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Research Article

Down-regulation of miR-15a/b accelerates fibrotic remodelling in the Type 2 diabetic human and mouse heart

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Aim: Myocardial fibrosis is a well-established cause of increased myocardial stiffness and subsequent diastolic dysfunction in the diabetic heart. The molecular regulators that drive the process of fibrotic events in the diabetic heart are still unknown. We determined the role of the microRNA (miR)-15 family in fibrotic remodelling of the diabetic heart.
Methods and results: Right atrial appendage (RAA) and left ventricular (LV) biopsy tissues collected from diabetic and non-diabetic (ND) patients undergoing coronary artery bypass graft surgery showed significant down-regulation of miR-15a and -15b. This was associated with marked up-regulation of pro-fibrotic transforming growth factor-β receptor-1 (TGFβR1) and connective tissue growth factor (CTGF), direct targets for miR-15a/b and pro-senescence p53 protein. Interestingly, down-regulation of miR-15a/b preceded the development of diastolic dysfunction and fibrosis in Type 2 diabetic mouse heart. Therapeutic restoration of miR-15a and -15b in HL-1 cardiomyocytes reduced the activation of pro-fibrotic TGFβR1 and CTGF, and the pro-senescence p53 protein expression, confirming a causal regulation of these fibrotic and senescence mediators by miR-15a/b. Moreover, conditioned medium (CM) collected from cardiomyocytes treated with miR-15a/b markedly diminished the differentiation of diabetic human cardiac fibroblasts.
Conclusion: Our results provide first evidence that early down-regulation of miR-15a/b activates fibrotic signalling in diabetic heart, and hence could be a potential target for the treatment/prevention of diabetes-induced fibrotic remodelling of the heart.

Introduction

Cardiac fibrosis, a hallmark of cardiac remodelling is a pathological condition resulting from excess accumulation of extracellular matrix (ECM) components in the interstitial and perivascular regions of the heart [1,2]. Excessive accumulation of ECM components and hence fibrosis is indeed multifactorial, which collectively increases the stiffness of the heart and eventually impairs cardiac contractility, and can increase the propensity for re-entry arrhythmias [3]. Diabetes leads to progressive loss of cardiomyocytes. Due to the limited regenerative capacity of the adult heart, dead cardiomyocytes are replaced with collagen-based scar, leading to extensive fibrotic remodelling in the diabetic heart [4,5]. We recently demonstrated a large increase in collagen deposition (60%) accompanied by impaired relaxation in the human diabetic myocardium of patients with diastolic dysfunction [6], a defect possibly due to the increased fibrosis [7]. However, the molecular regulators that drive the process of fibrotic events in diabetic myocardium are yet unknown.

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MicroRNAs (miRs) are the key molecular regulators responsible for several physiological and pathological processes. The overall significance of miRs in myocardial fibrosis was first shown by Martins et al. [8], who demonstrated that conditional deletion of Dicer, an enzyme that regulates miR biogenesis, in the mouse myocardium resulted in the development of cardiomyocyte hypertrophy and profound ventricular fibrosis. In support of this Tijsen et al. [9], recently showed a causal role of the miR-15 family, particularly miR-15b in regulating interstitial fibrosis in murine models of hypertension and pressure overloaded hypertrophy. Recently, Wang et al. [10] demonstrated the anti-inflammatory role for miR-15a in diabetic retinopathy. The miR-15 family consists of six highly conserved miRs (miR-15a, miR-15b, miR-16, miR-195, miR-497 and miR-322), which are abundantly expressed in cardiomyocytes [9,11,12]. However, knowledge on the role of miR-15 in the development of fibrosis in Type 2 diabetes is missing, which is important to be able to therapeutically target the fibrosis, one of the most common features of the diabetic heart.

In the present study, we demonstrate for the first time marked dysregulation of both miR-15a and -15b in Type 2 human diabetic myocardium. Moreover, using a mouse model of Type 2 diabetes, we confirmed that dysregulation of both the miRs precedes the activation of pro-fibrogenic mediators and the development of structural changes in the heart. Finally, we also demonstrate that *in vitro* normalization of miR-15a and -15b in adult mouse cardiomyocytes reduced high glucose (HG)-induced activation of pro-fibrotic and pro-senescent gene expression.

Materials and methods

A detailed description of the experimental procedure and statistical analysis is provided in the electronic supplementary file.

Ethics

The human myocardium study was approved by the Health and Disability Ethics Committee of New Zealand and the Dunedin microRNA study was approved by the Human Ethics Committee at University of Otago, New Zealand. All the patients provided written consent for collection and use of samples in the present study. The animal study was approved by the Animal Ethics Committee at University of Otago, New Zealand.

Human myocardial tissue collection

Right atrial appendage (RAA) and epicardial left ventricular (LV) biopsies were collected from Type 2 diabetic (D) and non-diabetic (ND) patients (n=8 each) undergoing on-pump coronary artery bypass graft surgery for ischaemic heart disease (IHD). In addition, to confirm the changes in miR-15 expression observed in the present study were specifically due to diabetes, we also collected RAA tissue samples from ND patients with mitral regurgitation, preserved ejection fraction, but without IHD as established by the presence of normal coronary arteries and no evidence of LV functional abnormalities who were undergoing mitral valve replacement (ND-H, n=5). The ND-H samples were used as the reference control. Tissue samples were snap-frozen and stored at -80°C immediately after collection for molecular analysis.

Human plasma sample collection

Peripheral blood samples were collected from individuals with Type 2 diabetes without any known history of cardiovascular diseases to determine the circulating level of miR-15a/b. Age- and gender-matched ND healthy volunteers served as controls (n=10, Supplementary Table S1). Samples were centrifuged at 2000 rpm to separate the plasma. Plasma was stored in -80°C until further analysis.

Animal model of Type 2 diabetes

To determine the role of miR-15a and -15b in fibrotic remodelling of diabetic heart Type 2 diabetic (BKS.Cg-m + / + Leprdb/J) mice (db/db) and their ND (C57BL/ksJ-lepr +) littermates (db/ +) were used at seven different time points [8, 12, 16, 20, 24, 28 and 32 weeks (W) of age, n=9–10 each following echocardiography to measure the diastolic function] [13]. Age-matched lean animals served as controls.

Molecular analyses

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted and RT-PCR was carried out as described by us earlier [14].

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Western blot analysis

Western blots were performed on protein extracted from human and mouse tissue and cells to determine the expression levels of p53 (Cell signaling, U.S.A.), sirtuin 1 (SIRT1) (Cell signaling, U.S.A.), transforming growth factor-β receptor-1 (TGFβR), connective tissue growth factor (CTGF) (both from Abcam, Australia) and SMAD2 (SantaCruz Biotechnologies, U.S.A.) [15–17].

Picrosirius Red staining for fibrosis

Five microns thick cryosections were stained with 1% Picrosirius Red (see ESM methods for details) [16].

In vitro cell culture

HL-1 cardiomyocytes

HL-1 mouse adult cardiomyocytes were a kind gift from Professor William Claycomb (Louisiana State University Medical Center). Cells were cultured as per the original protocol [18].

Isolation of human primary cardiac fibroblasts

The human primary cardiac fibroblasts were obtained by outgrowth from RAA biopsy samples collected from non-diabetic (ND-IHD) and diabetic (D-IHD) patients [19].

High glucose treatment

For the experiments, cells (3×10^5/well in six-well plates or 1×10^4/well in 96-well plates) were exposed to high D-glucose (HG, 30 mM) [20,21] or D-mannitol [normal glucose (NG), 30 mM, used as osmotic control] for 24 h.

miR mimic transfection

HL-1 cardiomyocytes were transfected with miR-15a/b mimics/scrambled sequence (Scr) using Lipofectamine RNAiMAX (ThermoFisher Scientific, NZ), according to the manufacturer's instructions. At the end of 48 h post-transfection, conditioned medium (CM) was collected, centrifuged at 1000 rpm to remove cell debris and stored at $-80\,^{\circ}$C until used for experiments on fibroblasts. Further, samples were collected for RT-PCR analysis to confirm the overexpression of miRs and Western blot analysis to determine the effect of miR-15a/b overexpression on pro-fibrotic proteins as above [16].

Differentiation of fibroblasts

For differentiation assay, 1×10^4 fibroblasts were plated in eight-chamber slides. After 24 h, cells were supplemented with CM collected as above at a ratio of 1:1 with normal growth medium. Simultaneously cells were treated with angiotensin II (100 nM) to induce differentiation. Twenty four hours later, the cells were fixed with freshly prepared 4% PFA and stained with α-smooth muscle actin (α-SMA) to identify the differentiated cells. Images were captured using a fluorescence microscope (Olympus) and quantified using ImageJ software (NIH, U.S.A.) [22].

Statistical analysis

Data are presented as mean $\pm$ SEM. Comparisons between the groups were made using analysis of variance (ANOVA) followed by Holm-Sidak's test for multiple comparisons. Unpaired *t*-test was used to compare two groups. Correlations between various variables were done using Pearson′s correlation equations. A probability value (*P* value) less than 0.05 was considered statistically significant.

Results

miR-15a and miR-15b as potential contributing factors in fibrotic remodelling of diabetic myocardium

Our first aim was to determine if diabetes affects the expression of miR-15a/b in the human heart. As shown in Figure 1, the expression of miR-15a/b was reduced in the ND-IHD patients compared with the ND-H patients, indicating that ischaemia alone significantly reduced the expression of miR-15a/b. Interestingly, the expression of miR-15a/b was further reduced in the samples from the D-IHD patients compared with ND-IHD, indicating that diabetes further down-regulated miR-15a and -15b in both atria and the ventricle (Figure 1).

Next, we used a Type 2 diabetic mouse model to determine the exact time point for the dysregulation of miR-15a/b expression during the progression of disease. Of note, these mice developed diastolic dysfunction (the functional

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Table 1 Diastolic function measured as E/A ratio in Type 2 diabetic mice during the evolution of the disease

DecT, deceleration time; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with ND of corresponding age group; n=9–10 in each group.

	Measurements					
	E/A ratio		E/e' ratio		DecT (ms)	
Age (weeks)	ND	D	ND	D	ND	D
8	1.8 ± 0.25	1.77 ± 0.13	8.2 ± 0.33	9.1 ± 1.10	28.94 ± 1.27	29.14 ± 1.67
12	1.79 ± 0.12	1.67 ± 0.17	8.91 ± 0.2	9.89 ± 0.37	30.61 ± 3.36	30.21 ± 4.04
16	1.82 ± 0.36	1.62 ± 0.41	9.4 ± 0.18	12.2 ± 1.88	27.31 ± 2.38	33.56 ± 3.11
20	1.75 ± 0.19	1.38 ± 0.20****	9.1 ± 0.16	18.5 ± 3.14*	28.08 ± 3.96	35.22 ± 1.46**
24	1.65 ± 0.22	1.70 ± 0.26	11.1 ± 1.20	17.32 ± 1.81**	27.06 ± 2.31	30.76 ± 3.25
28	1.85 ± 0.22	2.88 ± 0.33****	10.08 ± 0.73	20.08 ± 1.74***	31.76 ± 0.61	23.26 ± 0.82****
32	1.68 ± 0.15	2.78 ± 0.33****	10.77 ± 1.32	17.72 ± 0.92***	30.24 ± 0.79	20.53 ± 0.28****

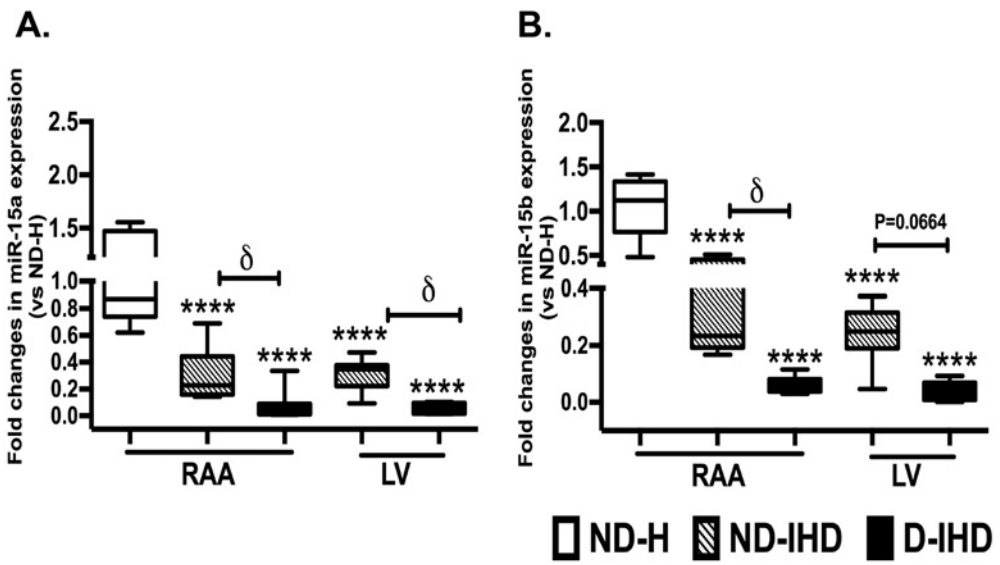

Figure 1. Diabetes down-regulates miR-15a/b expression in diabetic human heart

Box plots showing differential expression of miR-15a (**A**) and miR-15b (**B**) by quantitative RT-PCR analysis in the RAA and LV regions of the cardiac tissue samples collected from D-IHD ($n = 8$), age-matched ND-IHD, ($n = 8$) patients. ND-H was used as ND and non-ischaemic control ($n = 5$). Data were expressed as mean ± SEM. Whiskers indicate 90th and 10th percentiles. $^{****}P < 0.0001$ compared with ND-H and $^{\delta}P < 0.05$ compared with corresponding ND-IHD group, by two-way ANOVA with Bonferroni's post test.

indicator of fibrosis) from 20 weeks of age. This was evidenced by a decrease in E/A ratio (mitral valve inflow pattern) and an increase in LV filling pressure (increased E/e') in diabetic mice at 20 weeks of age that changes to pseudonormalization at 24 weeks followed by a restrictive filling pattern at 28 and 32 weeks of age (Table 1 and Supplementary Figure S1) [23]. RT-PCR analysis showed a significant down-regulation of miR-15a, starting from 12 weeks of age in the diabetic heart compared with age-matched ND heart (Figure 2A). Importantly, there was a significant down-regulation of miR-15b as early as 8 weeks of age in diabetic myocardium (Figure 2B), which continued to decrease with the progression of diastolic dysfunction (Table 1). Importantly, regression analysis until 20 weeks of age in diabetic animals showed significant negative correlation between miR-15a/b and the development of LV diastolic dysfunction (E/A ratio, miR-15a: $r^2 = 0.7118$, $P = 0.0001$, Figure 2C and miR-15b: $r^2 = 0.5188$, $P = 0.01$, Figure 2D). The correlation data were plotted until only 20 weeks of age because the E/A ratio changes to pseudonormal and restrictive patterns from 24 weeks of age in diabetic heart.

Interestingly, the first significant increase in fibrosis (both perivascular and interstitial fibrosis) was observed at only 20 weeks of age in diabetic myocardium (Figure 3C and E) and thereafter at all time points of the study ($P < 0.0001$ compared with ND counterparts, Figure 3E). Using polarized light microscopy, we also observed an accumulation

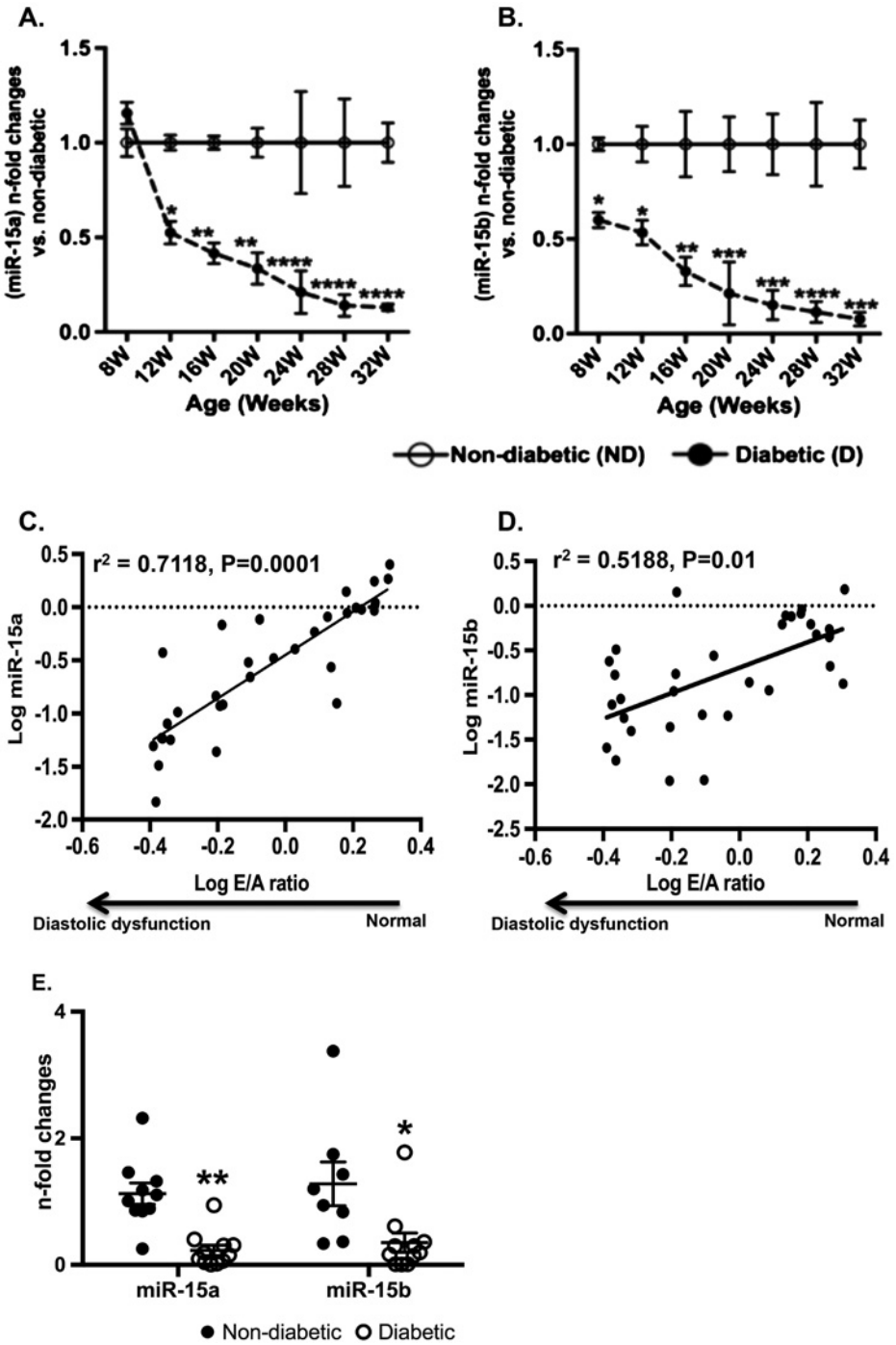

Figure 2. Diabetes induces early down-regulation of miR-15a/b

(**A** and **B**) Quantitative line graphs showing the myocardial expression level of miR-15a (A) and miR-15b (B) at different time points. Data were expressed as mean ± SEM. One-way ANOVA was used for comparison between groups, followed by Holm-Sidak's test for multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with age-matched ND control (n=10 each group). (**C** and **D**) Scatter plots of E/A ratio and miR-15a (C) or miR-15b (D) in diabetic heart irrespective of age. The correlation data were plotted only up to 20 weeks of age because the E/A ratio changes to pseudonormal and restrictive patterns from 24 weeks of age. (**E**) Scatter plots showing the expression pattern of miR-15a and miR-15b in plasma samples collected from Type 2 diabetic and age- and gender-matched ND volunteers without any known history of cardiovascular disease. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with corresponding ND group, by two-way ANOVA with Bonferroni's post test; n = at least 9 in each group.

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of thick collagen fibres (collagen I, which appeared as yellowish orange fibres) that are responsible for increased myocardial stiffness [24] from 20 weeks of age in diabetic myocardium (Figure 3A–D and Supplementary Figure S2). Further, regression analysis showed a significant negative correlation between both miRs and the development of fibrosis in mouse heart irrespective of the age and duration of diabetes (miR-15a: $r^2 = 0.4103$, $P < 0.0001$, Figure 3F and miR-15b: $r^2 = 0.3304$, $P = 0.0002$, Figure 3G).

These observations were striking as the changes in expression of miR-15a/b started much before (Figure 2) the structural changes could be detected, suggesting that the early modulation of miR-15a and miR-15b at molecular level might provide the foundation for the onset of fibrotic remodelling at the structural level. This was further supported by significant down-regulation of circulating levels of miR-15a and miR-15b in plasma samples collected from diabetic volunteers without any known history of cardiovascular disease (Figure 2E, $P < 0.05$), suggesting the potential to use miR-15a/b as a non-invasive biomarker to identify the fibrotic status of the heart, although more detailed studies are required to confirm this notion.

Down-regulation of miR-15a and miR-15b activates the pro-fibrogenic proteins to promote cardiac fibrosis

Next to explore the consequences of down-regulation in miR-15a/b expression in diabetic myocardium, we measured the expression of pro-fibrotic TGFβR1 and CTGF, known protein targets for miR-15a/b [9]. Western blotting analyses of human heart tissue revealed a significant up-regulation of TGFβR1 (Figure 4A) and CTGF (Figure 4B) in ND-IHD patients, an effect that was severely exaggerated in samples from D-IHD patients, and corresponded with the down-regulation of both miR-15a/b (Figure 1). Further, we also confirmed the activation of TGFβ signalling pathway by measuring the expression of one of its key downstream targets SMAD2 [25], which was significantly up-regulated in diabetic heart (Figure 4C). In mice, significant increases in TGFβR1 (Figure 4D), CTGF (Figure 4E) and SMAD2 (Figure 4F) protein expression ($P < 0.0001$ compared with ND at all time points, Figure 4D–F) were observed as early as 12 weeks of age (8 weeks for TGFβR1), and this remained consistently high until 28 weeks of age. Regression analysis showed a significant negative correlation between miR-15a and CTGF and miR-15b and TGFβR1, although there was no such correlation observed between miR-15a and TGFβR1 or between miR-15b and CTGF, suggesting that miR-15a regulates CTGF and miR-15b regulates TGFβR1 (Figure 4G–J).

In vitro modulation of miR-15a and miR-15b alleviates fibrogenic signalling in HG-treated HL-1 cardiomyocytes

Interstitial fibroblasts respond to signals released from dying cardiomyocytes and synthesize new matrix components aimed at replacing the damaged cells [26]. This was confirmed in our study, where HG treatment of cardiomyocytes for 24 h showed a marked increase in both TGFβR1 (Supplementary Figure S3A) and CTGF expressions (Supplementary Figure S3B). Importantly, HG markedly reduced the expression of both miR-15a and -15b in the cardiomyocytes (Figure 5A and B). To determine if therapeutic restoration of miR-15a and -15b in cultured cardiomyocytes is sufficient to attenuate the activation of fibrotic mediators under HG, cardiomyocytes were transiently transfected with either miR-15a/b mimic or Scr after exposing them to HG stress for 24 h. The efficacy of transfection was verified by increased levels of miR15-a/b following overexpression using quantitative real-time PCR (Figure 5A and B). While HG-treated cells transfected with the Scr showed comparable changes to that of HG treatment (Figure 5C–H), the miR-15a/b mimic markedly attenuated the up-regulation of TGFβR1 (Figure 5C and F), CTGF (Figure 5D and G) and SMAD2 (Figure 5E and H). Together, the results of *in vitro* experiments support the concept that miR-15a and miR-15b are the upstream regulators of fibrotic remodelling in diabetic heart by regulating the expression of TGFβR1 and CTGF in cardiomyocytes.

Secreted miR-15a inhibits differentiation of myofibroblasts from human diabetic heart

Secreted miRs represent a new mode of cell-to-cell communications. In order to understand if cardiomyocytes secrete miR-15a and -15b, we measured the expression levels of both the miRs in the growth medium CM collected from cardiomyocytes. miR-15a was highly secreted into the CM, while the expression level of miR-15b was barely detected under basal conditions (Figure 6A). HG treatment markedly reduced the secretion of miR-15a, whereas miR-15b was undetectable (Figure 6A). Transfection of cardiomyocytes with miR-15a mimic induced (206 ± 26)-fold and (110 ± 33)-fold increases in secretion of miR-15a in NG- and HG-treated cells respectively (Figure 6A). In contrast, miR-15b mimic markedly increased the expression of secreted miR-15b only in NG-treated

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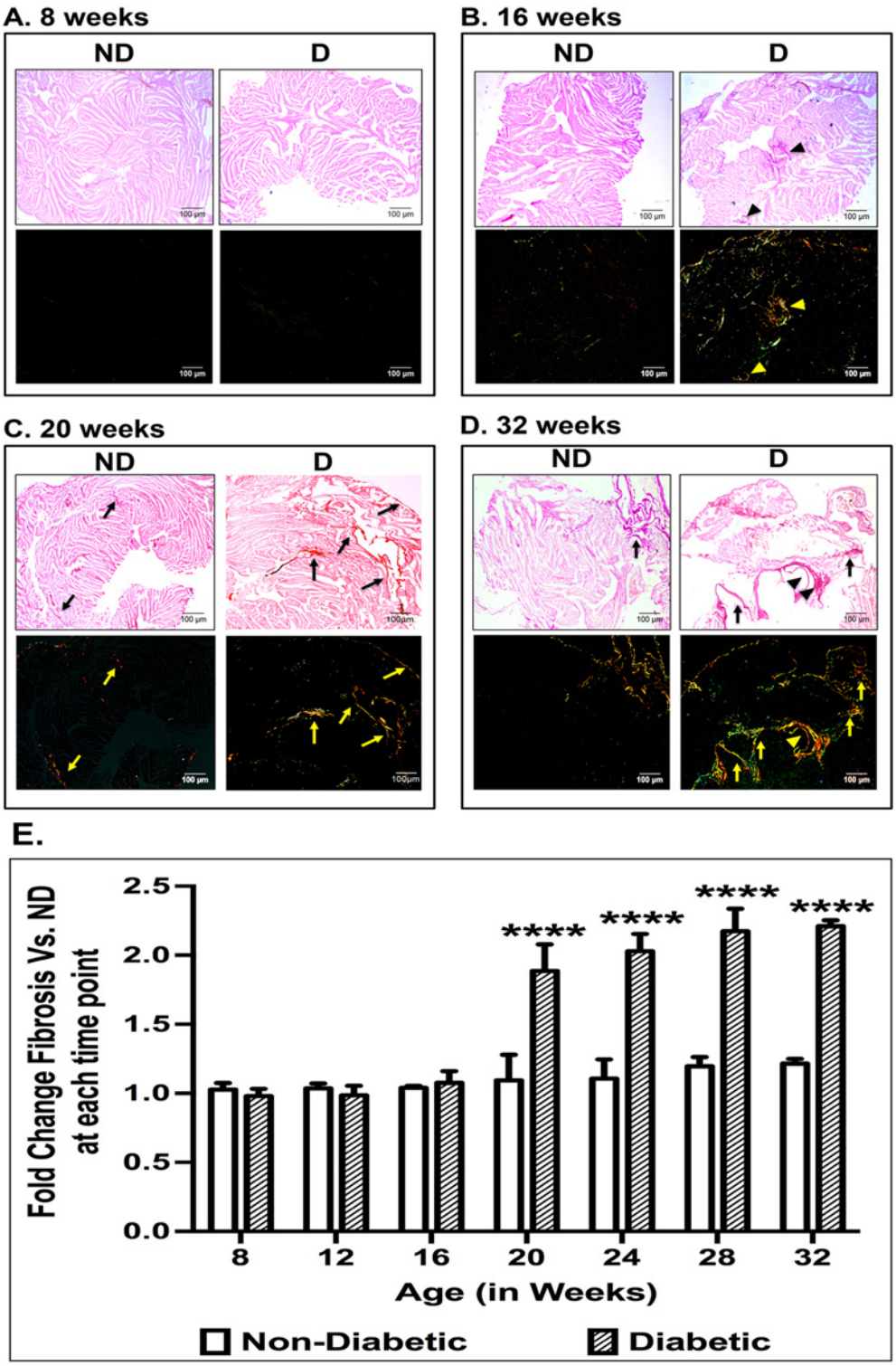

Figure 3. Increased fibrosis in the diabetic heart correlates with down-regulation of miR-15a/b

(**A**–**D**) Representative microphotographs of myocardial tissue sections from 8 (A), 16 (B), 20 (C) and 32 (D) weeks old diabetic and ND mice. All sections were stained with Picrosirius Red and imaged at ×20 magnification using standard bright field (top panel in each group) or polarized light (bottom panel in each group) microscopy. Perivascular (arrowhead) and interstitial fibrosis (open arrow) were observed in 16 weeks (B) and 20 weeks (C) old diabetic mouse heart, followed by more widespread interstitial fibrosis with the progression of diabetes (D). Thick collagen fibres were stained red (top panels) and appeared yellowish orange under polarized light (bottom panels). (**E**) Quantitative

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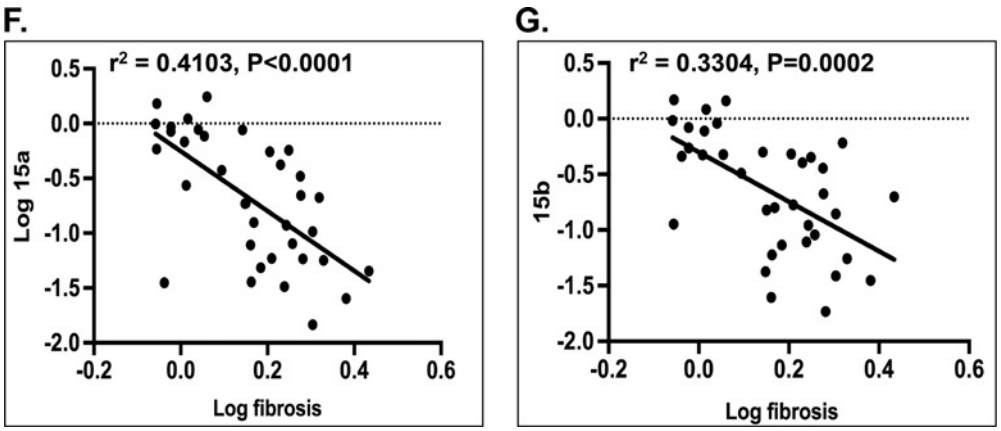

Figure 3. Continued

bar graph showing the fold changes in fibrotic area in diabetic heart at different time points with respect to the age-matched ND (n=5 for each group). Data were expressed as mean ± SEM. Two-way ANOVA was used for comparisons between groups at different time points, followed by Holm-Sidak's test for multiple comparisons. ****$P < 0.0001$ compared with age-matched ND control. (**F** and **G**) Scatter plots of fibrosis and miR-15a (F) or miR-15b (G) in diabetic heart irrespective of age.

cardiomyocytes [(189 ± 55)-fold increase], with only a minimal secretion in HG-treated cardiomyocytes [(1.4 ± 0.3)-fold increase]. Next, to confirm if secreted miR-15a/b is able to inhibit the differentiation of cardiac fibroblasts to myofibroblasts phenotype, a key process in the development of fibrosis [27], human diabetic and ND fibroblasts were treated with CM before treatment with angiotensin II to induce differentiation. As shown in Figure 6B and C, CM from HG-treated cardiomyocytes markedly increased the differentiation of fibroblasts, while this was attenuated in cells treated with CM collected from miR-15a/b mimic-treated cardiomyocytes. Although miR-15b was secreted at low levels, the effect on differentiation was comparable to the CM from miR-15a transfection (Figure 6C). These results provide evidence that secreted miR-15a/b plays an important role in differentiation of cardiac fibroblasts, a key process in cardiac fibrosis.

Possible role of miR-15a/b in regulating cardiac ageing in diabetic myocardium via the p53 network

A study by Fabbri et al. [28] reported the miR-15 family as an important player in the pathogenesis of chronic lymphocytic leukaemia (CLL) via regulating p53 protein expression. Recent studies showed that HG activates p53 in cardiomyocytes to induce apoptotic cell death [29]. Western blot analysis of murine diabetic hearts showed a significant increase in p53 expression starting from 8 weeks of age (Figure 7A and B) that was associated with repression of its downstream target SIRT1, a key regulator of cell survival and senescence (Figure 7A and C). Similarly, treating HL-1 cells with HG markedly increased the expression of p53 (Supplementary Figure S4A), while repressing SIRT1 (Supplementary Figure S4B). This was reverted following miR-15a and -15b overexpression (Figure 7D and E), indicating a possible role of miR-15a/b in controlling cardiac ageing in diabetic myocardium.

As a proof of concept, we also investigated the effect of miR-15a/b overexpression on other key senescent genes, cdkn2a (p16) and shc1 (p66), which are previously known to be involved in acceleration of the ageing process [30]. While HG markedly increased the expression level of both p16 (Figure 7F and G) and p66 (Figure 7H and I), miR-15a and -15b overexpression significantly down-regulated both genes (Figure 7F–I). These results provide a proof-of-concept that miR-15a/b regulates cardiac ageing, at least, in part by controlling the expression of senescent genes.

Discussion

Our study provides evidence for the dysregulation of miR-15a and -15b in both human and mouse Type 2 diabetic heart, and our results indicate that miR-15a/15b play a key role in fibrotic remodelling and ageing of the diabetic heart. Our *in vitro* experiments where restoration of miR-15a/b in HG cultured cardiomyocytes prevented the activation of the fibrotic signalling pathway support this concept.

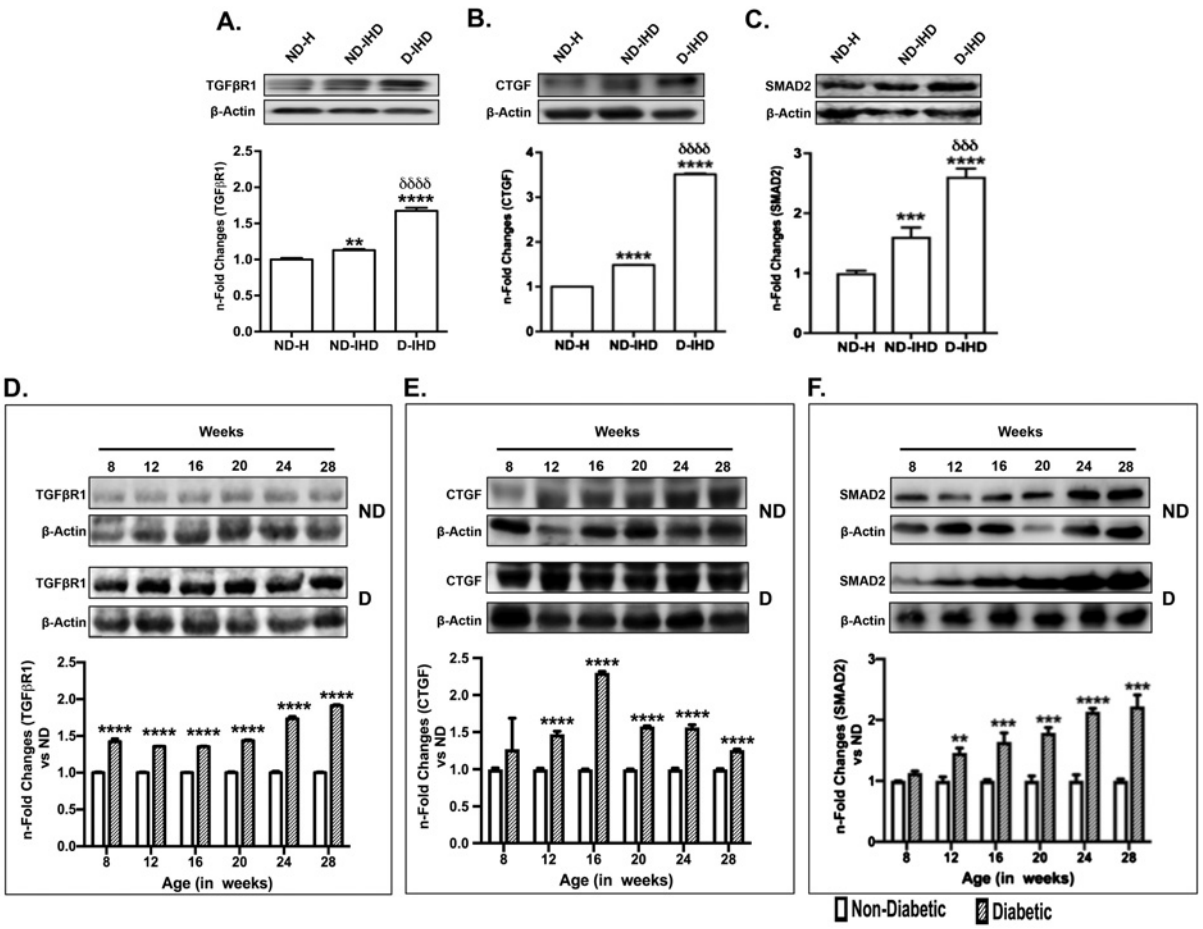

Figure 4. Activation of fibrotic signaling cascade in diabetic heart correlates with down-regulation of miR-15a/b

(**A–F**) Representative immunoblots and quantitative bar graphs showing the expression level of TGFβR1 (A and D), CTGF (B and E) and SMAD2 (C and F) protein expression in human (A–C) and mouse (D–F) myocardium. Data are Mean $\pm$ SEM. One way ANOVA was used for comparison between groups in human myocardium, two-way ANOVA was used for comparisons between groups at different time points in mice myocardium, followed by Holm Sidak's test for multiple comparisons. $n = 7$ in each group. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with ND-H (human) or age-matched ND control (mice); $^{\delta\delta\delta\delta}P < 0.0001$ compared with ND-IHD (human). (**G–J**) Scatter plots of TGFβR1 and CTGF vs. miR-15a (G and H) or miR-15b (I and J) in diabetic heart irrespective of age. There was a strong negative correlation between both the fibrogenic proteins and miR-15a/b expression, and this association was statistically significant.

Extensive evidence has documented the major role for myocardial fibrosis in inducing contractile dysfunction in individuals with diabetes [31]. While both miR-15a and -15b are expressed in cardiomyocytes [9,12], their functional role in the heart and, specifically, fibrosis is still under debate. Using a transgenic mouse model of miR-195 (a member of the miR-15 family), Porrello et al. [32] suggested that miR-15 could be regulating the withdrawal of cardiomyocytes from the cell cycle during the neonatal period. In another study, the same group showed that inhibition of miR-15 was able to increase cardiomyocyte proliferation and cardiac function following experimental myocardial infarction (MI), suggesting that miR-15 inhibition could be beneficial in patients with MI [33]. In contrast, a recent study by Tijsen et al. [9] showed that the miR-15 family negatively regulates the pro-fibrotic TGFβ pathway by targeting TGFβR1; hence, miR-15 could be anti-fibrotic. Interestingly, in our study, we found marked down-regulation of both miR-15a and -15b in the diabetic heart starting from the early stages of the disease when there was no structural evidence for fibrosis. Importantly, this was associated with significant up-regulation of the pro-fibrotic proteins and increased fibrosis. While it can be argued that miR-15 down-regulation increases the proliferation of cardiomyocytes to replace those killed by HG stress, we along with others have shown that diabetes adversely affects the proliferation of cardiovascular cells [30,34]. Moreover, in our study diabetic myocardium demonstrated a marked increase in thick collagen

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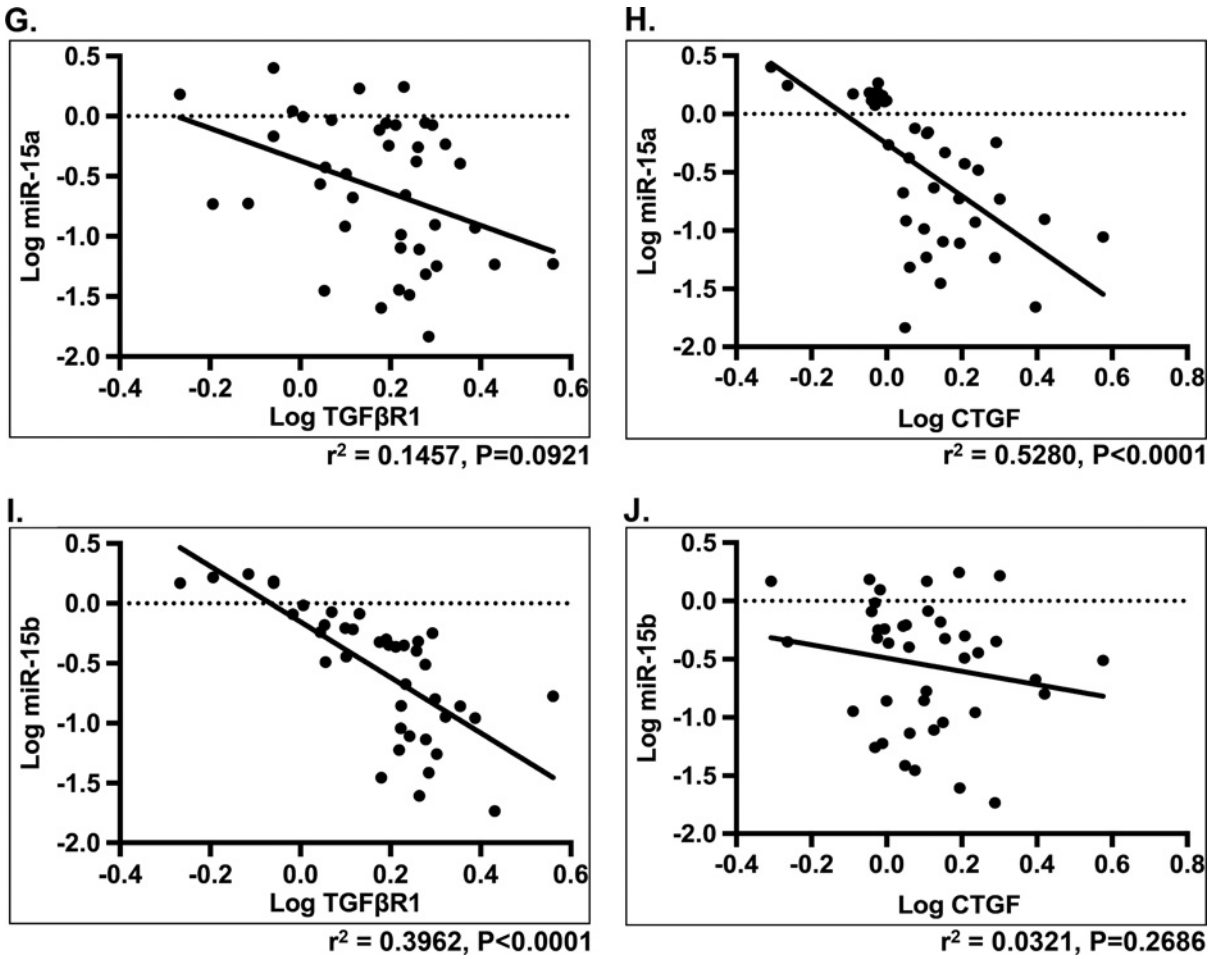

Figure 4. Continued

I fibres, which underlie diastolic stiffness of cardiac muscle in mouse (Figure 3A–D) along with increased expression of collagen type 1 (Supplementary Figure S5). Of note, overexpression of miR-15a has been demonstrated to induce down-regulation of collagen I expression [35]. Based on this, it is very likely that down-regulation of miR-15a/b is involved in the fibrotic remodelling of the diabetic heart.

Studies have shown that interstitial fibroblasts respond to signals released from dying cardiomyocytes and synthesize new matrix components aimed at replacing the damaged cells [26]. Cardiomyocytes and fibroblasts work in a regulatory network where they communicate through exchange of a variety of signals in the form of secretory proteins, growth factors, hormones and exosome-containing miRs that act as paracrine signalling molecules to regulate fibroblast phenotypes [36]. In our study HG increased the expression of pro-fibrotic TGFβR1 and CTGF through down-regulation of miR-15a/b in cardiomyocytes, which in turn activated the downstream SMAD pathway. This was reverted by restoring the expression of miR-15a/b. Activation of the TGFβ pathway induces differentiation of fibroblasts to myofibroblasts, which are responsible for the development of cardiac fibrosis [37]. As cardiomyocytes are the first responder cells to pathological stress and given the higher expression of miR-15a/b in cardiomyocytes, based on our results we speculate that the combination of increased pro-fibrotic factors and reduced miR-15a secretion from HG-stressed cardiomyocytes plays a crucial role in inducing the differentiation of fibroblasts in diabetic myocardium, leading to fibrotic remodelling.

In addition to its role in the fibrotic pathway, recent studies have shown that activation of p53 is controlled by the miR-15 family [28]. The tumour suppressor p53, one of the most frequently mutated genes in cancer [38], is also shown to be up-regulated in experimental heart failure, causing premature senescence and apoptosis in cardiomyocytes [39]. Moreover, increased cellular senescence in diabetic myocardium may trigger apoptosis via p53-mediated suppression of SIRT1, as observed in our study. This, in turn, provokes an inflammatory response (such

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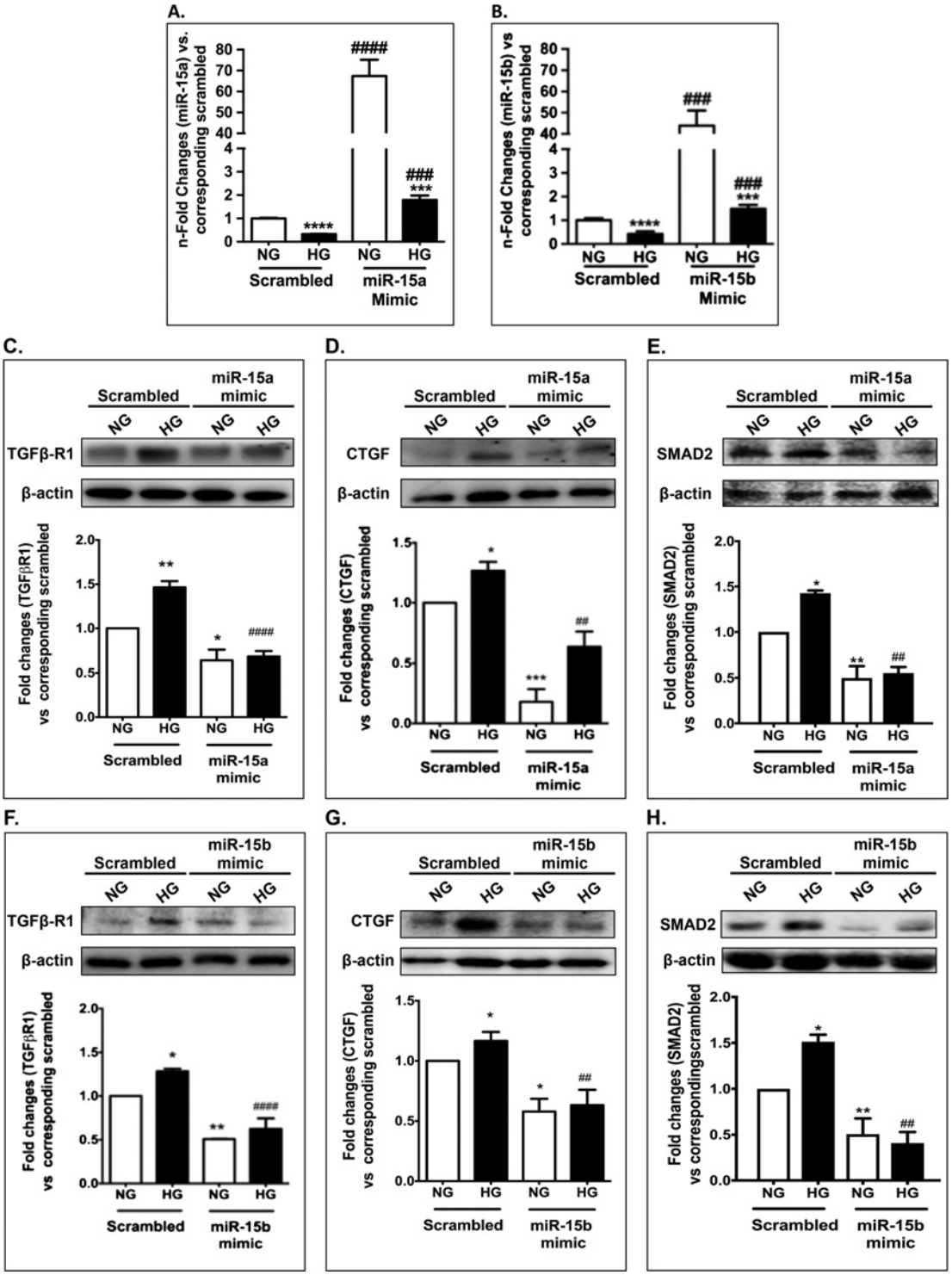

Figure 5. Therapeutic modulation of miR-15a/b in high glucose cultured cardiomyocytes prevented activation of fibrotic signaling cascade

(**A** and **B**) Quantitative bar graphs showing the expression level of miR-15a (A) and -15b (B) in HL-1 cardiomyocytes cultured in NG or HG and transfected with either Scr or miR-15a (A) or miR-15b (B) mimic. All data were expressed as mean ± SEM. U6 was used as internal control. $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with cells cultured in NG; $^{###}P < 0.001$ and $^{####}P < 0.00001$ compared with corresponding Scr transfected cells. (**C**–**H**) Representative immunoblot images and quantitative bar graphs showing the expression of TGFβR1 (C and F), CTGF (D and G) and SMAD2 (E and H) proteins in NG- and HG-treated HL-1 cardiomyocytes that were transfected with either scrambled or miR-15a/b mimic. All the data represented were quantified from three independent experiments.

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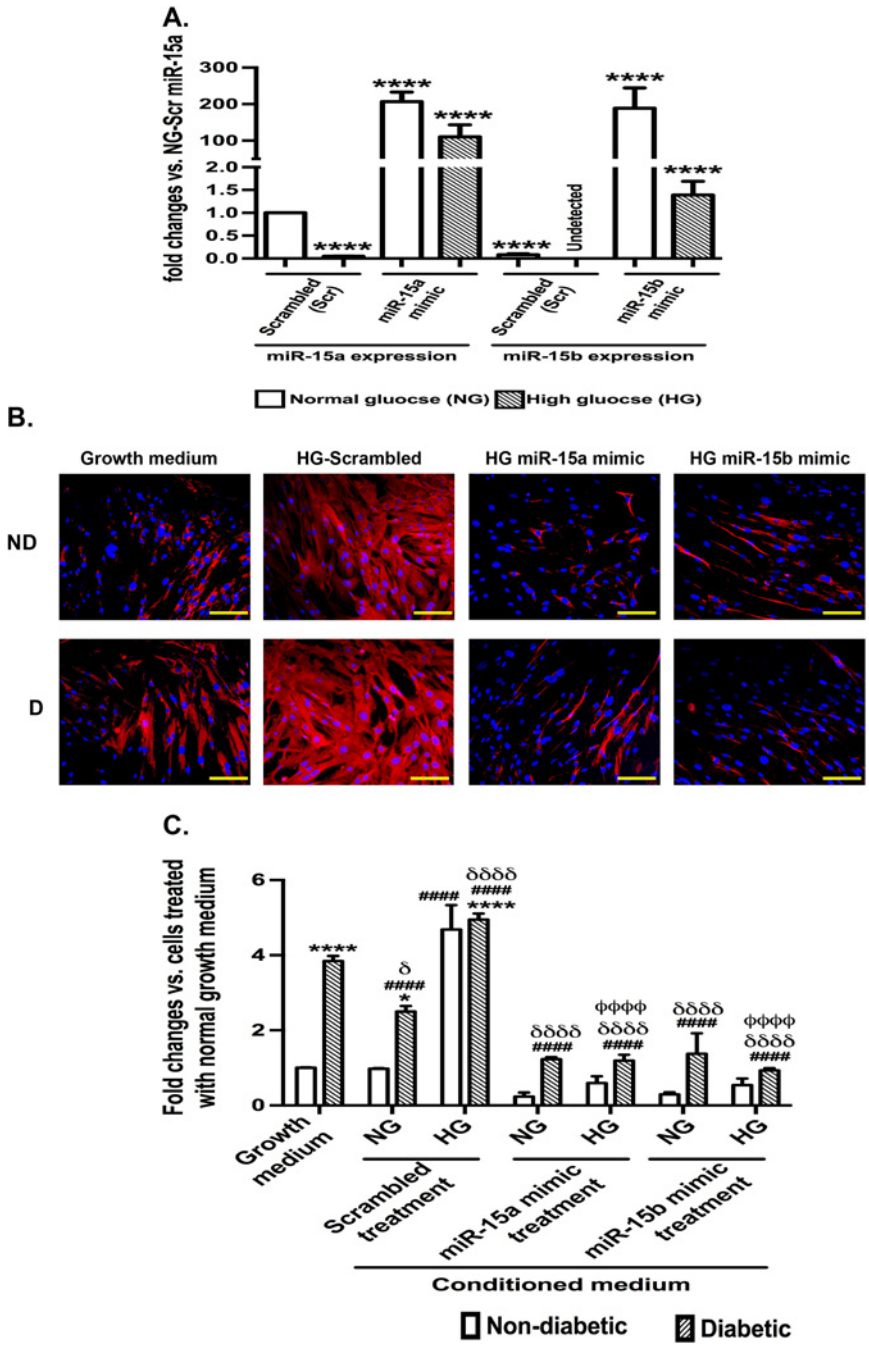

Figure 6. Secreted miR-15a/b inhibit myofibroblast differentiation

(**A**) Quantitative bar graph showing the expression of miR-15a and miR-15b in CM collected from cardiomyocytes cultured in NG or HG following transfection with Scr or miR-15a or miR-15b mimic. Data were expressed as mean ± SEM against miR-15a expression in CM collected from NG-Scr treated cells. U6 was used as internal control. $^{****}P < 0.0001$ compared with miR-15a expression in CM collected from NG-Scr treated cells. (**B**) Representative immunofluorescence images showing the differentiated myofibroblasts following angiotensin II treatment in fibroblasts collected from ND and diabetic (D) human heart. Differentiated myofibroblasts were identified by the positive staining for α-SMA. DAPI was used to stain the nuclei. Scale bars are 100 μm. (**C**). Quantitative bar graph showing the fold change in myofibroblast differentiation in diabetic and ND fibroblasts when exposed to CM from cardiomyocytes cultured in NG or HG following transfection with Scr and miR-15a or miR-15b mimic. Data were expressed in fold changes as mean ± SEM against cells cultured in normal growth medium. $^{*}P < 0.05$ and $^{****}P < 0.00001$ compared with corresponding ND group; $^{\#\#\#\#}P < 0.00001$ compared with ND fibroblasts cultured in growth medium; $^{\delta}P < 0.05$ and $^{\delta\delta\delta\delta}P < 0.00001$ compared with diabetic fibroblasts cultured in growth medium; $^{\Phi\Phi\Phi\Phi}P < 0.00001$ compared with diabetic fibroblasts grown in CM from cardiomyocytes cultured in HG following transfection with Scr.

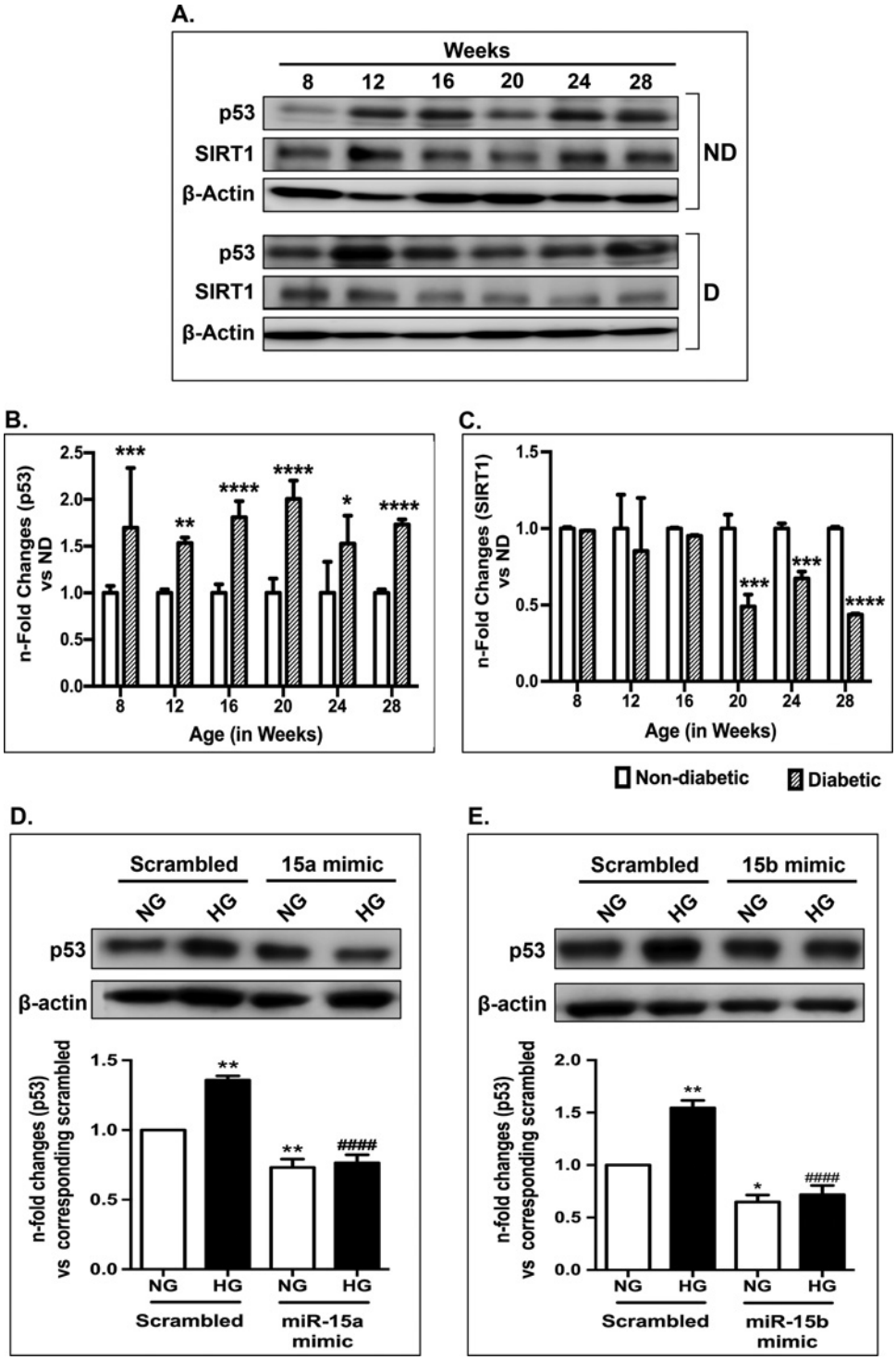

Figure 7. Possible role for miR-15a/b in regulating cardiac aging

(**A**–**C**) Representative immunoblot images (A) and quantitative bar graphs (B and C) showing the expression level of p53 (A and B) and SIRT1 (A and C) at different time points in mouse myocardium. Data were expressed as mean ± SEM. β-Actin was used as internal control. Two-way ANOVA was used for comparisons between groups at different time points, followed by Holm-Sidak's test for multiple comparisons. ****$P < 0.0001$ compared with age-matched ND control; $n=7$ in each group. (**D** and **E**) Representative immunoblot images and quantitative bar graphs showing the expression level of p53 in cardiomyocytes cultured in NG or HG and transfected with either miR-15a (D) or miR-15b (E) mimic. All data were expressed as mean ± SEM and represented as fold changes to the corresponding Scr-treated group. β-Actin was used as internal control. One-way ANOVA was used for comparison between groups, followed by Holm-Sidak's test for multiple

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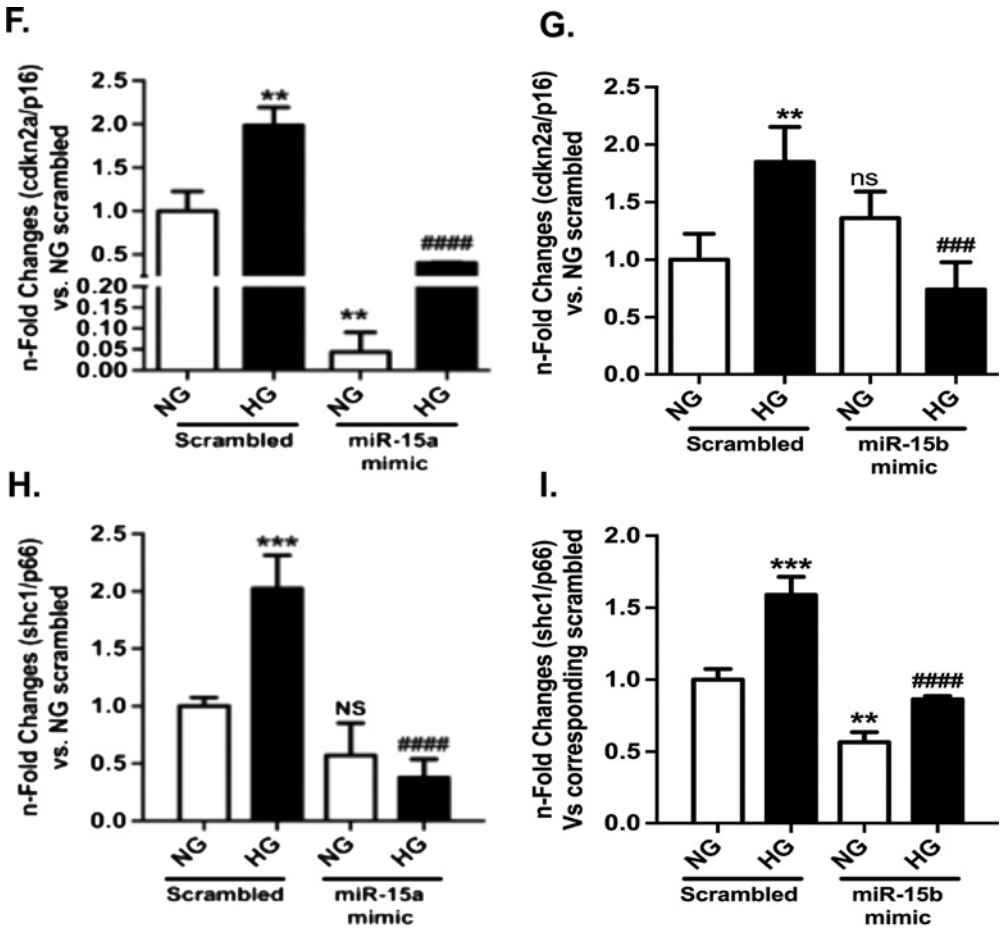

Figure 7. Continued

comparisons, $^{****}P < 0.0001$ compared with NG-Scr treated group; $^{\#\#\#\#}P < 0.0001$ compared with HG-Scr treated group. All the experiments were performed in triplicate and repeated at least three independent times. (**F–I**) Quantitative bar graphs showing the expression level of cdkn2a/p16 (F and G) and shc1/p66 (H and I) in NG or HG and transfected with either miR-15a (F and H) or miR-15b (G and I). All data were expressed as mean $\pm$ SEM and represented as fold changes to the corresponding Scr-treated group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with corresponding Scr-treated group; $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ compared with HG Scr-treated group; ns = not significant. All the experiments were performed in triplicate and repeated at least four independent times.

as activation of TGFβR1) that ultimately results in fibroblast activation and replacement of dead cardiomyocytes with fibrous tissue [40,41].

In summary, our results provide first evidence for the down-regulation of miR-15a and -15b as an early molecular alteration in the process of fibrotic remodelling of the diabetic heart. We also showed the multifaceted regulation of miR-15a/b in fibrosis by controlling fibrogenic mediators TGFβR1 and CTGF and in senescence via regulation of p53 in HG-stressed cardiomyocytes (summarized in Supplementary Figure S6). These molecular alterations eventually lead to fibrotic remodelling and premature senescence in the diabetic heart, suggesting that therapeutic restoration of the miR-15 family could be a novel tool for treatment/prevention of fibrosis in the diabetic heart. Although future studies are still warranted to show that complementation of miR-15a and/or miR-15b in an animal model of diabetes is able to prevent or treat the fibrotic remodelling, our study provides evidence that therapeutic restoration of miR-15 family could be a novel tool for treatment/prevention of fibrosis in the diabetic heart. Further, results from human plasma suggest that consistent monitoring of circulating levels of miR-15a could potentially identify those individuals with pre-clinical fibrotic remodelling of the heart, thereby providing a novel tool for the clinicians in timely management of these patients, which needs to be confirmed with long-term follow-up studies.

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Clinical perspectives

- Myocardial fibrosis increases myocardial stiffness leading to diastolic dysfunction in the diabetic heart. This study determined the role of the role of the microRNA (miR)-15 family in fibrotic remodelling of the diabetic heart, with the aim to develop novel therapies to combat diabetes induced cardiovascular complications.

- Diabetes downregualted miR-15a/b expression in the human heart, which preceded the development of structural fibrosis in mouse diabetic heart. Down-regulation of miR-15a/b was associated with significant up-regulation of pro-fibrotic factors. Therapeutic restoration of miR-15a/b level in *in vitro* cultured adult cardiomyocytes, reversed the high glucose induced activation of pro-fibrotic and pro-senescent factors. Importantly, miR-15a/b released from cardiomyocytes were able to prevent differentiation of diabetic human cardiac fibroblasts.

- This is the first evidence that early down-regulation of miR-15a/b activates fibrotic signaling in diabetic heart, hence could be a potential target for the treatment/prevention of diabetes induced fibrotic remodelling of the heart.

Author contribution

S.R. helped in conceiving and designing the study and carried out the *in vivo* and *in vitro* experiments, analysed the data and wrote the first draft of the manuscript; P.E.M carried out histological analysis; P.T.N. carried out part of the Western blotting; J.K.S.L. performed echocardiography (E/e') measurement; G.T.J. collected heart tissue samples from those who died due to non-ischaemic heart disease; M.J.A.W. performed echocardiography on human participants; P.D., D.B. and I.F.G. were involved in human study in writing the ethics and collecting the samples during cardiac surgery; P.M. was involved in recruiting the volunteers for 'Dunedin microRNA study' and provided plasma samples; R.R.L. holds ethics for collection of human myocardial samples and edited the manuscript; R.K. conceived and designed the study, collected the samples, analysed the results and wrote the manuscript.

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

α-SMA, α-smooth muscle actin; CM, conditioned medium; CTGF, connective tissue growth factor; ECM, extracellular matrix; HG, high glucose; IHD, ischaemic heart disease; LV, left ventricle; MI, myocardial infarction; miR, microRNA; ND, non-diabetic; NG, normal glucose; RAA, right atrial appendage; Scr, scrambled sequence; SIRT1, sirtuin 1; TGFβR1, transforming growth factor-β receptor-1.

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