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Simvastatin reduces TGF- β 1-induced SMAD2/3-dependent human ventricular fibroblasts differentiation: role of protein phosphatase activation

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Abstract

Background: Excessive cardiac fibrosis due to maladaptive remodeling leads to progression of cardiac dysfunction and is modulated by TGF- β 1-activated intracellular phospho-SMAD signaling effectors and transcription regulators. SMAD2/3 phosphorylation, regulated by protein-phosphatases, has been studied in different cell types, but its role in human ventricular fibroblasts (hVFs) is not defined as a target to reduce cytokine-mediated excessive fibrotic response and adverse cardiac remodeling. Statins are a class of drugs reported to reduce cardiac fibrosis, although underlying mechanisms are not completely understood. We aimed to assess whether simvastatin-mediated reduction in TGF- β 1-augmented profibrotic response involves reduction in phospho-SMAD2/3 owing to activation of protein-phosphatase in hVFs.

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All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation

AUTHOR CONTRIBUTIONS

F.R., conception and design of research; F.R., R.S., A.D., L.E., G.R., E.H., and P.H. performed experiments; F.R. and A.J. analyzed data; F.R., G.R., E.H., and A.J. interpreted results of experiments; F.R., R.S., A.D. and P.H. prepared figures; F.R. drafted the manuscript; A.J., A.J.T., G.R., E.H. and L.E. edited and revised the manuscript; all authors approved the final version of the manuscript.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

Methods and results: Cultures of hVFs were used. Effect of simvastatin on TGF- β 1-treated hVF proliferation, cytotoxicity, myofibroblast differentiation/activation, profibrotic gene expression and protein-phosphatase activity was assessed. Simvastatin (1 μ M) reduced effect of TGF- β 1 (5 ng/mL) on hVF proliferation, myofibroblast differentiation (reduced α -smooth muscle actin [α -SMA-expression]) and activation (decreased procollagen-peptide release). Simvastatin also reduced TGF- β 1-stimulated time-dependent increases in SMAD2/3 phosphorylation and nuclear translocation, mediated through catalytic activation of protein-phosphatases PPM1A and PP2A, which physically interact with SMAD2/3, thereby promoting their dephosphorylation. Effect of simvastatin on TGF- β 1-induced fibroblast activation was annulled by okadaic acid, an inhibitor of protein-phosphatase.

Conclusions: This proof-of-concept study using an *in vitro* experimental cell culture model identifies the protective role of simvastatin against TGF- β 1-induced hVF transformation into activated myofibroblasts through activation of protein phosphatase, a novel target that can be therapeutically modulated to curb excessive cardiac fibrosis associated with maladaptive cardiac remodeling.

Keywords

fibrosis; human ventricular fibroblasts; phospho-SMAD2/3; protein phosphatases; statins; heart

1. Introduction

Cardiac fibroblasts constitute about two-thirds of the total cell number in the human myocardium, and are involved in the maintenance of normal cardiac structure, function, and extracellular matrix (ECM) homeostasis and in the regulation of structural integrity and scar formation following myocardial injury [1,2]. Excessive cardiac fibrosis plays an important role in promoting heart failure (HF) and cardiac dysrhythmias, such as atrial fibrillation (AF), by altering the contractility and conductivity of the myocardium, with deposition of ECM proteins replacing cardiac tissue with fibrous scar [3–5]. In response to cardiac injury, resident cardiac fibroblasts, under the influence of chemotactic factors and cytokines such as transforming growth factor beta 1 (TGF- β 1), migrate to the site of the injury, proliferate, and transform into secretory and contractile myofibroblasts characterized by the polymerization of α -smooth muscle actin (α -SMA) contractile fibers; this modification is associated with the synthesis, secretion, and deposition of collagen and ECM proteins involved in cardiac remodeling [1–3,6,7]. These profibrotic processes are modulated by phosphorylation of receptor-regulated R-SMADs [8], an important step in intracellular TGF- β 1 canonical pathway signaling in inducing profibrotic genes, and further regulated by dephosphorylation by serine-threonine protein phosphatase (PP), Mg²⁺/Mn²⁺ 1A (PPM1A)/PP2C and protein phosphatase 2A (PP2A) [9–14]. Protein phosphatases interfere with TGF- β 1-SMAD signaling and have been demonstrated to play a role in idiopathic pulmonary fibrosis and in murine cardiac fibrosis [15–18]. Although anomalies in the expression of PP2A and PPM1A phosphatase activity and related post-translational modifications in intracellular TGF- β 1 signaling have been shown to contribute to cardiac remodeling associated with arrhythmias, AF, HF, and cardiac hypertrophy [14,15,17,19,20], the role of protein phosphatases in human cardiac fibroblasts has not been fully investigated.

Statins — 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors — are a class of cholesterol-lowering drugs that also reduce the incidence of postoperative AF, left ventricular dysfunction, and maladaptive tissue remodeling with their pleiotropic effects independent of cholesterol-lowering [21–24]. Statins recently were demonstrated to dephosphorylate signaling proteins by activating PP2A or PPM1A in extra-cardiac liver cell lines and neurons [25–28]. Because simvastatin prevents SMAD-phosphorylation-mediated fibrosis [29], activates protein phosphatases [28], and differentially affects cardiac atrial versus ventricular fibroblasts [21], we sought to investigate the effect of simvastatin on TGF- β 1 signaling in human ventricular fibroblasts (hVFs) and assess the role of protein phosphatase in regulating hVF activation. We hypothesize that the protective effect of simvastatin against TGF- β 1-induced hVF transformation into activated myofibroblasts and profibrotic transcriptional changes with collagen production involve the activation of protein phosphatase, which can serve as a novel therapeutic target to reduce cardiac fibrosis.

2. Methods and Materials

An expanded Materials and Methods section is available in the Online Data Supplement.

All protocols in this study were performed in compliance with institutional guidelines for human research and approved by the Aurora Health Care Institutional Review Board (IRB Protocol #12–03E).

2.1 In vitro culture of hVF

TGF- β 1-dependent proliferation, differentiation, and activation were studied on commercially available cultured hVFs extracted from the hearts of trauma victims free of cardiac disease, obtained from Lonza, ScienCell Research Laboratories, or Cell Applications, Inc., as previously described [21,30]. Information about age, gender, and absence of cardiac diseases and other comorbidities, such as diabetes, was provided by the vendor. Additionally, primary hVF lines from 5 different patients with advanced HF (New York Heart Association class III to IV) who underwent either cardiac transplantation or left ventricular assist device implantation at Aurora St. Luke's Medical Center (Milwaukee, USA) also were used.

2.2 Statistical analysis

Statistical analysis was performed using Sigma Plot or SAS. Data are presented as mean $\pm$ SEM and values were compared using Student's t-test, or analysis of variance (ANOVA) or covariance (ANCOVA) where it was deemed necessary. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 Simvastatin reduced TGF- β 1-induced proliferation of hVFs

To assess the effect of simvastatin on TGF- β 1-induced increases in hVF proliferation, cells were treated with TGF- β 1 for 96 hours with or without simvastatin and the rate of proliferation was determined by cell count and compared with an untreated control (Figure

1A, 1B). Treatment with TGF- β 1 (5 ng/mL) resulted in a significant ($P=0.002$) increase in proliferation ($\Delta=96.6\%$) compared with the control and a 2.33-fold higher count (87500 ± 2887) from the baseline 0 hour count (37530 ± 1119); simvastatin (1 μ M) significantly ($P=0.003$) reduced the TGF- β 1-stimulated proliferation rate ($\Delta=47.5\%$ vs 96.6%), with only a 1.7-fold increase (64167 ± 2205) from the baseline count compared to a 2.33-fold increase by TGF- β 1, a 60% increase in fibroblast proliferation (Figure 1C). The effect of simvastatin in reducing TGF- β 1-induced hVF proliferation was most prominent in the initial 24 hours (Figures 1B, 1D). The overall decrease in total hVF number by simvastatin treatment was not due to cellular toxicity, as determined by cell viability assay with or without simvastatin in the presence of TGF- β 1 (Figures 1E, 1F). No cytotoxicity was detected across the concentration range of 0.3 μ M-10 μ M simvastatin for 48 hours, and there was no significant difference in relative fluorescence intensity of propidium iodide-positive cells over this period (Figure 1F).

3.2 Simvastatin inhibits differentiation of hVFs

Differentiated and undifferentiated populations of hVFs characterized by the presence or absence of α -SMA actin fibers were measured by flow cytometry (Figure 2A). Treatment with TGF- β 1 (5 ng/mL) increased the differentiated (α -SMA positive) cell population from 27.4% to 75.4% ($P<0.01$) as illustrated in the second quadrant of Figure 2A and in 2B. The transformation of hVFs to myofibroblasts assessed by an increase in intracellular α -SMA expression was further confirmed by confocal microscopy. TGF- β 1 treatment of cultured hVFs for 72 hours resulted in an increase in the relative proportion of cells expressing α -SMA along with vimentin from 3.14% in control untreated cells to 48% in treated cells ($P<0.01$) (Figures 2C, 2D). TGF- β 1 induced a morphological change in hVFs from thin, spindle-shaped cells to a plump phenotype with large, bulging nuclei and increased cytoplasmic smooth muscle actin fibers compared with their absence in cytokine-untreated cells. TGF- β 1 significantly increased the α -SMA mRNA expression level by 3.4-fold ($P<0.01$; Figure 2E) in 24 hours and protein levels by 84% ($P=0.03$, Figure 2F) in 48 hours. Treatment with simvastatin (1 μ M) for 72 hours in the presence of TGF- β 1 decreased myofibroblast differentiation, and the cell population mostly consisted of a mixture of ramified structures and spindle-shaped cells with relatively low proportion of myofibroblasts expressing α -SMA that declined to 11% from 47% (Figure 2C, 2D). Simvastatin prevented increase in the expression of α -SMA mRNA that remained close to the control levels at 24 hours ($P<0.01$) (Figure 2E) without an increase in protein levels ($P=0.012$) by 48 hours (Figures 2F, 2G).

3.3 Effect of simvastatin on TGF- β 1-induced ECM protein secretion from activated myofibroblasts

The cardiac ECM, predominantly composed of collagen types I and III, provides tensile strength and maintains the shape and size of the heart. Both fibrillar collagen types I and III synthesized by cardiac myofibroblasts are released in the form of procollagen and, following pro-peptide cleavage, are deposited as collagen types I and III in the ECM. TGF- β 1-mediated increase in activation of myofibroblasts was associated with a 2.42-fold increase in *COL1A1* level compared with the control ($P<0.01$) and was inhibited by simvastatin with suppression of *COL1A1* level to 68% ($P<0.01$) in the presence of TGF- β 1 (Figure 2H). To

estimate markers of collagen types I and III syntheses by hVFs after treatment with TGF- β 1, PICP, and PIIINP, the release of peptide cleavage products in the media after 48 hours of treatment was measured by ELISA. TGF- β 1 increased the PICP level from 520 ± 43.4 ng/ μ L to 946 ± 69.9 ng/ μ L (81% increase; $P < 0.01$), and this was inhibited by simvastatin, with a reduction of the level in the media to 324 ± 45.8 ng/ μ L (65% decrease; $P < 0.01$) (Figure 2I). Likewise, the level of PIIINP increased from 585 ± 55.7 pg/ μ L to 856 ± 27.1 pg/ μ L (31%; $P < 0.01$) in the presence of TGF- β 1 but was inhibited by simvastatin, with a decrease in the PIIINP level to 146 ± 15.8 pg/ μ L, a reduction of 82% ($P < 0.01$) from the TGF- β 1-stimulated level (Figure 2J).

3.4 Simvastatin dephosphorylates TGF- β 1-induced phosphorylated SMAD2/3

Phosphorylation of SMADs by the activated TGF- β 1 receptor complex is an essential event in initiation of TGF- β 1 signal transduction; as a result, SMADs family members are oligomerized and SMAD2/3 is translocated into the nucleus to regulate expression of profibrotic genes involved in differentiation and activation of myofibroblasts. SMAD2 and SMAD3 are known to shuttle between cytoplasm and the nucleus in response to stimulatory signals [31]; therefore, the effect of simvastatin on TGF- β 1-stimulated increase in fluorescence intensity and translocation of phospho-SMAD2/3 into the nuclei of hVFs was determined. Simvastatin (1 μ M) treatment prevented TGF- β 1-mediated increased immunofluorescence and nuclear translocation of phospho-SMAD2/3 within 2 hours as revealed by reduced nuclear immunofluorescence with decreased localization of phospho-SMAD2/3 immunofluorescence signal, mostly confined within the cytoplasm (Figure 3A) and significant reduction of percentage population of phospho-SMAD2/3 positive pink nuclei (Figure 3B). A time-dependent increase in phosphorylation of SMAD2 ($n=3$, $P < 0.001$) and SMAD3 ($n=3$, $P < 0.001$) in hVFs was observed within 30 minutes of stimulation by TGF- β 1 (5 ng/mL) and remained elevated at 2 hours (Figure 3C). Simvastatin reduced time-dependent reduction in the Western blot intensities of phosphorylated SMADs (pSMADs) at 2 hours ([pSMAD2, TGF- β 1 vs. TGF- β 1+Sim; $P < 0.05$]; [pSMAD3, TGF- β 1 vs. TGF- β 1+Sim; $P < 0.05$]), without a significant effect on immediate phosphorylation after TGF- β 1 treatment (0.5 and 1 hour, Figure 3C). To determine whether the effect of simvastatin on phosphorylation of SMAD2/3 was mediated through inhibition of a TGF- β receptor or post-receptor event, SD-208, a specific inhibitor of TGF- β R1 kinase, was used to assess the effect on early and delayed phosphorylation. TGF- β 1-stimulated early phosphorylation of SMAD2/3 at 0.5 and 1 hour was not reduced by simvastatin, which reduced phospho-SMAD2/3 only later, at 2 hours, compared with SD-208 (100 nM), which inhibited TGF- β 1-stimulated SMAD 2/3 phosphorylation at all time points (0.5, 1 and 2 hours), ($n=3$; $P < 0.001$) (Figures 3C, 3D), suggesting an effect of simvastatin on SMAD2/3 dephosphorylation rather than TGF- β 1 receptor-mediated inhibition of SMAD phosphorylation.

3.5 Simvastatin-induced SMAD dephosphorylation by protein phosphatase is facilitated by PP2A and PPM1A interaction with SMAD2/3

Protein phosphorylation signaling pathways are dynamically regulated by systematic activation of kinases and phosphatases and are reported to involve PP2A/PPM1A as part of TGF- β 1-dependent phospho-SMAD signaling [13,21]. We reasoned that attenuation of

phospho-SMAD2/3 by simvastatin could be due to mediation of protein phosphatases, as suggested by the results in Figures 3C and 3D. Therefore, the association between SMAD2/3 and PP2A and PPM1A in hVFs in the absence and presence of TGF- β 1 with or without simvastatin treatment was determined on cellular lysates using an immunoprecipitation reaction with SMAD2/3 antibody immunoblotted with PP2A and PPM1A antibodies. Co-immunoprecipitation studies revealed that both PPM1A and PP2A physically interact with SMAD2/3, and the interaction appeared to be endogenous. No changes in the signal intensities of PP2A and/or PPM1A interaction with SMAD2/3 were observed in the lysates from TGF- β 1 or TGF- β 1 with simvastatin treated hVFs for 2 hours compared with non-treated controls (Figure 4A, top). Because no differences in the interactions were revealed by CoIP, we assessed whether simvastatin influenced the catalytic activity of PPM1A and PP2A for SMAD dephosphorylation. Cellular lysates treated with TGF- β 1 with or without simvastatin in the presence or absence of PP2A- and PPM1A-phosphatase-specific inhibitor okadaic acid (OA) (10 nM) were incubated with PP2A and PPM1A phosphopeptide substrate for 3 hours, and the amount of released phosphate was measured. The phosphatase activity increased in the lysates obtained from TGF- β 1 and simvastatin-treated cells compared with simvastatin-untreated cells (8.6 ± 0.12 vs 6.7 ± 0.52 pmol phosphate released/min/ μ g protein, respectively, $P < 0.05$) and was significantly inhibited by OA ($P < 0.01$, Figure 4A, bottom). No significant change in the enzymatic activity as determined by phosphate release from phosphopeptide substrate was seen in the hVF lysates treated with TGF- β 1 or unstimulated cells treated with simvastatin alone in comparison with the control, yet, the activity was significantly reduced by OA, ($P < 0.01$, Figure 4A, bottom). This indicates the presence of a low level of constitutively activated phosphatase activity (in TGF treated or untreated cells) that was not affected by the tested concentration of simvastatin but inhibited by OA.

3.6 Pharmacological inhibition of protein phosphatases prevents SMAD2/3 dephosphorylation and restores simvastatin-reduced expression of α -SMA and COL1A1 in the presence of TGF- β 1

Next, we assessed whether the pharmacological inhibition of protein phosphatase can prevent SMAD dephosphorylation induced by simvastatin and release simvastatin inhibition of hVF differentiation and activation by determining the expression of α -SMA and COL1A1 proteins. We pretreated the hVFs with OA (10 nM) for 3 hours before introducing TGF- β 1 (5 ng) and simvastatin (1 μ M) for another 2 hours to study SMAD2/3 phosphorylation and for 48 hours to assess α -SMA and COL1A1 protein expression. Western blotting revealed that treatment with protein phosphatase inhibitor prevented the simvastatin-induced dephosphorylation of SMAD2/3 by maintaining the sustained phosphorylation and preventing the reduction in pSMAD2/3 protein levels at 2 hours (Figure 4B), which was associated with maintained levels of α -SMA and COL1A1 protein expression after 48 hours in TGF- β 1 with simvastatin-treated hVF lysates (Figure 4C). Thus, OA restored the protein levels of TGF- β 1-induced α -SMA and COL1A1 that had been reduced by simvastatin.

3.7 Protein phosphatase enzymatic activity is decreased in failing heart hVFs

We previously found that HF in humans is associated with increased ventricular fibrosis due to the increased capacity of fibroblasts for collagen secretion [32]; therefore, we assessed

whether this was also related to decreased protein phosphatase activity in hVFs from patients with advanced HF. We measured the levels of protein-phosphatase activity in the lysates of cultured hVFs obtained from patients with a failing heart undergoing left ventricular assist device implantation or cardiac transplantation who have a high collagen deposition to myocardial ratio (13.32 ± 0.59 ; $n=5$), indicative of excessive fibrosis in their ventricular tissue as determined by mason trichrome staining. We found a significant reduction of protein phosphatase enzymatic activity ($P=0.037$) (Figure 4D) with enhanced collagen secretion ($P=0.04$) in those failing hearts' hVFs compared with hVFs obtained from non-failing, disease-free control hearts (Figure 4E).

4. Discussion

In this study, we show that simvastatin decreased TGF- β 1-stimulated proliferation of hVFs, SMADs phosphorylation, myofibroblast differentiation (α -SMA expression and fiber deposition) and activation (COL1A1 protein expression and release of procollagen PICP and PIIINP). This inhibitory effect of simvastatin was not mediated through inhibition of the TGF- β 1 receptor but downstream activation of phosphatases (PP2A and PP2C/PPM1A) that reduced SMAD phosphorylation, differentiation, and collagen synthesis and release, indicating the role of these protein phosphatases in regulating fibrosis in the human heart. This was supported by demonstrating a reduction in phosphatase activity in hVFs from patients with advanced HF associated with enhanced collagen synthesis. These data highlight the role of PP2A and PP2C/PPM1A protein phosphatases as important players in regulating myocardial fibrosis and therefore, identify them as potential therapeutic targets that can help prevent adverse cardiac remodeling and its consequences on cardiac mechanical and electrical dysfunction associated with aging and aging-associated cardiac fibrosis.

Data for hVFs in our study indicate a proliferative response to TGF- β 1, in accordance with findings reported for fibroblasts from various tissues and species [33–37]. Using antibodies specific to PP2A and PPM1A, we demonstrated that protein phosphatase (PP2A and PP2C/PPM1A) physically interacts with SMAD2/3 endogenously with or without TGF- β 1 stimulation, which is also in agreement with earlier studies that demonstrated the ability of protein phosphatase to interact with SMADs [14,15]. We speculate this interaction might be facilitating the dephosphorylation of SMAD2/3 by a simvastatin-induced increase in the catalytic activity of protein phosphatase in the presence of TGF- β 1. This is supported by the observation that protein phosphatase inhibitor OA countered simvastatin-mediated inhibition of TGF- β 1 effect on α -SMA and COL1A1 expression by preventing dephosphorylation with sustained elevation of phospho-SMAD2/3. This is in agreement with the report in lung fibroblasts in which already-inhibited TGF- β 1 downstream signaling for α -SMA induction was restored by PP2A/PP1inhibitors [18]. Our data suggest that simvastatin's effect is mediated, at least in part, by increasing protein phosphatase activity in the presence of TGF- β 1, promoting dephosphorylation of phospho-SMAD2/3, and thereby aids in countering the influence of TGF- β 1 on hVF activation. The clinical significance of these findings is supported by our observation that hVFs from patients with advanced HF that exhibit a profibrotic state demonstrated a significantly reduced level of protein phosphatase enzymatic

activity associated with increased collagen synthesis compared with hVFs from non-diseased hearts (Figures 4D, 4E).

Fibroblast differentiation into myofibroblasts and secretion of ECM proteins are part of the essential reparative process after myocardial injury to prevent infarct expansion and ventricular dysfunction as part of the normal physiological wound healing program aimed at restoring the mechano-protective function of ECM in the heart [2,32,38]. However, during aging or pathophysiological processes, persistent activation of resident fibroblasts in infarcted or non-infarcted myocardium may contribute to excessive or patchy fibrosis that could lead to maladaptive ventricular remodeling over time, increasing predisposition to myocardial mechanical and electrical dysfunction and progressive development of the substrate for HF and arrhythmogenesis [5,32, 38,39]. Our data demonstrate that exposure to TGF- β 1 promoted hVF proliferation and transformation to activated phenotype with increased collagen I and III production, reflected by increased release of pro-collagen peptides in the media. These processes can be inhibited by countering the effect of TGF- β 1-mediated phosphorylation of SMAD signaling through activation of specific phosphatases. Phosphorylated SMAD2/3 in cytoplasm binds to SMAD4 and transports into the nucleus to promote transcription of genes involved in cardiac remodeling and fibrosis [18,40]. TGF- β 1-induced phosphorylation of SMAD2/3 in our study was observed within 30 minutes of treatment and remained elevated at least for the next 2 hours (Figures 3A–3C), fostering translocation of phospho-SMAD2/3 into the nucleus (Figure 3A) and increasing the transcription and expression of α -SMA (Figure 2C), reflecting differentiation of hVFs to myofibroblasts after TGF- β 1 treatment (Figure 2A) [38]. The observation that phosphorylation of SMAD2/3 was reduced by simvastatin by a mechanism different from TGF- β R1 kinase inhibitor SD-208 (Figures 2C, 2F) has potential therapeutic significance. Several mechanisms of receding phosphorylation of SMADS have been proposed, including 1) TGF- β receptor internalization and degradation, ubiquitination, and proteasome-mediated degradation of SMAD2/3 or 2) catalytic activity of serine/threonine phosphatases switching off intracellular SMAD signaling [12,14,31]. Our data indicate that increase in the catalytic activity of serine/threonine phosphatases and dephosphorylation of phospho-SMAD2/3 might be involved in the statin-mediated reduction in hVF differentiation and activation.

Cardiovascular remodeling is a complex process involving a cascade of events tightly regulated by the balancing act of kinase and phosphatase activities [2,19]. PPM1A/PP2C and PP2A, belonging to the serine/threonine family of phosphatases, consist of scaffolding, catalytic, and regulatory subunits with coinciding functions [14,40,41]. These phosphatases endogenously bind to serine/threonine sites of SMAD2/3, for which the catalytic activity is not necessary [14,15,40] but can be activated and regulated by statins in dephosphorylating serine/threonine sites [12,15,25,27,28,42]. Protein phosphatase modulation of TGF- β 1/R-SMAD signaling may play a critical role in regulating a variety of cellular processes involved in regulation of the cardiac remodeling and fibrosis associated with myocardial injury and HF; however, mechanisms that support this paradigm in human cardiac fibroblasts remain undefined [16,18,19,27,43,44]. Therefore, our data in hVFs demonstrating that PP2A and PPM1A proteins physically interacted with SMAD2/3 (Figure 4A, top) and that protein phosphatase enzymatic activity could be modulated by simvastatin in the presence of TGF- β 1 (Figure 4A, bottom) are of therapeutic interest. The precise

mechanism underlying the increased phosphatase activity by simvastatin in the presence of TGF- β 1-treated hVFs promoting phospho-SMAD2/3 dephosphorylation is unclear and needs further investigation. In addition, the proof-of-concept data reported here using an *in vitro* experimental approach indicate involvement of PP2A and PPM1A phosphatases [13,14,40] in the fibrotic pathway, which is consistent with recently published reports [13–15,17,18] that identify this novel pathway as another target for modifying pathological processes. However, further investigation and *in vivo* molecular characterization using targeted approaches in established models of cardiac fibrosis [45,46] are needed to confirm definitive involvement of specific phosphatases in the pathological ECM remodeling in causing fibrosis associated with cardiac dysfunction before the full potential clinical relevance can be assessed.

5. CONCLUSIONS

The molecular basis for excessive fibrosis predisposing to HF and enhanced arrhythmogenesis is only partially understood [21,22,47–52]. Improving insights into mechanisms underlying adverse cardiac remodeling after injury or aging-associated cardiac fibrosis predisposing to progressive myocardial dysfunction and diseases could help in finding novel therapeutic targets [5,47–49,53]. This is important because existing therapies, although promising, have demonstrated only limited efficacy in halting the fibrosis process and molecules that specifically target ameliorating maladaptive cardiac remodeling and dedifferentiation of myofibroblasts to naïve phenotype are not yet available or safe for clinical use [54,55]. Therefore, the quest to discover novel antifibrotic targets and agents that are effective and safe continues.

Protein phosphatases are gaining increased attention for their likely role in HF; however, most studies have focused on their effects on cardiac myocytes [17,19,20]. Our study, to our knowledge, is the first in hVFs from non-diseased and failing hearts to investigate the role of protein phosphatases in cardiac remodeling and, therefore, could have therapeutic potential. Identifying protein phosphatases within fibroblasts as a target for manipulating the SMAD-dependent TGF pathway to prevent pathological ECM remodeling for limiting excessive fibrosis is a novel approach, but because protein phosphatases, like protein kinases, have multiple substrates and control several pathways critical in cell cycle and growth, their safety and specificity need further investigation in *in vivo* models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Fibroblast (hVF) PP2A/PPM1A role in ECM-remodel-linked cardiac dysfunction is unknown
- Data show simvastatin's pleiotropic role in hVF to myoFB differentiation/activation
- Simvastatin and TGF β 1 increase phosphatase activity and dephosphorylate SMAD2/3
- Protein phosphatase in hVF to control SMAD-dependent pathological ECM remodeling is novel
- Targeting phosphatase may have potential to prevent excessive cardiac fibrosis

Fig. 1.

Simvastatin reduced TGF- β 1-induced proliferation of human ventricular fibroblasts. (A) Phase contrast images of human ventricular fibroblasts (control, TGF- β 1 [5 ng/mL] and TGF- β 1+simvastatin [1 μ M-treated]). (B-D) TGF- β 1 (5 ng/mL in 2.5% fetal bovine serum-containing media)-induced proliferation (cell count by hemacytometer) compared with control showed a significant decrease by 1 μ M simvastatin compared with TGF- β 1 in 24 to 96 hours (Mean (N=3) $\pm$ S.E.M; * P < 0.01). (E) Immunofluorescent images of live/dead cell staining based on 4 mM calcein-AM and 2 mM ethidium homodimer-I (Eth-D1) nuclear staining following 1 μ M simvastatin treatment showed inhibition in proliferation, but no cell death was observed. (F) Assessment of cytotoxicity assay by intercalating propidium iodide assay. Propidium iodide will bind to nucleic acid of the dying cells. Cells grown in cultures treated with 0–10 μ M simvastatin did not show significant cytotoxicity of human ventricular fibroblasts in all the groups. PI, propidium iodide; Sim, simvastatin; TGF, transforming growth factor beta 1.

Fig. 2.

Simvastatin inhibits differentiation of human ventricular fibroblasts and suppressed TGF-β1 stimulated collagen synthesis and release. (A) Flow cytometry analysis of undifferentiated and differentiated human ventricular fibroblasts (hVFs) characterized by the absence or presence of α-SMA fibers. hVFs were stained with anti-α-SMA/ Alexa Fluor 594 and anti-vimentin/ Alexa Fluor488 are shown in the histogram. (B) The bar graph show mean ± standard error of the mean analyzed by Student's t test is a representative of three separate experiments with similar results. (C) Human ventricular fibroblast to myofibroblast differentiation determined by confocal microscopy using ICC: TGF-β1 treatment for 72 hours increased the transformation of human ventricular fibroblasts (vimentin/green) to myofibroblasts (αSMA/red), simvastatin reduced the TGF-β1 effect. (D) Data are presented as mean ± standard error of the mean. Bar graphs representing the relative proportion of the fibroblast and myofibroblast population from 5 fields (N=3; [*P<0.01 compared with

control, # $P<0.001$ compared with TGF- $\beta 1$). (E) Data are presented as mean $\pm$ standard error of the mean. Bar graphs representing α -smooth muscle actin (α -SMA) steady state mRNA pool determined by quantitative polymerase chain reactions (N=3; * $P<0.01$). (F) α -SMA protein determined by Western blotting. (G) Ratio of signal intensities of α -SMA and GAPDH immunoblot (N=3; † $P<0.05$). (H) COL1A1 protein expression determined by Western blotting. (I) PICP and (J) PIIINP release in media determined by ELISA after 48 hours of treatment with TGF- $\beta 1$ and simvastatin. Data are presented as the mean $\pm$ standard error of the mean of triplicate samples, each assayed in duplicate representative of 3 independent experiments, analyzed by Student's t test. α -SMA, alpha smooth muscle actin; Ctrl, control; DAPI, 4', 6-diamidino-2-phenylindole; FB, fibroblasts; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; myoFB, myofibroblasts; PICP, procollagen type 1 C-terminal peptide; PIIINP, type III N-terminal peptide; Sim, simvastatin; TGF, transforming growth factor beta 1.

Fig. 3.

Effect of simvastatin on TGF- β 1 induced SMAD2/3 phosphorylation and phospho-SMAD nuclear translocation. (A) Nuclear translocation of phospho-SMAD2/3 (pink nuclei) determined by immunocytochemistry using immunofluorescence microscopy prevented by simvastatin (blue nuclei), (B) Bar graph representing percent red/pink nuclei out of total blue nuclei as the mean $\pm$ standard error of the mean of 5 fields, assayed in triplicate representative of 2 independent experiments (* P <0.05). (C-D) Western blot analysis of time-dependent TGF- β 1-induced increased in phosphorylation of SMAD2/3 (P <0.001) reduced by simvastatin only at 2 hours' time point compared with TGF receptor kinase inhibitor SD-208; TGF vs TGF+Sim groups P <0.05 for pSMAD3 and P <0.001 for pSMAD2. The data were expressed as mean $\pm$ SEM from three independent experiments and analyzed by two-way ANOVA. *** P <0.001, * P <0.05 significant compared to control group; ### P <0.001, # P <0.05, significant compared to TGF- β 1 treated group. GAPDH, glyceraldehyde-3-

phosphate-dehydrogenase; pSMAD, phosphorylated SMAD; Sim, simvastatin; TGF, transforming growth factor beta 1.

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Fig. 4.

The okadaic acid-inhibitable protein phosphatase activity associated with simvastatin in SMAD2/3 dephosphorylation restored simvastatin-mitigated TGF- β 1-induced SMAD2/3 phosphorylation and reduced COL1A1 and α -SMA expression. (A, top) Co-immunoprecipitation study on hVF lysate from 2h of TGF- and simvastatin-treatment revealed the presence of physical association between SMAD2/3, PP2A, and PPM1A. (A, bottom) OA-inhibitable phosphatase activity measured in the presence and absence of simvastatin and TGF- β 1 stimulation for 3 hours. Phosphatase activity was calculated as the amount of phosphate/min released from a synthetic phosphothreonine peptide. Data presented as mean $\pm$ standard error of the mean, analyzed by Student's t test. (B) Western blot analysis showed restoration of SMAD2/3 phosphorylation by protein phosphatase inhibitor OA (10 nM). (C) Protein phosphatase inhibitor OA prevented the action of simvastatin on TGF- β 1-induced increased α -SMA and COL1A1 protein expression in human ventricular fibroblast lysates from various treatments. (D) Phosphatase activity was calculated as the amount of phosphate/min released from a synthetic phosphothreonine peptide. Box plot graph displays the distribution of values of phosphatase enzymatic activity in the lysates isolated from the hVFs of normal disease-free and failing heart ventricles with excessive fibrosis. (E) Collagen secretion and deposition measured by soluble collagen assay

in hVFs from disease-free and failing hearts after a 48-hour culture. Box plot graph displays the distribution of values of collagen secreted/deposited for each group. Outlier outside the extended whisker is denoted by a dot. Boxes extend from the 25th to 75th percentiles. Data (A-C) presented as mean $\pm$ standard error of the mean. A value of $P < 0.05$ was considered significant, analyzed by Student's t test. α -SMA, alpha smooth muscle actin; Con, control; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; hVF, human ventricular fibroblast; OA, okadaic acid; pSMAD, phosphorylated SMAD; Sim, simvastatin; TGF, transforming growth factor beta 1.