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Cytokine receptor-like factor 1 (CRLF1) promotes cardiac fibrosis via ERK1/2 signaling pathway

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Abstract: Cardiac fibrosis is a cause of morbidity and mortality in people with heart disease. Anti-fibrosis treatment is a significant therapy for heart disease, but there is still no thorough understanding of fibrotic mechanisms. This study was carried out to ascertain the functions of cytokine receptor-like factor 1 (CRLF1) in cardiac fibrosis and clarify its regulatory mechanisms. We found that *CRLF1* was expressed predominantly in cardiac fibroblasts. Its expression was up-regulated not only in a mouse heart fibrotic model induced by myocardial infarction, but also in mouse and human cardiac fibroblasts provoked by transforming growth factor-β1 (TGF-β1). Gain- and loss-of-function experiments of *CRLF1* were carried out in neonatal mice cardiac fibroblasts (NMCFs) with or without TGF-β1 stimulation. *CRLF1* overexpression increased cell viability, collagen production, cell proliferation capacity, and myofibroblast transformation of NMCFs with or without TGF-β1 stimulation, while silencing of *CRLF1* had the opposite effects. An inhibitor of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway and different inhibitors of TGF-β1 signaling cascades, comprising mothers against decapentaplegic homolog (SMAD)-dependent and SMAD-independent pathways, were applied to investigate the mechanisms involved. CRLF1 exerted its functions by activating the ERK1/2 signaling pathway. Furthermore, the SMAD-dependent pathway, not the SMAD-independent pathway, was responsible for *CRLF1* up-regulation in NMCFs treated with TGF-β1. In summary, activation of the TGF-β1/SMAD signaling pathway in cardiac fibrosis increased *CRLF1* expression. CRLF1 then aggravated cardiac fibrosis by activating the ERK1/2 signaling pathway. CRLF1 could become a novel potential target for intervention and remedy of cardiac fibrosis.

Key words: Cytokine receptor-like factor 1 (CRLF1); TGF-β1/SMAD signaling pathway; ERK1/2 signaling pathway; Cardiac fibrosis; Myofibroblast transformation; Extracellular matrix (ECM)

1 Introduction

Cardiovascular disease is a serious disease that endangers people's lives, and its incidence is increasing year by year. Cardiac fibrosis most commonly occurs in the wake of many kinds of cardiovascular diseases (Brown et al., 2005; Schuetze et al., 2014). A key characteristic of cardiac fibrosis is that extracellular matrix (ECM) components are deposited in myocardial stroma (Janicki and Brower, 2002; Berk et al., 2007; Bacmeister et al., 2019). During the process of cardiac fibrosis, multiple fibrosis-associated components in the ECM, especially type I collagen (Col-1) and type III collagen (Col-3), change significantly in stimulated hearts (Shirwany and Weber, 2006; Disertori et al., 2017; López et al., 2021). Collagen proteins are synthesized and secreted mainly by cardiac fibroblasts, and maintain a dynamic balance of synthesis and degradation under physiological conditions, which is broken under pathological conditions. This quantitative or proportional imbalance of collagen proteins in the ECM subsequently damages heart functions including cardiac systolic and diastolic functions (Fernández-Alfonso and Ruilope, 2014; Nguyen et al., 2014, 2017; Perestrelo

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et al., 2021). Although it is well known that increased collagen deposition and myofibroblast transformation occur during cardiac fibrosis, the underlying events and regulation mechanisms are still unclear and need to be explored urgently. In this study, we chose cytokine receptor-like factor 1 (CRLF1) as our target protein in the hope of discovering a new action molecule in cardiac fibrosis.

CRLF1 is a member of the cytokine family proteins. Mutation of *CRLF1* is proposed as a clinical auxiliary diagnostic index of the cold-induced sweating syndrome (CISS) (Herholz et al., 2011; Busch et al., 2017). Subsequent research has shown that CRLF1 plays an important role in controlling facial muscles, heat production, and other autonomic nervous systems (Buers et al., 2020). Some studies have shown that CRLF1 can participate in the physiological and pathological processes of various tissues and organs (Yu ST et al., 2018, 2020; Li et al., 2021). In addition, the expression of *CRLF1* is positively correlated with the level of liver fibrosis, and *CRLF1* overexpression can up-regulate Col-3 expression and the α-smooth muscle actin (α-SMA) level in quiescent hepatic stellate cells (HSCs) (Stefanovic and Stefanovic, 2012). There has been very little research on the role of CRLF1 in the heart. Acute cocaine exposure can cause an increased expression of *CRLF1* in both the ventricular tissue of the rabbit and H9C2 cell lines (Lattanzio et al., 2005). Here, we analyzed the expression of *CRLF1* in two important kinds of heart cells (fibroblasts and cardiomyocytes) and found that *CRLF1* was expressed in the heart, mostly in cardiac fibroblasts rather than cardiomyocytes (Tucker et al., 2020; Chaffin et al., 2022; Koenig et al., 2022). This finding suggested that CRLF1 might affect the regulation of cardiac fibrosis. Unfortunately, little was known about the functions of CRLF1 in cardiac fibrosis.

In this study, we carried out detailed and in-depth research about CRLF1 in cardiac fibrosis. Of note, we found that *CRLF1* was predominantly expressed in cardiac fibroblasts, and its expression was induced during the fibrotic process both in vitro and in vivo. *CRLF1* overexpression promoted the cardiac fibrotic process, including excessive cell proliferation, collagen protein synthesis and secretion, and myofibroblast transformation, while silencing of *CRLF1* had the opposite effects. Furthermore, activation of the transforming growth factor-β1 (TGF-β1)/mothers against decapentaplegic homolog (SMAD) signaling pathway during the fibrotic process not only caused the production of collagen and myofibroblast transformation, but also increased *CRLF1* expression. Up-regulation of *CRLF1* in turn consolidated the outcome of the TGF-β1/SMAD signaling pathway in cardiac fibrosis by activating the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. In summary, we elucidated the function of CRLF1 in the development of cardiac fibrosis and its relationship with the TGF-β1/SMAD signaling pathway. Our study provides a theoretical basis for CRLF1 to be applied as a novel promising marker for cardiac fibrosis diagnosis as well as a useful target for cardiac fibrosis remedies.

2 Results

2.1 *CRLF1* expression during the cardiac fibrotic process

Firstly, the change in *CRLF1* expression during cardiac fibrosis was investigated in a mouse cardiac fibrosis model achieved by myocardial infarction (MI) for four weeks. Masson's trichrome staining suggested cardiac fibrosis had occurred in the mouse hearts after MI (Fig. 1a). The messenger RNA (mRNA) expression of *Col-1* (consisting of the *Col-1A1* and *Col-1A2* subunits) and *Col-3* (*Col-3A1* subunit) was increased in the MI group, suggesting enhanced collagen production (Fig. 1b). In line with the pathogenesis of cardiac fibrosis, the MI group showed increased *α-SMA* expression, implying elevated myofibroblast transformation (Fig. 1b). Interestingly, compared to the sham group, the cardiac mRNA expression and protein levels of CRLF1 were also increased during the fibrotic process in vivo (Figs. 1b and 1c).

Next, *CRLF1* expression was compared among neonatal mice cardiac fibroblasts (NMCFs), adult mice cardiac fibroblasts (AMCFs), neonatal mice cardiomyocytes (NMCMs), and endothelial cells (ECs). Compared to NMCFs, *CRLF1* expression was similar in AMCFs, while NMCMs and ECs expressed lower levels of *CRLF1* (Fig. 1d). Staining with 5-ethynyl-2'-deoxyuridine (EdU) suggested that NMCFs were undergoing a fibrotic process of excessive proliferation under TGF-β1 stimulation (Fig. 1e). In addition, TGF-β1 stimulation in NMCFs induced collagen production and myofibroblast transformation as shown

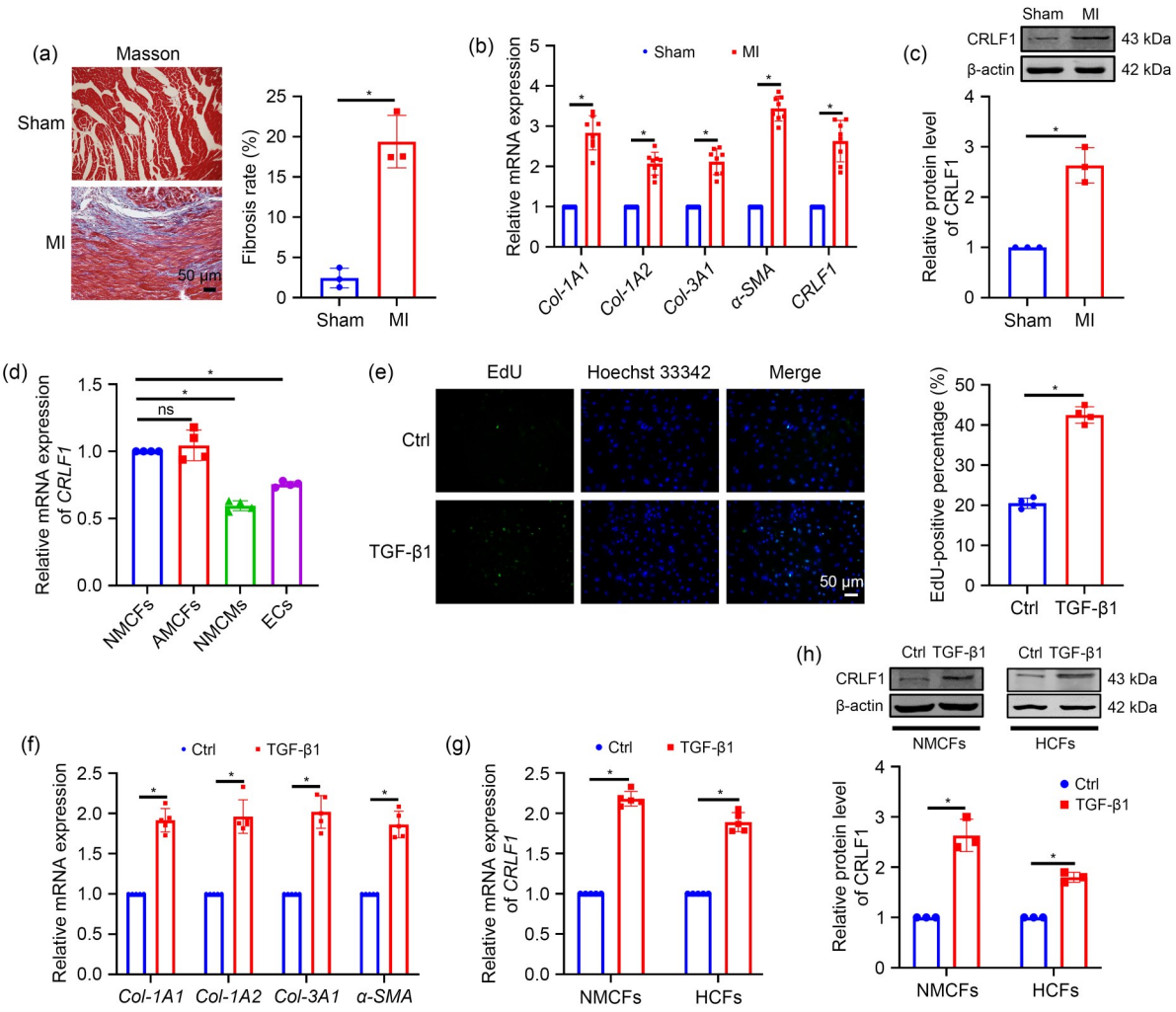

Fig. 1 Up-regulation of *CRLF1* in the fibrotic process in vivo and in vitro. (a) Comparison of interstitial fibrosis detected by Masson's trichrome staining in the sham and MI groups. *n*=3. (b) mRNA expression of *Collagen* and *α-SMA* in mice hearts. *n*=8. (c) Protein level of CRLF1 in heart tissues. *n*=3. (d) mRNA expression of *CRLF1* in NMCFs, AMCFs, NMCMs, and ECs. *n*=4. (e) Comparison of EdU staining of NMCFs in the Ctrl and TGF-β1 stimulation groups. *n*=4. (f) mRNA expression of *Collagen* and *α-SMA* in NMCFs under TGF-β1 stimulation. *n*=5. (g) mRNA expression of *CRLF1* in NMCFs and HCFs under TGF-β1 stimulation. *n*=5. (h) Protein level of CRLF1 in NMCFs and HCFs under TGF-β1 stimulation. *n*=3. Data are shown as mean±SD. * *P*<0.05; ns: not significant. CRLF1: cytokine receptor-like factor 1; MI: myocardial infarction; mRNA: messenger RNA; α-SMA: α-smooth muscle actin; Col-1: type I collagen; Col-3: type III collagen; NMCFs: neonatal mice cardiac fibroblasts; AMCFs: adult mice cardiac fibroblasts; NMCMs: neonatal mice cardiomyocytes; ECs: endothelial cells; Ctrl: control; TGF-β1: transforming growth factor-β1; EdU: 5-ethynyl-2'-deoxyuridine; HCFs: human cardiac fibroblasts; SD: standard deviation.

by increased expression of *Col-1A1*, *Col-1A2*, *Col-3A1*, and *α-SMA* (Fig. 1f). During this fibrotic process, *CRLF1* expression was significantly increased in NMCFs and human cardiac fibroblasts (HCFs) under TGF-β1 stimulation (Fig. 1g). In addition, the CRLF1 protein level was detected by western blotting in NMCFs and HCFs under TGF-β1 stimulation. Compared to the control (Ctrl) group, CRLF1 had a higher level under TGF-β1 stimulation in both NMCFs and HCFs (Fig. 1h).

2.2 Effects of *CRLF1* overexpression or silencing on fibrotic process in NMCFs under a physiological state

Gain- and loss-of-function strategies were used to explore the functions of CRLF1 in NMCFs by transfection of overexpression plasmids and small interfering RNAs (siRNAs). We screened the transfection concentrations of plasmids and finally selected 100 ng/mL as the concentration for subsequent study (Fig. 2a).

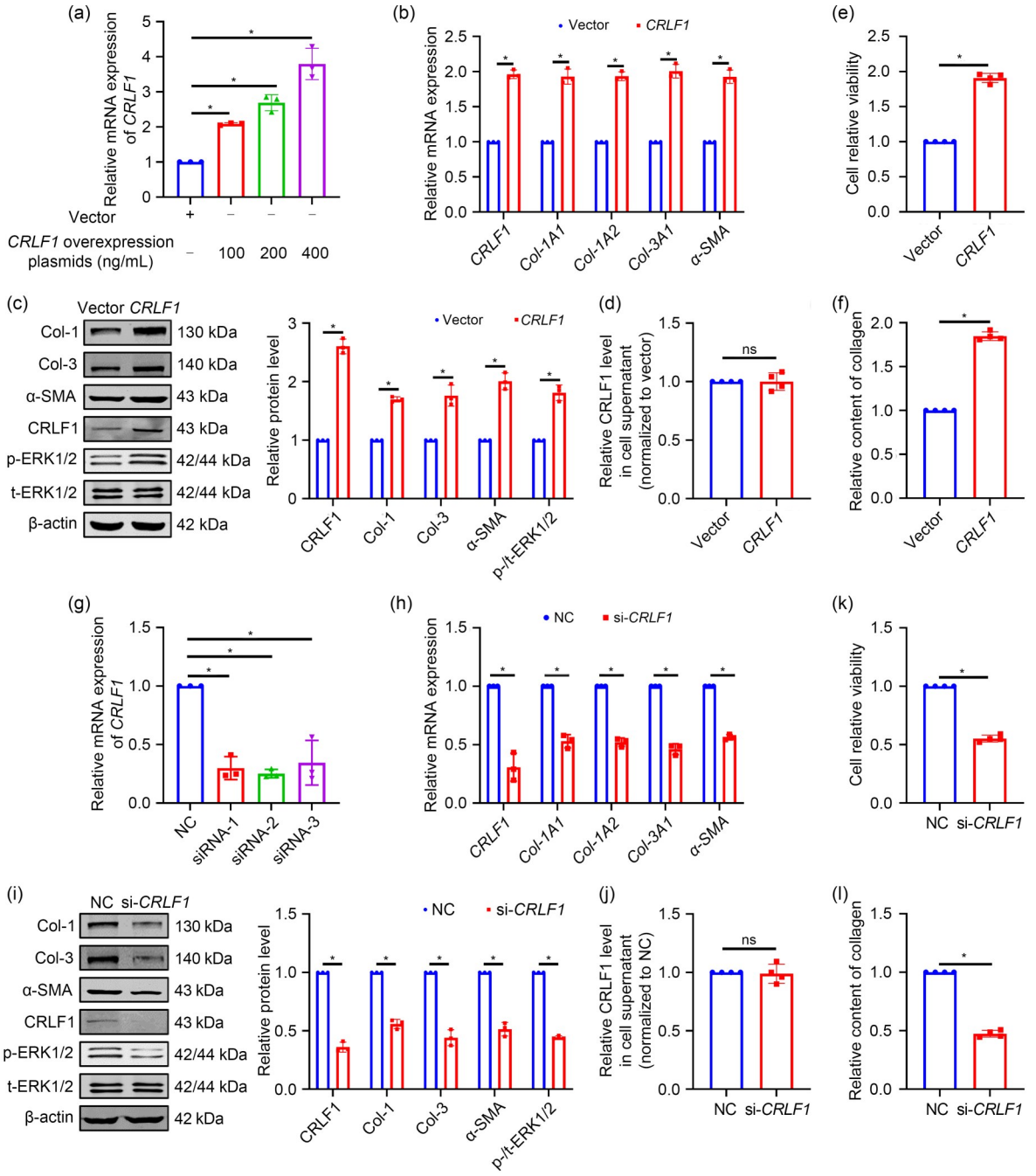

Fig. 2 Effects of *CRLF1* overexpression or silencing on fibrotic process in NMCFs. (a) mRNA expression of *CRLF1* after transfection of its overexpression plasmids with different concentrations. *n*=3. (b) mRNA expression of *Collagen* and *α-SMA* in NMCFs after *CRLF1* overexpression. *n*=3. (c) Protein levels in NMCFs after *CRLF1* overexpression. *n*=3. (d) CRLF1 level detected by ELISA in cell supernatant of NMCFs after *CRLF1* overexpression. *n*=4. (e) Cell viability of NMCFs after *CRLF1* overexpression. *n*=4. (f) Collagen content of NMCFs after *CRLF1* overexpression. *n*=4. (g) mRNA expression of *CRLF1* after transfection of its different siRNAs. *n*=3. (h) mRNA expression of *Collagen* and *α-SMA* in NMCFs after *CRLF1* silencing. *n*=3. (i) Protein levels in NMCFs after *CRLF1* silencing. *n*=3. (j) CRLF1 level detected by ELISA in cell supernatant after *CRLF1* silencing. *n*=4. (k) Cell viability of NMCFs after *CRLF1* silencing. *n*=4. (l) Collagen content of NMCFs after *CRLF1* silencing. *n*=4. Data are shown as mean±SD. * *P*<0.05; ns: not significant. CRLF1: cytokine receptor-like factor 1; NMCFs: neonatal mice cardiac fibroblasts; mRNA: messenger RNA; si-*CRLF1*: small interfering RNAs (siRNAs) for *CRLF1*; α-SMA: α-smooth muscle actin; Col-1: type I collagen; Col-3: type III collagen; ERK1/2: extracellular signal-regulated kinase 1/2; p-ERK1/2: phospho-ERK1/2; t-ERK1/2: total-ERK1/2; ELISA: enzyme-linked immunosorbent assay; NC: negative control; SD: standard deviation.

Furthermore, transfection efficiency was verified at mRNA and protein levels accompanied by increased transcription and translation of CRLF1, collagen proteins (Col-1 and Col-3), and α-SMA (Figs. 2b and 2c). Cell counting kit-8 (CCK-8) assay results showed that *CRLF1* overexpression increased the cell viability of NMCFs, which indirectly indicated that NMCFs had a higher proliferative capacity after *CRLF1* overexpression (Fig. 2e). Similarly, *CRLF1* overexpression increased collagen production of NMCFs (Fig. 2f).

In the following loss-of-function experiments, we screened for siRNAs that could stably and efficiently silence *CRLF1* expression (Fig. 2g). We ultimately chose siRNA-2 (si-*CRLF1*) for *CRLF1* silencing, and its silencing efficiency was verified by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting experiments (Figs. 2h and 2i). Meanwhile, transcription levels of *Col-1A1*, *Col-1A2*, *Col-3A1*, and *α-SMA* were decreased (Fig. 2h). In addition, Col-1, Col-3, and α-SMA protein levels were also decreased after *CRLF1* silencing in NMCFs (Fig. 2i). Moreover, cell viability (Fig. 2k) and collagen content (Fig. 2l) of the *CRLF1* silencing group were significantly reduced. No change in CRLF1 was detected in the cell supernatant after *CRLF1* overexpression or silencing (Figs. 2d and 2j). This suggested that CRLF1 might function in the cytoplasm rather than in the extracellular space.

2.3 Effect of *CRLF1* overexpression on fibrotic process in NMCFs under a pathological state

Having detected the role of CRLF1 in a physiological state, we investigated whether CRLF1 also played a role in cells under a pathological state. We provoked NMCFs with TGF-β1 to proliferate excessively and synthesize collagen to imitate the fibrotic process in vitro. *CRLF1* overexpression aggravated the increase of cell viability and collagen production activated by TGF-β1 (Figs. 3a and 3b). The EdU results directly showed that *CRLF1* overexpression resulted in further proliferation induced by TGF-β1 (Fig. 3c). Furthermore, we tested the mRNA and protein levels of fibrosis-related molecules. The qRT-PCR results showed that the mRNA expression levels of *Col-1* (*Col-1A1* and *Col-1A2*), *Col-3* (*Col-3A1*), and *α-SMA* were all up-regulated in response to TGF-β1 stimulation after *CRLF1* overexpression (Fig. 3d). In addition, *CRLF1* overexpression obviously raised the protein levels of Col-1, Col-3, and α-SMA compared with those of the TGF-β1 stimulation group (Fig. 3e).

2.4 Effect of *CRLF1* silencing on fibrotic process in NMCFs under a pathological state

In contrast, silencing of endogenous *CRLF1* by si-*CRLF1* inhibited the up-regulation of cell viability (Fig. 4a), collagen content (Fig. 4b), and cell proliferation (Fig. 4c) induced by TGF-β1. Moreover, *CRLF1* silencing decreased the mRNA and protein levels of Col-1, Col-3, and α-SMA (Figs. 4d and 4e). These results suggested that *CRLF1* overexpression promoted NMCFs to proliferate and synthesize collagen excessively, and transform myofibroblasts, while *CRLF1* silencing had the opposite effects under physiological and pathological states.

2.5 Activation of ERK1/2 signaling pathway after *CRLF1* overexpression or silencing in NMCFs

The function of CRLF1 is regulated by the ERK1/2 signaling pathway in other tissues (Yu ST et al., 2018, 2020; Zheng et al., 2020), and the ERK1/2 signaling pathway also participates widely in the regulation of cardiac fibrosis (Yamaguchi et al., 2004; Westermann et al., 2007; Wang XW et al., 2021; Wen et al., 2022). Therefore, we examined whether the ERK1/2 signaling pathway changed when *CRLF1* was overexpressed or silenced in NMCFs with or without TGF-β1 stimulation. Enzyme-linked immunosorbent assay (ELISA) results showed that *CRLF1* overexpression increased the phospho-ERK1/2 (p-ERK1/2) protein level in NMCFs without TGF-β1 stimulation (Fig. 5a). Amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) results also showed that the ratio of p-ERK1/2 to total-ERK1/2 (t-ERK1/2) was increased by *CRLF1* overexpression in NMCFs without TGF-β1 stimulation (Fig. 5b). Then, NMCFs were co-treated with TGF-β1 and *CRLF1* overexpression plasmids. The ELISA and AlphaLISA results showed that *CRLF1* overexpression increased p-ERK1/2 protein activation in TGF-β1-treated NMCFs (Figs. 5c and 5d). Furthermore, western blotting results indicated that *CRLF1* overexpression increased the p-/t-ERK1/2 protein level after TGF-β1 stimulation (Fig. 5e).

In contrast, *CRLF1* silencing showed the opposite effects of *CRLF1* overexpression (Figs. 5f–5j). We also detected the changes of the p-ERK1/2 protein level after *CRLF1* overexpression or silencing in NMCFs

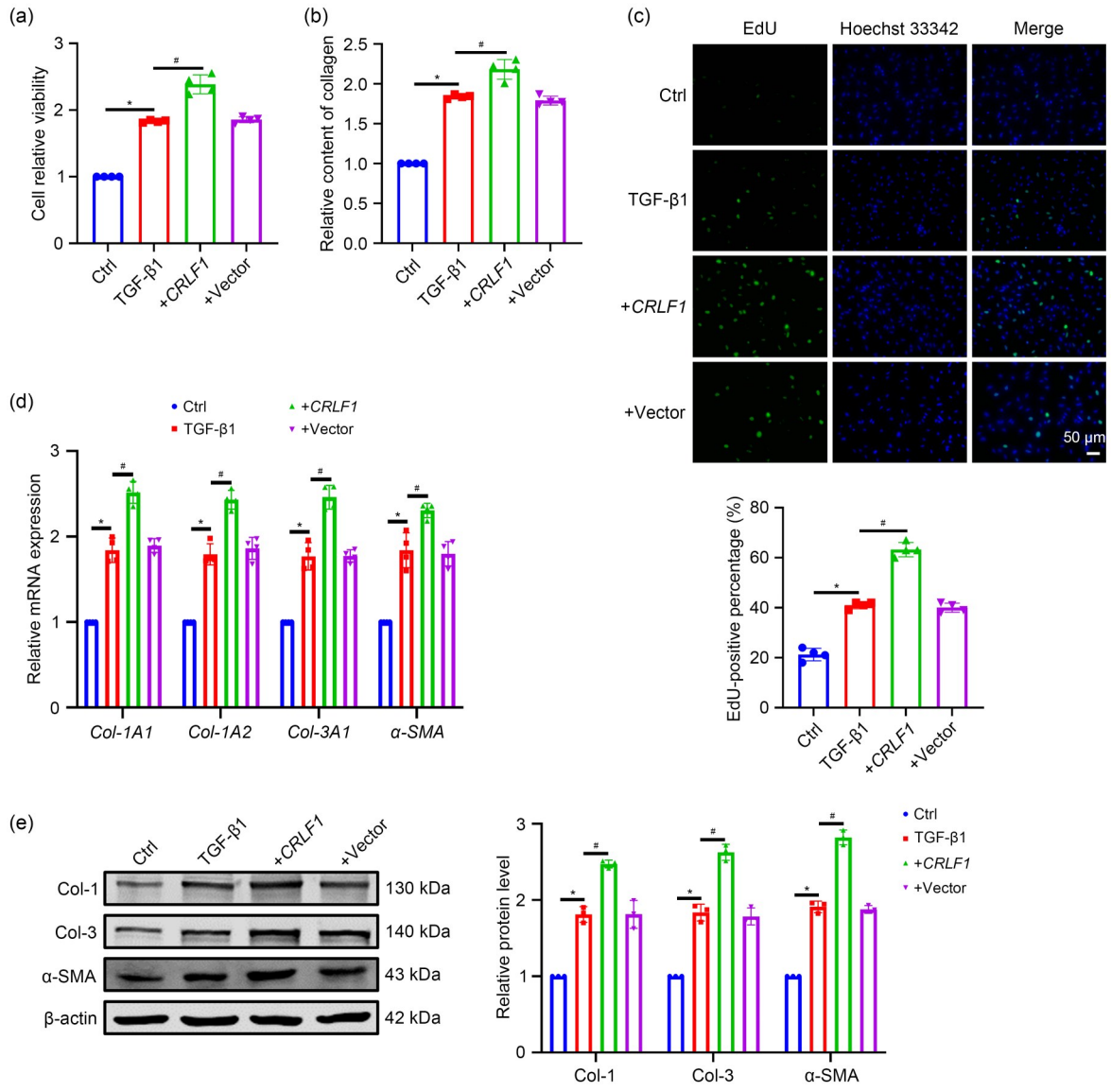

Fig. 3 Effect of *CRLF1* overexpression on fibrotic process in NMCFs under TGF-β1 stimulation. (a) Cell viability of NMCFs after *CRLF1* overexpression under TGF-β1 stimulation. *n*=4. (b) Collagen content of NMCFs after *CRLF1* overexpression under TGF-β1 stimulation. *n*=4. (c) EdU staining of NMCFs after *CRLF1* overexpression under TGF-β1 stimulation. *n*=4. (d) mRNA expression of *Collagen* and *α-SMA* after *CRLF1* overexpression in TGF-β1-treated NMCFs. *n*=4. (e) Protein levels of collagen and α-SMA after *CRLF1* overexpression in TGF-β1-treated NMCFs. *n*=3. Data are shown as mean±SD. * *P*<0.05; # *P*<0.05. CRLF1: cytokine receptor-like factor 1; NMCFs: neonatal mice cardiac fibroblasts; TGF-β1: transforming growth factor-β1; EdU: 5-ethynyl-2'-deoxyuridine; mRNA: messenger RNA; α-SMA: α-smooth muscle actin; Col-1: type I collagen; Col-3: type III collagen; Ctrl: control; SD: standard deviation.

without TGF-β1 stimulation. The ratio of p-ERK1/2 to t-ERK1/2 was also increased after *CRLF1* overexpression, but decreased after *CRLF1* silencing in non-activated NMCFs (Figs. 2c and 2i). These results suggested that CRLF1 could influence the ERK1/2 signaling pathway in NMCFs under pathological and physiological states.

2.6 Functions of CRLF1 via the ERK1/2 signaling pathway in NMCFs

We wondered if the ERK1/2 signaling pathway could mediate the functions of CRLF1 in NMCFs. Firstly, by consulting the medical literature (Wang ZY et al., 2019; Wang JC et al., 2021), we tested 1 and

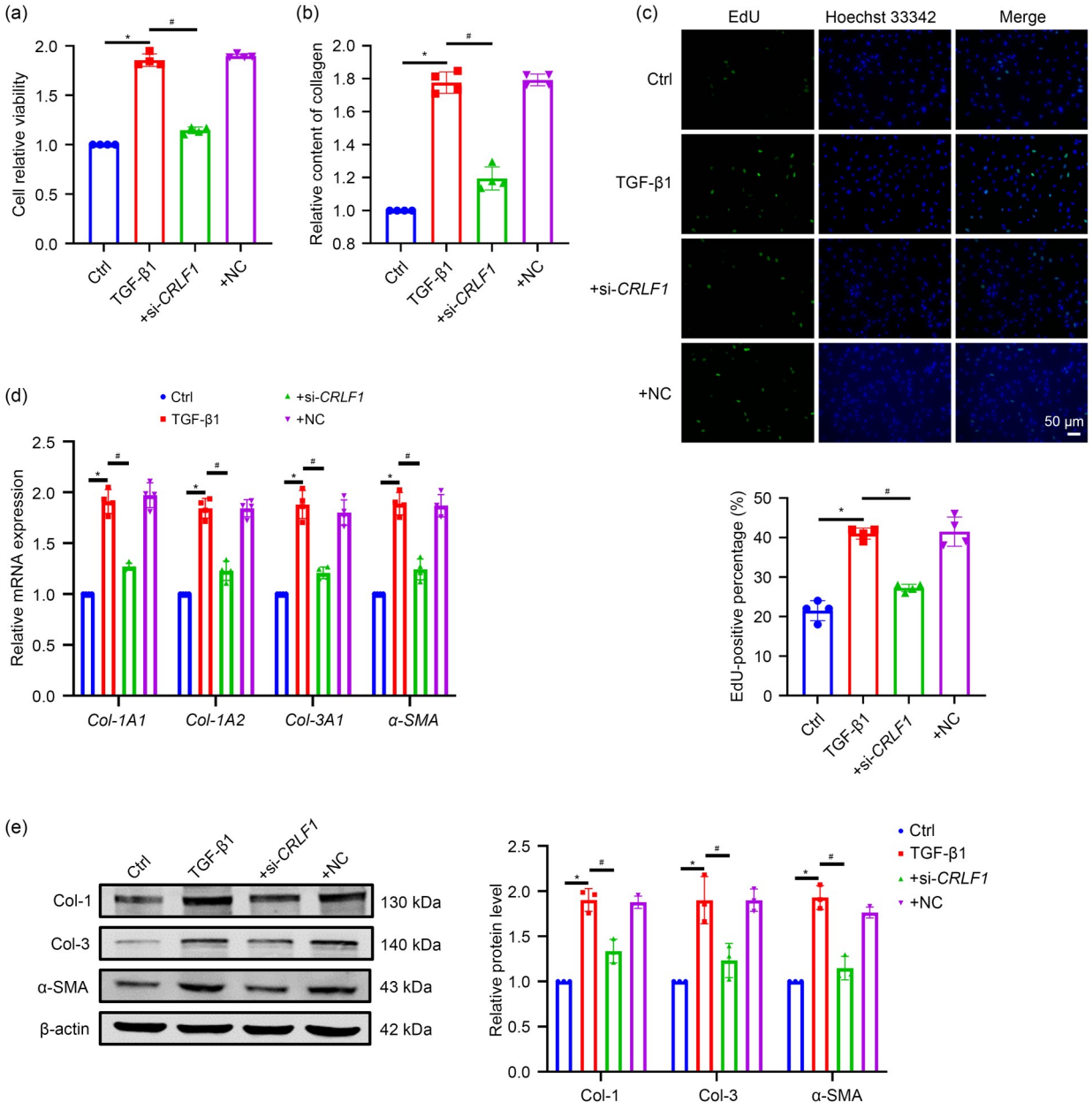

Fig. 4 Effect of *CRLF1* silencing on fibrotic process in NMCFs under TGF-β1 stimulation. (a) Cell viability of NMCFs after *CRLF1* silencing under TGF-β1 stimulation. *n*=4. (b) Collagen content of NMCFs after *CRLF1* silencing under TGF-β1 stimulation. *n*=4. (c) EdU staining of NMCFs after *CRLF1* silencing under TGF-β1 stimulation. *n*=4. (d) mRNA expression of *Collagen* and *α-SMA* after *CRLF1* silencing in TGF-β1-treated NMCFs. *n*=4. (e) Protein levels of collagen and α-SMA after *CRLF1* silencing in TGF-β1-treated NMCFs. *n*=3. Data are shown as mean±SD. * *P*<0.05; # *P*<0.05. CRLF1: cytokine receptor-like factor 1; NMCFs: neonatal mice cardiac fibroblasts; TGF-β1: transforming growth factor-β1; si-*CRLF1*: small interfering RNAs for *CRLF1*; EdU: 5-ethynyl-2'-deoxyuridine; mRNA: messenger RNA; α-SMA: α-smooth muscle actin; Col-1: type I collagen; Col-3: type III collagen; Ctrl: control; NC: negative control; SD: standard deviation.

2 μmol/L doses of the ERK1/2 inhibitor Temuterkib (LY3214996) to select an appropriate inhibition concentration. Both 1 and 2 μmol/L of Temuterkib significantly reduced the basal p-ERK1/2 and t-ERK1/2 protein levels (Fig. 6a). Both doses of Temuterkib clearly reduced activation of the ERK1/2 signaling pathway. We finally chose 1 μmol/L because of its low dose and favorable potency. After treating NMCFs with *CRLF1* overexpression plasmids, qRT-PCR showed that *CRLF1* overexpression no longer increased the expression of

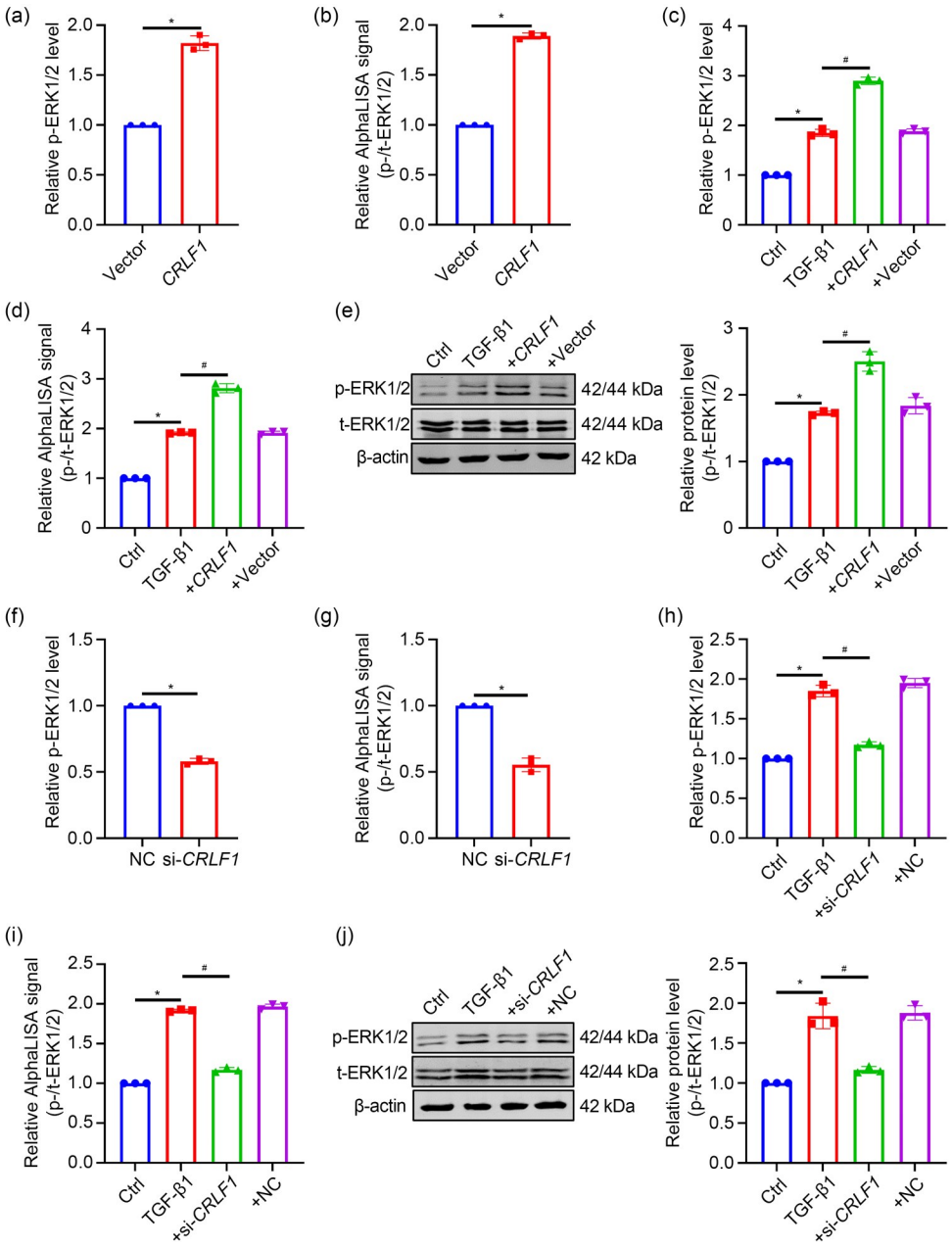

Fig. 5 ERK1/2 protein activation after *CRLF1* overexpression or silencing in NMCFs with or without TGF-β1 stimulation. (a) p-ERK1/2 level detected by ELISA in NMCFs after *CRLF1* overexpression without TGF-β1 stimulation. *n*=3. (b) Relative AlphaLISA signal (p-/t-ERK1/2) in NMCFs after *CRLF1* overexpression without TGF-β1 stimulation. *n*=3. (c) p-ERK1/2 level detected by ELISA in NMCFs after *CRLF1* overexpression with TGF-β1 stimulation. *n*=3. (d) Relative AlphaLISA signal (p-/t-ERK1/2) in NMCFs after *CRLF1* overexpression with TGF-β1 stimulation. *n*=3. (e) p-/t-ERK1/2 level detected by western blotting in NMCFs after *CRLF1* overexpression under TGF-β1 stimulation. *n*=3. (f) p-ERK1/2 level detected by ELISA in NMCFs after *CRLF1* silencing without TGF-β1 stimulation. *n*=3. (g) Relative AlphaLISA signal (p-/t-ERK1/2) in NMCFs after *CRLF1* silencing without TGF-β1 stimulation. *n*=3. (h) p-ERK1/2 level detected by ELISA in NMCFs after *CRLF1* silencing with TGF-β1 stimulation. *n*=3. (i) Relative AlphaLISA signal (p-/t-ERK1/2) in NMCFs after *CRLF1* silencing with TGF-β1 stimulation. *n*=3. (j) p-/t-ERK1/2 level detected by western blotting in NMCFs after *CRLF1* silencing under TGF-β1 stimulation. *n*=3. Data are shown as mean±SD. * *P*<0.05; # *P*<0.05. ERK1/2: extracellular signal-regulated kinase 1/2; p-ERK1/2: phospho-ERK1/2; t-ERK1/2: total-ERK1/2; CRLF1: cytokine receptor-like factor 1; NMCFs: neonatal mice cardiac fibroblasts; TGF-β1: transforming growth factor-β1; si-*CRLF1*: small interfering RNAs for *CRLF1*; ELISA: enzyme-linked immunosorbent assay; AlphaLISA: amplified luminescent proximity homogeneous assay-linked immunosorbent assay; Ctrl: control; NC: negative control; SD: standard deviation.

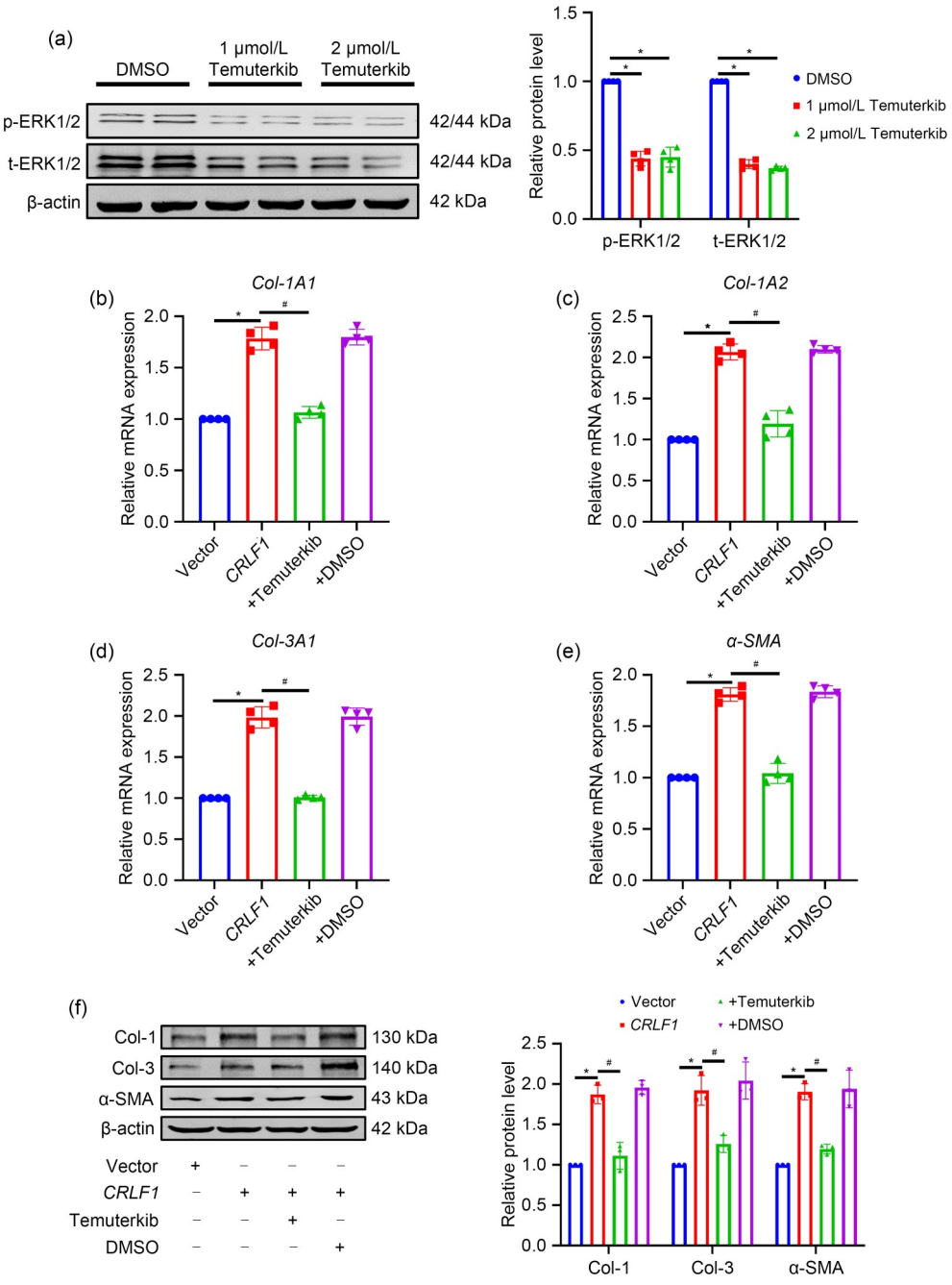

Fig. 6 Functions of CRLF1 via the ERK1/2 signaling pathway in NMCFs. (a) Protein levels of p-ERK1/2 and t-ERK1/2 under different stimulation concentrations of the ERK1/2 inhibitor Temuterkib. *n*=4. (b–e) mRNA expression of *Col-1A1* (b), *Col-1A2* (c), *Col-3A1* (d), and *α-SMA* (e) in NMCFs. *n*=4. (f) Protein levels of collagen and α-SMA in NMCFs. *n*=3. Data are shown as mean±SD. * *P*<0.05; # *P*<0.05. CRLF1: cytokine receptor-like factor 1; ERK1/2: extracellular signal-regulated kinase 1/2; p-ERK1/2: phospho-ERK1/2; t-ERK1/2: total-ERK1/2; NMCFs: neonatal mice cardiac fibroblasts; DMSO: dimethyl sulfoxide; mRNA: messenger RNA; α-SMA: α-smooth muscle actin; Col-1: type I collagen; Col-3: type III collagen; SD: standard deviation.

Col-1 (*Col-1A1* and *Col-1A2*), *Col-3* (*Col-3A1*), or *α-SMA* mRNAs after inhibition of the ERK1/2 signaling pathway (Figs. 6b–6e). Col-1, Col-3, and α-SMA protein levels showed the same trend (Fig. 6f). These results suggested that CRLF1 worked in NMCFs by activating the ERK1/2 signaling pathway.

2.7 *CRLF1* up-regulation via TGF-β1/SMAD signaling pathway in cardiac fibrosis

Through the above research, we had identified p-ERK1/2 as the downstream target in the regulation of CRLF1 function, but it was unclear why *CRLF1* was up-regulated in NMCFs under TGF-β1 stimulation. TGF-β1 is the best characterized fibrogenic growth factor (Frangogiannis, 2020; Lodyga and Hinz, 2020). TGF-β1 signaling cascades are divided into SMAD-dependent and SMAD-independent pathways (Derynck and Zhang, 2003; Schmierer and Hill, 2007; Hu et al., 2018). In SMAD-dependent pathways (SMAD signaling pathways), the phosphorylated SMAD2 and SMAD3 complicate with SMAD4 and translocate to the nucleus to induce transcription of profibrotic molecules (Meng et al., 2016; Hanna and Frangogiannis, 2019). We speculated that the SMAD signaling pathway could regulate the expression of *CRLF1* because it is widely involved in cardiac fibrosis (Li et al., 1997; Dewald et al., 2004; Xia et al., 2011; Biernacka et al., 2015; Nagaraju et al., 2019). Following a comprehensive review of the medical literature (Tu et al., 2011; Sundararaj et al., 2016; Meng et al., 2020; Shen et al., 2020), we first screened 1 and 2 μmol/L doses of the SMAD signaling pathway inhibitor, specific inhibitor of SMAD3 (SIS3), to test its effect on the mRNA expression of *Col-1* (*Col-1A1* and *Col-1A2*), *Col-3* (*Col-3A1*), and *α-SMA* (Fig. 7a). Both doses of SIS3 had good potency. Then, NMCFs were treated with TGF-β1, followed by treatment with DMSO (a solvent for SIS3) or SIS3 (1 μmol/L). Up-regulated expression of *CRLF1* induced by TGF-β1 was blocked by SIS3 in mRNA and protein levels (Figs. 7b and 7c).

In addition, in SMAD-independent pathways, TGF-β1 can regulate the activation of three known mitogen-activated protein kinase (MAPK) pathways,

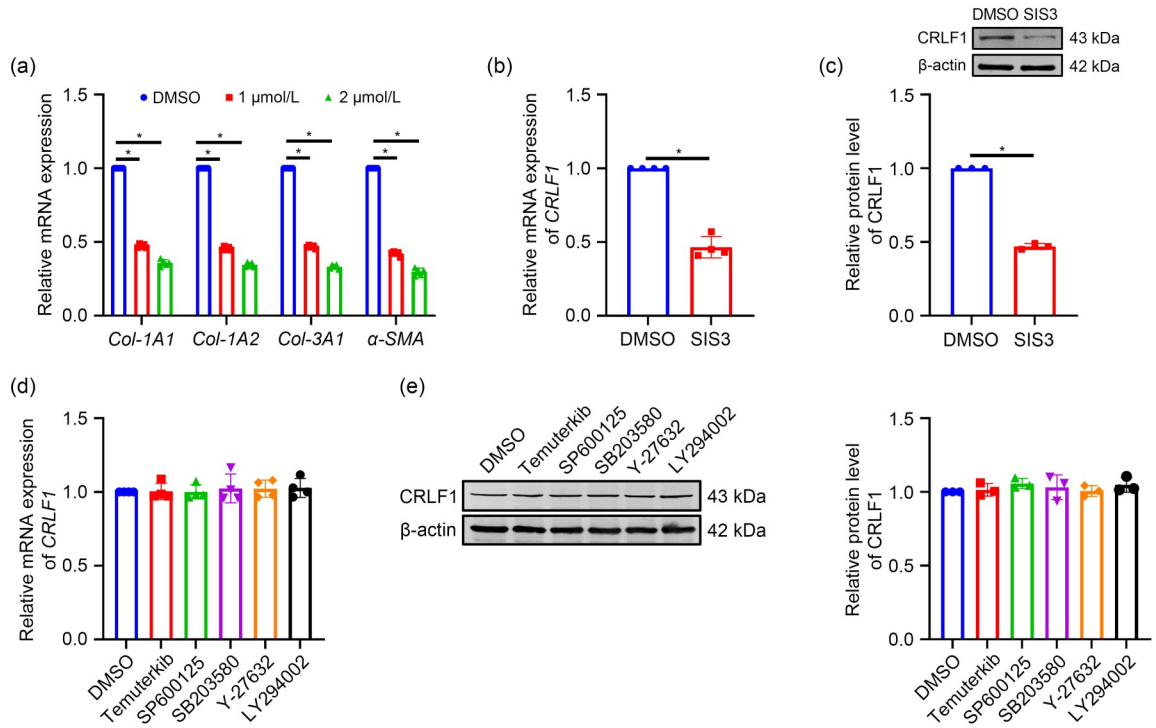

Fig. 7 *CRLF1* up-regulation via TGF-β1/SMAD signaling pathway. (a) mRNA expression in NMCFs under different concentrations of SIS3. *n*=5. (b) mRNA expression of *CRLF1* under SIS3 treatment in TGF-β1-treated NMCFs. *n*=4. (c) Protein level of CRLF1 under SIS3 treatment in TGF-β1-treated NMCFs. *n*=3. (d) mRNA expression of *CRLF1* under treatment of different inhibitors of SMAD-independent pathways in TGF-β1-treated NMCFs. *n*=4. (e) Protein level of CRLF1 under treatment with different inhibitors of SMAD-independent pathways in TGF-β1-treated NMCFs. *n*=3. Data are shown as mean±SD. * *P*<0.05. CRLF1: cytokine receptor-like factor 1; TGF-β1: transforming growth factor-β1; SMAD: mothers against decapentaplegic homolog; mRNA: messenger RNA; NMCFs: neonatal mice cardiac fibroblasts; SIS3: specific inhibitor of SMAD3; DMSO: dimethyl sulfoxide; Col-1: type I collagen; Col-3: type III collagen; α-SMA: α-smooth muscle actin; SD: standard deviation.

namely ERK1/2, c-Jun N-terminal kinase (JNK), and protein 38 (p38) MAPK, as well as the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and Ras homolog gene family, member A (RhoA)/Rho-associated kinase (ROCK) pathways (Horbelt et al., 2012; Scalise et al., 2021). Therefore, we applied the ERK1/2 inhibitor Temuterkib (1 μmol/L) and other inhibitors within the same category, at a concentration based on the literature: SP600125 (25 μmol/L), SB203580 (10 μmol/L), Y-27632 (10 μmol/L), and LY294002 (10 μmol/L). These inhibitors were used to inhibit the following pathways in NMCFs under TGF-β1 stimulation: SP600125 for JNK (Singh et al., 2021), SB203580 for p38 MAPK (Stratton et al., 2019), Y-27632 for ROCK (Childers et al., 2019), and LY294002 for PI3K (Lei et al., 2015; Yu P et al., 2020). We then applied qRT-PCR and western blotting to detect CRLF1 levels after these inhibitors were used under TGF-β1 stimulation. The results of qRT-PCR (Fig. 7d) and western blotting (Fig. 7e) showed that CRLF1 mRNA and protein levels did not change after these inhibitors were used. These results suggested that up-regulation of *CRLF1* in NMCFs under TGF-β1 stimulation depended on the activation of the SMAD-dependent pathway rather than SMAD-independent pathways.

3 Discussion

In this study, we identified the function and mechanism of action of CRLF1 in cardiac fibrosis and provided a theoretical basis for the development of CRLF1 as a potential pharmaceutical target of cardiac fibrosis. Firstly, we clarified the expression pattern of *CRLF1* in different in vivo and in vitro models of cardiac fibrosis in humans and mice. CRLF1, which was enriched in cardiac fibroblasts, was up-regulated not only in a mouse heart fibrotic model induced by MI, but also in NMCFs and HCFs provoked by TGF-β1. We then explored the roles of CRLF1 in NMCFs in detail. CRLF1 increased collagen production and aggravated the cardiac fibrotic process under physiological and pathological conditions. Mechanistically, up-regulation of *CRLF1* was attributed to the activation of the TGF-β1/SMAD signaling pathway. CRLF1 increased fibrosis-related proteins and up-regulated the activity of the ERK1/2 signaling pathway by increasing the p-ERK1/2 level in the cytoplasm of NMCFs. Finally, we summarized our research and drew a schematic diagram (Fig. 8).

Cardiac fibrosis often accompanies various cardiac pathological conditions and may initially be an adaptive

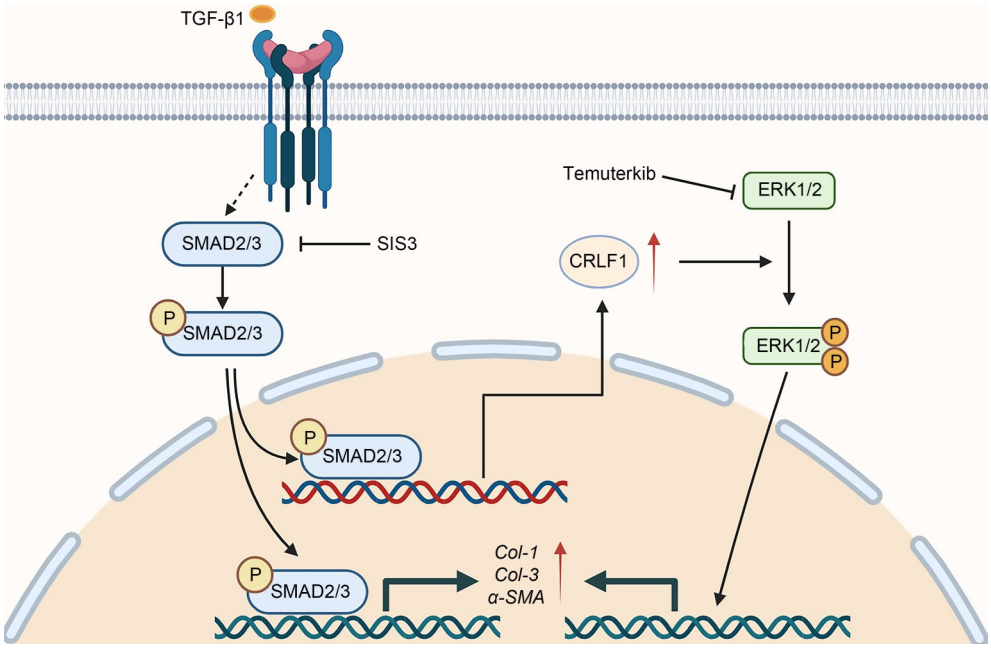

Fig. 8 Role of CRLF1 in cardiac fibrosis. CRLF1: cytokine receptor-like factor 1; TGF-β1: transforming growth factor-β1; SMAD: mothers against decapentaplegic homolog; SIS3: specific inhibitor of SMAD3; P: phosphorylation; Col-1: type I collagen; Col-3: type III collagen; α-SMA: α-smooth muscle actin; ERK1/2: extracellular signal-regulated kinase 1/2. Created with BioRender.com.

activation of cardiac maladaptive processes (Berk et al., 2007; Frangogiannis, 2019; Umbarkar et al., 2021). However, when this adaptive activation becomes inappropriate, excessive, or unrestrained, cardiac fibrosis can contribute to cardiac dysfunction, eventually leading to heart failure (Wynn, 2008; Gupta et al., 2021; Umbarkar et al., 2021). TGF-β1 is a key factor of collagen production and cell proliferation in the heart, and induces fibroblasts to differentiate into myofibroblasts, thereby promoting the transcription and translation of α-SMA (Scalise et al., 2021). We applied TGF-β1 in NMCFs to induce cardiac fibrosis in vitro. Under TGF-β1 stimulation, collagen production and cell proliferation were increased, and *CRLF1* was up-regulated. These results indicated that NMCFs were undergoing fibrotic process, and this process was associated with an increase in the expression of *CRLF1*. The fibrotic process was further up-regulated by *CRLF1* overexpression, and down-regulated by *CRLF1* silencing. These results suggested that CRLF1 obviously participated in the regulation of cardiac fibrosis induced by TGF-β1. In addition, a change of *CRLF1* expression alone in NMCFs could also influence the fibrotic process. In particular, the inhibition of the fibrotic process by *CRLF1* silencing indicated that *CRLF1* silencing could be used not only as a therapeutic strategy but also as a prevention method for cardiac fibrosis. Anti-fibrosis therapy can be a useful treatment for heart diseases (Roubille et al., 2014; Schuetze et al., 2014; Tuleta and Frangogiannis, 2021). Our findings provide strong support for CRLF1 becoming a novel prevention and remedy target for heart diseases.

Clarifying the regulation of collagen synthesis and degradation is the key to clarify the basis of the regulatory mechanism of cardiac fibrosis. In addition, Col-1 and Col-3 are the major structural components of the collagenous matrix in the heart. In particular, Col-1 accounts for about 85% to 90% of the matrix (Weber, 1989; Bashey et al., 1992; Jugdutt, 2003; Frangogiannis, 2017). In our research, the mRNA and protein levels of Col-1 and Col-3 were further increased by *CRLF1* overexpression compared with the levels in the TGF-β1 stimulation group. Conversely, *CRLF1* silencing inhibited the up-regulation of Col-1 and Col-3 mRNA expression and protein levels induced by TGF-β1. Activated fibroblasts and myofibroblasts are the main sources of matrix proteins (Umbarkar et al., 2021). In our study, α-SMA, a myofibroblast transformation indicator, showed the same trend as Col-1 and Col-3 when *CRLF1* was overexpressed or silenced under TGF-β1 stimulation. Moreover, *CRLF1* overexpression increased Col-1, Col-3, and α-SMA mRNA and protein levels in cardiac fibroblasts without TGF-β1 stimulation, while *CRLF1* silencing showed the opposite effects. These results suggest that CRLF1 not only increases collagen production and myofibroblast transformation, but also functions in cardiac fibroblasts under pathological and physiological states. To confirm and extend our findings, further research in vivo needs to be carried out in the future. Although we have found that *CRLF1* is increased in HCFs treated with TGF-β1, the function of CRLF1 in HCFs with or without TGF-β1 stimulation is yet to be elucidated. The wider use of human specimens in future studies would help to confirm the clinical potential of CRLF1 as a remedy for cardiac fibrosis.

We attempted to find a target for CRLF1 in cardiac fibroblasts. Previous studies in other organs or cells have shown that CRLF1 can activate the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT), PI3K/Akt, and MAPK signal pathways via its membrane receptor (Looyenga et al., 2013; Yu et al., 2018). However, *CRLF1* overexpression or silencing in NMCFs did not change the CRLF1 protein level in the cell supernatant, which suggested that CRLF1 might function in the cytoplasm via a new regulation mode. Using western blotting, ELISA, and the highly sensitive AlphaLISA methods, we found that CRLF1 could change the p-ERK1/2 level in NMCFs with or without TGF-β1 stimulation. Therefore, we chose to study the ERK1/2 signaling pathway as a downstream target of CRLF1, although many other signaling pathways could also regulate cardiac fibrosis (Chou et al., 2018; Magaye et al., 2020, 2021a, 2021b). To confirm the uniqueness of the ERK1/2 signaling pathway in the process of CRLF1 regulation, inhibitors of other pathways should be applied in the future to assess whether CRLF1 exerts its function also via these pathways. Fortunately, we found that *CRLF1* overexpression no longer had a stimulatory effect in NMCFs when the ERK1/2 signaling pathway was inhibited. Therefore, we concluded that the ERK1/2 signaling pathway was the most likely downstream target of CRLF1 regulating cardiac fibrosis.

CRLF1 was up-regulated in NMCFs provoked by TGF-β1. However, the downstream signaling cascades

of TGF-β1 are divided into SMAD-dependent and SMAD-independent pathways (Derynck and Zhang, 2003; Schmierer and Hill, 2007; Hu et al., 2018). TGF-β1 binds to its type I and type II cell surface receptors (TGFβRI and TGFβRII) to form heteromeric complexes to activate SMAD-dependent or SMAD-independent pathways (Horbelt et al., 2012; Meng et al., 2016; Hanna and Frangogiannis, 2019; Scalise et al., 2021). Therefore, we investigated which kind of molecule or signaling pathway was the most immediate upstream of CRLF1 in cardiac fibrosis. Firstly, we checked whether SMAD-dependent pathways were the upstream pathways or not by using SIS3, because SMAD3 could directly regulate transcription in the nucleus. After treating NMCFs with TGF-β1 and SIS3, we found that *CRLF1* expression decreased. We also applied inhibitors of SMAD-independent pathways to clarify the upstream pathways of CRLF1. We separately treated NMCFs with different inhibitors under TGF-β1 stimulation. The CRLF1 mRNA and protein levels did not change after these inhibitors were used. Combining the above results, we concluded that *CRLF1* expression was up-regulated in NMCFs under TGF-β1 stimulation mainly through the TGF-β1/SMAD signaling pathway. Interestingly, CRLF1 was up-regulated under TGF-β1 stimulation via a SMAD-dependent pathway, namely the TGF-β1/SMAD signaling pathway, but in turn, CRLF1 strengthened the effects of TGF-β1 by influencing the SMAD-independent ERK1/2 signaling pathway in NMCFs.

Based on the above studies, we have shed light on the role and mechanism of action of CRLF1 in cardiac fibrosis. However, there were some limitations in our study. Firstly, we observed that *CRLF1* was up-regulated in a mouse heart fibrotic model induced by MI. Once we have ascertained the relationship between the level of CRLF1 and the degree of cardiac fibrosis, it would be useful to determine the most effective intervention time node. In addition, apart from TGF-β1, some inflammatory factors, such as interleukin (IL)-4, IL-6, and IL-13, could also activate the cardiac fibrosis pathway (Mantovani et al., 2005; Barron and Wynn, 2011; Kong et al., 2014; Zhao et al., 2021). In this study, we focused only on the effect of TGF-β1 on *CRLF1* expression in vitro and ignored the effect of inflammatory factors. Whether inflammatory factors are upstream or downstream targets of CRLF1 needs to be clarified in the future. This will contribute to a comprehensive elucidation of the mechanism of CRLF1 in cardiac fibrosis. Secondly, by examining published single cell sequences (Tucker et al., 2020; Chaffin et al., 2022) and searching a human database (The Human Protein Atlas, https://www.proteinatlas.org) and mice database (https://tabula-muris.ds.czbiohub.org) (The Tabula Muris Consortium et al., 2018), we found that *CRLF1* expressed mostly in cardiac fibroblasts, especially activated fibroblasts. Furthermore, we measured the expression of *CRLF1* in the heart and found that *CRLF1* was more highly expressed in NMCFs than in NMCMs and ECs. However, there are many cell types other than fibroblasts, cardiomyocytes, and ECs in heart. It may be useful to test *CRLF1* expression patterns in more types of cells, such as immune cells, in future studies. This will be helpful to identify the target cells of CRLF1. Thirdly, we have verified that the ERK1/2 signaling pathway is the downstream target of CRLF1. RNA sequencing (RNA-seq) could be used as a reliable and accurate method to find out whether there are other downstream pathways affected by CRLF1. Fourthly, we did not investigate how CRLF1 interacted with the p-ERK1/2 protein. Protein co-immunoprecipitation experiments are needed to determine whether CRLF1 directly interacts with p-ERK1/2. Finally, we speculated that CRLF1 worked in the cell cytosol because we did not detect a change of CRLF1 after its overexpression or silencing in the cell supernatant using ELISA. Other more sensitive measurements may be needed to detect CRLF1 in the cell supernatant. Taken together, these intriguing questions merit further research.

4 Conclusions

CRLF1 is up-regulated in cardiac fibrosis and aggravates cardiac fibrosis by increasing the p-ERK1/2 protein level. The activation of the TGF-β1/SMAD signaling pathway in cardiac fibrosis can increase *CRLF1* expression, and the up-regulation of *CRLF1* in turn can strengthen collagen production and myofibroblast transformation by increasing the p-ERK1/2 level in the cytosol. These findings imply that CRLF1 has the potential to become a novel prevention and remedy target for cardiac fibrosis.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Huijie ZHANG, Shenjian LUO, and Jin LI contributed to conception and designed this study. Zhi YANG and Ruxin CHEN carried out the experiments. Shenjian LUO performed the statistical analysis and revised the manuscript. Danming YOU, Fei TENG, and Wenhui LIU wrote the first manuscript version. Shenjian LUO, Zhi YANG, and Ruxin CHEN discussed and modified all sections of the manuscript. Youwen YUAN supervised the experiments. All authors have reviewed and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Shenjian LUO, Zhi YANG, Ruxin CHEN, Danming YOU, Fei TENG, Youwen YUAN, Wenhui LIU, Jin LI, and Huijie ZHANG declare that they have no conflict of interest.

The animal experiments were carried out under the approval of the Institutional Animal Care and Use Committee of Nanfang Hospital, Southern Medical University (No. NFYY-2021-1047).

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Supplementary information

Materials and methods