Y activates TGF-β1 promoter activity (Figure 3E). These findings reveal that SIRT6 might transcriptionally control expression of TGF-β signaling genes.
We next tested whether SIRT6 binds to the promoters of TGF-β signaling genes by chromatin immunoprecipitation and found that SIRT6 binds significantly to TGF-β1, TGFBRI, TGFBRII and SMAD3 promoters in cardiac fibroblasts (Figure 3F). Similarly, we found significant enrichment of SIRT6 at the promoters of TGF-β signaling genes in fibroblasts (Supplementary Figure 2A). Further, for the validation of anti-SIRT6 antibody used for ChIP, we tested the occupancy of SIRT6 at the TGFβ1 gene promoter in control and SIRT6 depleted cells. We find that SIRT6 displays significant binding only in control cells, but not in SIRT6 depleted cells (Supplementary Figure 2B), indicating the specificity of the antibody. Therefore, these data suggest that SIRT6 might regulate TGF-β signaling by binding to the promoters of TGF-β signaling genes. Since SIRT6 is known to deacetylate histone 3 at Lys 9 and Lys 56 and control gene expression at chromatin level (5,6), we tested the levels of acetylated H3K9 and H3K56 on promoters of TGF-β signaling genes. We found increased acetylated H3K9 and H3K56 at the promoters of TGF-β1, TGFBRI, TGFBRII and SMAD3 genes in SIRT6 depleted cardiac fibroblasts (Figure 3G & 3H). Similarly, we observed increased H3K9 acetylation at the promoters of TGF-β signaling genes in SIRT6 depleted 293 fibroblasts (Supplementary Figure 2C). These data demonstrate that SIRT6 may repress the expression of key TGF-β signaling genes by deacetylating H3K9 and H3K56. Collectively, our findings suggest that SIRT6 transcriptionally regulates expression of key TGF-β signaling pathway genes through deacetylation of histone 3 at Lys 9 and Lys 56.

SIRT6 suppresses transcriptional activity of SMAD3 transcription factor

We performed in silico analysis of TGF-β signaling genes to find the transcription factors that could be targeted by SIRT6. We identified conserved binding sites for SMAD3 in the promoters of TGF-β1, TGF-β2, TGFBRI, TGFBRII and SMAD3, but not in TGF-β3 (Supplementary File 1). Earlier studies indicate that autocrine TGF-β/SMAD3 transcription factor signal is necessary for TGF-β1 expression in dendritic cells (34). Thus, we hypothesized that SIRT6 might regulate SMAD3 transcription factor to control TGF-β signaling genes. SMAD3 has been shown to be an acetylated protein (35) and SIRT6 deacetylates proteins such as PKM2 (36). Thus, we tested whether SMAD3 acetylation is regulated by SIRT6. We immunoprecipitated SMAD3 from SIRT6−/− mice hearts and found increased acetylation of SMAD3 (Figure 4A). In addition, SIRT6 was found to interact with both endogenous as well as ectopically overexpressed SMAD3 (Figure 4B, 4C and 4D). Further, we tested whether catalytic activity of SIRT6 plays a role in SMAD3 deacetylation. Our data suggest that SMAD3 can be deacetylated by SIRT6-WT, but not SIRT6-H133Y (Figure 4E). Further, in vitro deacetylation assay suggests that SIRT6 regulates acetylation of SMAD3 in NAD+ dependent manner (Figure 4F). Hence, SIRT6 mediated deacetylation might regulate the transcriptional activity of SMAD3. To further verify our findings, we assessed the interplay between SIRT6 and SMAD3 by using a reporter construct carrying SMAD3 binding elements (SBE). Luciferase reporter assay suggested an increase in transcriptional activity of SMAD3 in SIRT6 depleted cells, which was abrogated by SB-505124 treatment (Figure 4G). On the other hand, SIRT6, but not SIRT6-H133Y or SIRT6-S56Y, was found to attenuate the transcriptional activity of SMAD3 (Figure 4H). Interestingly,
overexpression of SIRT6-H133Y and SIRT6-S56Y significantly upregulated the transcriptional activity of SMAD3 under basal conditions (Figure 4H). These findings suggest that SIRT6 regulates the transcriptional activity of SMAD3 in a deacetylation dependent manner. To test whether SIRT6-dependent deacetylation regulates the occupancy of SMAD3 on the promoters of TGF-β1, TGF-βR1, TGF-βR1I, fibronectin1 and collagen 3a which are targets of SMAD3, we performed chromatin immunoprecipitation experiments on SIRT6-depleted cardiac fibroblasts. Our results suggest that the occupancy of SMAD3 was significantly increased on the promoters of TGF-β1, TGF-βR1, TGF-βR1I, fibronectin1 and collagen 3a in SIRT6-depleted cardiac fibroblasts (Figure 4I). Further, we also observed similar results in SIRT6-depleted 293 fibroblasts (Supplementary Figure 3). These data suggest that the deficiency of SIRT6 may promote activation of TGF-β signaling by increase in SMAD3 binding to key TGF-β signaling genes.

**SIRT6 deficiency increases expression of fibrosis-associated markers in myofibroblasts**

TGF-β signaling is involved in transformation of fibroblasts to myofibroblasts as well as expression of fibrosis-associated markers such as α-smooth muscle actin (α-SMA), collagen 1a (Col1a), collagen 3a (Col3a) and fibronectin1 (FN1) (14-16). Since, SIRT6 deficiency was found to augment the TGF-β signaling, we assessed whether SIRT6 deficiency affects fibrotic markers such as α-SMA, Col1a and FN1 in fibroblasts. Immunostaining of SIRT6-/- fibroblasts suggested increased expression of fibrotic markers (Figure 5A, Supplementary Figure 4A). Similarly, depletion of SIRT6 in cardiac fibroblasts enhanced the expression of fibrosis-associated myofibroblast markers under basal conditions, which was further augmented by the treatment with TGF-β1 (Figure 5B, Supplementary Figure 4B). These findings indicate that SIRT6 deficiency promotes spontaneous and TGF-β1 induced transformation of fibroblasts to myofibroblasts. To test whether SIRT6 overexpression could inhibit transformation of fibroblasts, we overexpressed SIRT6 in cardiac fibroblasts and analyzed the expression of fibrosis-related myofibroblast markers. Our results suggest that TGF-β1 treatment increase the expression of fibrotic markers in control, but not in SIRT6 overexpressing cardiac fibroblasts (Figure 5C, 5D, Supplementary Figure 4C). Together, these findings reveal SIRT6 to be an endogenous negative regulator of transformation of fibroblasts to myofibroblasts.

**SIRT6 deficiency induces aging-dependent multi organ fibrosis in mice**

To verify the *in vitro* results of increased expression of fibroblast differentiation markers, we assessed fibrosis in multiple organs of SIRT6-/- mice by histology. Masson’s trichrome staining suggested increased fibrosis in heart, liver, kidney and lungs of SIRT6-/- mice (Figure 6A, Supplementary Figure 5A). Further, western blotting analysis suggested increased expression of α-SMA, FN1, Col1a and Col3a in heart and lung tissues of SIRT6-/- mice (Figure 6B, Supplementary Figure 5B). Since life span of whole body SIRT6-/- mice is only 4 weeks, we further verified our findings in SIRT6 +/- mice at 2 months and 12 months age. Although, 2 months old SIRT6 +/- mice showed mild fibrosis, we observed severe fibrosis in heart, liver, kidney and lung of 12 months old SIRT6 +/- mice (Figure 6C, 6D).
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Supplementary Figure 5C). Along the same lines, we found significantly increased expression of α-SMA, FN1, Col1a and Col3a in the heart samples of SIRT6+/− mice at 12 months of age as measured by western blotting (Figure 6D, Supplementary Figure 5D). Moreover, the phosphorylation of SMAD3 and the protein levels of TGF-β1 and TGFβRI were significantly high in 12 months old SIRT6+/− mice (Figure 6D, Supplementary Figure 5D), suggesting that augmented TGF-β/SMAD3 signaling might be the contributing factor for multi-organ fibrosis found in SIRT6+/− mice. These findings were consistent with our observations on SIRT6−/− mice (Figure 1A). Overall, our findings suggest that haploinsufficiency of SIRT6 is sufficient to activate TGF-β signaling, transformation of fibroblasts and tissue fibrosis in age dependent manner.

Organ fibrosis is generally associated with impaired functioning and hence, we tested whether fibrosis observed in 12 months old SIRT6+/− mice hearts impairs cardiac function. We performed transthoracic echocardiography to assess the structure and contractile functions of heart in 12 months old SIRT6+/− mice. We found that SIRT6+/− mice hearts show increased left ventricular chamber diameter and ventricular wall thickness, indicating cardiac hypertrophy (Figure 6E and 6F). Moreover, SIRT6+/− mice hearts showed contractile dysfunction as assessed by reduction in fractional shortening (Figure 6G). These data suggest that SIRT6 deficiency augments aging associated fibrosis and causes contractile dysfunction in mice hearts.

Inhibition of SMAD3 rescues fibrosis phenotype in SIRT6+/− fibroblasts

To test whether SMAD3 is responsible for the increase in expression of fibrosis-related markers observed in SIRT6 depleted cells, we transiently depleted SMAD3 in SIRT6 depleted cells and assessed their phenotype. Our results suggest that depletion of SMAD3 markedly reduces the expression of α-SMA and fibronectin-1 in SIRT6 depleted fibroblasts (Figure 7A, 7B, 7C, Supplementary Figure 6A, Supplementary Figure 6B). To further confirm our findings, we inhibited TGF-β/SMAD3 signaling in SIRT6 depleted fibroblasts with SB-505124 and tested the phenotype. Our findings reveal that treatment of SB-505124 reduces the expression of α-SMA, FN1, and Col3a in SIRT6 depleted fibroblasts (Figure 7D, Supplementary Figure 6C). We further verified these findings with SIS3, a small molecule inhibitor of SMAD3 (37). Our findings show that SIS3 reduces expression of α-SMA and fibronectin-1 in SIRT6-KO fibroblasts as well as causes attenuation of SMAD3 activation, as reflected in its reduced binding to SMAD binding element (Figure 7E & 7F, Supplementary Figure 6D). These findings suggest that hyperactive TGF-β/SMAD3 signaling is responsible for the expression of fibrosis-related myofibroblast markers in SIRT6-depleted fibroblasts. Previous studies suggest that acetylation of SMAD3 regulates its activity (38). In line with this, our data suggests that K378R, which mimics deacetylated state of Smad3 and reduces SMAD3 activity, failed to show enhanced activity in SIRT6-KD fibroblasts (Figure 7G). This finding indicate that SIRT6 might target K378 of Smad3. However, future work is required for confirming this observation. Next, we tested whether SIRT6 reconstitution could rescue the fibrotic phenotype observed in SIRT6−/− fibroblasts. We, therefore, cultured SIRT6+/− fibroblasts
SIRT6 represses SMAD3 transcription factor and then infected them with adenovirus expressing SIRT6. We found that reconstitution of SIRT6 in SIRT6−/− fibroblasts markedly reduces the expression of α-SMA and FN1 (Figure 7H, Supplementary Figure 6E). Further, western blotting analysis revealed that reconstitution of SIRT6 into the SIRT6−/− myofibroblasts markedly reduced the phosphorylation of SMAD3 as well as the expression of TGF-β1, TGFβRI, α-SMA, FN1, and Col3a in SIRT6−/− fibroblasts (Figure 7I). Furthermore, reconstitution of SIRT6 into SIRT6−/− myofibroblasts reduced the mRNA expression of α-SMA, and FN1 (Figure 7J). These data suggest that SIRT6 is sufficient to reverse the fibrotic phenotype in SIRT6−/− myofibroblasts by attenuation of TGF-β/SMAD3 signaling.

Inhibition of TGF-β/SMAD3 signaling improves cardiac function in SIRT6−/− mice

Since we found fibrosis in 12 months old SIRT6−/− mice hearts where their cardiac structural and functional parameters were compromised, we treated SIRT6−/− mice with TGF-β/SMAD3 signaling inhibitor. SIRT6−/− mice were injected intraperitoneally with either vehicle or SB-505124 at a dose of 10mg/kg at every alternate day for 15 days. SB-505124 treatment did not significantly change the body weight, but rescued the cardiac hypertrophy observed in 12 months old SIRT6−/− mice as depicted by HW/TL, left ventricular wall thickness and left ventricular internal diameter (Figure 8A, 8B, 8C, 8D). Moreover, cardiac contractile function, as assessed by fractional shortening, improved after SB-505124 treatment in 12 months old SIRT6−/− mice (Figure 8E). Further, western blotting analysis suggested that TGF-β/SMAD3 signaling, as assessed by the phosphorylation of SMAD3 was attenuated following SB-505124 treatment in 12 months old SIRT6−/− mice. In addition, the levels of TGF-β1 and TGFβRI were reduced in heart of 12 months old SIRT6−/− mice following SB-505124 treatment (Figure 8F). These data suggest that inhibition of TGF-β/SMAD3 signaling rescues cardiac function in 12 months old SIRT6−/− mice hearts.

Reduced SIRT6 levels might cause aging-related activation of TGF-β/SMAD3 signaling

Aging activates TGF-β signaling in the brain (19), corneal epithelium (20), heart (21), lung (22), kidney (23) and liver (24). We previously found low SIRT6 levels in the tissues of heart failure patients (8). Hence, we tested whether SIRT6, which is considered to be longevity promoting sirtuin, has a role in fibrosis associated with physiological aging. Western blotting results suggest increased phosphorylation of SMAD3 and increased levels of TGFβ1 and TGFβRI in hearts of 12 months old mice as compared to 2 months old mice, while the levels of SIRT6 was markedly reduced in the 12 months old mice (Figure 9A, Supplementary Figure 7). Further, we observed that the mRNA levels of SIRT6 were also significantly reduced in 12 months old mice hearts (Figure 9B). Since SIRT6 levels were reduced in heart samples of 12 months old mice, we tested the occupancy of SIRT6 on the promoters of TGF-β/SMAD3 signaling genes in young and old mice. Our chromatin immunoprecipitation assay results suggest reduced binding of SIRT6 to the promoters of TGF-β1, TGFβRI, and TGFβRII in heart samples of 12 months old mice, when compared to 2 months old mice (Figure 9C). Since our findings suggest that SIRT6 represses SMAD3, we tested whether SMAD3 occupancy increased 12 months old mice hearts. Our results suggest increased
binding of SMAD3 to promoters of TGF-β1, TGFβRI, TGFβRII in heart tissues of 12 months old mice as compared to 2 months old mice (Figure 9D). These data indicate that the binding of SIRT6 deacetylase to TGF-β signaling pathway genes significantly reduced with age, which corresponds to the increased binding of SMAD3 to these promoters. As a result, SIRT6 deficient fibroblasts express increased levels of TGF-β1, TGFβRI and TGFβRII, and activate TGF-β signaling in a cell autonomous manner. Enhanced TGF-β signaling transforms fibroblasts to myofibroblasts and causes deposition of fibrous material in aged organs leading to organ dysfunction (Figure 9E).

DISCUSSION

Whole-body SIRT6−/− mice show features of accelerated aging and age related fibrosis (10). However, the exact mechanism of SIRT6 in the development of fibrosis is not well elucidated. Our work provides evidence that SIRT6 regulates fibrosis via TGF-β/SMAD3 signaling. We have shown that mRNA levels of TGF-β1, TGF-β2 and TGFβRI are upregulated in SIRT6-deficient hearts and SIRT6 transcriptionally regulates these genes by deacetylation of SMAD3 and Histone 3 at Lys 9 and Lys 56.

Previous studies have shown that TGF-β1 and TGFβRI activation is sufficient to induce TGF-β signaling almost in any cell type (14-16). Our findings suggest a similar phenomenon in SIRT6 deficient fibroblasts, which show increased levels of TGF-β1 and TGFβRII and transformation of fibroblasts to myofibroblasts in a cell autonomous manner. Recently, SIRT6 was shown to inhibit cardiac fibrosis by inhibiting the transcriptional activity of NF-κB (39). Furthermore, SIRT6 inhibits inflammation and fibrosis in liver by c-Jun and H3K9 deacetylation (40). Reduced SIRT6 levels leading to hyperacetylation of H3K9 and H3K56 is also implicated in early neurodegenerative events in diabetic retinopathy (41). Similarly, SIRT6 inhibits TGF-β induced epithelial senescence, a key feature associated with idiopathic pulmonary fibrosis in human bronchial epithelial cells (42). SIRT6 silencing in human dermal fibroblasts activates NF-κB signaling leading to decreased collagen 1 expression (43). In contrast, SIRT6 was found to promote TGF-β signaling in systemic sclerosis (44) and in cancer (45). SIRT6 expression along with SMAD2, SMAD5 and other contractile markers, increased when vascular smooth muscle cells were stimulated with cyclic strain (46). Thus, it is possible that SIRT6 controls TGF-β signaling as well as fibrosis in a cell and tissue specific manner.

Loss of heart elasticity is one of the major causes for contractile dysfunction in cardiovascular diseases such as hypertension, myocardial infarction, diabetic cardiomyopathy and drug-induced cardiotoxicity (47-49). It is well proven that loss of elasticity occurs due to severe interstitial fibrosis in cardiac tissue (50,51). During cardiac fibrosis, the resident cardiac fibroblasts transform into myofibroblasts and secrete several fibrosis-associated proteins such as collagen, fibronectin and laminin, resulting in remodeling of extracellular matrix (48). Although several signal transduction pathways are believed to be associated with fibroblast transformation and fibrosis, one of the well-proven mechanisms involved in fibrosis is TGF-β signaling (52). In the present study, we show that SIRT6 regulates TGF-β signaling in order to control the process of fibrosis in mice. Similarly, other sirtuin isoforms are known to modulate TGF-β signaling and fibrosis. Studies indicate that SIRT1 inhibits renal fibrosis by inhibiting TGF-β/SMAD3 signaling and resveratrol, a
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SIRT1 activator, inhibits extracellular matrix deposition by decreasing the acetylation of SMAD3 (53). Similarly, activation of SIRT1 has been shown to attenuate doxorubicin-induced cardiac fibrosis by downregulating TGF-β/SMAD3 pathway (54). On the other hand, inhibition of SIRT2 suppresses hepatic fibrosis (55). Our previous work demonstrated that SIRT3 inhibits age related tissue fibrosis (56). Similarly, SIRT5 deficiency induces cardiac fibrosis (57). In contrast, SIRT4 accelerates angiotensin II-induced cardiac fibrosis by inhibiting SIRT3 mediated deacetylation of MnSOD (58). SIRT7 deficiency downregulates TGF-β signaling and impairs wound healing (59). These findings indicate that SIRT6 may not be the only deacetylase regulating the TGF-β signaling and fibrosis. Hence, from our work, we show evidence that SIRT6 deficiency is sufficient to cause transformation of fibroblasts and fibrosis, and the mechanism is distinct from other sirtuins. Since, TGF-β signaling is a master regulator of fibrosis and warrants multilayer control and sirtuins may regulate TGF-β signaling at multiple levels to control its activation.

Earlier studies indicate that transcription factor Sp1 is essential for basal expression of TGF-β1 (60). Interestingly, we recently reported that SIRT6 deficiency upregulates the expression of Sp1 target genes (61). Moreover, AP-1 sites are found in the promoter of TGF-β1 and transcription factors c-Jun and c-Fos are required for the induction of TGF-β1 (62,63). Consistent with the previous findings, we found that SMAD3 binds to and activates the promoters of key TGF-β signaling genes such as TGF-β1, TGF-β2 and FN1 (34). Our previous work demonstrated that SIRT6 represses the activity of c-Jun (8). SMAD3 forms a complex with either c-Jun/c-Fos (64) or Sp1 (65) to increase the transcription of TGF-β signaling target genes involved in the fibrotic program. Since, SMAD3/c-Jun complex may be the functional unit, inhibition of SMAD3 might inactivate the complex and rescue fibrosis in SIRT6-deficient cells.

We show, here that SMAD3 inhibition can rescue expression of fibrosis markers in SIRT6 depleted cells. Published works suggest that SMAD3−/− mice are protected from fibrosis and suppression of SMAD3 is sufficient to attenuate the process of fibrosis (66,67). SMAD3 is an acetylated protein and p300/CBP-mediated acetylation positively regulates its transcriptional activity (35). In line with the previous findings, our work confirms that prevention of acetylation of SMAD3 at Lys 378 reduces its transcriptional activity in SIRT6 depleted cells. ERK5-induced increase in acetylation of SMAD3 augments pulmonary fibrosis (68). Studies also indicate that SIRT1 deacetylates and represses SMAD3 transcription factor to protect mice from renal and cardiac fibrosis (53,54). Our results suggest that SIRT6 deacetylates SMAD3 and H3K9 to control SMAD3 activation.

Our previous work show that SIRT6 levels reduced in heart tissues of patients with heart failure (8). Cardiac specific deletion of SIRT6 causes cardiac hypertrophy and fibrosis leading to heart failure, whereas cardiac specific overexpression of SIRT6 protects mice from hypertrophy and fibrosis (8). SIRT6−/− mice hearts exhibit increased TGF-β1 and TGFBRI receptor expression. Similarly, SIRT6 deficiency leads to increased expression of TGF-β1 in fibroblasts. Further, we noted that SIRT6 overexpression suppresses the expression of TGF-β1, TGF-βRI, and TGF-βRII in cardiomyocytes as well as fibroblasts. It is possible that SIRT6 might regulate TGF-β
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signaling in both cardiomyocytes and fibroblasts. Previous studies corroborate these findings, emphasizing the importance of TGFβ signaling in cardiomyocyte hypertrophy (69).

Our work suggests that inhibition of TGF-β signaling by specific small molecule inhibitors can reverse fibrosis markers in SIRT6 deficient fibroblasts. Upon inhibition of TGF-β signaling, the expression of TGF-β1 and TGFβRI was observed to reduce in SIRT6 depleted fibroblasts, possibly due to prevention of SMAD3 occupancy at the gene promoters of TGF-β1 and TGFβRI and thus inhibition of a feed-forward activation loop for TGF-β signaling. Our work elucidates a novel feed forward loop involving transcriptional regulation of TGF-β signaling. SIRT6 deacetylase plays a major role in antagonizing this feed forward loop in resting fibroblasts under basal conditions. However, loss of SIRT6 in fibroblasts during aging amplifies this feed forward loop to hyperactivate TGF-β signaling to favor the fibroblast transformation. However, further experiments are needed to support the hypothesis if TGF-β1 depletion or that antagonizing TGFβRI can affect SIRT6/SMAD3 association with chromatin at these gene promoters.

Our result of transcriptional upregulation of TGF-β pathway in mice model mirrors the observations noted in aged human. We found that increased TGF-β signaling is associated with reduced levels and occupancy of SIRT6 at the promoters of TGF-β signaling genes. Our earlier work revealed that SIRT6 levels were decreased in the tissue samples of heart failure patients (8). Similarly, SIRT6 levels were significantly low in human dermal fibroblasts isolated from older human subjects (70), senescent 2BS cells and in liver, spleen and kidney tissues of 18 months old BALB/c mice (71). We believe that SIRT6 plays a critical role in maintenance of tissue architecture by regulating a fine balance of fibroblasts and myofibroblasts. Loss of SIRT6 protein could be major determining factor causing multiple-organ fibrosis. We believe that SIRT6 can be a potential therapeutic target for the treatment of aging-related fibrotic diseases.

EXPERIMENTAL PROCEDURES

Animal experiments and cell culture

Wild type (WT) and SIRT6 heterozygous (SIRT6+/−) mice were purchased from the Jackson Laboratories, USA. All animal protocols were carried out with the approval of Institutional animal ethics committee of Indian Institute of Science, Bengaluru. Animal experiments were carried out as per the strict accordance with the recommendations of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The animals were housed under 12-hour light/dark cycle in individually ventilated cages in the clean air facility of Indian Institute of Science, with food and water ad libitum. Male mice were used for in-vivo experiments. Mice were sacrificed with CO₂ gas inhalation from compressed CO₂ gas cylinder. Neonatal mice pups were anaesthetized with 1-2% isoflurane. In vitro experiments were carried out in primary neonatal cardiac fibroblasts isolated from wild type and SIRT6−/− mice pups. Cardiac fibroblasts were then isolated using standard protocols (72). Third passage cardiac fibroblasts were used for experiments. The cells were cultured in high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

Animal Echocardiography
Transthoracic echocardiography was performed by Vevo 1100 Imaging System (FUJIFILM Visual Sonics Inc) as per previously published protocol (73). Briefly, 1-2% isoflurane was used for anesthetizing mice. Heated imaging platform was used to maintain the temperature of mice throughout the procedure. Chest hair were removed prior to recording. Images were captured at both long and short axis at parasternal projection. M-mode recordings were captured for both the views at midventricular level. All the echocardiography readings were taken for at least three beats from each projection. An in-built software was used for data analysis of left ventricular wall thickness, left ventricular cavity diameter and fractional shortening.

**Histology**

Age matched WT, SIRT6 +/− and SIRT6 −/− mice heart, liver, lung, muscle and kidney were collected in 10% neutral buffered formalin. Tissues were then processed with automated tissue processor (Leica, Germany). Paraffin embedded 4µm thick tissue sections were prepared and stained with Masson’s trichrome stain (Sigma) as per the protocols described in our previous publication (8).

**Chromatin Immunoprecipitation assay**

Detailed protocol for Chromatin Immunoprecipitation assay (ChIP) from young and old mice are described in our previous publication (8). Protocol for ChIP assay from 293 cells and cardiac fibroblasts were adapted from previously published work (74). SimpleChIP® Enzymatic Chromatin IP Kit (CST, Magnetic Beads) was used for this assay. HPRT gene promoter was used as the negative control. Primers used for ChIP are following: human TGF-β1 forward 5' -GARGGCACAGTGCTCAAGAG-3' , reverse 5'- AGGATGGAAGGTCAGGAG-3'; human TGF-β2 forward 5'- CATCCAGGAAACAAGCTGAG-3' , reverse 5'- TGCCAGCAGATAACATCAC-3'; human TGF-β3 forward 5'- CCGAGGTGCTGGTACCCTG-3', reverse 5'-CCAGTGAGTGAGTGAGGAG-3'; human TGFβRI forward 5'- CATAGTGAAACTTGACC-3', reverse 5'- GCTCTTCTTCTCAGAC-3'; human TGFβRII forward 5'- ATCCACCGCAGTTCAAGAGT-3', reverse 5'- GACTGTCAAGCAGCGGAGA-3'; human SMAD3 forward 5'- GCGTGTGTGAGAGTGG-3', reverse 5'- AGGTGTGGAAGCCAGAGT-3'; human HPRT forward 5'- GCCACAGGTAGTCAAGGTTT-3', reverse 5'- TCTATATATATATATATAT-3'; human TGFβRI forward 5'- CACGCAGATACCATCTACAGC-3', reverse 5'- ACCCATGAGATAATACGCTT-3'; mouse TGFβRI forward 5'- CACCCAGATAACCCTACAGC-3', reverse 5'- ACCCATGAGATAATACGCTT-3'; mouse TGFβRII forward 5'- TCTGAGCTCATTTGGGCT-3', reverse 5'- AGCGGGAGCAGTCATAGGA-3'; mouse TGFβRII forward 5'- TGAGCAGAGGCTGGGGCTC-3', reverse 5'- TCTGAGCTCATTTGGGCT-3'; mouse TGFβRII forward 5'- ATGACTTGTTCCTCTCTC-3', reverse 5'- GCTAGGAGTCTGTCCTCC-3'; mouse SMAD3 forward 5'- TGTAAGAGATGGGCCTAA-3', reverse 5'- ATGCCACGAGATTCCAGATA-3'.

**Real time qPCR**

Real time qPCR analysis was performed as described previously (8). Briefly, total RNA was isolated from tissues using Trizol reagent as per manufacturer’s instructions (Invitrogen). Corresponding cDNA was synthesized from 1µg RNA using ReverTaid first strand cDNA synthesis kit (Thermo Scientific). The real time PCR was then carried out for TGF-β1, TGF-β2, TGF-β3,
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TGFβRI, TGFβRII, SMAD3 and HPRT. GAPDH was used as the housekeeping reference gene. The primer sequences used were TGF-β1 Forward: TTGCTTCAGCTCCACAGAGA, Reverse: TGGTTGTAGAGGGCAAGGAC, TGF-β2 Forward: CCGCATCTCCTGCTAATGTG, Reverse: AATAGGCCGATCCAAAGC, TGF-β3 Forward: AAGAGAAGCAAGGGACAGA, Reverse: GCTGGATGAGGGATTATGTAC, TGFβRI Forward: TGCCATAACCGCCTACGTCA, Reverse: AATGAAAGGGCCTACTAGTGA, TGFβRII Forward: CCGCTGCATATCGTCCTGTG, Reverse: AGTGGATGGATGGTCCTATTACA, SMAD3 Forward: GAGGGGAGGTCTTTGCG, Reverse: GCTGGGATGAGGGATTATGTAC, GAPDH Forward: TATGTCGTGGAGTCTACTGGT, Reverse: GAGTTGTCATATTCTCGGT.

Western blot analysis

Western blot was performed as per previously published protocol (8). The cell lysates or tissue lysates were prepared in lysis buffer containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP40, 1% Sodium dodecyl sulfate and 0.5% sodium deoxycholate, 10mM Sodium fluoride, 2.5mM sodium pyrophosphate, 1mM sodium ortho vanadate , 1mM PMSF, 1X protease inhibitor cocktail (Roche). Protein concentration was determined by Bradford assay. Cell or tissue lysates were mixed with 2X laemml buffer (Bio-rad). Samples were mixed and boiled for 5 min at 96°C. The proteins were resolved by SDS-PAGE and western blotting performed to transfer resolved proteins onto PVDF membrane. Membrane was blocked with a solution of 5% non-fat dried milk in TBST buffer (25mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% Tween 20) for 1 hr at room temperature. Blots were probed overnight with primary antibodies at 4°C. The membrane was washed thrice with TBST and HRP-conjugated secondary antibody probed at room temperature for 1 hr. The antibodies used were p-SMAD3 (Abcam), SMAD3 (Abcam), α-SMA (Sigma), Fibronectin-1(Santa Cruz), Collagen-1a (Millipore), Collagen-3a (Santa Cruz) and SIRT6 (Cell Signaling). GAPDH (Santa Cruz), β-Actin (Cell Signaling) was used as loading control. The blots were developed with Clarity ECL western blotting substrate (BioRad) or SuperSignal West Pico chemiluminescent Substrate (ThermoScientific) in a chemiluminescence imager (Chemidoc Touch, Biorad).

Co-immunoprecipitation experiments

Immunoprecipitation assay was performed as per previously published protocol (73). Tissues were homogenized in modified RIPA lysis buffer [50mM Tris-Cl, pH 7.4 150mM NaCl, 1% Triton-X-100, 1mM EDTA, 10mM Sodium fluoride, 2.5mM sodium pyrophosphate, 1mM Na3VO4, 1mM PMSF, 1X protease inhibitor cocktail (Roche)]. Cells were transfected with pcDNA FLAG-SMAD3 plasmid vector. pcDNA-FLAG plasmid served as control. Cells were lysed, and proteins harvested in RIPA buffer. Cell or tissue homogenates were centrifuged at 12,000 rpm at 4°C for 10 min and the supernatant collected. 500-1000 µg protein was incubated with 2 µg of SMAD3 or SIRT6 or control IgG antibodies. Immune complex was incubated with protein A conjugated agarose beads for 2 hr. Beads were washed and the immunoprecipitated proteins subjected to SDS-PAGE. Western blotting was performed to detect the protein-protein interactions.
Confocal microscopy

For confocal microscopy, cells were washed twice with 1X PBS followed by fixing with 3.7% formaldehyde for 15 minutes at room temperature. Fixed cells were then washed thrice with 1X PBS and then permeabilized with 0.2% triton X-100 prepared in PBS. The cells were washed thrice with 1X PBS and blocked with 5% BSA prepared in PBST (PBS containing 1% Tween 20). Blocked cells were incubated overnight with specific primary antibodies prepared in 1% BSA in PBST at 4°C. The cells were then incubated with the respective secondary antibodies conjugated with Alexa flour 488 and/or Alexa flour 546 prepared in 1% BSA in PBST for 1 hour at room temperature. The cells were washed thrice with 1X PBS followed by incubation with Hoechst 33342 prepared in PBS for 10 minutes at room temperature for staining the nucleus. The cells were finally washed thrice with 1X PBS before mounting with Fluoromount G on a clean glass slide. Images were captured with Zeiss LSM 710 or 880 confocal microscope.

Luciferase assay

The luciferase reporter assay was performed using TGF-β1/Activin response element-luc (TARE-luc), TGF-β1 promoter-luc, SMAD binding element-luc (SBE-luc), and alpha smooth muscle actin-luc (SMA-luc)reporter plasmid constructs as described previously (56). Cardiac fibroblasts were transfected with the above plasmid constructs. Renilla luciferase was used for normalizing the luciferase signal and pGL3 vector was used as non-responding negative control reporter. Luminescence was measured using a luminometer (Pharmingen Moonlight 3010; BD Biosciences, San Jose, CA, USA).

SMAD3 deacetylation assay

Detailed protocol for deacetylation assay is adapted from our previous publication (73). Flag tagged SIRT6-WT or SIRT6-H133Y were transfected to 293 cells by lipofectamine using manufacturer’s instructions. Cells were harvested in lysis buffer, the homogenate was centrifuged at 12,000 rpm at 4°C for 10 min and the supernatant was collected in fresh micro-centrifuge tubes. Recombinant SIRT6 was purified using agarose beads conjugated to Flag antibody. Beads were washed, and the protein eluted. Recombinant SMAD3 protein overexpressed in 293 was incubated with eluted SIRT6-WT or SIRT6-H133Y protein in deacetylation buffer (250mM Tris-Cl, pH 9.0, 20mM MgCl₂, 250mM NaCl, 2.5mM DTT, 5mM NAD⁺, 2.5μM TSA) for 2h at 30°C. SMAD3 acetylation was tested by western blotting using acetyl-lysine specific antibody.

Enzyme linked immunosorbrant assay

Sandwich enzyme linked immunosorbrant assay (ELISA) was performed as per standard protocol. Briefly, 96-well ELISA plate was coated with 1 μg of TGFβ antibody (Thermo MAI-21479) overnight at room temperature in 100 μl PBS. The plate was then blocked with 0.5% gelatin in PBS for 1 hr. 1μg of antigen was added and incubated for 2hr; the plate was washed thrice with PBST followed by three washings with PBS and incubation with 1μg of TGFβ antibody (Sc-146, Santacruz) for 1 hr. Plate was again washed and incubated with 100μl HRP-conjugate rabbit anti-mouse secondary antibody (1:2000) in PBS for 45 min. The plate was washed and 100μl of substrate, 0.03 % H₂O₂ in citrate phosphate buffer, pH 5.5 and chromogen 3,3',5,5'-tetra methyl benzidine (60μg/ml) was added to detect the HRP activity. The reaction was quenched by the addition of 50μl of 1M H₂SO₄ and...
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absorbance measured at 450nm using an ELISA microplate reader.

**Statistics**

Student’s *t*-test was performed for pairwise comparison. One-way ANOVA or two-way ANOVA was used to calculate the statistical significance for multiple comparisons between experimental groups using GraphPad Prism software version 6.04.

Western blots were processed using Image-lab software (Bio-Rad). Densitometric analyses of western blots were performed by ImageJ. Confocal images processing was performed ZEN-Black software and signal quantified using ImageJ. The promoter sequences were retrieved using the Genomatix Software Suite and sequence alignment performed using Clustal Omega from EMBL.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**Funding:** N.R.S. is the recipient of Ramalingaswami Re-entry Fellowship and the Innovative Young Biotechnologist Award (IYBA) from the Department of Biotechnology, Government of India. N.R.S laboratory is supported by research funding from Department of Science and Technology (EMR/2014/000065), the Department of Biotechnology (BRB/10/1294/2014 and MED/30/1454/2014), the Council for Scientific and Industrial Research (37(1646)/15/EMR-II) and the Department of Biotechnology–Indian Institute of Science partnership program for advanced research.

J.M. is supported by the Department of Biotechnology- Research Associateship.

S.M. and M.S.K. are supported by the DST-SERB-National Post-Doctoral Fellowship (N-PDF). B.P.A. is supported by Dr.D.S.Kothari Postdoctoral fellowship (DKPDF). P.A.D. is a DST-INSPIRE faculty fellow.

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Figure legends

Figure 1 (A) Western blotting analysis of kidney, heart, lung and liver lysates from 25 days old wild type (WT) and SIRT6 knockout (SIRT6-KO) mice littermates for phosphorylated SMAD3 (p-SMAD3), SMAD3, transforming growth factor β1 (TGF-β1), TGF-β receptor 1 (TGFβR1), Sirtuin 6 (SIRT6), and β-Actin. n = 3 mice per group. Arrow pointers are used to indicate specific bands. (B) Western blotting analysis of lysates from cardiac fibroblasts, isolated from WT and SIRT6-KO littermate pups for the indicated proteins. (C) Western blotting analysis of the indicated proteins from control or SIRT6-depleted (SIRT6-KD) cardiac fibroblasts (D, E) Luciferase activity assay for TGF-β1/Activin response elements (TARE-Luciferase, D) and α-SMA-reporter Luciferase (E). Luciferase activity was measured with or without the treatment of SB-505124 (2.5μg/mL) for 24 hours, with the help of a luminometer. pGL3 basic vector was used as a negative control. n = 3. Data presented as Mean±SD, *p<0.05 One-way ANOVA was used to calculate p values. (F) Western blotting analysis to confirm knockdown of SIRT6 in cardiac fibroblasts, upon transfection with SIRT6-siRNA. (G) Representative confocal images of control or SIRT6-KD cardiac fibroblasts, with or without SB-505124 treatment (2.5 μg/mL) for 24 hours, for the indicated proteins. Scale bar - 20μm. (H) Quantitative ELISA for estimating the levels of TGF-β1 in WT or SIRT6 heterozygous (SIRT6+/−) mice at 12 months of age. n = 11 mice per group.

Figure 2. (A) Western blotting analysis of cardiac fibroblasts transfected with FLAG-control, FLAG-SIRT6 WT, FLAG-SIRT6 H133Y or FLAG-SIRT6 S56Y-SIRT6. n = 3. (B) Western blotting analysis to confirm overexpression of SIRT6 in cardiac fibroblasts, upon infection with Ad-Null or Ad-SIRT6 for 24 hours (C) Representative confocal images of cardiac fibroblasts infected with either Ad-Null or Ad-SIRT6, with or without TGF-β1 treatment (10ng/mL) for 24 hours, for the indicated proteins. Scale bar - 20 μm. (D, E) Luciferase activity assay for TGF-β1/Activin response elements (TARE-Luciferase, D) and α-SMA-reporter Luciferase reporter (E). Luciferase plasmid was transfected in cardiac fibroblasts, along with FLAG-control, FLAG-SIRT6 WT, FLAG-SIRT6 H133Y or FLAG-SIRT6 S56Y using Lipofectamine 2000 for 48 hours. Luciferase activity was
measured with or without the treatment of SB-505124 (2.5μg/mL) for 24 hours. pGL3 basic vector was used as a negative control. n = 3. Data presented as Mean±SD, *p<0.05 One-way ANOVA test was used to calculate p values.

Figure 3. (A, B & C) Scatterplot representing real time qPCR analysis of heart tissue samples from WT and SIRT6-KO mice (A), cardiomyocytes infected with Ad-Null or Ad-SIRT6 for 24 hours (B), and cardiac fibroblasts (C). GAPDH was used to normalize the mRNA levels. Data presented as Mean±SD, n = 6-7. *p<0.05 Student’s t test was used to calculate p values. (D & E) Luciferase activity assay for TGF-β promoter-Luciferase. TGF-β-Luciferase plasmid was transfected in control and SIRT6-KD cardiac fibroblasts with or without SB-505124 (2.5μg/mL) treatment (D) or after transfection with either FLAG-control or FLAG-SIRT6 WT or FLAG-SIRT6 H133Y or FLAG-SIRT6 S56Y, with or without SB-505124 treatment (E). pGL3 basic vector was used as a negative control. n = 3. Data presented as Mean±SD, *p<0.05, Student’s t test was used to calculate p values. (F) Chromatin Immunoprecipitation analysis to check for the binding of SIRT6 to the promoters of the indicated genes. Hypoxanthine phosphoribosyltransferase (HPRT) used as the negative control. n = 3-5 data presented as Mean±SD. *p<0.05, Student’s t test was used to calculate p values. (G & H) Chromatin immunoprecipitation analysis for the indicated genes in control or SIRT6-depleted (SIRT6-KD) mouse cardiac fibroblasts. Immunoprecipitation was performed with Anti-H3 specific and Anti-acetylated H3K9 and H3K56 antibodies. Acetylated H3K9 and H3K56 levels shown relative to total H3 levels. HPRT used as the negative control. n = 3-5. Data presented as Mean±SD. *p<0.05, Student’s t test was used to calculate p values.

Figure 4. (A) Smad3 was immunoprecipitated to assess its acetylation status in heart samples of 25 days old WT and SIRT6-KO littermate mice using anti-SMAD3 antibody. Western blotting was performed to detect SMAD3 acetylation using anti-Ac-Lysine antibody. Whole cell lysates (WCL) were probed for SIRT6 and Actin by western blotting. (B) Co-immunoprecipitation analysis to assess the interaction between SIRT6 and SMAD3 in heart tissue lysates of wild type mice. Western blotting was performed for SMAD3 and SIRT6. IgG was used as the control. Whole cell lysates (WCL) were probed for Actin. (C) Co-immunoprecipitation analysis to assess the interaction between SIRT6 and SMAD3 in heart tissue lysates of wild type mice. Western blotting was performed for SMAD3 and SIRT6 using specific antibodies. IgG was used as the control. Whole cell lysates (WCL) were probed for indicated proteins. (D) Assessment of the interaction in vitro between SIRT6 and SMAD3 in FLAG-control or FLAG-SMAD3 plasmid transfected cells.
IgG was used as the control. Whole cell lysates (WCL) were probed for SMAD3, SIRT6 and Actin by western blotting. (E) Western blotting analysis showing changes in the acetylation levels of SMAD3 upon SIRT6 overexpression. Cells were transfected with FLAG-SIRT6 H133Y (catalytically inactive mutant) or FLAG-SIRT6 (wild type). SMAD3 was immunoprecipitated from the cell lysate using anti-SMAD3 antibody. Western blotting was performed to detect SMAD3 acetylation using anti-Ac-Lysine antibody. Whole cell lysates (WCL) were probed for SIRT6 and Actin by western blotting. (F) In vitro deacetylation assay showing SIRT6 as SMAD3 deacetylase. SMAD3 was overexpressed by transfection of the plasmid pcDNA3-HA-SMAD3. SMAD3 was immunoprecipitated and then incubated with FLAG-tagged SIRT6 or FLAG-SIRT6-H133Y. Deacetylation reaction was carried out either in the presence or absence of NAD⁺ in HDAC buffer. BSA was used as control. Anti-Ac-Lysine antibody was used to analyze SMAD3 acetylation. (G & H) Luciferase activity assay for SMAD binding element (SBE-Luciferase). SBE-Luciferase plasmid was transfected in control and SIRT6-KD cardiac fibroblasts, with or without SB-505124 (2.5μg/mL) treatment (G) or after transfection with either FLAG-control or FLAG-SIRT6 WT or FLAG-SIRT6 H133Y or FLAG-SIRT6 S56Y, with or without SB-505124 treatment (H). pGL3 basic vector was used as a negative control. n = 3. Data presented as Mean±SD, *p<0.05, One-way ANOVA test was used to calculate p values. (I) Chromatin immunoprecipitation analysis of SMAD3 binding to the promoters of the indicated genes in cardiac fibroblasts. HPRT used as the negative control. n = 4; data presented as Mean±SD. *p<0.05, Student’s t test was used to calculate the p values.

**Figure 5.** (A) Representative confocal images of cardiac fibroblasts isolated from WT or SIRT6-KO littermate mice pups, for α-smooth muscle actin (α-SMA), Collagen 1a (COL1A), Fibronectin 1 (FN1), and Sirtuin 6 (SIRT6). Scale bar - 20μm. n = 3. (B) Representative confocal images of control or SIRT6-KD cardiac fibroblasts, with or without TGF-β1 treatment (10ng/mL) for 24 hours. Scale bar - 50μm. n = 3. (C) Representative confocal images of cardiac fibroblasts infected with Ad-Null or Ad-SIRT6, with or without TGF-β1 treatment (10ng/mL) for 24 hours. Scale bar - 50μm. n = 3. (D) Western blotting analysis of cardiac fibroblasts infected with Ad-Null or Ad-SIRT6 for 24 hours, with or without TGF-β1 treatment (10ng/mL) for 24 hours.

**Figure 6.** (A& C) Histology of heart, liver, kidney and lung tissue samples from 25 days old WT and SIRT6-KO littermate mice (A), and 2 months and 12 months old WT and SIRT6⁺⁻ mice (C) showing tissue fibrosis. Scale bar - 50μm. n = 3. (B) Western blotting analysis of heart and lung lysates from 25 days old WT and SIRT6-KO mice littermates for the indicated proteins. n = 3. (D) Western blotting analysis of heart lysates from 12 months old WT and SIRT6⁺⁻ mice littermates...
SIRT6 represses SMAD3 transcription factor

for the indicated proteins. n = 4 mice per group. (E, F & G) Scatterplot showing the left ventricular internal diameter (LVID), left ventricular posterior wall thickness and cardiac contractile functions, as measured by fractional shortening of 12 months old WT and SIRT6+/− mice. n = 5; data presented as Mean±SD, *p<0.05 Student’s t test was used to calculate the p values.

**Figure 7.** (A & B) Representative confocal images of control, SIRT6-depleted (SIRT6-KD), SMAD3-depleted (SMAD3-KD) or both SIRT6 and SMAD3-depleted (SIRT6-KD+SMAD3-KD) cardiac fibroblasts for α-SMA and FN1. Scale bar - 20μm. n = 3. (C) Western blot analysis of control, SIRT6-KD, SMAD3-KD or SIRT6-KD+SMAD3-KD cardiac fibroblasts for the indicated proteins. (D) Representative confocal images of control or SIRT6-KD cardiac fibroblasts, with or without SB-505124 treatment (2.5μg/mL). Scale bar - 20μm. n = 3. (E) Representative confocal images of control or SIS3 treated cardiac fibroblasts isolated from SIRT6-KO mice pups. Scale bar - 20μm. n = 3. (F & G) Scatterplots showing luciferase activity assay for SMAD binding element (SBE-Luciferase). SBE-Luciferase plasmid was transfected in control and SIRT6-KD cardiac fibroblasts, with or without SIS3 treatment. (F) or after transfection with either pcDNA or pcDNA-SMAD3 WT or pcDNA-SMAD3 K378R (G). (H) Representative confocal images of cardiac fibroblasts infected with Ad-Null or Ad- SIRT6 for the indicated proteins. Scale bar for α-SMA - 50μm. Scale bar for FN1 - 20μm. n = 3. (I) Western blotting analysis of cardiac fibroblasts isolated from WT or SIRT6-KO littermate mice pups, infected with Ad-Null or Ad-SIRT6 for the indicated proteins. (J) Scatter plot representing real time qPCR analysis of α-SMA and FN1 from fibroblasts isolated from SIRT6-KO mice and then infected with either control or SIRT6 overexpressing adenovirus. GAPDH was used to normalize the mRNA levels. Data presented as Mean±SD, n = 5; *p<0.05, Student’s t test was used to calculate the p values.

**Figure 8.** (A - E) Scatter plot depicting body weight, heart weight to tibia length ratio (HW/TL), left ventricular posterior wall thickness, left ventricular internal diameter (LVID) and cardiac contractile functions respectively of WT and SIRT6+/− mice, before and after the treatment with SB-505124. SB-505124 was injected intraperitoneally at a concentration of 10mg/kg every alternate day for 15 days. Sterile peanut oil was used as the vehicle. n = 5. *p<0.05. One-way ANOVA test was used to calculate the p values. (F) Western blotting analysis of heart lysates from WT and SIRT6+/− mice, before and after the treatment with SB-505124 (10mg/kg).

**Figure 9.** (A) Western blotting analysis of heart lysates from 2- and 12-months old WT mice for the indicated proteins. n = 4. (B) Scatter plot representing real time qPCR analysis of SIRT6 in
heart samples of 2 months and 12 months old mice. GAPDH was used to normalize the mRNA levels. Data presented as Mean ±SD, n = 4; *p<0.05. Student's t test was used to calculate the p values. (C&D) Chromatin Immunoprecipitation analysis of heart tissue samples to check for the binding of SMAD3 and SIRT6 to the promoters of the indicated genes in 2 months and 12 months old mice. HPRT is used as the negative control. n = 3–7 mice per group, data presented as Mean ±SD. *p<0.05 Student’s t test was used to calculate the p values. (E) Representative model depicting the findings of the study. Reduction in levels of SIRT6 with aging results in increased binding of SMAD3 to TGF-β signalling genes, hence leading to enhanced transformation of fibroblasts to myofibroblasts and development of multi-organ fibrosis. SIRT6 acts as a transcriptional repressor of TGF-β/SMAD signalling to regulate the aging-related fibrosis
Figure 1
Figure 2
Figure 3
Figure 4

G. SBE-Luciferase Assay

H. SBE-Luciferase Assay

I. SBE-Luciferase Assay
Figure 5
Figure 6

A

Heart
Liver
Kidney
Lung

WT
SIRT6−/−

B

Heart
Lung

WT
SIRT6−/−

α-SMA
FN1
COL1A
COL3A
SIRT6
β-Actin

KDa
50
37
250
150
100
50
37
25
10

D

WT
SIRT6+/−

p-SMAD3
SMAD3
TGF-β1
TGFβR1
α-SMA
FN1
COL1A
COL3A
SIRT6
GAPDH

KDa
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C

Heart
Liver
Kidney
Lung

2 months
12 months

WT
SIRT6+/−

E

LV/ID (mm)

WT
SIRT6−/−

F

Wall thickness (mm)

WT
SIRT6−/−

G

% FS

WT
SIRT6−/−

*
Figure 7
Figure 8
Sirtuin 6 deficiency transcriptionally up-regulates TGF-β signaling and induces fibrosis in mice
Sangeeta Maity, Jaseer Muhamed, Mohsen Sarikhani, Shweta Kumar, Faiz Ahamed, Kondapalli Mrudula Spurthi, Venkatraman Ravi, Aditi Jain, Danish Khan, Bangalore Prabhshankar Arathi, Perumal Arumugam Desingu and Nagalingam R. Sundaresan

*J. Biol. Chem.* published online November 19, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.007212

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