British Journal of Pharmacology

DOI:10.1111/bph.13002
www.brjpharmacol.org

Themed Section: Chinese Innovation in Cardiovascular Drug Discovery

RESEARCH PAPER

Genistein alleviates pressure overload-induced cardiac dysfunction and interstitial fibrosis in mice

Wei Qin[1,*], Ning Du[1,*], Longyin Zhang[1,*], Xianxian Wu[1], Yingying Hu[1], Xiaoguang Li[1], Nannan Shen[1], Yang Li[1], Baofeng Yang[1,2], Chaoqian Xu[1], Zhiwei Fang[1], Yanjie Lu[1,2], Yong Zhang[1,2] and Zhimin Du[3]

[1]*Department of Pharmacology (State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, Harbin, Heilongjiang, China,* [2]*Institute of Cardiovascular Research, Harbin Medical University, Harbin, Heilongjiang, China, and* [3]*Institute of Clinical Pharmacy, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China*

Correspondence
Professor Yong Zhang, Department of Pharmacology, Harbin Medical University, 157 Baojian Rd, Harbin, Heilongjiang 150081, China. E-mail: hmuzhangyong@hotmail.com or Professor Zhimin Du, Institute of Clinical Pharmacy, The Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Rd, Harbin, Heilongjiang 150086, China. E-mail: dzm1956@126.com

*These authors contributed equally to this work.

Received
4 June 2014
Revised
23 October 2014
Accepted
28 October 2014

BACKGROUND AND PURPOSE

Pressure overload-induced cardiac interstitial fibrosis is viewed as a major cause of heart failure in patients with hypertension or aorta atherosclerosis. The purpose of this study was to investigate the effects and the underlying mechanisms of genistein, a natural phytoestrogen found in soy bean extract, on pressure overload-induced cardiac fibrosis.

EXPERIMENTAL APPROACH

Genisten was administered to mice with pressure overload induced by transverse aortic constriction. Eight weeks later, its effects on cardiac dysfunction, hypertrophy and fibrosis were determined. Its effects on proliferation, collagen production and myofibroblast transformation of cardiac fibroblasts (CFs) and the signalling pathways were also assessed *in vitro*.

KEY RESULTS

Pressure overload-induced cardiac dysfunction, hypertrophy and fibrosis were markedly attenuated by genistein. In cultured CFs, genistein inhibited TGFβ1-induced proliferation, collagen production and myofibroblast transformation. Genistein suppressed TGFβ-activated kinase 1 (TAK1) expression and produced anti-fibrotic effects by blocking the TAK1/MKK4/JNK pathway. Further analysis indicated that it up-regulated oestrogen-dependent expression of metastasis-associated gene 3 (MTA3), which was found to be a negative regulator of TAK1. Silencing MTA3 by siRNA, or inhibiting the activity of the MTA3-NuRD complex with trichostatin A, abolished genistein's anti-fibrotic effects.

CONCLUSIONS AND IMPLICATIONS

Genistein improved cardiac function and inhibited cardiac fibrosis in response to pressure overload. The underlying mechanism may involve regulation of the MTA3/TAK1/MKK4/JNK signalling pathway. Genistein may have potential as a novel agent for prevention and therapy of cardiac disorders associated with fibrosis.

LINKED ARTICLES

This article is part of a themed section on Chinese Innovation in Cardiovascular Drug Discovery. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-23

Abbreviations

CFs, cardiac fibroblasts; CTGF, connective tissue growth factor; ECM, extracellular matrix; EdU, 5-ethynyl-2′-deoxyuridine; EF, ejection fraction; ER, oestrogen receptors; FS, fractional shortening; MTA3, metastasis-associated gene 3; NuRD, nucleosome remodelling and deacetylase; TAC, transverse aortic constriction; TAK1, TGFβ-activated kinase 1; TSA, trichostatin A

Tables of Links

TARGETS	
Nuclear hormone receptors[a]	**Enzymes**[b]
Oestrogen receptor: ERα	Endothelial (e) NOS
Oestrogen receptor: ERβ	Histone deacetylase (HDAC)
Other protein targets	Inducible (i) NOS
Kelch-like ECH-associated protein 1 (Keap1)	JNK
	P38-MAPK
	Smad2/3
	TAK1

LIGANDS	
Aliskiren	ICI182780
Col1a1	Isoprenaline
Col3a1	TGFβ1
Curcumin	Trichostatin A (TSA)
Genistein	Wnt4

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Cardiac fibrosis is a crucial pathological process occurring in a variety of heart diseases that are associated with myocardial stiffness and cardiac systolic and diastolic dysfunction (Creemers and Pinto, 2011). This process is characterized by excess production and deposition of extracellular matrix (ECM) proteins such as collagen I, collagen III and fibronectin (Cavalera *et al.*, 2014; Kong *et al.*, 2014), which cause cardiac stiffness and eventually heart failure. Hence, the discovery of agents that can control cardiac fibrosis is useful for the treatment of heart disease.

Genistein (4′,5,7-trihydroxy isoflavone, Figure 1), a natural phytoestrogen found in soy extract, has been reported to produce anti-cancer and anti-inflammatory effects (Si and Liu, 2007; Valsecchi *et al.*, 2008). It also possesses cardioprotective properties, such as attenuating isoprenaline-induced cardiac hypertrophy (Maulik *et al.*, 2012). However, the role of genistein in pressure overload-induced cardiac fibrosis has yet to be explored.

Cardiac fibrosis can be triggered by many signalling molecules, of which, TGFβ1 plays a central role in activating the proliferation, collagen production and myofibroblast transformation of cardiac fibroblasts (CFs; Dobaczewski *et al.*, 2011; Meyer *et al.*, 2012). In fibroblasts, TGFβ-activated kinase 1 (TAK1) mediates TGFβ1-induced fibronectin expression, and depletion of TAK1 inhibits the pro-fibrotic effect of TGFβ1 (Shi-wen *et al.*, 2009). TAK1 functions by activating the downstream MKK3/p38-MAPK cascade or MKK4/JNK cascade (Hocevar *et al.*, 2005; Kim *et al.*, 2007).

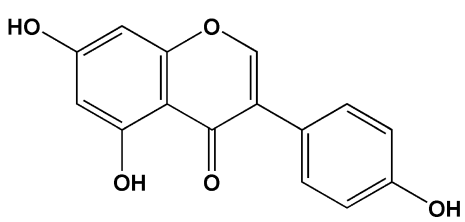

Figure 1
Chemical structure of genistein.

Metastasis-associated gene 3 (MTA3), an oestrogen-dependent component of the nucleosome remodelling and deacetylase (NuRD) complex, was identified as a transcriptional repressor of multiple genes, such as Snail and CGB5 (Chen *et al.*, 2013). Studies have shown that MTA3 plays a critical role in cancer metastasis (Chu *et al.*, 2014), lymphocyte differentiation (Fujita *et al.*, 2004) and embryo development (Li *et al.*, 2009). However, the expression and function of MTA3 in heart is unclear.

In this study, we found that genistein attenuated pressure overload-induced cardiac dysfunction and fibrosis. Our results also suggest that genistein exerts its anti-fibrotic effect by inhibiting TAK1 expression and the subsequent phosphorylation of MKK4/JNK. Moreover, the up-regulation of MTA3 by genistein is responsible for the down-regulation of TAK1 and its anti-fibrotic effects. These findings allow us to conclude that genistein inhibits pressure overload-induced cardiac fibrosis through the MTA3/TAK1/MKK4/JNK pathway, and it might be a promising therapeutic agent for adverse cardiac remodelling.

Methods

Animal welfare and ethical statements

All the experimental procedures were in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039), UK, and the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The procedures were approved by the Institutional Animal Care and Use Committee and Ethics Committee of the Harbin Medical University [SYXK 2011-033 (2011.12.13–2016.12.12)] and complied with the standards of animal welfare in China. All experimental procedures were conducted as humane as possible. A total of 49 healthy male Kunming mice weighing 20–25 g were housed in conditions in which an artificial 12 h dark–light cycle was maintained, and food and water were available *ad libitum*.

The animals were kept in standard animal rooms with temperature 20–22°C and humidity 55–60%.

Mice model of transverse aortic constriction (TAC) and genistein administration

A total of 49 animals were randomly divided into seven groups: control, genistein (100 mg·kg^{-1}·day^{-1}), sham, TAC and TAC+genistein (10, 50 and 100 mg·kg^{-1}·day^{-1}) groups. The genistein dosage was determined according to the dosage used in previous studies (Hool *et al.*, 1998; Cong *et al.*, 2009; Qi *et al.*, 2011; Fan *et al.*, 2013; Chinigarzadeh *et al.*, 2014). Animals were anaesthetized with pentobarbital sodium (6 mg·kg^{-1}, i.p.) as assessed by interdigital reflex. Then, TAC was performed as previously described (Yasuno *et al.*, 2013). Sham-operated animals underwent open chest procedures without aorta constriction. Genistein was administered to animals daily, intragastrically, three days post-surgery for 8 consecutive weeks. Other groups were given equal volumes of saline.

Cardiac echocardiography

Eight weeks after drug administration, mice were anaesthetized with pentobarbital sodium (6 mg·kg^{-1}, i.p.) for cardiac echocardiography using an ultrasound machine (Vivid 7, GE Medical System, Milwaukee, WI, USA). The left ventricular ejection fraction (EF) and fractional shortening (FS) were calculated from the M-mode recording.

Measurements of collagen deposition and cardiomyocyte area

After echocardiographic recording, the hearts of mice were excised and fixed in 4% paraform, and then embedded in paraffin. The preparations were cut cross-sectionally into 5 μm thick sections. Masson's trichrome staining was performed to view collagen accumulation as previously described (Shan *et al.*, 2009). Haematoxylin and eosin (HE) staining was applied to view the cardiomyocytes. The stained sections were viewed with a microscope and the images were analysed with ImagePro Plus software (Media Cybernetics, Bethesda, MD, USA) to quantify collagen percentage and cardiomyocyte area.

Fibroblast cultures and transfection

The cultured CFs were obtained from 1- to 3-day-old neonatal rat hearts as described previously (Chen *et al.*, 2014). Briefly, after being treated with 0.25% trypsin, CFs were collected by discarding the cloudy medium containing cardiomyocytes after 1–2 h incubation at 37°C. Fresh DMEM containing 10% FBS was added into the culture flask. CFs at the second or third passages were used in our experiments. Cells were transfected with MTA3 siRNA as specified. The siRNA was constructed by Shanghai GenePharma Co. Ltd (Shanghai, China) and transfected following the manufacturer's instructions.

Luciferase reporter activity assay

The promoter regions of TAK1 were amplified and subcloned into pGL3-basic (Promega Corporation, Madison, WI, USA). Next, CFs cultured in a 24-well plate were transiently transfected with siRNA along with 1 μg of plasmid and 0.05 μg of the internal control plasmid (SV40, expressing *Renilla* luciferase) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega Corporation) 24 h post-transfection. Assays were repeated three times and promoter activity was determined by the ratio of firefly over *Renilla*.

5-Ethynyl-2′-deoxyuridine (EdU) labelling

To determine the proliferation rate of CFs, sterilized coverslips were placed into 24-well plates seeded with CFs. After incubation, the medium was discarded and 200 μL EdU was added to the wells. Then cells were incubated at 37°C for 2 h. Hoechest33342 was used to stain the nuclei. Cells were examined under a fluorescence microscope.

Measurement of cell viability and collagen production

The MTT assay was used to measure CFs viability as previously described (Pan *et al.*, 2012; Xu *et al.*, 2014). Briefly, 20 μL of MTT solution was added to a 96-well plate seeded with CFs and incubated for 4 h. The medium was then discarded, followed by the addition of 150 μL of DMSO. Finally, absorbance at 570 nm was recorded. Collagen production was detected using a Sircol soluble collagen assay kit (Biocolor Ltd., Carrickfergus, County Antrim, UK) according to the manufacturer's protocol. Briefly, collagen that was produced by the CFs was dissolved in cold acid. Then the Sircol Dye Reagent was used to stain the collagen. Absorbance at 555 nm was recorded and the collagen content was calculated.

Immunofluorescence microscopy

Immunofluorescent staining was performed on CFs to determine α-SMA expression. Briefly, cells were fixed with 4% paraformaldehyde for 10 min at 37°C, and then treated with 0.4% Triton x-100 for 1 h. After being blocked with goat serum in PBS, the cells were incubated with α-SMA antibody at 4°C overnight. Secondary antibody conjugated with Alex Fluro 594 was added and incubated with the cells for 1 h. The cells were then incubated with DAPI (1:100) for another 1 h. Cells were examined and photographs were taken by fluorescence microscope.

Real-time RT-PCR

RNA was isolated using TRIZOL reagent as previously described (Lu *et al.*, 2009; Zhang *et al.*, 2013b), and was reverse transcribed. The first-strand cDNA was used for real-time PCR to quantify mRNA expression with GAPDH as an internal control. The relative mRNA level was presented as values of $2^{-\Delta Ct}$ [ΔCt = Ct (gene of interest) – Ct (GAPDH)]. The sequences of primers were connective tissue growth factor (CTGF), forward: 5′-CTTCTGCAGACTGGAGAAGC-3′, reverse: 5′-CAGCCAGAAAGCTCAAACTTG-3′. Collagen 1a1 (Col1a1), forward: 5′-CAATGGCACGGCTGTGTGCG-3′, reverse: 5′-CACTCGCCCTCCCGTCTTTGG-3′. Collagen 3a1 (Col3a1), forward: 5′-TGAATGGTGGTTTTCAGTTCAG-3′, reverse: 5′-GATCCCATCAGCTTCAGAGACT-3′. MTA3, forward: 5′-CAACGACATTCGTCAGGACT-3′,reverse: 5′-TTCGGCATG TCTGTCTGC-3′. GAPDH, forward: 5′-AAGAAGGTGGTGAAG CAGGC-3′, reverse: 5′-TCCACCACCCAGTTGCTGTA-3′.

Western blot analysis

Heart tissues and cultured CFs were lysed in RIPA buffer. Proteins were isolated as described previously by our laboratory (Zhang *et al.*, 2010; 2014). Protein extracts (100 μg) were subjected to electrophoresis in 10% SDS-PAGE and transferred to nitrocellulose membranes. Then, the nitrocellulose membranes were probed with TAK1, MKK4, JNK, p-TAK1 ($Thr^{184/187}$), p-MKK4 (Ser^{257}/Thr^{261}), p-JNK (Thr^{183}/Tyr^{185}; Cell Signaling Technology, Inc., Danvers, MA, USA), α-SMA or MTA3 (Abcam, Inc., Cambridge, MA, USA) and GAPDH (internal control; Zhongshanjinqiao, Inc., Beijing, China) antibodies overnight at 4°C. Membranes were then incubated with a secondary antibody and scanned by Odyssey Imaging System (LI-COR, Inc., Lincoln, NE, USA).

Design and statistical analysis

Experimental subjects/preparations were randomized to groups and equal group sizes were obtained. Group assignments, data recording and data analysis were blinded to the operator. Data are shown as mean ± SEM from at least five independent experiments (Curtis *et al.*, 2014). ANOVA in conjunction with Turkey multiple-comparisons test (GraphPad Prism version 5.0) was used for comparisons between groups. $P < 0.05$ was considered as statistically significant.

Materials

Genistein (purity > 95%) was obtained from Xi'an QingYue Biotechnology Co, Ltd (Xi'an, Sha'anxi, China). TGFβ1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). ICI 182,780 was purchased from Abcam, Inc. Trichostatin A (TSA) and TAK1 inhibitor (5Z)-7-oxozeaenol were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Effect of genistein on pressure overload-induced cardiac dysfunction

We performed echocardiographic measurements 4 and 8 weeks after TAC surgery and genistein administration. The results showed that EF and FS values were unchanged in the 4-week TAC-treated hearts, suggesting that heart function was still in a compensatory state (Supporting Information Fig. S1), whereas in the 8-week TAC treated hearts these values were markedly decreased, being indicative of heart failure. Genistein administration significantly alleviated these changes in a dose-dependent manner (Figure 2A–C, Supporting Information Table S1). Genistein had no effect on the echocardiographic parameters of normal mice.

Effect of genistein on pressure overload-induced cardiac hypertrophy

It is known that pathological pressure overload can cause cardiac hypertrophy. We calculated the heart weight/tibia length (HW/TL) ratio of mice as an index of change in heart size. The results showed that HW/TL ratio was dramatically increased in the TAC group, indicating increased weight of the heart. As expected, genistein reduced the HW/TL ratio in a dose-dependent manner (Table 1). Moreover, the hearts

Table 1
Tissue weights in normal or TAC mice with or without treatment with genistein

	Control	Genistein (100 mg·kg^{-1}·day^{-1})	Sham	TAC	TAC+genistein (10 mg·kg^{-1}·day^{-1})	TAC+genistein (50 mg·kg^{-1}·day^{-1})	TAC+genistein (100 mg·kg^{-1}·day^{-1})
BW (g)	36.37 ± 2.26	36.68 ± 1.42	37.17 ± 1.77	36.78 ± 1.27	36.19 ± 1.16	36.52 ± 1.50	37.27 ± 2.45
HW/TL (mg·mm^{-1})	9.39 ± 0.28	9.42 ± 0.46	9.49 ± 0.28	13.94 ± 0.57*	11.60 ± 0.57$^{\#}$	10.58 ± 0.35$^{\#}$	9.84 ± 0.40$^{\#}$
HW/BW (mg·g^{-1})	4.63 ± 0.09	4.65 ± 0.21	4.58 ± 0.17	6.88 ± 0.14*	5.80 ± 0.19$^{\#}$	5.19 ± 0.24$^{\#}$	4.93 ± 0.15$^{\#}$
LW/BW (mg·g^{-1})	8.19 ± 0.57	8.19 ± 0.54	8.22 ± 0.49	8.78 ± 0.16	8.64 ± 0.08	8.47 ± 0.27	8.28 ± 0.92

Data are expressed as mean ± SEM.
$n = 7$.
*$P < 0.05$ vs. sham.
$^{\#}P < 0.05$ vs. TAC.
BW, body weight; HW, heart weight; LW, lung weight; TAC, transverse aortic constriction; TL, tibia length.

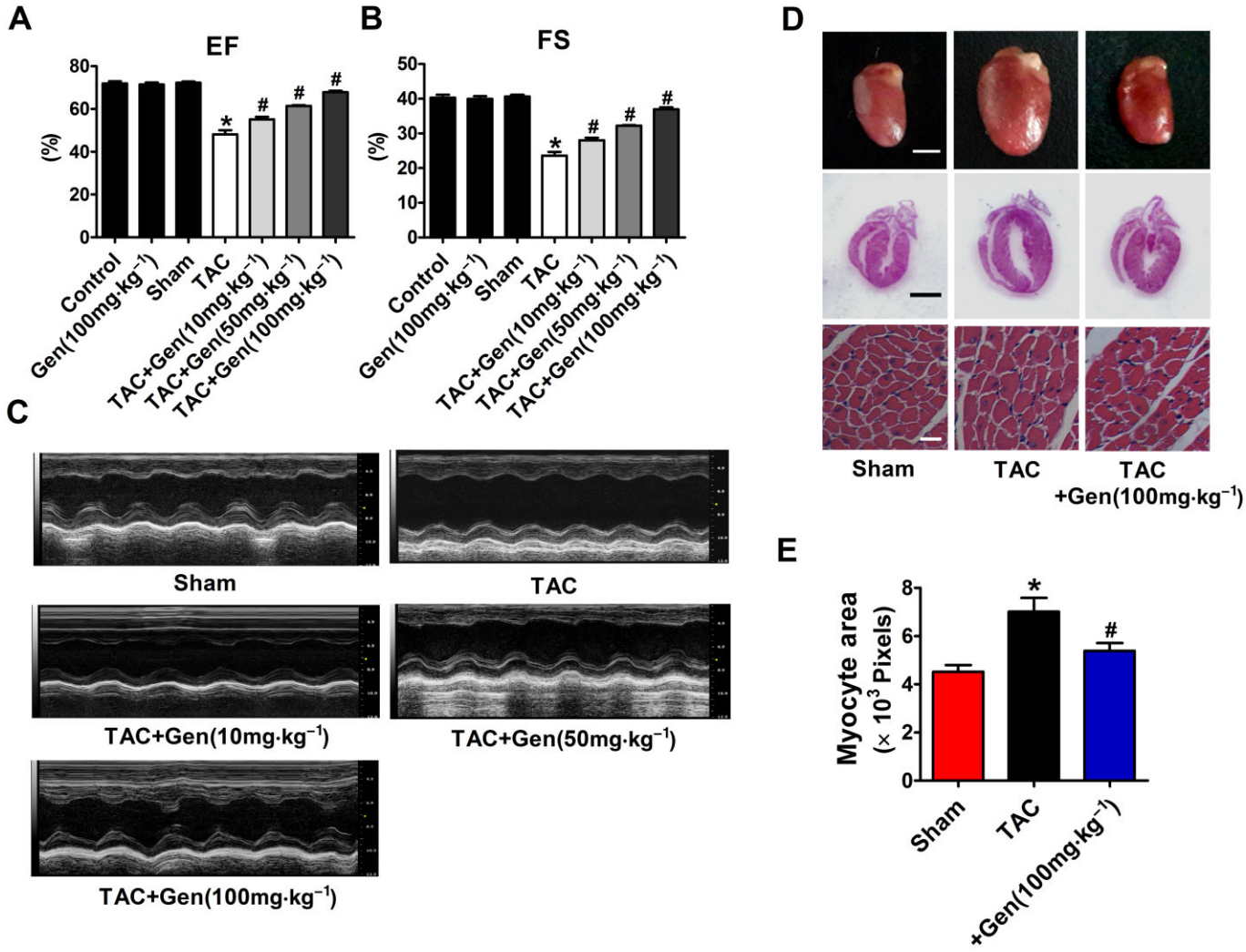

Figure 2

Genistein alleviates pressure overload-induced cardiac dysfunction and hypertrophy in mice. (A and B) EF and FS levels evaluated by echocardiography. $n = 7$. (C) M-mode echocardiographic photos. (D) Hearts from mice subjected to a sham operation or pressure overload (TAC) or TAC with genistein (Gen) administration are shown at the top. Histological sections stained with HE are shown at the bottom. Scale bars: 2 mm (top panel); 2 mm (middle panel), 100 μm (bottom panel). (E) Summarized data of cardiomyocyte cross section area measured from the HE sections. $n = 5$. $^{*}P < 0.05$ vs. sham; $^{\#}P < 0.05$ vs. TAC.

from the TAC group were obviously larger than those from the sham group, while genistein reversed this change (Figure 2D). Histological sections stained with HE show the typical phenotypes of hypertrophy (Figure 2D). The photographs showed that TAC-induced eccentric hypertrophy as indicated by the enlarged left ventricular chamber, while genistein treatment attenuated this change (Figure 2D). Analysis of myocardial cross section area confirmed the ability of genistein to suppress cardiac hypertrophy induced by TAC. As shown in Figure 2D and E, TAC treatment caused the cell area of cardiomyocytes to increase; this was inhibited by genistein administration.

Effect of genistein on cardiac interstitial fibrosis

Next, to explore the possible role of genistein in modulating ECM deposition, we assessed the fibrosis area using Masson's trichrome staining. We found that the cardiac interstitial fibrotic area was markedly increased in the TAC group compared with the sham group ($P < 0.05$). Treatment with genistein was sufficient to abolish TAC-induced ECM deposition in a dose-dependent manner ($P < 0.05$ vs. TAC; Figure 3). The same results were obtained from picrosirius red staining (Supporting Information Fig. S2).

Genistein regulates proliferation and collagen production of cultured CFs

We performed EdU assay in cultured CFs at baseline and after TGFβ1 stimulation (10 ng·mL^{-1}, 24 h) in the presence or absence of genistein (100 μM). The proliferation of CFs was increased by TGFβ1 treatment as indicated by calculating the percentage of EdU positive cells and this change was attenuated significantly by genistein (Figure 4A). The results from the MTT assay further confirmed the anti-proliferation effect of

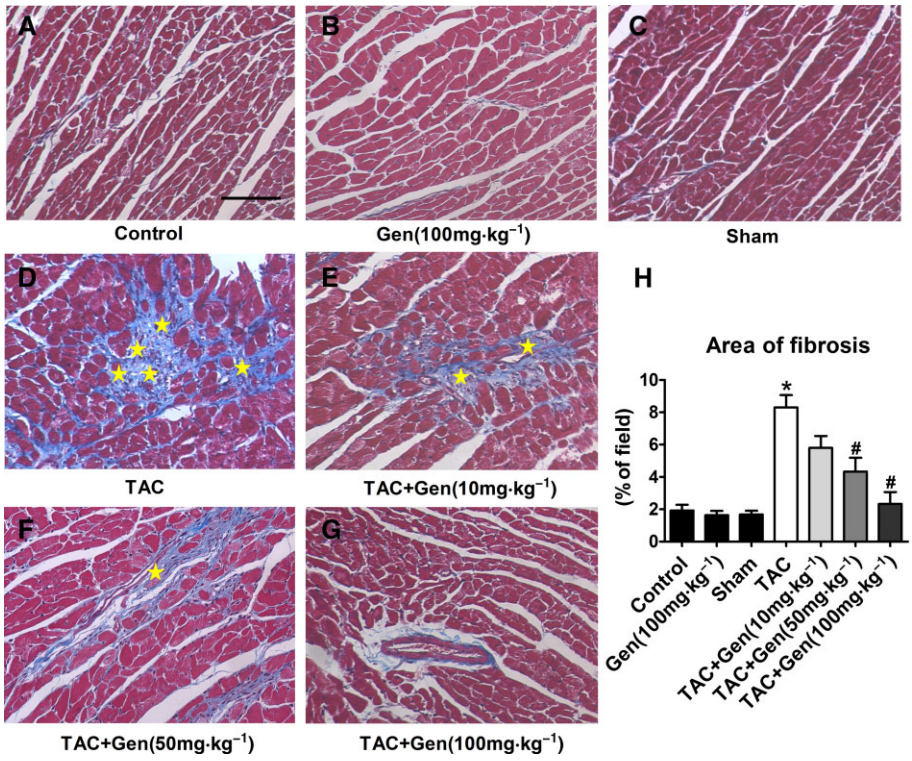

Figure 3

Genistein attenuates pressure overload-induced cardiac interstitial fibrosis. (A–G) Representative photographs of Masson trichrome-stained sections. The collagens are stained blue and indicated by yellow stars. Scale bar indicates 100 μm. (H) Summarized data of cardiac fibrosis ratio measured from the Masson trichrome-stained sections. $n = 6$. $^{*}P < 0.05$ vs. sham; $^{\#}P < 0.05$ vs. TAC.

genistein (Figure 4B). We next assessed the effect of genistein on collagen synthesis of CFs and found that all three doses of genistein markedly inhibited TGFβ1-mediated collagen production (Figure 4C). In addition, genistein also dramatically suppressed the expression of pro-fibrotic genes, such as Col1a1, Col3a1 and CTGF, at the mRNA level (Figure 4D–F).

Genistein regulates the transformation from fibroblast to myofibroblast

Because the process of fibroblast to myofibroblast transformation, characterized by α-SMA up-regulation, plays an important role in cardiac fibrosis, we performed an immunostaining assay to verify whether genistein could repress the expression of α-SMA. Our results showed that TGFβ1 significantly induced the fibroblast to myofibroblast transformation as indicated by the increased percentage of α-SMA-positive cells. Co-application of genistein markedly attenuated this change (Figure 5A). Moreover, genistein also attenuated TGFβ1-mediated up-regulation of α-SMA protein levels (Figure 5B), consistent with the results obtained from the mouse model (Figure 5C).

Genistein regulates the TAK1/MKK4/JNK pathway

To explore the pathway through which genistein exerts its anti-fibrotic effects, we assessed the changes in expression of the fibrotic mediators at the protein level in cultured CFs using Western blot analysis. Since TGFβ1/Smads are the most well-known pro-fibrogenic mediators, we first examined the effects of genistein on p-Smad2/3 and Smad2/3 protein levels. As shown in Supporting Information Fig. S3, genistein did not inhibit the up-regulation of p-Smad2/3 induced by TGFβ1, suggesting that the anti-fibrotic effect of genistein was not mediated by the Smads' signalling. Recent studies have demonstrated that TAK1 plays a critical role in TGFβ1 induced pro-fibrotic responses (Ono *et al.*, 2003; Guo *et al.*, 2013). In this study, we confirmed this effect by treating cells with a TAK1 inhibitor. We found that TAK1 inhibition significantly suppressed TGFβ1 induced proliferation and collagen production of CFs (Supporting Information Fig. S4). As shown in Figure 6A, TAK1 expression was significantly up-regulated in TGFβ1 treated CFs, while genistein inhibited this change in a dose-dependent manner. Moreover, the change in expression of p-TAK1 was consistent with that of the total TAK1 (Figure 6B). Studies have shown that TAK1 can function through the MKK4/JNK signalling cascade to regulate ECM deposition and remodelling (Hocevar *et al.*, 2005). Our data indicated that genistein substantially inhibited the up-regulation of p-MKK4 and p-JNK, while having no effect on the total MKK4 and JNK in TGFβ1-treated CFs (Figure 6C and D). These results were consistent with the data obtained from the mouse model (Supporting Information Fig. S5).

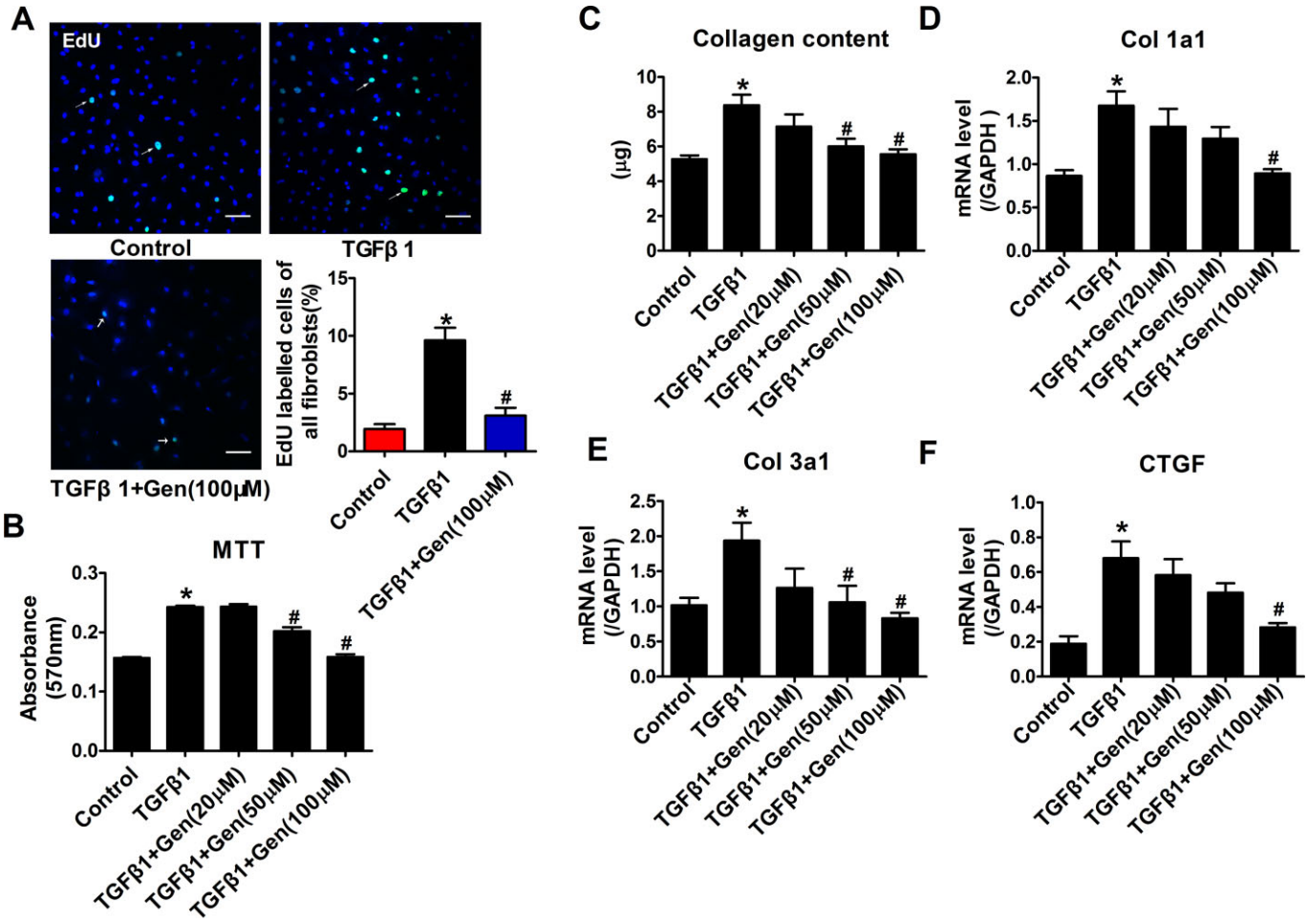

Figure 4

Genistein suppresses TGFβ1-induced proliferation and collagen production of cultured CFs. (A) Representative photographs of EdU assay for cell proliferation and the summarized data of EdU labelled cells ratio. Scale bar indicates 100 μm. Blue indicates Hoechest33342. Arrows indicate EdU labelled cells. $n = 5$. (B) MTT test. $n = 5$. (C) Collagen production assay. $n = 5$. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TGFβ1.

MTA3 expression correlates with TAK1 repression in genistein-treated CFs

We also further investigate the upstream regulator of TAK1 in genistein-treated CFs. While genistein caused a dramatic decline in TAK1 protein level, the addition of the oestrogen receptor (ER) antagonist ICI 182,780 (1 μM) reversed this change (Figure 7A). As expected, ICI 182,780 also significantly abolished the anti-fibrotic effect of genistein (Figure 7B–E). These results indicated that genistein regulated TAK1 in an ER-dependent manner. MTA3, a component of the NuRD transcriptional corepressor complex, was tightly linked to oestrogen's action (Fujita *et al.*, 2003). Therefore, we hypothesized that the down-regulation of TAK1 induced by genistein might result from a gain of MTA3 function. In contrast to TAK1, MTA3 was decreased at both protein and mRNA levels by TGFβ1. However, addition of genistein restored MTA3 expression to control levels and this effect was blocked by ICI 182,780 (Figure 7F). To further validate TAK1 as a regulatory target of MTA3, we designed a siRNA specific for MTA3 and demonstrated that transfection of MTA3 siRNA resulted in a significant decrease in MTA3 protein level (Figure 7G). Analysis of TAK1 expression revealed that depletion of MTA3 increased the TAK1 level in genistein-treated CFs (Figure 7G). Furthermore, the TAK1 promoter-luciferase fusion vector cotransfected with MTA3 siRNA increased TAK1 promoter activities in CFs (Figure 7H). In addition, treatment with the histone deacetylase (HDAC) inhibitor TSA (100 ng·mL^{-1}) restored TAK1 levels in genistein-treated CFs, but did not affect MTA3 levels (Figure 7I), suggesting that MTA3 functions as a component of the NuRD complex.

MTA3 is essential for the anti-fibrotic activity of genistein

The above results suggested that MTA3 acted as a downstream effector of genistein and played an important role in regulating TAK1 expression. Next, we designed experiments to address the role of MTA3 in mediating the anti-fibrotic effect of genistein. The mRNA levels of Col1a1 and Col3a1 were both dramatically decreased after exposure to genistein, whereas transfection of MTA3 siRNA reversed these changes,

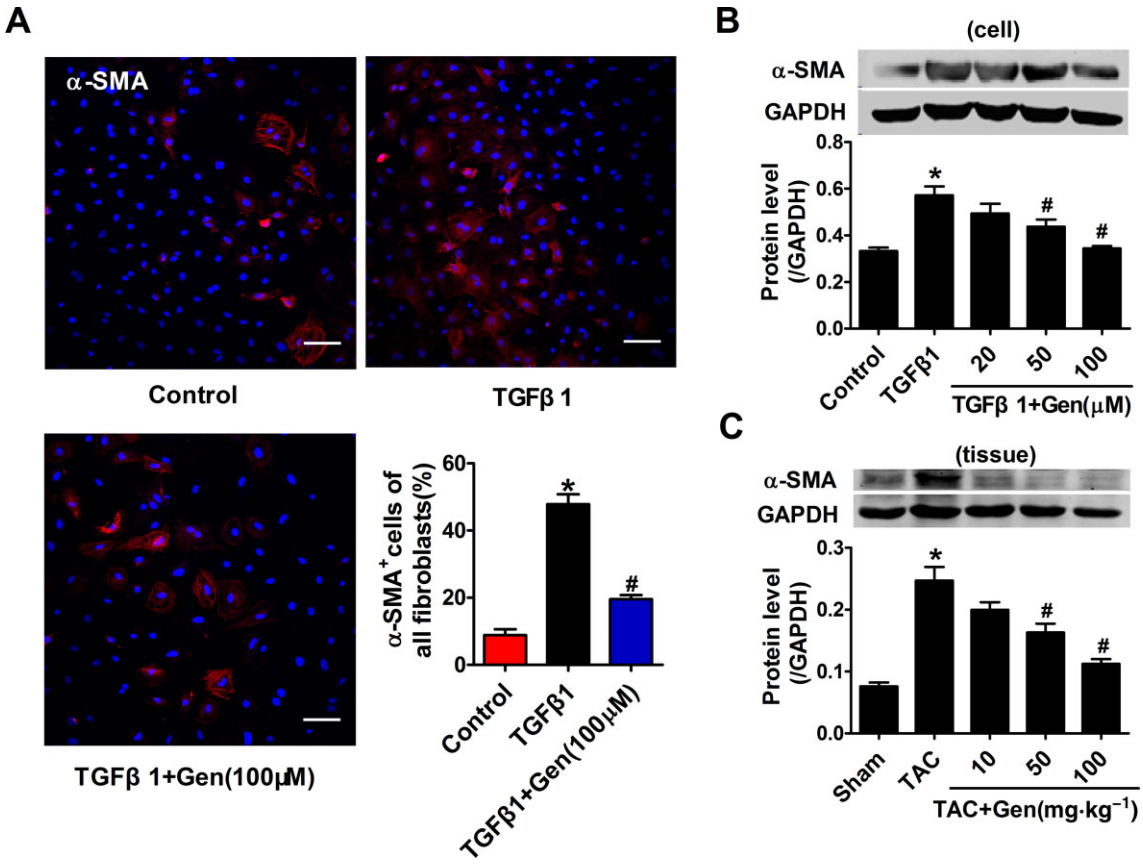

Figure 5

Genistein inhibits TGFβ1-induced fibroblast to myofibroblast transformation. (A) Representative photographs of CFs stained with α-SMA (red) and the summarized data of α-SMA$^+$ cells ratio. Blue indicates DAPI. Scale bar indicates 100 μm. $n = 5$. *$P < 0.05$ vs. control or sham; $^{\#}P < 0.05$ vs. TGFβ1 or TAC.

so did TSA (Figure 8A). Similar results were obtained from the collagen synthesis assay (Figure 8B). Genistein induced a significant decrease in the proliferation of CFs as measured by MTT assay and EdU assay, and this effect was inhibited by MTA3 siRNA or by TSA (Figure 8C–E). Our results also showed that the inhibitory effect of genistein on fibroblast to myofibroblast transformation was abolished by MTA3 depletion or TSA treatment as indicated by α-SMA immunostaining and protein expression (Figure 8D–F). Taken together, these results indicate that the MTA3-NuRD complex plays an important role in mediating the anti-fibrotic effects of genistein.

Discussion and conclusions

In the present study, we assessed the effects of genistein on the pressure overload-induced cardiac dysfunction and interstitial fibrosis. Firstly, we showed that genistein treatment ameliorated the cardiac dysfunction and hypertrophy in TAC mice. Secondly, we showed that genistein effectively attenuated interstitial fibrosis by inhibiting CFs proliferation, collagen production and myofibroblast formation. Finally, we found that the MTA3/TAK1/MKK4/JNK pathway was involved in the anti-fibrotic effects of genistein. The possible mechanism is summarized in Figure 9.

Genistein, a phytoestrogen found in soy, has been demonstrated to afford beneficial effects on the cardiovascular system partially by reducing blood pressure and improving lipid profile (Schwab *et al.*, 2012). A recent study has shown that genistein can reverse pulmonary hypertension and prevent right ventricular failure (Matori *et al.*, 2012). Our previous work demonstrated that genistein can attenuate As_2O_3-induced of apoptosis cardiomyocytes (Fan *et al.*, 2013). However, the effects of genistein on pressure overload-induced cardiac dysfunction and interstitial fibrosis have not been previously characterized. Our study confirmed that the treatment of TAC mice with genistein significantly improved cardiac function.

Cardiac fibrosis, characterized by excess collagen production and deposition in the interstitium (Creemers and Pinto, 2011; Wang *et al.*, 2014), is an important hallmark of pressure overload and a main cause of decreased myocardial compliance and heart failure (Chen *et al.*, 2011). Thus, therapies that inhibit cardiac fibrosis are viewed as one of the most important goals for the treatment of heart diseases (Vanhoutte *et al.*, 2006). Recently, agents such as FT011, aliskiren, curcumin and aminoguanidine were found to be able to attenu-

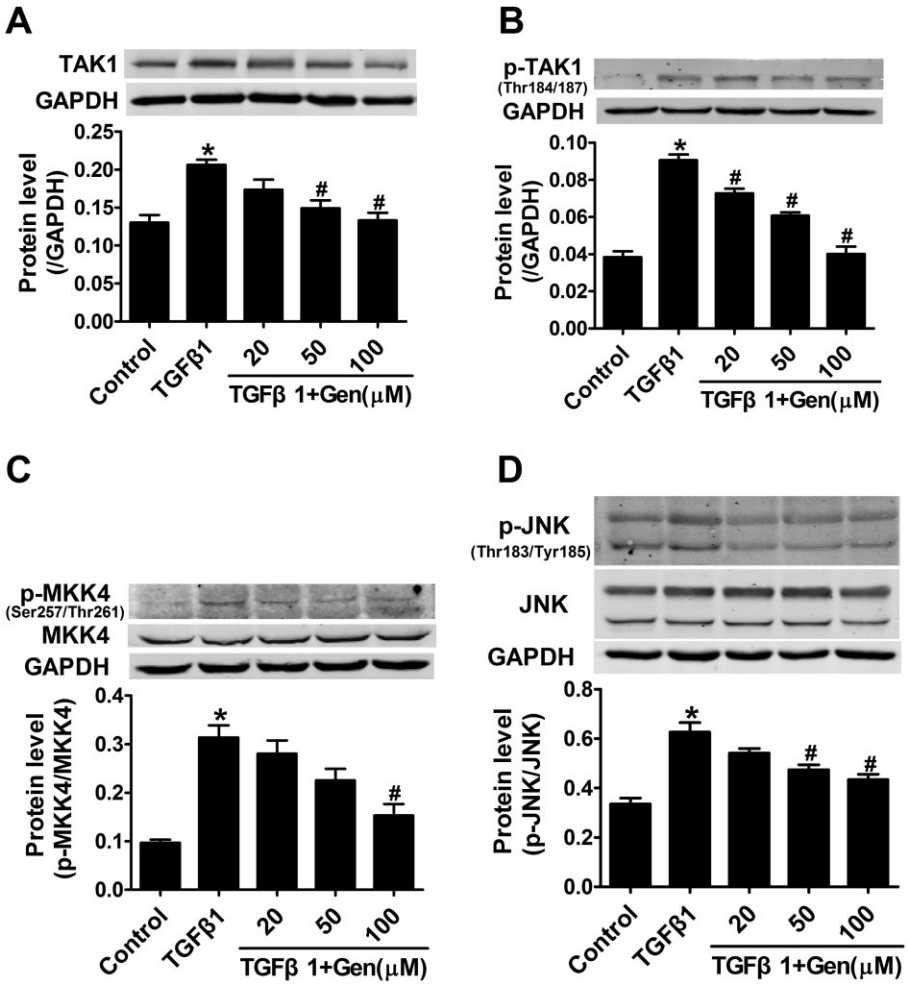

Figure 6

Genistein suppresses TAK1 expression and blocks the TAK1/MKK4/JNK pathway. (A) TAK1 protein level in CFs. (B) p-TAK1 protein level in CFs. (C) p-MKK4 and MKK4 protein levels in CFs. (D) p-JNK and JNK protein levels in CFs. $n = 5$. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TGFβ1.

ate fibrosis and improve cardiac function (Ma *et al.*, 2012; Vadla and Vellaichamy, 2012; Wang *et al.*, 2012; Zhang *et al.*, 2013a). Strikingly, results from our laboratory showed that scutellarin, a herbal compound extracted from Chinese medicine, produces beneficial effects on myocardial infarction-induced cardiac remodelling (Pan *et al.*, 2011). In the present study, we found that genistein significantly attenuated cardiac fibrosis in the TAC mice, which may contribute to its ability to preserve cardiac function.

It is known that accelerated proliferation of CFs is closely associated with deposition of ECM and fibrosis. In our study, we found that genistein markedly reduced the rapid proliferation and excess collagen production of CFs induced by TGFβ1, which may explain the inhibitory effects of genistein on fibrosis. Increasing evidence suggests that fibroblast to myofibroblast transformation, characterized by α-SMA expression, allows the cells to acquire the capacity to produce more ECM components and thus plays a key role in cardiac fibrosis (Swaney *et al.*, 2005). In this study, we found that genistein significantly inhibited fibroblast to myofibroblast transformation induced by TGFβ1, which may partially underlie the beneficial effects of genistein on cardiac fibrosis.

Progression of fibrosis is associated with biochemical signalling activation. Among the complicated signalling pathways, TGFβ1 is the most important regulator of ECM production in many organ systems (Yi *et al.*, 2014). TGFβ1 can signal through TAK1-dependent signalling to regulate fibrosis (Ma *et al.*, 2011; Choi *et al.*, 2012). In this study, we found that genistein treatment decreased TAK1 expression in both the mouse model and the cultured CFs. Moreover, TAK1 has been defined as a vital activator of MKK4/JNK signalling and the TAK1/MKK4/JNK pathway has been reported to mediate TGFβ1-induced fibronectin expression (Hocevar *et al.*, 2005). Based on this information, we measured p-TAK1, p-MKK4, MKK4, p-JNK and JNK expression, and found that the down-regulation of TAK1 induced by genistein prevented the activation of the TAK1/MKK4/JNK pathway, indicating that inhibition of TAK1/MKK4/JNK signalling might account for the anti-fibrotic action of genistein.

Genistein is a phytoestrogen that can bind to ER-α and -β and function like oestrogens. Studies have shown that ER activation plays a critical role in preventing cardiac fibrosis (Pedram *et al.*, 2010). In this study, we revealed that genistein suppressed TAK1 expression and produced its anti-fibrotic

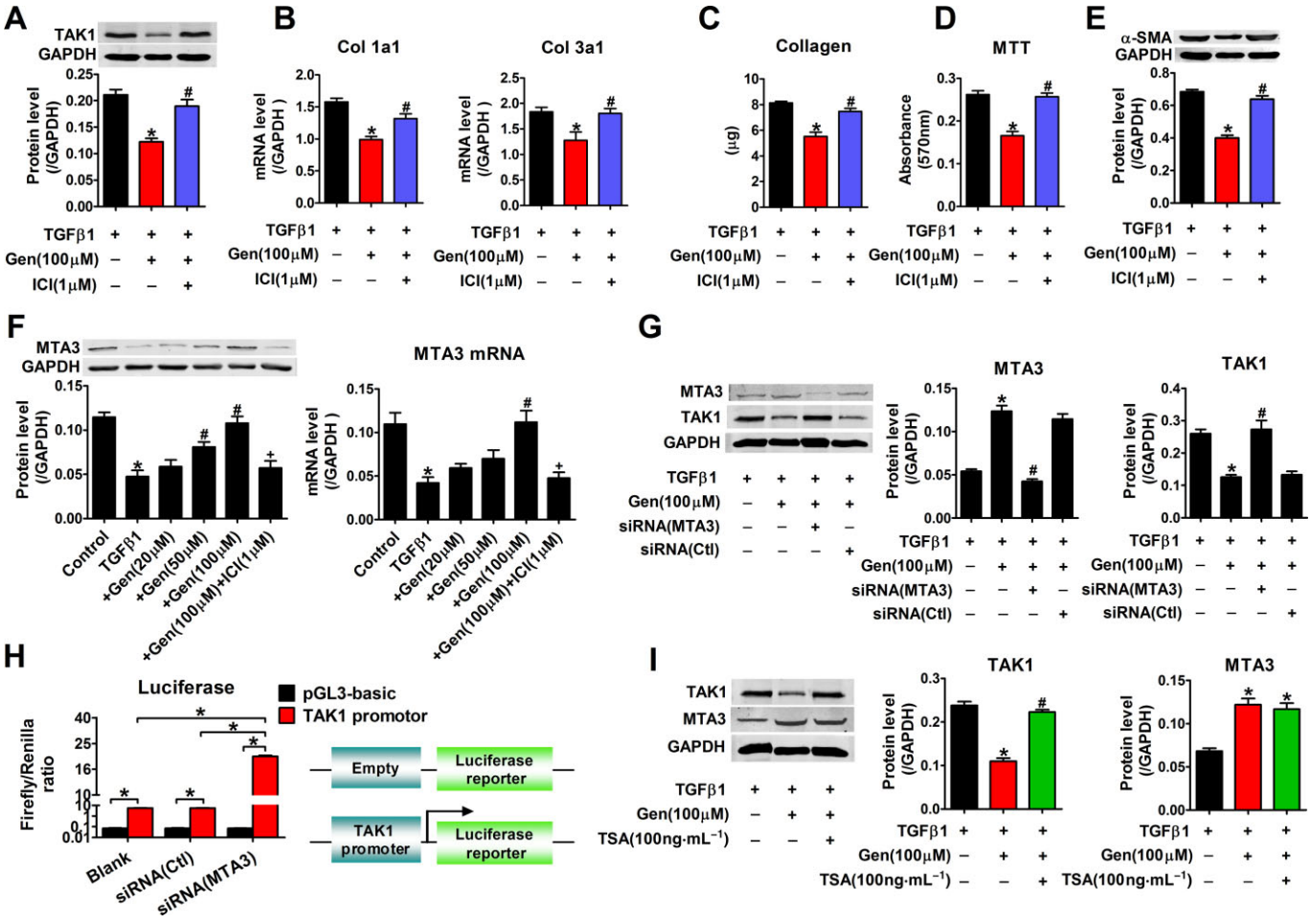

Figure 7

Genistein regulates TAK1 expression and fibrosis in an ER-dependent manner and MTA3 is essential for genistein-induced TAK1 repression. (A–E) Effect of ER antagonist ICI 182,780 (1 μM) on genistein's anti-fibrotic effect. $n = 5$. $^{*}P < 0.05$ vs. TGFβ1; $^{\#}P < 0.05$ vs. TGFβ1+genistein (100 μM). (A) Protein expression of TAK1. (B) Col 1a1 and Col 3a1 mRNA expressions. (C) Collagen production assay. (D) MTT test. (E) Protein expression of α-SMA. (F) Effect of genistein on the protein and mRNA levels of MTA3. $n = 5$. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TGFβ1; $^{+}P < 0.05$ vs. TGFβ1+genistein (100 μM). (G) Effect of MTA3 knockdown on protein level of TAK1. siRNA(MTA3) indicates siRNA to MTA3. siRNA(Ctl) is scrambled RNA as a negative control. $n = 5$. $^{*}P < 0.05$ vs. TGFβ1; $^{\#}P < 0.05$ vs. TGFβ1+genistein (100 μM). (H) TAK1 promoter luciferase reporter assay. $n = 3$. $^{*}P < 0.05$. (I) Effect of histone deacetylase inhibitor TSA (100 ng·mL^{-1}) on protein levels of TAK1 and MTA3. $n = 5$. $^{*}P < 0.05$ vs. TGFβ1; $^{\#}P < 0.05$ vs. TGFβ1+genistein (100 μM).

effects in an ER-dependent manner, suggesting that the downstream signalling molecules of ER might be an important mediator of the effects of genistein. MTA3 is an oestrogen-dependent component of the Mi-2/NuRD transcriptional corepressor complex, belonging to a group of proteins associated with histone deacetylase activities. In breast cancer cells, MTA3 inhibits Snail transcription and thus regulates cell invasion and tumour metastasis (Fujita *et al.*, 2003). MTA3 was also reported to suppress Wnt4 signalling by inhibiting Wnt4 transcription and secretion (Zhang *et al.*, 2006). Although MTA3 transcripts have been detected in mouse heart (Simpson *et al.*, 2001), the function of MTA3 in normal heart or heart diseases has not been previously identified. In this study, we found that the expression of MTA3 was up-regulated by genistein in cultured CFs. We further used the loss-of-function techniques on MTA3 expression to explore the regulatory effect MTA3 on TAK1 expression. Depletion of MTA3 by siRNA enhanced the expression of TAK1 in genistein-treated CFs. In addition, knockdown MTA3 led to increased TAK1 promoter activity, indicating that MTA3 does directly repress TAK1. Our results also suggested that the repressive activity of MTA3 was sensitive to an HDAC inhibitor, indicating MTA3 functions through the NuRD complex. Furthermore, when MTA3 expression was silenced by siRNA, genistein lost the ability to inhibit fibrosis, suggesting that MTA3/TAK1 is a downstream signalling pathway for the effects of genistein.

The present study identified genistein as an anti-fibrotic agent in TAC-induced cardiac dysfunction. Other studies also reported the anti-fibrotic effects of genistein in other pathological factors-induced cardiac dysfunction, such as isoprenaline-induced hypertrophy (Maulik *et al.*, 2012). However, the mechanisms for the anti-fibrotic properties of genistein were poorly understood. Probably, the most striking

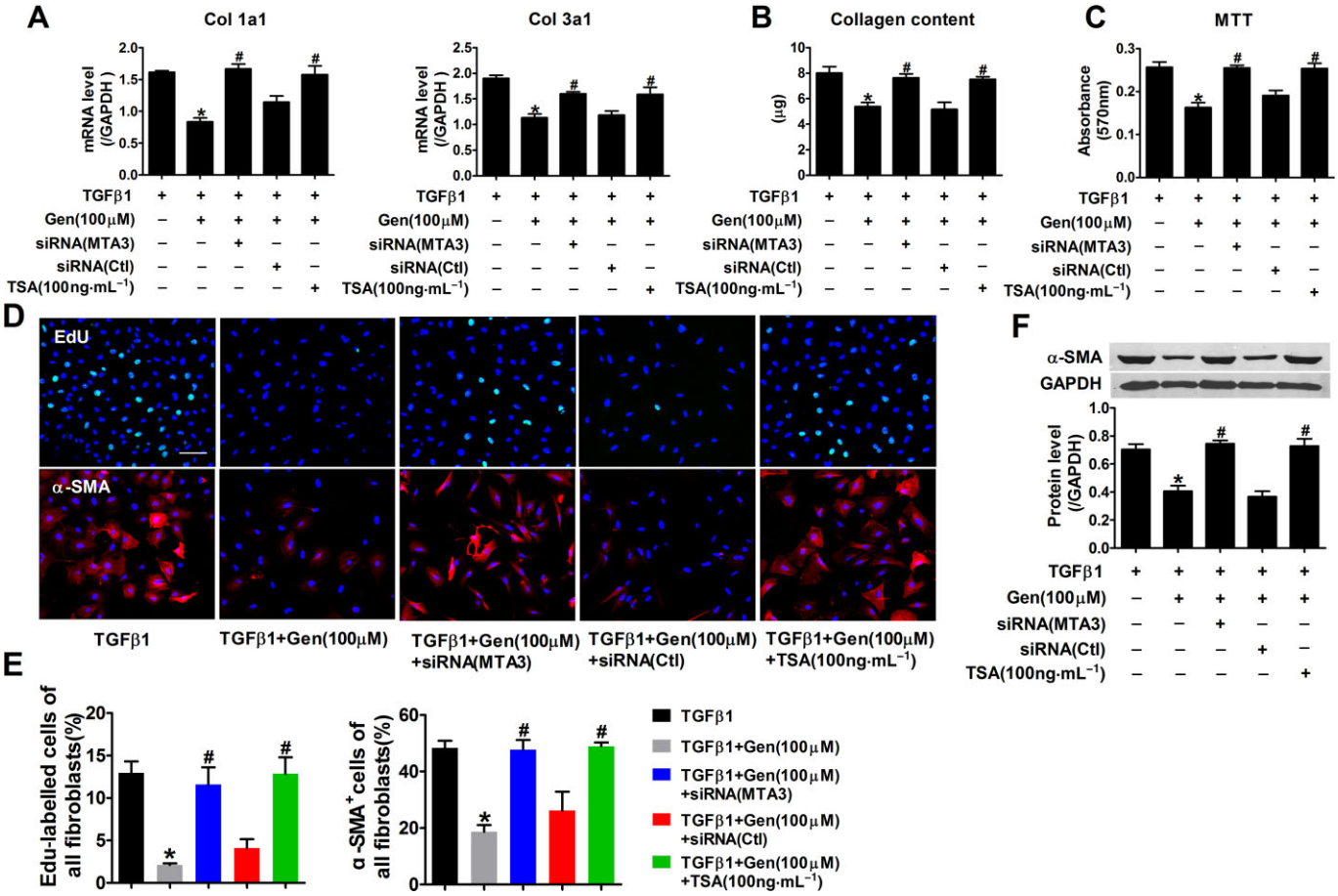

Figure 8

MTA3 is necessary for the anti-fibrotic effects of genistein (Gen). (A) Col 1a1 and Col 3a1 mRNA expressions. (B) Collagen production assay. (C) MTT test. (D) Representative photographs of EdU (green) assay and α-SMA (red) immunostaining. Blue indicates Hoechest33342 or DAPI. Scale bar indicates 100 μm. (E) Summarized data of EdU labelled cells ratio and α-SMA$^+$ cells ratio. (F) Protein expression of α-SMA. $n = 5$. $^*P < 0.05$ vs. TGFβ1; $^{\#}P < 0.05$ vs. TGFβ1+genistein (100 μM).

finding of our study is that genistein inhibits proliferation, collagen production and myofibroblast transformation of CFs and the discovery that MTA3 and TAK1/MKK4/JNK signalling are crucial mediators of the anti-fibrotic effects of genistein. However, it is possible that other mechanisms may account for the cardioprotective effects of genistein. For instance, genistein inhibits isoprenaline-induced cardiac hypertrophy through inhibition of iNOS and potentiation of eNOS activities (Maulik *et al.*, 2012). Isoflavones (including genistein) activate the Nrf2-Keap1 signalling pathway, leading to an up-regulation of detoxifying and antioxidant defence genes in cardiovascular disease (Mann *et al.*, 2009). Genistein protects against the adverse cardiac effects of As_2O_3 partly by inhibiting phosphorylated JNK and p38-MAPK (Fan *et al.*, 2013), which is in line with our findings on the relationship between genistein and JNK. However, a previous study by Hu *et al.* (2013) showed that genistein suppresses apoptosis-associated JNK signalling activation in isoprenaline-treated H9c2 cardiomyoblast cells. There are at least two explanations for the discrepancy between our findings and the study by Hu *et al.* Firstly, it is likely that JNK is not the direct downstream molecule of genistein, and alternatively, different cells and treatments were used in the study by Hu *et al.* and ours: H9c2 in their study and CFs in ours and isoprenaline in their study and TGFβ1 in ours, which may well have different apoptotic and survival signalling pathways or mediators as targets for genistein, thereby different effects on JNK signalling.

In this study, we characterized the anti-fibrotic property of genistein. Genistein inhibited pressure overload-induced cardiac dysfunction, hypertrophy and interstitial fibrosis. It inhibited TGFβ1-induced CFs proliferation, collagen production and myofibroblast formation. MTA3 served as a mediator of the anti-fibrotic effect of genistein through direct negative regulation of TAK1 and the TAK1/MKK4/JNK pathway. This study provides evidence that genistein may be a promising anti-fibrotic agent for cardiac diseases associated with fibrosis.

Acknowledgements

This work was supported by the Funds for Creative Research Groups (81421063) and the Major Program (81230081) of the National Natural Science Foundation of China, the National

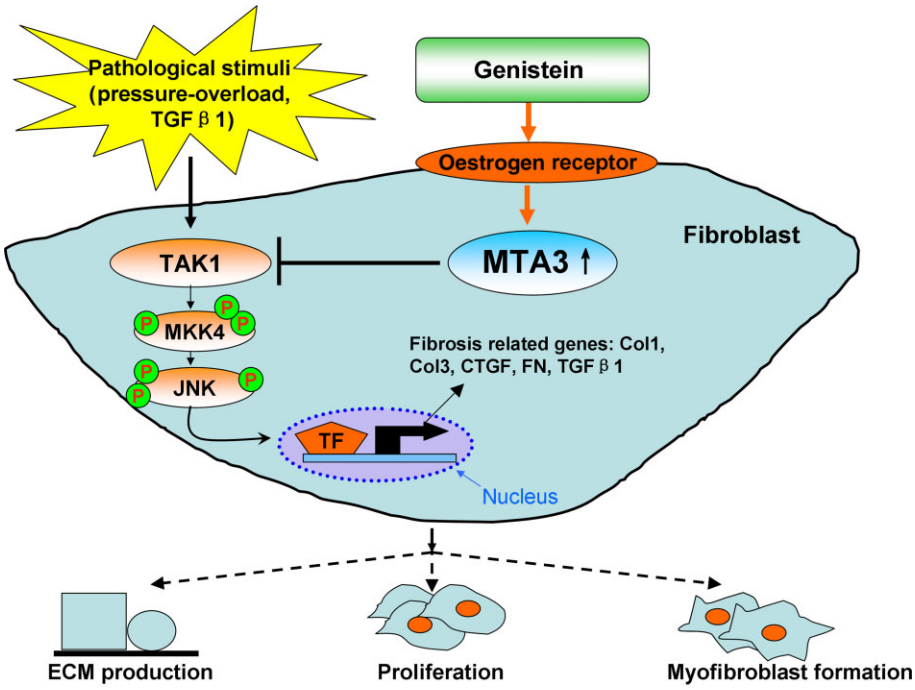

Figure 9
Schematic diagram of proposed mechanisms underlying the beneficial effects of genistein on pressure overload-induced cardiac fibrosis.

Nature Science Foundation of China (81270042, 30901208), Specialized Research Fund for the Doctoral Program of Higher Education (20112307130004) and the Program for New Century Excellent Talents In Heilongjiang Provincial University (1254-NCET-01).

Author contributions

Y. Z. and Z. D. supervised the project and wrote the manuscript; W. Q., N. D. and Z. F. designed the experiments and conducted the animal model; L. Z. and X. W. performed the cell culture and Western blot; Y. H. and X. L. performed real-time RT-PCR experiments; N. S. performed immunofluorescent staining; Y. L. and C. X. participated in statistical analysis; B. Y. and Y. L. reviewed and edited the manuscript.

Conflict of interest

The authors have no competing interests.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013a). The Concise Guide to PHARMACOLOGY 2013/14: Nuclear hormone receptors. Br J Pharmacol 170: 1652–1675.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Cavalera M, Wang J, Frangogiannis NG (2014). Obesity, metabolic dysfunction, and cardiac fibrosis: pathophysiological pathways, molecular mechanisms, and therapeutic opportunities. Transl Res 164: 323–335.

Chen B, Lu D, Fu Y, Zhang J, Huang X, Cao S *et al.* (2014). Olmesartan prevents cardiac rupture in mice with myocardial infarction by modulating growth differentiation factor 15 and P53. Br J Pharmacol 171: 3741–3753.

Chen Y, Miyazaki J, Nishizawa H, Kurahashi H, Leach R, Wang K (2013). MTA3 regulates CGB5 and Snail genes in trophoblast. Biochem Biophys Res Commun 433: 379–384.

Chen YW, Pat B, Gladden JD, Zheng J, Powell P, Wei CC *et al.* (2011). Dynamic molecular and histopathological changes in the extracellular matrix and inflammation in the transition to heart failure in isolated volume overload. Am J Physiol Heart Circ Physiol 300: H2251–H2260.

Chinigarzadeh A, Kasim NF, Muniandy S, Kassim NM, Salleh N (2014). Genistein induces increase in fluid pH, Na+ and HCO3(-) concentration, SLC26A6 and SLC4A4 (NBCe1)-B expression in the uteri of ovariectomized rats. Int J Mol Sci 15: 958–976.

Choi ME, Ding Y, Kim SI (2012). TGF-beta signaling via TAK1 pathway: role in kidney fibrosis. Semin Nephrol 32: 244–252.

Chu H, Chen X, Wang H, Du Y, Wang Y, Zang W *et al.* (2014). MiR-495 regulates proliferation and migration in NSCLC by targeting MTA3. Tumour Biol 35: 3487–3494.

Cong R, Sun Q, Yang L, Gu H, Zeng Y, Wang B (2009). Effect of Genistein on vasculogenic mimicry formation by human uveal melanoma cells. J Exp Clin Cancer Res 28: 124.

Creemers EE, Pinto YM (2011). Molecular mechanisms that control interstitial fibrosis in the pressure-overloaded heart. Cardiovasc Res 89: 265–272.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SPA, Giembycz MA *et al*. (2014). Time for blinded, randomized, unbiased, fully-powered studies: new journal requirements. Br J Pharmacol.

Dobaczewski M, Chen W, Frangogiannis NG (2011). Transforming growth factor (TGF)-beta signaling in cardiac remodeling. J Mol Cell Cardiol 51: 600–606.

Fan Y, Wang C, Zhang Y, Hang P, Liu Y, Pan Z *et al*. (2013). Genistein ameliorates adverse cardiac effects induced by arsenic trioxide through preventing cardiomyocytes apoptosis. Cell Physiol Biochem 31: 80–91.

Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA (2003). MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell 113: 207–219.

Fujita N, Jaye DL, Geigerman C, Akyildiz A, Mooney MR, Boss JM *et al*. (2004). MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation. Cell 119: 75–86.

Guo F, Hutchenreuther J, Carter DE, Leask A (2013). TAK1 is required for dermal wound healing and homeostasis. J Invest Dermatol 133: 1646–1654.

Hocevar BA, Prunier C, Howe PH (2005). Disabled-2 (Dab2) mediates transforming growth factor beta (TGFbeta)-stimulated fibronectin synthesis through TGFbeta-activated kinase 1 and activation of the JNK pathway. J Biol Chem 280: 25920–25927.

Hool LC, Middleton LM, Harvey RD (1998). Genistein increases the sensitivity of cardiac ion channels to beta-adrenergic receptor stimulation. Circ Res 83: 33–42.

Hu WS, Lin YM, Ho TJ, Chen RJ, Li YH, Tsai FJ *et al*. (2013). Genistein suppresses the isoproterenol-treated H9c2 cardiomyoblast cell apoptosis associated with P-38, Erk1/2, JNK, and NFkappaB signaling protein activation. Am J Chin Med 41: 1125–1136.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kim SI, Kwak JH, Zachariah M, He Y, Wang L, Choi ME (2007). TGF-beta-activated kinase 1 and TAK1-binding protein 1 cooperate to mediate TGF-beta1-induced MKK3-p38 MAPK activation and stimulation of type I collagen. Am J Physiol Renal Physiol 292: F1471–F1478.

Kong P, Christia P, Frangogiannis NG (2014). The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 71: 549–574.

Li X, Jia S, Wang S, Wang Y, Meng A (2009). Mta3-NuRD complex is a master regulator for initiation of primitive hematopoiesis in vertebrate embryos. Blood 114: 5464–5472.

Lu Y, Zhang Y, Shan H, Pan Z, Li X, Li B *et al*. (2009). MicroRNA-1 downregulation by propranolol in a rat model of myocardial infarction: a new mechanism for ischaemic cardioprotection. Cardiovasc Res 84: 434–441.

Ma FY, Tesch GH, Ozols E, Xie M, Schneider MD, Nikolic-Paterson DJ (2011). TGF-beta1-activated kinase-1 regulates inflammation and fibrosis in the obstructed kidney. Am J Physiol Renal Physiol 300: F1410–F1421.

Ma L, Hua J, He L, Li Q, Zhou J, Yu J (2012). Anti-fibrotic effect of Aliskiren in rats with deoxycorticosterone induced myocardial fibrosis and its potential mechanism. Bosn J Basic Med Sci 12: 69–73.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Mann GE, Bonacasa B, Ishii T, Siow RC (2009). Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. Curr Opin Pharmacol 9: 139–145.

Matori H, Umar S, Nadadur RD, Sharma S, Partow-Navid R, Afkhami M *et al*. (2012). Genistein, a soy phytoestrogen, reverses severe pulmonary hypertension and prevents right heart failure in rats. Hypertension 60: 425–430.

Maulik SK, Prabhakar P, Dinda AK, Seth S (2012). Genistein prevents isoproterenol-induced cardiac hypertrophy in rats. Can J Physiol Pharmacol 90: 1117–1125.

Meyer A, Wang W, Qu J, Croft L, Degen JL, Coller BS *et al*. (2012). Platelet TGF-beta1 contributions to plasma TGF-beta1, cardiac fibrosis, and systolic dysfunction in a mouse model of pressure overload. Blood 119: 1064–1074.

Ono K, Ohtomo T, Ninomiya-Tsuji J, Tsuchiya M (2003). A dominant negative TAK1 inhibits cellular fibrotic responses induced by TGF-beta. Biochem Biophys Res Commun 307: 332–337.

Pan Z, Zhao W, Zhang X, Wang B, Wang J, Sun X *et al*. (2011). Scutellarin alleviates interstitial fibrosis and cardiac dysfunction of infarct rats by inhibiting TGFbeta1 expression and activation of p38-MAPK and ERK1/2. Br J Pharmacol 162: 688–700.

Pan Z, Sun X, Shan H, Wang N, Wang J, Ren J *et al*. (2012). MicroRNA-101 inhibited postinfarct cardiac fibrosis and improved left ventricular compliance via the FBJ osteosarcoma oncogene/transforming growth factor-beta1 pathway. Circulation 126: 840–850.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al*.; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl Acids Res 42 (Database Issue): D1098–D1106.

Pedram A, Razandi M, O'Mahony F, Lubahn D, Levin ER (2010). Estrogen receptor-beta prevents cardiac fibrosis. Mol Endocrinol 24: 2152–2165.

Qi W, Weber CR, Wasland K, Savkovic SD (2011). Genistein inhibits proliferation of colon cancer cells by attenuating a negative effect of epidermal growth factor on tumor suppressor FOXO3 activity. BMC Cancer 11: 219.

Schwab K, Stein R, Scheler C, Theuring F (2012). Dietary genistein enhances phosphorylation of regulatory myosin light chain in the myocardium of ovariectomized mice. Electrophoresis 33: 1795–1803.

Shan H, Zhang Y, Lu Y, Pan Z, Cai B, Wang N *et al*. (2009). Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. Cardiovasc Res 83: 465–472.

Shi-wen X, Parapuram SK, Pala D, Chen Y, Carter DE, Eastwood M *et al*. (2009). Requirement of transforming growth factor beta-activated kinase 1 for transforming growth factor beta-induced alpha-smooth muscle actin expression and extracellular matrix contraction in fibroblasts. Arthritis Rheum 60: 234–241.

Si H, Liu D (2007). Phytochemical genistein in the regulation of vascular function: new insights. Curr Med Chem 14: 2581–2589.

Simpson A, Uitto J, Rodeck U, Mahoney MG (2001). Differential expression and subcellular distribution of the mouse metastasis-associated proteins Mta1 and Mta3. Gene 273: 29–39.

Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA (2005). Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. Proc Natl Acad Sci U S A 102: 437–442.

Vadla GP, Vellaichamy E (2012). Anti-fibrotic cardio protective efficacy of aminoguanidine against streptozotocin induced cardiac fibrosis and high glucose induced collagen up regulation in cardiac fibroblasts. Chem Biol Interact 197: 119–128.

Valsecchi AE, Franchi S, Panerai AE, Sacerdote P, Trovato AE, Colleoni M (2008). Genistein, a natural phytoestrogen from soy, relieves neuropathic pain following chronic constriction sciatic nerve injury in mice: anti-inflammatory and antioxidant activity. J Neurochem 107: 230–240.

Vanhoutte D, Schellings M, Pinto Y, Heymans S (2006). Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: a temporal and spatial window. Cardiovasc Res 69: 604–613.

Wang NP, Wang ZF, Tootle S, Philip T, Zhao ZQ (2012). Curcumin promotes cardiac repair and ameliorates cardiac dysfunction following myocardial infarction. Br J Pharmacol 167: 1550–1562.

Wang Q, Ma S, Li D, Zhang Y, Tang B, Qiu C *et al.* (2014). Dietary capsaicin ameliorates pressure overload-induced cardiac hypertrophy and fibrosis through the transient receptor potential vanilloid type 1. Am J Hypertens 27: 1521–1529.

Xu C, Hu Y, Hou L, Ju J, Li X, Du N *et al.* (2014). Beta-blocker carvedilol protects cardiomyocytes against oxidative stress-induced apoptosis by up-regulating miR-133 expression. J Mol Cell Cardiol 75C: 111–121.

Yasuno S, Kuwahara K, Kinoshita H, Yamada C, Nakagawa Y, Usami S *et al.* (2013). Angiotensin II type 1a receptor signalling directly contributes to the increased arrhythmogenicity in cardiac hypertrophy. Br J Pharmacol 170: 1384–1395.

Yi X, Li X, Zhou Y, Ren S, Wan W, Feng G *et al.* (2014). Hepatocyte growth factor regulates the TGFbeta1induced proliferation, differentiation and secretory function of cardiac fibroblasts. Int J Mol Med 34: 381–390.

Zhang H, Singh RR, Talukder AH, Kumar R (2006). Metastatic tumor antigen 3 is a direct corepressor of the Wnt4 pathway. Genes Dev 20: 2943–2948.

Zhang Y, Zhang L, Chu W, Wang B, Zhang J, Zhao M *et al.* (2010). Tanshinone IIA inhibits miR-1 expression through p38 MAPK signal pathway in post-infarction rat cardiomyocytes. Cell Physiol Biochem 26: 991–998.

Zhang Y, Elsik M, Edgley AJ, Cox AJ, Kompa AR, Wang B *et al.* (2013a). A new anti-fibrotic drug attenuates cardiac remodeling and systolic dysfunction following experimental myocardial infarction. Int J Cardiol 168: 1174–1185.

Zhang Y, Sun L, Liang H, Li X, Cai R, Wang L *et al.* (2013b). Overexpression of microRNA-1 causes atrioventricular block in rodents. Int J Biol Sci 9: 455–462.

Zhang Y, Li X, Zhang Q, Li J, Ju J, Du N *et al.* (2014). Berberine hydrochloride prevents postsurgery intestinal adhesion and inflammation in rats. J Pharmacol Exp Ther 349: 417–426.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13002

Figure S1 Effects of genistein (Gen) on EF, FS values and LV mass of 4-week TAC-treated hearts. (A) Eject fraction (EF) levels evaluated by echocardiography. n = 7. (B) Fraction shortening (FS) levels evaluated by echocardiography. n = 7. (C) Left ventricular (LV) mass evaluated by echocardiography. n = 7. *P < 0.05 vs. sham; #P < 0.05 vs. TAC.

Figure S2 Effects of genistein (Gen) on pressure overload-induced cardiac interstitial fibrosis. (A–G) Representative fields of Sirius red–stained heart sections in control, Gen, sham, TAC and TAC+Gen animals. Scale bar indicates 100 μm. (H) The summarized data showed that genistein treatment dose-dependently abolished TAC-induced cardiac interstitial fibrosis. n = 5. *P < 0.05 vs. sham; #P < 0.05 vs. TAC.

Figure S3 Genistein (Gen) treatment has no effects on Smad signalling. The expression of p-Smad2/3 was increased after TGFβ1 treatment. Co-application with Gen did not inhibit p-Smad2/3/Smad2/3 ratio. n = 5. *P < 0.05 vs. control.

Figure S4 TAK1 inhibition suppresses TGFβ1-induced proliferation and collagen production of cultured CFs. Cells were treated with TGFβ1 in the presence of DMSO or 500 nM TAK1 inhibitor (5Z)-7-oxozeaenol for 24 h. (A) MTT test. (B) Collagen production assay. (C, D) Col 1a1 and Col 3a1 mRNA expressions. n = 5. *P < 0.05 vs. TGFβ1.

Figure S5 Genistein (Gen) represses TAK1 expression and blocks TAK1/MKK4/JNK signalling in TAC-treated mice. (A) Representative Western blots and analysed data for total TAK1 in heart tissue. (B) Representative Western blots and analysed data for phosphorylated TAK1 (p-TAK1) in heart tissue. (C) Representative Western blots and analysed data for p-MKK4 and MKK4 in heart tissue. (D) Representative Western blots and analysed data for p-JNK and JNK in heart tissue. n = 5. *P < 0.05 vs. sham; #P < 0.05 vs. TAC.

Table S1 Echocardiography of mice before and after TAC and treatment with genistein (Gen).