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Inhibition of EGFR-STAT3 attenuates cardiomyopathy in streptozotocin-induced type 1 diabetes

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Abstract

Emerging evidence implicates elevated activity of STAT3 transcription factor in driving the development and progression of diabetic cardiomyopathy (DCM). We hypothesized that the fibrosis-promoting and hypertrophic actions of STAT3 are linked to the activation by epidermal growth factor receptor (EGFR). We tested this hypothesis by challenging cultured cardiomyocytes to high-concentration glucose and heart tissues of streptozotocin (STZ)-induced type 1 diabetic mice. Our results indicated that, in diabetic mice, the blockade of STAT3 or EGFR using selective inhibitors S3I-201 and erlotinib, respectively, abrogated the increased activating STAT3 phosphorylation and the induction of genes regulating fibrosis and hypertrophy in myocardial tissue. S3I-201 and erlotinib significantly reduced myocardial structural and functional deficits in diabetic mice. In cultured cardiomyocytes, high-concentration glucose induced EGFR-mediated STAT3 phosphorylation. We further showed that blockade of STAT3 or EGFR using selective inhibitors and siRNAs significantly reduced the increased expression of genes known to promote fibrosis and hypertrophy in cardiomyocytes. These results provide novel evidence that the EGFR-STAT3 signaling axis likely plays a crucial role in the development and progression of DCM.

Key Words

- ▶ diabetic cardiomyopathy
- ▶ STAT3
- ▶ EGFR
- ▶ type 1 diabetes
- ▶ fibrosis
- ▶ cardiac hypertrophy

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Introduction

Diabetic cardiomyopathy (DCM) is a major cardiovascular complication of diabetes mellitus, in which heart structural impairment and functional deficits lead to heart failure (Zhou *et al.* 2018). DCM is characterized by interstitial and perivascular fibrosis, ventricular hypertrophy, as well as diastolic and systolic dysfunction, all occurring in the absence of coronary artery disease and hypertension (Bugger & Abel 2014). The underlying factors driving the

development of DCM are multiple and complex, and include oxidative stress, inflammation, mitochondrial function and cardiomyocyte apoptosis (Zhang *et al.* 2012, Westermeier *et al.* 2016, Wang *et al.* 2017, Yu *et al.* 2018). However, the precise molecular mechanisms mediating these cellular and biochemical changes remain unclear.

Increasing evidence indicates a potential link between diabetic secondary complications to the activities of

signal transducer and activator of transcription 3 (STAT3). There are reports of both a positive and a negative role of STAT3 in driving the development of DCM. Activated STAT3 is detected in heart tissues from streptozotocin (STZ)-induced diabetic rats (Wang *et al.* 2015) and mice (Sun *et al.* 2014). *In vitro* challenge of cardiomyocytes (Lo *et al.* 2017) and cardiac fibroblasts (Fiaschi *et al.* 2014) with high-concentration glucose (HG) also induces STAT3 activation and leads to the induction of connective tissue growth factor and extracellular matrix proteins fibronectin and collagen. Moreover, STAT3 has been shown to be upregulated in the tubulointerstitial compartment of renal samples from diabetic patients with progressive nephropathy (Berthier *et al.* 2009) and in animal models of diabetic nephropathy (Matsui & Meldrum 2012). In contrast, there are reports that STAT3 mediates cardioprotective activity against injury from acute and chronic stresses, such as smoking, aging, hypertension and obesity (Zouein *et al.* 2015, Pipicz *et al.* 2018). Several studies have also reported decreased STAT3 phosphorylation and/or activation in heart tissues of various experimental models of diabetes (Pipicz *et al.* 2018). Therefore, the exact role of STAT3 in DCM remains an enigma.

STAT3 is expressed in different cell types of the heart tissue, including cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells and inflammatory cells and cardiac neurons. Therefore, differential activation of STAT3 in various cardiac cell types likely contributes to the pleomorphic functions. Additionally, a variety of extracellular polypeptide ligands acting on their cognate receptors activate STAT3 through phosphorylation of Tyr-705 or Ser-727. STAT3 phosphorylated on Tyr-705 leads to formation of homo- and heterodimers with other STAT family members, and subsequent nuclear translocation to activate transcription of target genes. Whereas Ser-727 phosphorylation causes the monomer to translocate to mitochondria for the regulation of activities such as ATP production (Pipicz *et al.* 2018). Therefore, the outcome of STAT3 activation may be dependent on the activating ligand.

In the context of diabetes, upstream events leading to STAT3 activation remain unknown. Much of the evidence indicates that HG induces the production and secretion of mediators such as cytokines, reactive oxygen species and growth factors, which then can activate their cognate receptors or target molecules, leading to STAT3 activation. EGFR is a potential activator of STAT3. EGFR activation has been shown in experimental diabetes and in cultured cells exposed to HG (Saad *et al.* 2005, Wu *et al.* 2009). We also

know that EGFR plays a role in cardiomyocyte function. The global or conditional knockout of the EGFR member, ErbB2, results in faulty heart development in which the mice die *in utero* (Lee *et al.* 1995) or develop heart failure (Crone *et al.* 2002). EGFR overexpression in the heart also induces hypertrophic cardiomyopathy (Sysa-Shah *et al.* 2012). We previously found that in mouse models of diabetes, EGFR activity was increased in cardiomyocytes, and blocking EGFR reduced the severity of DCM (Zou *et al.* 2017). These findings are consistent with the notion that dysregulated EGFR signaling is likely coupled to heart dysfunction in diabetes. We, therefore, hypothesized that development and progression of DCM may be driven by an activated EGFR-STAT3 signaling axis.

Our findings show increased EGFR-STAT3 signaling in heart tissues of diabetic mice. Inhibition of either STAT3 or EGFR was associated with normalization of factors critical to cardiac fibrosis and hypertrophy. Furthermore, inhibition of EGFR-STAT3 axis prevented the development of functional cardiac deficits in diabetic mice. We confirmed these findings by utilizing cardiomyocytes and cardiac fibroblasts exposed to HG. Our findings provide a novel pathogenic mechanism in the development and progression of DCM.

Materials and methods

Reagents and cell culture

Streptozotocin (STZ) was purchased from Sigma-Aldrich. S3I-201 (STAT3 inhibitor) (Pang *et al.* 2010, Ahmad *et al.* 2018) and erlotinib (EGFR inhibitor) were purchased from Sigma-Aldrich. S3I-201 and erlotinib were dissolved in dimethyl sulfoxide (DMSO; 10 mM stock concentration) for *in vitro* experiments and in 0.5% carboxymethylcellulose sodium (CMC-Na) buffer for *in vivo* experiments. Nitrocellulose membrane and chemiluminescence reagents for Western blotting were purchased from Bio-Rad Laboratory. Antibodies directed against STAT3 (Cat. #9139S), phosphorylated Tyr-705 STAT3 (p-STAT3; Cat. #9145), EGFR (Cat. #4267), phosphorylated EGFR (p-EGFR; Cat. #3777), collagen-I (COL1; Cat. #84336) and transforming growth factor- β (TGF- β ; Cat. #3711) were obtained from Cell Signaling Technology. Antibodies directed against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat. ab8245), cardiac myosin heavy chain, alpha isoform (MyHC-alpha, MYH6; Cat. ab185967) and lamin B (Cat. #ab16048) were purchased from Abcam. Goat anti-rabbit IgG-HRP

(Cat. sc-2004) and atrial natriuretic peptide antibodies (ANP; Cat. #SC-20158) were purchased from Santa Cruz Biotechnology. DAPI (4',6-diamidino-2-phenylindole) was purchased from Invitrogen.

The immortalized rat cardiomyocyte cell line (H9C2) was obtained from ATCC. The cells were cultured in DMEM medium (Gibco) containing 5.5mM D-glucose (low-concentration glucose, LG) and supplemented with 10% FBS (Gibco), 100 U/mL of penicillin and 100 mg/mL of streptomycin. Isolation and culture of neonatal rat primary cardiomyocytes were performed as described previously (Nakamura *et al.* 1998).

siRNA transfections

Gene silencing in H9C2 cells was performed by transfecting cells with siRNA target sequences synthesized by Genepharma Co. LTD. The siRNA sequence for rat *STAT3*: sense, 5'-GCAGGAUCUAGAACAGAAATT-3'; and antisense, 5'-UUUCUGUUCUAGAUCUGCTT-3'. The siRNA sequence for rat *EGFR*: sense, 5'-CGAGAGUUGAUUCUCGAAUTT-3'; and antisense, 5'-AUUCGAGAAUCAACUCUCGTT-3'. Negative control siRNA sequence: sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Briefly, cells were plated at sub-confluent densities (5×10^3 cells/cm²) and cultured for 24 h. Target siRNA or negative control siRNA were transfected at a final concentration of 50 pmol/mL using Lipofectamine 2000 (Invitrogen). Culture medium was replaced with fresh growth medium after 6 h, and knockdown efficiency was evaluated by Western blotting.

Streptozotocin-induced diabetic mouse model

Six-week-old male C57BL/6 mice were obtained from the Animal Center of Wenzhou Medical University. Animal procedures were approved by the Ethics Committee of Wenzhou Medical University Animal Policy and Welfare Committee. The mice were acclimated in an environmentally controlled room at $22 \pm 2.0^\circ\text{C}$ with $50 \pm 5\%$ humidity, exposed to a 12-h:12-h light/darkness cycle, and fed food and water *ad libitum*. Diabetes was induced in mice at 8 weeks of age (approximately 18–22 g body weight) by intraperitoneal (i.p.) injection of STZ (50 mg/kg dissolved in 100 mM citrate buffer; pH 4.5) for 5 consecutive days. Non-diabetic control mice received the same volume of citrate buffer. Blood was collected by mandibular vein puncture to measure glucose using a Glucometer. Mice with a fasting-blood glucose

>12 mmol/L were considered diabetic. Treatment with S3I-201 or Erlotinib was initiated after establishment of diabetes, by oral gavage every other day for 16 weeks. S3I-201 was administered at 5 mg/kg and erlotinib at 10 mg/kg ($n=7$). Untreated mice received the same volume of CMC-Na buffer ($n=7$). At the indicated time points, blood glucose and body weights were recorded. At 18 weeks, mice were killed by sodium pentobarbital anesthesia, and the final body weight and heart weight was measured. Blood samples and heart tissues were collected. Heart tissues were either fixed in 4% paraformaldehyde for pathological analysis or flash-frozen in liquid nitrogen for gene and protein expression analysis. Blood samples were used to obtain serum for the measurement of creatine kinase-muscle/brain (CK-MB) as well as lactate dehydrogenase (LDH) by ELISA (Nanjing, Jiancheng, Jiangsu, China).

Echocardiography analysis

Systolic and diastolic cardiac function was determined non-invasively by transthoracic echocardiography in anesthetized mice, 1 day before the killing. Ultrasound heart function was assessed using a Vevo 770 high-resolution imaging system (Visual Sonics, Canada) equipped with a high-frequency ultrasound probe (RMV-707B). Ejection fraction (EF) was calculated from LV (left ventricle) end-diastolic volume (LVEDV) and end-systolic volume (LVESD) using the equation: $(\text{LVEDV} - \text{LVESD}) / \text{LVEDV} \times 100\%$. Fractional shortening (FS) was calculated using the equation: $\text{FS} = ((\text{LVIDd} - \text{LVIDs}) / \text{LVIDd}) \times 100\%$.

Reverse transcription and real-time quantitative PCR

Total RNA was isolated from cells and tissues using TRIZOL (Invitrogen). Reverse transcription and quantitative PCR (RT-qPCR) were performed using M-MLV Platinum RT-qPCR Kit (Invitrogen). Real-time qPCR was carried out using the Eppendorf Real-plex 4 instrument (Eppendorf). Primers for genes of interest and house-keeping gene, β -actin, were obtained from Invitrogen (Invitrogen) and are shown in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

Western blot analysis

Cell and tissue lysate homogenates were prepared. Total proteins (30–60 μg) were separated by 8–12% SDS-PAGE and electro-transferred onto a nitrocellulose membrane.

The membrane was blocked for 1 h at room temperature in TBST (Tris-buffered saline with 0.05% Tween 20, pH 7.4) plus 5% non-fat milk and incubated overnight at 4°C with primary antibodies to p-STAT3, STAT3, p-EGFR, EGFR, COL1, TGF- β , MYH6, ANP, GAPDH and laminB. After three washes with TBST, membranes were incubated with the appropriate secondary antibody at 1:3000 dilution for 1 h at room temperature. The signals were visualized using enhanced chemiluminescence reagents, and band intensities quantified using ImageJ software (NIH).

Histopathology

The fixed mouse heart tissues were embedded in paraffin and sectioned at 5 μ m thickness. After dehydration, sections were stained with hematoxylin and eosin (H&E). Myocardial fiber morphology was evaluated and recorded by bright field microscopy (Nikon). Heart tissue sections were stained Masson's Trichrome stain and Sirius red to assess fibrosis and collagen deposition. Heart connective tissue and collagen fiber content were quantified by a single-blinded observer, using the ImageJ program (NIH).

Inflammatory cytokine production

Cardiomyocytes were challenged with 33 mM glucose (HG) for 2 h. Condition media was then collected. Secreted interleukin-6 (IL6) was determined in the medium using rat IL6 ELISA (Thermo Fisher). The absorbance was measured at 450 nm and values normalized to the standard curve to calculate the concentration of the cytokine.

Cell staining

H9C2 cell size was calculated by labeling cells with rhodamine-conjugated phalloidin. Cells were grown onto slides, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with rhodamine-conjugated phalloidin at a concentration of 50 μ g/mL for 30 min. Nuclei were stained with the DAPI at room temperature for 5 min.

The nuclear localization of p-STAT3 was determined by immunofluorescence staining. The cells were processed as described above. Fixed and permeabilized cells were blocked with 1% fetal bovine serum for nonspecific antigens. Cell slides were incubated with anti-p-STAT3 antibody (1:1000) overnight at 4°C, followed by PE-conjugated secondary antibody (1:200) at room temperature for 1 h. DAPI was used to counterstain. Images were captured

using a Nikon epifluorescence microscope (Nikon). The quantification/morphometric of cells was performed using ImageJ software (NIH).

Statistical analysis

Data were collected from at least three independent experiments for *in vitro* studies and seven mice in each group for *in vivo* studies. Values were presented as mean $\pm$ S.E.M. GraphPad Pro (GraphPad) was used to perform statistical testing. We used ANOVA followed by Dunnett's *post hoc* test for parametric testing and Kruskal–Wallis test followed by Dunn's *post hoc* test when comparing non-parametric data. Differences were considered to be significant at $P < 0.05$.

Results

Inhibition of EGFR-STAT3 signaling improves cardiac function in mouse model of STZ-induced type 1 diabetes

We tested the effect of modulating the EGFR-STAT3 signaling pathway using specific small-molecule inhibitors in a mouse model of STZ-induced type 1 diabetes (see 'Materials and methods' section). S3I-201 is a selective STAT3 inhibitor with an IC_{50} of 86 μ M to STAT3 (Sen *et al.* 2012) and has been used in animal models at doses reaching 10 mg/kg (Pang *et al.* 2010, Matsui *et al.* 2017) without signs of toxicity. Erlotinib is a selective EGFR inhibitor with an IC_{50} of 2 nM in cell-free assay (Moyer *et al.* 1997) and previous studies have shown attenuation of features of diabetic nephropathy, including fibrosis, in mice with doses of 80 mg/kg daily (Chen *et al.* 2012, Zhang *et al.* 2014). Based on these studies, mice that presented with hyperglycemia (fasting-blood glucose >12 mmol/L) within 2 weeks of the last STZ injection, were subsequently given S3I-201 (5 mg/kg) or erlotinib (10 mg/kg) by oral gavage. Our results show that hyperglycemia was two-fold greater in diabetic mice compared to the non-diabetic controls for the duration of the 20-week study, and neither S3I-201 nor erlotinib affected hyperglycemia (Fig. 1A). All untreated diabetic mice and diabetic mice treated with S3I-201 or erlotinib showed about 20% weight loss relative to the non-diabetic control mice (Fig. 1B). Western blot analysis of cardiac tissue indicated robust STAT3 phosphorylation in the diabetic mice, and reduced levels in diabetic mice treated with S3I-201 and erlotinib (Fig. 1C and Supplementary Fig. 1).

Figure 1

STAT3 activation in cardiac tissue is associated with increased markers of cardiac injury in STZ-induced diabetic mice. The *in vivo* effects of blocking EGFR-STAT3 signaling by S3I-201 (5 mg/kg) or erlotinib (10 mg/kg) were investigated in the STZ-diabetic mouse model. (A) Blood glucose levels and (B) body weights are shown for up to 20 weeks of study. (C) Representative Western blot analysis of heart tissue for phosphorylated STAT3 (p-STAT3) and total STAT3 ($n = 7$). Serum levels of creatine kinase-muscle/brain (CK-MB) (D) and (E) lactate dehydrogenase (LDH) in diabetic mice and mice treated with EGFR and STAT3 inhibitors (Ctrl = non-diabetic control mice; T1DM = STZ-induced type 1 diabetic mice; T1DM + S3I-201 = diabetic mice treated with 5 mg/kg S3I-201, T1DM + erlotinib = diabetic mice treated with 10 mg/kg erlotinib). Data shown as mean $\pm$ S.E.M. in panels A, B, D and E; $n = 6$; * $P < 0.05$ compared to Ctrl; # $P < 0.05$, ## $P < 0.01$ compared to T1DM.

These results show that diabetes activates STAT3 in the heart which can be prevented by EGFR inhibition.

Diabetic mice presented with elevated levels of CK-MB and LDH (Fig. 1D and E). Treatment of diabetic mice with either S3I-201 or Erlotinib abrogated these increases (Fig. 1D and E). These findings suggest that inhibition of the activated EGFR-STAT3 pathway in the type 1 model of diabetes reduces cardiac tissue injury. To verify this notion, we performed transthoracic echocardiography in mice to assess cardiac function. Our results showed reduced FS and EF indicative of cardiac dysfunction. These functional deficits were associated with increased ventricular diameter, and increases in the thickness of anterior, posterior and interventricular

walls (Table 1). Treatment of diabetic mice with S3I-201 and erlotinib prevented functional and structural impairments, providing *in vivo* evidence for EGFR-STAT3 signaling in mediating the deleterious cardiac outcomes in diabetic mice.

EGFR-STAT3 signaling promotes cardiac fibrosis and hypertrophy in diabetic mice

DCM is characterized by interstitial and perivascular fibrosis (Boudina & Abel 2010), driven through sustained hyperglycemia and hyperlipidemia (Jia *et al.* 2016). We determined whether blockade of EGFR-STAT3 signaling reduces cardiac remodeling in the diabetic mice.

Table 1 Effects of S3I-201 and erlotinib on cardiac structure and function in T1DM mice.

	T1DM			
	Control $n = 7$	Vehicle $n = 7$	S3I-201 $n = 7$	Erlotinib $n = 7$
FS%	44.02 $\pm$ 1.828	37.43 $\pm$ 1.736 ^a	43.73 $\pm$ 1.632 ^b	39.48 $\pm$ 0.9106 ^b
EF%	82.2 $\pm$ 1.646	74.12 $\pm$ 1.858 ^a	81.37 $\pm$ 1.659 ^b	81.37 $\pm$ 1.659 ^b
LVIDd, mm	13.43 $\pm$ 0.1581	16.00 $\pm$ 0.4451 ^a	13.40 $\pm$ 0.04082 ^b	14.33 $\pm$ 0.1549 ^b
LVIDs, mm	12.5 $\pm$ 0.7868	13 $\pm$ 1.414 ^a	13 $\pm$ 1.140	14.3 $\pm$ 1.506
FWd, mm	6.825 $\pm$ 0.1768	7.356 $\pm$ 0.2911 ^a	6.867 $\pm$ 0.2582 ^b	7.017 $\pm$ 0.2041
FWs, mm	8.638 $\pm$ 0.01844	8.886 $\pm$ 0.1944	8.533 $\pm$ 0.03162	8.500 $\pm$ 0.08333
PWd, mm	6.771 $\pm$ 0.04880	7.633 $\pm$ 0.4761 ^a	6.95 $\pm$ 0.07071 ^b	7.017 $\pm$ 0.2041 ^b
PWs, mm	8.588 $\pm$ 0.2532	8.967 $\pm$ 0.4664	8.600 $\pm$ 0.1265 ^b	8.583 $\pm$ 0.2229 ^b
IVSd, mm	6.775 $\pm$ 1.000	7.614 $\pm$ 1.952 ^a	6.883 $\pm$ 2.066 ^b	7.000 $\pm$ 0.8367 ^b
IVSs, mm	8.663 $\pm$ 0.2615	9.011 $\pm$ 0.5622	8.600 $\pm$ 0.2530 ^b	8.667 $\pm$ 0.2658 ^b

Transthoracic echocardiography was performed on control and diabetic mice at the conclusion of the study. Cardiac parameters evaluated: FS, fractional shortening %; EF, ejection fraction %; LVIDd and LVIDs, left ventricular internal diameter end diastole and end systole, respectively; FWd and FWs, diastole and systole forward wall thickness, respectively; PWd and PWs, diastole and systole posterior wall thickness, respectively; IVSd and IVSs, respective diastole and systole interventricular septal thickness. Data presented as mean $\pm$ S.E.M.

^a $P < 0.05$, compared to control; ^b $P < 0.05$, compared to vehicle-treated diabetic mice.

Figure 2

Inhibition of EGFR-STAT3 signaling reduces cardiac fibrotic and hypertrophic responses in diabetic mice. (A) Brightfield images of mouse heart tissue stained with hematoxylin and eosin (H&E; top row, $n = 7$); Masson's Trichrome stain (2nd row from top, $n = 7$); Sirius red staining (3rd row from top, $n = 7$). (B) Quantification of fibrotic changes as assessed through Masson's Trichrome staining. Data was normalized to Ctrl. (C) Quantification of collagen deposition as assessed through Sirius red staining. Data was normalized to Ctrl. (D) mRNA analysis showing levels of *COL1*, *TGFβ*, *MYH6* and *ANP* in heart tissues of mice (values normalized to β-actin and reported relative to Ctrl). (E) Western blot analysis of COL1, TGF-β, MYH6 and ANP protein levels in heart tissues. GAPDH was used as loading control. Data shown as mean ± s.e.m. in panels B, C, and D; $n = 7$; Treatment groups are as shown in Fig. 1; * $P < 0.05$, ** $P < 0.01$ compared to Ctrl; # $P < 0.05$, ## $P < 0.01$ compared to T1DM alone.

Histological evaluation of cardiac tissues indicated disorganized and enlarged myofibers in diabetic mice (Fig. 2A, top panel). Similar morphology was seen in heart tissues of diabetic mice treated with S3I-201 and erlotinib as non-diabetic control mice (Fig. 2A, top panel). Staining of heart tissues with Mason's Trichrome stain (Fig. 2A, middle panel and Fig. 2B) and Sirius Red (Fig. 2A, bottom panel; Fig. 2C), showed 2- to 3-fold increased level of fibrosis and collagen deposition in diabetic mouse tissues compared to non-diabetic mice and diabetic mice treated with S3I-201 and erlotinib. These results indicate that inhibiting EGFR or STAT3 was able to prevent diabetes-induced cardiac fibrosis. We confirmed these findings by measuring transcript and protein levels of COL1 and TGFβ. In agreement with our histological findings, heart tissues of diabetic mice exhibited increased levels of *COL1* and *TGFβ* mRNA (Fig. 2D, left two panels) as well

as significant increases in protein levels (Fig. 2E, top two rows). These increases were not seen in tissues of diabetic mice treated with S3I-201 and erlotinib (Fig. 2D, E and Supplementary Fig. 2A, B).

To supplement our studies, we measured markers of cardiac hypertrophy, alpha isoform of myosin heavy chain (MYH6) and ANP. Transcripts (Fig. 2D, right two panels) and protein levels (Fig. 2E, 3–4th rows) of both hypertrophy markers were elevated in heart tissues of diabetic mice. As expected, treatment of diabetic mice with the STAT3 inhibitor or EGFR inhibitor was associated with a lack of hypertrophy factor induction (Fig. 2D, E and Supplementary Fig. 2C, D). Overall, these results suggest that the progression and severity of DCM likely involved EGFR-STAT3 activation, as inhibiting the axis preserved cardiac function and prevented molecular and structural manifestation of diabetes.

High glucose activates EGFR-STAT3 signaling in cardiomyocytes

To confirm our animal model findings, we challenged cultured cardiomyocytes with HG (33 mM) and assessed the phosphorylation of Tyr-705 residue of STAT3 (p-STAT3) as an indicator of its activation (Pipicz *et al.* 2018). In the rat cardiomyocyte cell line H9C2 and primary cardiomyocytes, HG increased p-STAT3 maximally within 30 min of the exposure to HG, and the levels remained elevated up to 120 min (Fig. 3A and Supplementary Fig. 3A). HG-induced increased p-STAT3 levels were associated with increased p-STAT3 localization to cardiomyocyte nuclei (Fig. 3B and Supplementary Fig. 3B), indicating nuclear translocation of the activated STAT3. Pretreatment of cells with the selective STAT3 inhibitor S3I-201 (Siddiquee *et al.* 2007) for 1 h prevented HG-induced increases in p-STAT3 (Fig. 3C and Supplementary Fig. 3C) as well as the nuclear p-STAT3 (Fig. 3D). These results provide

evidence that HG-activated STAT3 in cardiomyocytes. As previous studies indicated that a potential mechanism of STAT3 activation was through interleukin-6 (IL6) (Kodama *et al.* 1998), we determined whether HG-induced STAT3 activation was also associated with the production of IL6 by cardiomyocytes. Interestingly, our results show that HG treatment for up to 120 min (time corresponding to STAT3 activation) did not alter the mRNA or protein levels of IL6 (Fig. 3E). These results suggest that HG-induced STAT3 activation may be independent of the classical IL6 mechanism.

Our previous study showed a rapid activation of EGFR in cardiomyocytes within 5 min of exposure to HG (Liang *et al.* 2015). This observation, together with the findings from our diabetic mouse studies, led us to postulate that HG-induced STAT3 activation in cardiomyocytes may be mediated through EGFR activation. Consistent with our previous study, stimulation of H9C2 with HG resulted in the rapid onset of EGFR phosphorylation by 5 min,

Figure 3

HG activates STAT3 in cardiomyocytes. (A) Rat cardiomyocyte cell line H9C2 and primary rat cardiomyocytes (PMC) were exposed to HG (33 mM) for up to 120 min. Levels of STAT3 or STAT3 phosphorylated at Tyr-705 (p-STAT3) were determined by Western blotting. Representative figure of three separate determinations is shown. (B) Levels of nuclear p-STAT3 in H9C2 cells were determined from nuclear cell fractions. Cell treatments were as outlined in (A). Lamin B was used as loading control ($n = 3$). (C) H9C2 cells were pretreated with the STAT3 selective inhibitor, S3I-201, for 1 h. Cells were then exposed to HG and the levels of p-STAT3 were determined by Western blot analysis. DMSO as vehicle control. A representative image from three independent studies is shown. (D) Immunofluorescence staining for p-STAT3 (red) was performed in H9C2 cells exposed to HG for 1 h, following pretreatment with S3I-201 (1 h at 10 μ M). DMSO as vehicle control (DAPI = nuclear fluorescence stain (blue); $n = 3$). (E) Measurement of IL6 mRNA and protein levels in H9C2 cells exposed to HG for up to 2 h. Data are reported as mean $\pm$ s.e.m. and relative to '0 min'; $n = 3$.

Figure 4

HG activates STAT3 through EGFR signaling. (A) H9C2 cells were stimulated with HG (33 mM) for up to 30 min, and Western blot analysis was performed to assess EGFR and phosphorylated EGFR (p-EGFR) ($n = 3$). (B) H9C2 cells were pretreated with erlotinib at 10 μ M for 30 min or S3I-201 at 10 μ M for 30 min. Cells were then exposed to HG for 5 min and levels of p-EGFR and p-STAT3 were determined ($n = 3$). (C) Treatment and analysis of primary rat cardiomyocytes (PMC) was performed to confirm the results presented in panel B ($n = 3$). (D) Western blot analysis for EGFR content in H9C2 cells following transfection with EGFR siRNA (si-EGFR) or negative control siRNA (NC). GAPDH was used as loading control ($n = 3$). (E) Western blot analysis for p-STAT3 in HG-stimulated H9C2 cells following transfection with si-EGFR or NC. GAPDH was used as loading control ($n = 3$). The statistical data were shown in the Supplementary file.

which was sustained for up to 30 min (Fig. 4A and Supplementary Fig. 4A). When H9C2 cells were pretreated with 10 μ M erlotinib, the levels of HG-induced EGFR and STAT3 phosphorylation were reduced (Fig. 4B). In contrast, S3I-201 pretreatment reduced HG-induced STAT3 phosphorylation but not EGFR phosphorylation (Fig. 4B and Supplementary Fig. 4B). These results potentially highlight that STAT3 activation is downstream of EGFR. Similar findings were obtained when assessing primary cardiomyocytes (Fig. 4C and Supplementary Fig. 4C). We confirmed the regulation of STAT3 by EGFR by knocking down the expression of EGFR using siRNA transfection. Our results show that siRNA transfection led to approximately 70% decrease in EGFR protein levels (Fig. 4D and Supplementary Fig. 4D), and this level of reduction was sufficient to prevent HG-induced STAT3 phosphorylation (Fig. 4E and Supplementary Fig. 4E). These results are consistent with the notion that HG-stimulated EGFR activation, which in turn, activated STAT3 in cardiomyocytes.

High glucose promotes fibrotic and hypertrophic gene expression in cardiomyocytes through STAT3

After showing that HG challenge of cardiomyocytes induces STAT3 activation through EGFR, findings consistent with our *in vivo* data, we determined whether STAT3 was involved in regulating genes involved in extracellular matrix deposition and cellular hypertrophy. We pretreated H9C2 cells with a range of S3I-201 concentrations (2.5–10.0 μ M) for 1 h and then challenged the cells with HG. RT-qPCR analyses showed

that HG-induced *COL1*, *TGFB*, *MYH6* and *ANP* mRNA levels after 8 h (Fig. 5A). These inductions were not seen when H9C2 cells were pretreated with increasing concentrations of S3I-201. Moreover, increased protein levels of these factors were also suppressed by S3I-201 pretreatment, 24 h after HG exposure (Fig. 5B and Supplementary Fig. 5). Evaluation of the H9C2 cell actin cytoskeleton using rhodamine-phalloidin indicated that HG treatment for 24 h increased the cell size by five-fold over control cells (Fig. 5C and D). S3I-201 pretreatment at 10 μ M significantly reduced HG-induced increases in cardiomyocyte size (Fig. 5C and D). These results indicated that STAT3 inhibition effectively prevents HG-induced fibrosis and hypertrophy in cardiomyocytes. In addition, cardiomyocyte apoptosis is also an important index in diabetic cardiomyopathy. We also examined the apoptotic marker Bcl-2/Bax ratio. The data in Supplementary Fig. 6 showed that inhibition of STAT3 or EGFR by specific inhibitors reversed HG-induced apoptosis in cardiomyocytes. To avoid potential confounding off-target effects of small-molecule inhibitors, we tested the cells following knockdown of STAT3 in H9C2 cells. siRNA against STAT3 reduced protein levels to less than 10% compared to levels found in negative control transfected cells (Fig. 5E and Supplementary Fig. 7A). Transfected cells were then challenged with HG for 24 h and protein levels of COL1, TGFB and ANP were measured. Our results show significantly reduced levels of these factors relative to the negative control siRNA group exposed to HG (Fig. 5F and Supplementary Fig. 7B). However, levels of α MyHC were only modestly reduced. Overall, our results show that STAT3 promotes the expression of several key

Figure 5

HG-activated STAT3 promotes fibrotic and hypertrophic gene expression in cardiomyocytes. (A) H9C2 cells were pretreated with S31-201 at 2.5, 5 and 10 μ M for 1 h, and stimulated with HG (33 mM) for 8 h. mRNA levels of *COL1*, *TGF β* , *MYH6* and *ANP* were then measured by qPCR (data normalized to β -actin mRNA and reported as mean $\pm$ s.e.m. relative to Ctrl; $n = 3$; * $P < 0.05$, ** $P < 0.01$ compared to Ctrl; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to HG). (B) Protein levels of COL1, TGF- β , MYH6 and ANP in cells. Cells were treated as in panel A with the exception that HG exposure was carried out for 24 h. DMSO was used as vehicle control; GAPDH was as loading control ($n = 3$). (C) F-actin (red) was detected by rhodamine-phalloidin staining of H9C2 cells stimulated by HG alone or HG with S31-201 pretreatment (10 μ M for 1 h). DMSO was used as vehicle control for S31-201 pretreatment ($n = 3$). (D) Quantification of H9C2 cell size from rhodamine-phalloidin staining data (area values reported as mean $\pm$ s.e.m. relative to DMSO control; ### $P < 0.001$ compared to DMSO, ** $P < 0.01$ compared to HG). (E) STAT3 was knocked down in H9C2 cells by siRNA transfection or negative control sequences (NC). Representative Western blot showing levels of STAT3. GAPDH was as loading control ($n = 3$). (F) H9C2 cells with STAT3 knocked down were stimulated with HG for 24 h and Western blot was performed to detect COL1, TGF β , MYH6 and ANP (NC = negative control, GAPDH = loading control, $n = 3$).

tissue remodeling genes in cardiomyocytes, implicating the significance of STAT3 in development of DCM.

Discussion

We tested the hypothesis that the development and progression of DCM are driven by the EGFR-STAT3 signaling axis. The following central pieces of data support our hypothesis: (i) Increased STAT3 phosphorylation was found in myocardial tissue of diabetic mice and in cultured cardiomyocytes exposed to HG; (ii) blocking EGFR reduced increased STAT3 phosphorylation levels in diabetic mice and cardiomyocyte cultures, and blocking STAT3 or EGFR reduced the expression of genes known to promote fibrosis and hypertrophy; (iii) STAT3 or EGFR inhibition preserved cardiac function and structural integrity in diabetic mice. These results provide strong

evidence that the EGFR-STAT3 signaling axis is a crucial factor in the development and progression of DCM.

Our findings confirmed our previous report (Zou *et al.* 2017) showing activation of EGFR in HG-stimulated cardiomyocytes and in myocardial tissue of diabetic mice. Importantly, inhibition of the activated EGFR prevented STAT3 phosphorylation, whereas the inhibition of STAT3 had no effect on EGFR activation. These data indicated that STAT3 was a downstream target of EGFR signaling. Further support for this EGFR-STAT3 signaling axis was the observation that blockade of STAT3 resulted in comparable degrees of inhibition as the blockade of EGFR on cardiac fibrosis and hypertrophy responses, as well as deficits of myocardial structure and function.

The EGFR-STAT3 signaling may be particularly important in the myocardial tissue. Tyrosine kinase inhibitors, including erlotinib, used for cancer patients are commonly associated with the occurrence of adverse

cardiac toxicities such as heart failure and left ventricular dysfunction (Srikanthan *et al.* 2015, Vermeulen *et al.* 2016). It is quite possible that cardiac tissue has a relatively higher expression of EGFR than other tissues, which may contribute to this higher sensitivity to EGFR inhibitors. This is consistent with reports from experimental animal models in which the conditional overexpression (Sysa-Shah *et al.* 2012) or mutation of epidermal growth factor receptor 2 (ErbB2) (Ozcelik *et al.* 2002) in mice induces hypertrophic cardiomyopathy. An additional factor could be attributed to the ability of an expanding number of G-protein coupled receptors (GPCRs) that can transactivate EGFR in cardiac tissue (Grisanti *et al.* 2017). GPCRs constitute the largest receptor superfamily known, and because of its wide-ranging role in cardiovascular disorders, greater than 40% of all current therapeutics are targeted to GPCRs. Thus, in the diabetes context, a variety of GPCR ligands may activate their receptors (e.g., adenosine, adrenergic, aldosterone, angiotensin II, bradykinin, δ -opioid, endothelin-1, muscarinic, prostaglandin E₂, protease-activated, sphingosine-1-phosphate, urotensin II) (Grisanti *et al.* 2017), which in turn, can transactivate EGFR and induce cardiomyopathies. Even in endotoxemic heart failure, lipopolysaccharide-mediated EGFR transactivation has been implicated (Sun *et al.* 2015). Although it remains to be determined the specific mechanisms by which EGFR harbors cardiac-selective activity, these reports clearly indicate that the maintenance of appropriate EGFR signaling is vital for cardiac health.

Analysis of the echocardiographic data indicated that STAT3 activity induced significant increases in ventricular and interventricular wall thickness in diabetic mice. We suspect that these increases were from the combination of increased fibrosis as well as cardiomyocyte hypertrophy, as revealed by the histochemical findings. An important factor responsible for such changes is the increased expression of TGF- β in HG-stimulated cardiomyocytes and myocardial tissue in diabetic mice. Many different cell types in the heart may contribute to the elevation of TGF- β (Dobaczewski *et al.* 2011). Subsequent to its production, TGF- β may exert potent and diverse effects on the different cell types. For example, TGF- β may regulate cardiac fibrosis through the stimulation of fibroblast proliferation, fibroblast differentiation into myofibroblasts, extracellular matrix synthesis (i.e., collagen types I, III, and IV, proteoglycans, laminin and fibronectin), as well as the simultaneous inhibition of collagenase and degradative matrix metalloproteinases (Dobaczewski *et al.* 2011, Ma *et al.* 2017). Moreover, increased TGF- β and ANP

levels in our experimental models likely contributed to the cardiomyocyte hypertrophy observed. Our findings are consistent with previous reports showing that cardiac TGF- β is upregulated in patients with myocardial hypertrophy (Li *et al.* 1997) and is correlated with the development of fibrosis (Hein *et al.* 2003).

STAT3 plays a variable role in different heart diseases. STAT3 activation has been reported to mediate cardioprotection against ischemia/reperfusion injury (Oshima *et al.* 2005). Previous reports also showed that STAT3 mediates angiotensin II-induced heart injuries (Xue *et al.* 2015) and small-molecule inhibitor of STAT3 was found to reverse these injuries (Skoumal *et al.* 2011). However, a few studies using STAT3-knockout mice found aggravated heart injuries in mice lacking STAT3 (Zouein *et al.* 2013). Furthermore, cardiomyocyte-specific STAT3 deficiency has also been shown to impair cardiac contractility in hypertensive mice (Altara *et al.* 2016). However, these later findings may reflect a role of STAT3 in heart development and cardiomyogenesis (Foshay *et al.* 2005). Both whole body knockout and cardiomyocyte-specific knockout of STAT3 may affect the cardiac development and function, which may aggravate the cardiac injury under pathological conditions. It would be important to study inducible cardiomyocyte-specific STAT3 knockout during specific stresses to rule out the role of STAT3 during development.

In summary, the combined data obtained from studies using cultured cardiomyocytes and STZ-induced type 1 diabetic mouse model indicated that the EGFR-STAT3 signaling axis was important in promoting cardiac fibrosis and cardiomyocyte hypertrophy, and thereby resulting in heart structural and functional deficits in DCM. These findings also support small-molecule inhibitors of EGFR and STAT3 as a potential therapeutic strategy for the management of DCM.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0058>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors approved the final version of the manuscript. W L, L H, J W, F Z, X Z and H Y performed the research. Y W, C Z, G L and Y Q designed the research study. Y W, Y Q and J W analyzed the data. Y W and W L wrote the paper.

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