Rad GTPase inhibits cardiac fibrosis through connective tissue growth factor

Ji Zhang1, Lin Chang2, Chunlei Chen1, Meiling Zhang1, Yan Luo1, Milton Hamblin2, Luis Villacorta2, Jing-Wei Xiong1, Y. Eugene Chen2, Jifeng Zhang2*, and Xiaojun Zhu1*

1Institute of Molecular Medicine, Peking University, No. 5, Yi He Yuan Road, Beijing 100871, China; and 2Cardiovascular Center, Department of Internal Medicine, University of Michigan Medical Center, 7200 Medical Sciences Research Building III, 1150 W Medical Center Drive, Ann Arbor, MI 48109, USA

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1. Introduction

Rad (Ras associated with diabetes) is a 35 kDa GTPase that belongs to the RGK (Rad, Gem, and Kir) family of Ras-related small G protein, is significantly decreased in human failing hearts and plays an important role in attenuating cardiac hypertrophy. The goal of this study is to identify the effect of Rad on cardiac fibrosis and the underlying mechanisms.

METHODS AND RESULTS

Rad knockout (KO) mice showed more severe cardiac fibrosis compared with wild-type littermate controls as detected by Sirius Red staining. Western blot analyses demonstrated that the expression of connective tissue growth factor (CTGF), a key mediator of fibrosis, increased dramatically in Rad KO mice. Overexpression of Rad in cultured neonatal cardiomyocytes suppressed both basal and transforming growth factor-β1-induced CTGF expression. Elevated CTGF expression was observed in cardiomyocytes when Rad was reduced by RNA interference. Moreover, cardiac fibroblasts produced greater extracellular matrix (ECM) when stimulated with conditioned medium from Rad-knockdown cardiomyocytes. ECM production was completely abolished by adding a CTGF-neutralizing antibody into the medium. CCAAT/enhancer-binding protein δ (C/EBP-δ) was demonstrated to activate CTGF in cardiomyocytes. Chromatin immunoprecipitation assay and co-immunoprecipitation further demonstrated that Rad inhibited the binding of C/EBP-δ to the CTGF promoter via direct interaction with C/EBP-δ.

CONCLUSION

Our data reveal that Rad deficiency can lead to cardiac fibrosis. Rad inhibits CTGF expression through binding with C/EBP-δ, thus regulating ECM production in the heart. This study suggests a potential link between decreased Rad levels and increased cardiac fibrosis in human failing hearts.

KEYWORDS

Rad GTPase • Cardiac fibrosis • CTGF • C/EBP-δ

* Corresponding author. Tel/fax: +86 10 6275 5291, Email: zhuxiaojun@pku.edu.cn (X.Z.); Tel: +1 734 647 8975; fax: +1 734 936 2641, Email: jifengz@umich.edu (J.Z.)

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undergo a process known as cardiac remodelling, which includes a series of changes in heart size, shape, and function. Pathological cardiac remodelling is a key determinant of clinical outcomes in heart disease. It is characterized by a structural rearrangement of the cardiac chamber wall that involves cardiomyocyte hypertrophy and myocardial fibrosis. Cardiac fibrosis, which is not inevitably associated with cardiac hypertrophy, can be defined as an inappropriate accumulation of extracellular matrix (ECM) proteins in the heart. This process alters mechanical and electrical properties of cardiomyocytes, which adversely affects heart function. Cardiac fibrosis is an important contributor to the development of cardiac dysfunction in diverse pathological conditions, such as ischaemic and hypertensive cardiac diseases. There is evidence that increased fibrosis, rather than cardiac hypertrophy, may be the most significant cause of dystolic dysfunction in hypertrophic cardiac disease. Connective tissue growth factor (CTGF, also known as CCN2), which belongs to the CCN (Cyr61, CTGF, and Nov) family of immediate early genes, plays an important role in fibrogenic processes throughout the body, including the heart. CTGF is a matricellular molecule that has multiple effects on fibrosis, ECM production, hypertrophy, adhesion, proliferation, migration, angiogenesis, and apoptosis.

In our current study, we found that loss of Rad expression causes abnormal ECM deposition in the heart. Further investigation of Rad function in cardiac fibrosis by gain- and loss-of-function approaches both in vivo and in vitro demonstrates that Rad inhibits cardiac fibrosis through the inhibition of CTGF.

2. Methods

2.1 Animal model
Rad KO mice were generated as described previously. Ten-week-old male Rad KO and wild-type (WT) littermate control mice were subjected to transverse aortic constriction (TAC) or a sham operation as described previously. Fourteen days after surgery, all hearts were harvested. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experiments were approved by the Institutional Animal Care and Use Committee of the University of Michigan (Protocol number #09469).

2.2 Histology
Hearts were harvested from Rad KO and WT control mice that underwent a TAC or sham operation and fixed with 4% formaldehyde overnight. Heart samples were dehydrated and embedded in paraffin wax following standard laboratory procedures. Serial horizontal paraffin heart sections (5 µm) were mounted on glass slides and deparaffinized. Cardiac fibrosis was determined by Sirius Red staining by using a Picrosirius Red Stain Kit (Polysciences Inc.). Collagen fibres in the heart stained a red colour and cardiac fibrotic area was evaluated. An ABC staining system (Santa Cruz Biotechnology) was used for immunohistochemical detection of Rad. The ABC system for detection of Rad was performed as described previously. A neonatal cardiomyocyte isolation kit (Worthington Biochemical Corporation) was used to isolate rat cardiomyocytes from 1- to 2-day-old Sprague–Dawley rats. Cardiomyocytes were plated at a density of 1.0 × 10^6 cells/well in six-well plates and cultured in DMEM medium containing 10% foetal bovine serum.

Recombinant adeno viruses for Rad overexpression or knockdown and their respective control viruses (Ad-Rad and Ad-GFP or Ad-Rad-shRNA and Ad-Ctrl-shRNA) were generated as described previously. Ad-GFP and Ad-Rad infections were performed at a multiplicity of infection (MOI) of 5. Cells were infected with 40 MOI Ad-Rad-shRNA to reach 70–80% knockdown of Rad in cardiomyocytes. The Rad expression level after Ad-Rad or Ad-Rad-shRNA infection was detected by western blotting (see Supplementary material online, Figure S1B). The siRNA target sequence for knockdown of rat CCAAT/enhancer-binding protein α (C/EBP-α) was 5′-AACAGAGAAGCTCTAGGCTG-3′. Non-silencing siRNA 5′-GCAAGCTGACCCCGAATTT-3′ was used as a control. Cardiomyocytes were transfected with 50 nM siRNA using Lipofectamine 2000 (Invitrogen).

2.4 Real-time RT–PCR
Total heart RNA and cellular RNA were isolated using Trizol Reagent (Invitrogen). cDNA was synthesized from 1 µg RNA of each sample using SuperScript II reverse transcriptase (Invitrogen). A SYBR Green Supermix (Bio-Rad) was used for real-time quantitative PCR. Details of primers are indicated in Supplementary material online, Table S7.

2.5 Western blot analysis
Heart protein lysates and cellular protein lysates were prepared using T-PER tissue protein extraction buffer and M-PER mammalian protein extraction buffer (Thermo Scientific), respectively. The rabbit anti-Rad antibody was prepared and characterized as described previously. Goat anti-CTGF, rabbit anti-C/EBP-α, and goat anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, whereas rabbit anti-fibronectin and mouse anti-Flag antibodies were obtained from Sigma-Aldrich. Rabbit anti-phospho-Smad3 and anti-Smad3 antibodies were purchased from Cell Signaling Technology.

2.6 Enzyme-linked immunosorbent assay
About 1.0 × 10^6 cardiomyocytes were plated in each well of a six-well plate. Twenty-four hours after Ad-GFP or Ad-Rad infection, fresh DMEM without foetal bovine serum was applied to the cells (1.5 mL culture medium in each well). Twenty-four hours later, cardiomyocyte culture medium was collected. CTGF and transforming growth factor-β1 (TGF-β1) levels in the culture medium of each group were determined by ELISA (USCNLIFE and Cell Applications Inc., respectively) according to the manufacturer’s instructions.

2.7 Conditioned medium stimulation
Non-serum conditioned medium was obtained as described above. Cardiac fibroblasts were obtained during the preplating step of neonatal rat cardiomyocyte isolation and maintained in DMEM containing 10% foetal bovine serum. After confluence, cardiac fibroblasts were placed in serum-free DMEM for 24 h. Then, fibroblasts were exposed to the conditioned medium of cardiomyocytes and harvested after 24 h. For experiments involving the use of neutralizing antibodies, cells were incubated with conditioned medium containing 10 µg/mL anti-CTGF.

2.8 Co-immunoprecipitation
The C/EBP-α expression plasmid, pcDNA3.1-C/EBP-α, was generated by inserting mouse C/EBP-α cDNA into a pcDNA3.1 vector. pcDNA3.1-C/EBP-α and an expression vector for Flag-tagged Rad were transfected to HEK293 cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cell lysates were precipitated using anti-Flag M2 Agarose (Sigma-Aldrich) for 24 h at 4°C. Precipitated proteins were...
subjected to western blotting using an anti-C/EBP-δ antibody (Santa Cruz Biotechnology).

2.9 Plasmid transfection and luciferase assays
CTGF promoter–reporter plasmid constructs used in transfection experiments included a ~600 bp fragment of the human CTGF promoter (containing nucleotides −565 to +61 bp) as well as serial deletion of the promoter: −122 bp (containing nucleotides −122 to +61 bp), −80 bp (containing nucleotides −80 to +61 bp), and −27 bp (containing nucleotides −27 to +61 bp). These DNA fragments were amplified by PCR from human genomic DNA and cloned into a luciferase reporter vector pGL3-basic plasmid. A C/EBP reporter was generated by inserting a C/EBP response element (TTGCGGAAT × 3) into the pGL3-basic plasmid. Cultured rat cardiomyocytes were transiently transfected with 0.4 μg of the CTGF promoter reporter only or together with pcDNA3.1-C/EBP-δ or the pcDNA3.1 control plasmid using Lipofectamine 2000. pRL-CMV Renilla luciferase was co-transfected as a control reporter vector. Cells were lysed in cell lysis buffer (Promega) 48 h after transfection, and luciferase activity was monitored using a luminometer (TD-20/20 Luminometer, Turner Designs).

2.10 Chromatin immunoprecipitation assay
For chromatin immunoprecipitation (ChIP) assays, the EZ Chromatin Immunoprecipitation kit (Millipore) was used following the manufacturer’s protocol. Cardiomyocytes were transfected with pcDNA3.1-C/EBP-δ and infected with Ad-GFP or Ad-Rad. Sonicated DNA fragments were immunoprecipitated with the rabbit anti-C/EBP-δ antibody or normal mouse IgG (as the negative control), respectively. Immunoprecipitated DNA fragments were amplified by PCR. Primers located at −172 and +37 bp of the CTGF promoter were 5′-GACGGAGGAATGTGGAGTGT-3′ and 5′-TGAGTCTGGTGAGTCTTCT-3′.

2.11 Statistical analysis
Data are presented as mean ± SD. Differences between mean values were evaluated by Student’s t-test or one-way ANOVA, with values of \( P < 0.05 \) indicating a significant difference.

3. Results

3.1 Rad deficiency leads to cardiac fibrosis in mice
Ten-week-old Rad KO mice and their WT littermates were subjected to TAC as described previously.7 Sham operations were performed on sex- and age-matched WT or KO mice, and they served as controls for all experimental groups. Complete KO of the Rad protein in the heart was confirmed by western blotting (see Supplementary material online, Figure S1A). Fourteen days after surgery, hearts from Rad KO and WT mice were harvested and collagen was detected by Sirius Red staining. As shown in Figure 1A, Rad KO animals displayed a marked increase in collagen accumulation compared with WT littermates both in sham-operated mice and mice subjected to TAC. The ratio of the area of fibrosis to the entire LV area was significantly higher in Rad KO mice compared with WT mice (5.6 ± 0.7 vs. 1.2 ± 1.0% in the sham-operated groups and 17.8 ± 2.4 vs. 8.2 ± 3.8% in the TAC groups). The expression of another important ECM component, fibronectin,19 was also increased in Rad KO mouse hearts (Figure 1B; see Supplementary material online, Figure S2).

3.2 Rad deficiency leads to elevated CTGF expression in vivo and in vitro
To demonstrate the molecular mechanisms of Rad-deficient-induced fibrosis, microarray analyses were performed to compare the gene expression profile of hearts from Rad KO mice and their WT littermates. Our profiling data indicate that CTGF is one of the most remarkably increased genes in the heart when Rad is knocked out (data not shown). CTGF has been well-documented as an essential mediator in cardiac fibrosis and remodelling. To confirm the CTGF expression changes in Rad KO mice, we examined CTGF levels and found that CTGF increased significantly in 10-week-old Rad-KO mice compared with their WT littermates (Figure 2A; see Supplementary material online, Figures S3 and S4). Because abundant expression of Rad was detected only in isolated rat cardiomyocytes and not in cardiac fibroblasts (see Supplementary material online, Figure S5), we focused on how Rad regulates CTGF expression in cardiomyocytes. TGF-β1 is considered to be a strong inducer of fibrolysis by activating CTGF and other cytokines.20,21 Rad overexpression in neonatal rat cardiomyocytes resulted in significantly reduced CTGF expression at basal levels or upon TGF-β1 stimulation, whereas CTGF increased when Rad was knocked down (Figure 2B and C).

CTGF levels in cell culture supernatants from cardiomyocytes infected with Ad-GFP or Ad-Rad were analysed. Rad overexpression in cardiomyocytes significantly decreased CTGF levels (Figure 3A) without altering TGF-β1 levels (see Supplementary material online, Figure S6). Further experiments showed that this decrease also happens under stimulation as the cell culture supernatant of Rad-overexpressing cardiomyocytes contained less CTGF in the presence of exogenously added TGF-β1 (see Supplementary material online, Figure S7). Fibroblasts are the major source of ECM in the heart.22 To define whether Rad levels in cardiomyocytes affect fibroblast ECM production, we treated fibroblasts with conditioned medium from cardiomyocytes in which Rad was either overexpressed or knocked down, and then we examined fibronectin expression in fibroblasts. Consistent with CTGF level changes in Rad-overexpressed cardiomyocyte culture medium, fibronectin expression was decreased in cardiac fibroblasts stimulated with conditioned medium from Rad-knockdown cardiomyocytes. Meanwhile, elevated fibronectin generation was detected in fibroblasts stimulated with conditioned medium from Rad-knockdown cardiomyocytes (Figure 3B). Increased fibronectin expression in fibroblasts can be completely blocked by the presence of a CTGF-neutralizing antibody (Figure 3C), which indicates that CTGF is a mediator of this pro-fibrotic signalling communication between cardiomyocytes and cardiac fibroblasts.

3.3 C/EBP-δ activates CTGF in cardiomyocytes
Smad3 activation is important for TGF-β1-induced CTGF expression.16 Therefore, we tested whether Rad affects the Smad3 signalling pathway. Neither Smad3 phosphorylation nor Smad reporter activity was affected by Rad expression changes (see Supplementary material online, Figure S8). These data indicate that Rad regulates CTGF through an Smad3-independent signalling pathway.

To elucidate the cis-acting elements in the CTGF gene promoter that mediate CTGF activation when Rad is knocked down, a luciferase assay was performed using a p-CTGF-565-Luc plasmid and several
deleted promoter constructs. Reporter constructs were transfected into cardiomyocytes infected with Ad-Ctrl-shRNA or Ad-Rad-shRNA, and luciferase activity was measured 48 h after transfection. Rad knockdown led to increased CTGF promoter activity in all promoter constructs of different sizes except for the −27 bp CTGF promoter construct (Figure 4A). These results suggest that a transcription factor located between −27 and −80 bp of the CTGF promoter would be essential for Rad knockdown-mediated CTGF up-regulation.

A consensus C/EBP-binding site was predicted between −66 and −48 bp in the CTGF promoter. Compared with other transcription factors in the C/EBP family, C/EBP-δ showed the highest activity on the CTGF promoter in our preliminary experiments. Our preliminary data also showed that myocardial C/EBP-δ expression was increased in response to TAC, indicating that C/EBP-δ plays a role in TAC-induced cardiac remodelling. Furthermore, the microarray data also indicated that 14 days after TAC, C/EBP-δ was the most significantly induced C/EBP protein in Rad KO mice compared with WT mice (data not shown).

We generated two CTGF promoter constructs: p-CTGF-66-Luc (containing −66 to +61 bp of CTGF promoter) and p-CTGF-48-Luc (containing −48 to +61 bp of CTGF promoter). The −48 or −66 bp CTGF promoter reporters were co-transfected with a pcDNA3.1-C/EBP-δ plasmid or the pcDNA3.1 control plasmid. C/EBP-δ expression increased the −66 bp CTGF promoter activity but had no effect on the −48 bp CTGF reporter (Figure 4B). Induction of CTGF promoter activity by C/EBP-δ was also observed on −80, −122, and −565 bp CTGF promoter constructs (data not shown). These observations support the hypothesis that a C/EBP-δ-binding site is located within the −48 to −66 bp region of the CTGF promoter, further confirming that C/EBP-δ activates the CTGF promoter.

Western blots were performed to test CTGF expression in cardiomyocytes with C/EBP-δ overexpressed or knocked down. Induction of CTGF in cardiomyocytes was observed following transfection of a C/EBP-δ expression plasmid. On the other hand, knockdown of C/EBP-δ by siRNA decreased CTGF protein levels (Figure 4C). These results suggest that C/EBP-δ activates CTGF expression and may be the key factor in Rad-induced inhibition of CTGF.

3.4 Rad inhibits CTGF via binding with C/EBP-δ in cardiomyocytes

The above experiments indicate that C/EBP-δ activates CTGF expression. To test whether Rad inhibits C/EBP-δ-induced activity, a C/EBP reporter was transfected into cardiomyocytes together with pcDNA3.1-C/EBP-δ and infected with Ad-Rad or Ad-GFP. As shown in Figure 5A, C/EBP-δ dramatically induced C/EBP reporter activity, whereas Rad overexpression in cardiomyocytes abolished C/EBP-δ-induced activity. Similar experiments were performed with the CTGF promoter. Cardiomyocytes were co-transfected with p-CTGF-565-Luc and pcDNA3.1-C/EBP-δ and then infected with Ad-Rad or Ad-GFP as indicated in Figure 5B. CTGF promoter activity was up-regulated by C/EBP-δ, and Rad overexpression abolished this induction (Figure 5B).

Here, we demonstrate that C/EBP-δ activates CTGF in cardiomyocytes, and Rad abolishes C/EBP-δ-induced CTGF activation. ChIP assays were performed to test whether C/EBP-δ binds to the CTGF promoter in cardiomyocytes and whether Rad affects this binding. As shown in Figure 5C, C/EBP-δ binds to the −172 to +37 bp CTGF promoter region and this C/EBP-δ binding to the CTGF promoter is decreased when Rad is overexpressed. Furthermore, immunoprecipitation was performed to test whether there is
a direct interaction between Rad and C/EBP-δ. HEK293 cells were co-transfected with a pcDNA3.1-C/EBP-δ plasmid together with pcDNA3.1-Rad-Flag or pcDNA3.1-Flag. Forty-eight hours after transfection, Flag-tagged Rad was isolated by immunoprecipitation and the bound C/EBP-δ was visualized by immunoblotting. As seen in Figure 5D, C/EBP-δ could be detected in Flag-immunoprecipitated portions of pcDNA3.1-Rad-Flag-transfected cells. This suggests that the Rad protein can physically interact with C/EBP-δ.

Taken together, our data indicate that Rad can prevent cardiac fibrosis by inhibiting CTGF expression through suppression of C/EBP-δ-mediated signalling.

4. Discussion

In the present study, we demonstrate that the small G protein Rad is a powerful negative regulator of fibrosis in the heart. Our study shows for the first time that disruption of the Rad gene in mice results in severe myocardial fibrosis. Next, our in vitro and in vivo data demonstrate that Rad deficiency leads to elevated CTGF expression in cardiomyocytes. In addition, we identify C/EBP-δ as a mediator of Rad-induced inhibition of CTGF expression. This is the first time that the function of Rad on cardiac fibrosis is addressed and C/EBP-δ is identified as an important mediator in CTGF expression.

Rad has been shown recently to be highly expressed in the heart, and thus, considerable efforts have been made in elucidating its role in maintaining normal cardiac function. Rad, as well as other members of the RGK family, has been identified as a negative regulator of L-type calcium channel activity. Our recent findings extend these findings and demonstrate that Rad suppressed L-type calcium current (I<sub>Ca</sub>), [Ca<sup>2+</sup>]i transients, and contractility in adult cardiomyocytes. These results establish Rad as a novel endogenous regulator of cardiac EC coupling and β-AR signalling. Also, our previous observations demonstrated that Rad expression was significantly decreased in patients with end-stage heart failure and that Rad deficiency in mice led to cardiac hypertrophy.

Cardiac remodelling, which includes changes in heart size, shape, and function in response to injury or stress stimulation, can be classified as physiological remodelling and pathological remodelling. Unlike physiological remodelling, pathological remodelling involves reactivation of the foetal gene programme, cellular dysfunction, and cardiac fibrosis. Myocardial fibrosis and cardiac hypertrophy are characteristic features of pathological cardiac remodelling associated with
congestive heart failure. In response to pathological stimuli such as pressure loading, ischaemia, and inflammation, multiple cells in the heart are activated, resulting in altered metabolism of collagen and other ECM components. Eventually, ECM proteins accumulate excessively in the heart. Increased deposition of ECM components, along with the proliferation of fibroblasts, results in myocardial stiffness and diastolic dysfunction, which ultimately leads to heart failure. Numerous pro-fibrotic growth factors are involved in ECM accumulation. CTGF is considered to be a key molecule in controlling ECM synthesis and may serve as a diagnostic marker and therapeutic target for cardiac fibrosis and heart failure. Our data demonstrate that Rad KO mice can spontaneously develop fibrosis as early as 10 weeks of age. Although Rad deficiency is one of many factors inducing cardiac fibrosis, considering that Rad decreases significantly in patients with end-stage heart failure, Rad may serve as a good target to treat cardiovascular-related diseases. Furthermore, as Rad itself is related with diabetes and is abundant in the heart, this protein may participate in diabetic cardiomyopathy. Since fibrosis is an important feature of diabetic cardiomyopathy, it is possible that Rad may be a regulator of this cardiac fibrotic process in diabetic patients. Through microarray profiling and western blot analysis of Rad KO and WT mouse hearts, we found that CTGF is significantly up-regulated in Rad KO mice. Consistent with the in vivo data, Rad overexpression in cardiomyocytes inhibited CTGF expression, whereas Rad knockdown in cardiomyocytes increased CTGF levels. These data suggest that CTGF induction is responsible for Rad deficiency-induced fibrosis. Real-time PCR and western blot analysis demonstrate that Rad is dominantly expressed in cardiomyocytes but not fibroblasts, the major source of ECM, indicating that Rad deficiency-induced fibrosis may have developed as a result of paracrine signalling involving local communication between cardiomyocytes and fibroblasts. We used conditioned medium from Rad-overexpressing and Rad-knockdown cardiomyocytes to treat cardiac fibroblasts and found that the production of fibronectin, an important component of the ECM, decreased and increased, respectively. Blocking CTGF by using a neutralizing antibody completely abolished the pro-fibrotic effect of conditioned medium from Rad-knockdown cardiomyocytes. These results suggest that Rad deficiency resulted in elevated CTGF expression in cardiomyocytes and induced ECM production in cardiac fibroblasts.

Figure 3  Rad affects fibronectin production in fibroblasts through CTGF. (A) The CTGF level in cell culture supernatant was decreased when Rad is overexpressed. Non-serum conditioned medium from cardiomyocytes infected with Ad-GFP or Ad.Rad was collected and measured by ELISA (n = 6, *P < 0.05). (B) Cardiac fibroblasts were stimulated with non-serum conditioned medium from cardiomyocytes infected with Ad-GFP, Ad.Rad, Ad-Ctrl-shRNA, or Ad-Rad-shRNA, and fibronectin expression in fibroblasts was detected by western blotting. GAPDH expression was detected and utilized as a loading control. A representative western blot of four independent experiments is shown. Relative expression levels of fibronectin normalized to GAPDH are expressed as mean + SD (n = 4, **P < 0.01). (C) Non-serum conditioned medium from cardiomyocytes infected with Ad-Ctrl-shRNA or Ad-Rad-shRNA containing 10 μg/mL CTGF-neutralizing antibody (IgG as control) was used to stimulate cardiac fibroblasts. Fibronectin and GAPDH were detected by western blotting. Relative fibronectin expression levels of three independent experiments are expressed as mean + SD (n = 3, *P < 0.05, **P < 0.01).
Regulation of CTGF expression has been studied extensively. TGF-β1 strongly induces CTGF expression in many cell types, including cardiac myocytes and fibroblasts. Smad3 is the most important regulator for TGF-β1-induced expression of CTGF. However, Smad3 phosphorylation and Smad reporter activity remain consistent when Rad expression is altered. These results suggest that the Smad signalling pathway is not involved in Rad inhibition of CTGF expression.

Promoter deletion analysis identifies C/EBP-δ as the mediator of Rad-suppressed CTGF expression. Since we identified that Rad affects CTGF expression at the transcriptional level, promoter analyses were performed to determine the key mediators of Rad-regulated CTGF expression. The −80 bp CTGF promoter was activated by Rad knockdown; meanwhile, the −27 bp CTGF promoter activity was not affected. These results suggest that the region of the CTGF promoter between −27 and −80 bp is essential for Rad inhibition of CTGF expression. Within this region, a C/EBP-binding site was identified.

Figure 4 C/EBP-δ activates CTGF in cardiomyocytes. (A) Ad-Ctrl-shRNA- or Ad-Rad-shRNA-infected cardiomyocytes were transfected with different human CTGF promoter constructs as indicated. CTGF promoter activity was measured by firefly luciferase assay normalized to Renilla luciferase activity 48 h after transfection. Values are mean ± SD (n = 6, *P < 0.05). (B) A CTGF −48 or −66 bp promoter reporter was transfected into neonatal rat cardiomyocytes together with a pcDNA3.1-C/EBP-δ plasmid or pcDNA3.1 empty vector. Luciferase activity was measured 48 h after transfection. Values were normalized with Renilla luciferase activity. Values are mean ± SD (n = 6, **P < 0.01 vs. pcDNA3.1-transfected group). (C) Neonatal rat cardiomyocytes were transfected with pcDNA3.1, pcDNA3.1-C/EBP-δ, scrambled siRNA, or C/EBP-δ siRNA. CTGF and C/EBP-δ levels were detected by western blotting. GAPDH expression was detected and used as a loading control. Relative C/EBP-δ and CTGF expression levels of four independent experiments are expressed as mean ± SD (n = 4, **P < 0.01, *P < 0.05).

C/EBP-δ is a member of the C/EBP transcription factor family. It has been identified to be involved in cell growth and differentiation. C/EBP-δ regulates adipogenesis, participates in tumour suppression, and mediates various inflammatory responses. Although C/EBP-δ is characterized as a potential mediator in norepinephrine-induced cardiac hypertrophy, little is known about its function in regulating cardiac disease-related genes. Here, our data indicate for the first time that C/EBP-δ activates CTGF transcription through a predicted C/EBP-binding site located on the CTGF promoter. Knocking down C/EBP-δ can reduce CTGF expression in cardiomyocytes. Co-immunoprecipitation and ChIP assay experiments indicated that Rad binds with C/EBP-δ, and this interaction inhibits the binding of C/EBP-δ to the CTGF promoter, thus suppressing CTGF transcription. RGK proteins have been shown to be localized to the cytoplasm, plasma membrane, and nucleus. The distribution of Rad between the cytoplasm and nucleus could be regulated by calmodulin and 14-3-3 binding. According to our
observations, we found a novel mechanism by which Rad binds to C/EBP-δ and thereby regulates transcription of CTGF. This raises the possibility that the regulation of Rad transportation between the cytoplasm and nucleus is important for Rad function. However, there is a need for further investigation.

Taken together, our findings demonstrate that Rad is a potent inhibitor of cardiac fibrosis. By directly interacting with C/EBP-δ, Rad suppresses CTGF expression and subsequently reduces ECM deposition. This study suggests a potential link between decreased Rad expression and increased cardiac fibrosis in human failing hearts. Thus, further understanding of Rad function will help us elucidate the mechanisms responsible for diabetic cardiomyopathy and heart failure and provide new therapeutic targets for the treatment of cardiovascular diseases.

### Supplementary material

Supplementary material is available at [Cardiovascular Research online](#).

### Conflict of interest

None declared.

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