



Epigenetic Repression of Chloride Channel Accessory 2 Transcription in Cardiac Fibroblast: Implication in Cardiac Fibrosis

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Cardiac fibrosis is a key pathophysiological process that contributes to heart failure. Cardiac resident fibroblasts, exposed to various stimuli, are able to *trans*-differentiate into myofibroblasts and mediate the pro-fibrogenic response in the heart. The present study aims to investigate the mechanism whereby transcription of chloride channel accessory 2 (Clca2) is regulated in cardiac fibroblast and its potential implication in fibroblast-myofibroblast transition (FMyT). We report that Clca2 expression was down-regulated in activated cardiac fibroblasts (myofibroblasts) compared to quiescent cardiac fibroblasts in two different animal models of cardiac fibrosis. Clca2 expression was also down-regulated by TGF- β , a potent inducer of FMyT. TGF- β repressed Clca2 expression at the transcriptional level likely *via* the E-box element between -516 and -224 of the Clca2 promoter. Further analysis revealed that Twist1 bound directly to the E-box element whereas Twist1 depletion abrogated TGF- β induced Clca2 *trans*-repression. Twist1-mediated Clca2 repression was accompanied by erasure of histone H3/H4 acetylation from the Clca2 promoter. Mechanistically Twist1 interacted with HDAC1 and recruited HDAC1 to the Clca2 promoter to repress Clca2 transcription. Finally, it was observed that Clca2 over-expression attenuated whereas Clca2 knockdown enhanced FMyT. In conclusion, our data demonstrate that a Twist1-HDAC1 complex represses Clca2 transcription in cardiac fibroblasts, which may contribute to FMyT and cardiac fibrosis.

Keywords: transcriptional regulation, epigenetics, histone deacetylation, histone deacetylase, cardiac fibroblast, myocardial fibrosis

INTRODUCTION

Cardiac fibrosis is generally considered an adaptive response to adversarial stimuli when the heart is exposed to various injuries. A specialized cell type termed “myofibroblast,” typically absent from the normal myocardium under physiological conditions, emerges following injuries and mediates the fibrogenic response (Tomasek et al., 2002). On the one hand, myofibroblasts are capable of muscle-like contraction owing to the acquisition of the expression of genes encoding contractile

proteins (e.g., α -SMA). Contraction by myofibroblasts facilitates wound healing and prevents the incidence of cardiac rupture (Talman and Ruskoaho, 2016). On the other hand, myofibroblasts produce multiple extracellular matrix proteins (e.g., type I collagen, type III collagen, fibronectin) to promote ventricular remodeling and maintain myocardial integrity (van den Borne et al., 2010). After the recession of injurious stimuli, myofibroblasts are no longer needed and thus must be removed or resolved to terminate cardiac fibrogenesis. On the contrary, persistent presence of myofibroblasts in the heart or failure of resolution often leads to aberrant and averse cardiac remodeling and increased rigidity of the myocardium dampening heart function. In fact, cardiac fibrosis is frequently observed and associated with poor diagnosis in patients with heart failure (Gonzalez et al., 2018).

The origin of myofibroblasts in the stressed heart was a subject matter of great controversy and remained elusive prior to the development and utilization of genetic lineage tracing technique. It has been proposed that microvascular endothelial cells (Zeisberg et al., 2007), epicardial epithelial cells (Zhou et al., 2010), myelocytic fibrocyte (Mollmann et al., 2006), and perivascular mesenchymal cells (Kramann et al., 2015) may trans-differentiate into myofibroblasts *in vitro* and/or *in vivo* under different conditions. Landmark studies from the Molkentin laboratory (Kanisicak et al., 2016) and the Evans laboratory (Moore-Morris et al., 2014), aided by lineage tracing, have unequivocally demonstrated that cardiac resident fibroblasts are the predominant source of mature myofibroblasts and become the effector cell type of cardiac fibrosis following cardiac injury *via* fibroblast-myofibroblast transition (FMyT). Further analysis has revealed that cardiac myofibroblasts can be labeled by periostin (encoded by *postn*), a matricellular protein that can function as a ligand for integrins to promote cell migration (Stempien-Otero et al., 2016). One of the most convincing pieces of evidence that supports the pivotal role of myofibroblasts in cardiac fibrosis is provided by Kaur et al. (2016) who demonstrate that elimination of periostin-positive cells (mature myofibroblasts), by diphtheria toxin mediated killing, abrogates aberrant fibrogenic response and preserves heart function after myocardial infarction. Despite these advances, many transcriptional events taking place during FMyT remain to be investigated in detail.

Chloride channel accessory 2 (Clca2) belongs to the family of calcium sensitive chloride conductance proteins or regulators (Jentsch and Pusch, 2018). Clca2 plays versatile pathophysiological roles by regulating multiple distinct yet interconnected cellular processes including proliferation (Walia et al., 2009), differentiation (Ramena et al., 2016), migration (Sasaki et al., 2012), and apoptosis (Selmann et al., 2018). Early characterization of Clca2 protein structure and expression pattern indicated that Clca2 might be a regulator of cystic fibrosis (Gruber et al., 1999). More recently, Walia et al. (2012) have reported that Clca2 expression can be down-regulated by TGF- β , one of the most potent inducer of tissue fibrogenesis, in epithelial cells. In mammalian cells, gene expression is acutely influenced by the epigenetic machinery. Epigenetics mechanisms are heritable phenotypic changes that do not involve alterations

in the DNA sequence; these mechanisms play an important role in a wide spectrum of human diseases (Surguchov et al., 2017). These observations prompted us to investigate whether and, if so, how Clca2 expression might be regulated in the process of cardiac FMyT. We report here that Clca2 is transcriptionally repressed by a Twist1-HDAC1 epigenetic complex in cardiac fibroblasts by pro-fibrogenic stimuli. In addition, Clca2 is able to modulate TGF- β induced FMyT *in vitro*.

MATERIALS AND METHODS

Animals

All animal protocols were reviewed and approved the intramural Ethics Committee on Humane Treatment of Laboratory Animals of Jiangsu Health Vocational College. The mice were maintained in an SPF environment with 12 h light/dark cycles and libitum access to food and water. Cardiac fibrosis was induced by permanent ligation of left-anterior descending (LAD) coronary artery or transverse aortic constriction (TAC) as previously described (Yang et al., 2017; Liu et al., 2021b,c).

Cell Culture, Plasmids, Transient Transfection, and Reporter Assay

Primary cardiac fibroblasts were isolated and maintained in DMEM supplemented with 10% FBS as previously described (Gao et al., 2020; Liu et al., 2020; He et al., 2021; Zhao et al., 2021). Mouse embryonic fibroblasts (MEFs) were isolated and maintained in DMEM supplemented with 10% FBS as previously described (Angrisani et al., 2021). Clca2 promoter-luciferase construct was made by amplifying genomic DNA spanning the proximal promoter and the first exon of Clca2 gene (–1100/ + 91) and ligating into a pGL3-basic vector (Promega). Truncation mutants were made using a QuikChange kit (Thermo Fisher Scientific, Waltham, MA, United States) and verified by direct sequencing. Small interfering RNAs were purchased from Dharmacon. Transient transfection was performed with Lipofectamine 2000. Cells were harvested 48 h after transfection and reporter activity was measured using a luciferase reporter assay system (Promega) as previously described (Kong et al., 2021a,c; Liu et al., 2021c; Zhang et al., 2021). MS-275 and MC-1568 were purchased from Selleck. Mouse recombinant TGF- β was purchased from R&D.

Protein Extraction, Immunoprecipitation and Western Blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche) as previously described (Chen et al., 2020a,b,c; Wu X. et al., 2020; Yang et al., 2020b; Zhang et al., 2020; Chen B. et al., 2021; Dong et al., 2021). Nuclear proteins were extracted using the NE-PER Kit (Pierce) following manufacturer's recommendation. Specific antibodies or pre-immune IgGs were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz).

Precipitated immune complex was released by boiling with 1X SDS electrophoresis sample buffer. Western blot analyses were performed with anti-Clca2 (Proteintech, 19273-1, 1:500), anti- α -SMA (Sigma, A2547, 1:8000), anti-collagen type I (Proteintech, 14695-1, 1:2000), anti-Twist1 (Proteintech, 25465-1, 1:500), anti-HDAC1 (Santa Cruz, sc-7872, 1:1000), anti-HDAC2 (Santa Cruz, sc-7899, 1:1000), anti-HDAC3 (Santa Cruz, sc-11417, 1:1000), anti-HDAC8 (Santa Cruz, sc-11405, 1:1000), anti-FLAG (Sigma, F1804, 1:5000), and anti- β -actin (Sigma, A2228, 1:4000) antibodies.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assays were performed essentially as described before (Wang et al., 2020; Liu et al., 2021a). In brief, chromatin in control and treated cells were cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet and PMSF. DNA was fragmented into ~200 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μ g of protein were used for each immunoprecipitation reaction with anti-Twist1 (Proteintech, 25465-1), anti-Slug (Cell Signaling Technology, 9585), anti-Zeb1 (Cell Signaling Technology, 3396), anti-Snail (Cell Signaling Technology, 3879), anti-anti-acetyl H3 (Millipore, 06-599), anti-acetyl H4 (Millipore, 06-598), anti-HDAC1 (Santa Cruz, sc-7872), or pre-immune IgG. For re-ChIP, immune complexes were eluted with the elution buffer (1% SDS, 100 mM NaCO₃), diluted with the re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris pH 8.1), and subject to immunoprecipitation with a second antibody of interest.

RNA Isolation and Real-Time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen) as previously described (Dong et al., 2020; Hong et al., 2020; Wu T. et al., 2020; Yang et al., 2020a,b, 2021; Zhang et al., 2020; Kong et al., 2021b). Real-time PCR reactions were performed on an ABI Prism 7500 system. Ct values of target genes were normalized to the Ct values of housekeeping control gene (18s rRNA, 5'-CGCGGTTCTATTTTGGT-3' and 5'-TCGTCTCGAAACTCCGACT-3' for both human and mouse genes) using the $\Delta\Delta$ Ct method and expressed as relative mRNA expression levels compared to the control group which is arbitrarily set as 1.

5-Ethynyl-2'-Deoxyuridine Incorporation Assay

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was performed in triplicate wells with a commercially available kit (Thermo Fisher Scientific) per vendor instruction. Briefly, the EdU solution was diluted with the culture media and added to the cells for an incubation period of 2 h at 37°C. After several washes with 1XPBS, the cells were then fixed with 4% formaldehyde and stained with Alexa FluorTM 488. The nucleus

was counter-stained with DAPI. The images were visualized by fluorescence microscopy and analyzed with Image-Pro Plus (Media Cybernetics). For each group, at least six different fields were randomly chosen and the positively stained cells were counted and divided by the number of total cells. The data are expressed as relative EdU staining compared to the control group arbitrarily set as 1.

Statistical Analysis

One-way ANOVA with *post hoc* Scheffé analyses were performed by SPSS software (IBM SPSS v18.0, Chicago, IL, United States). Unless otherwise specified, values of $p < 0.05$ were considered statistically significant.

RESULTS

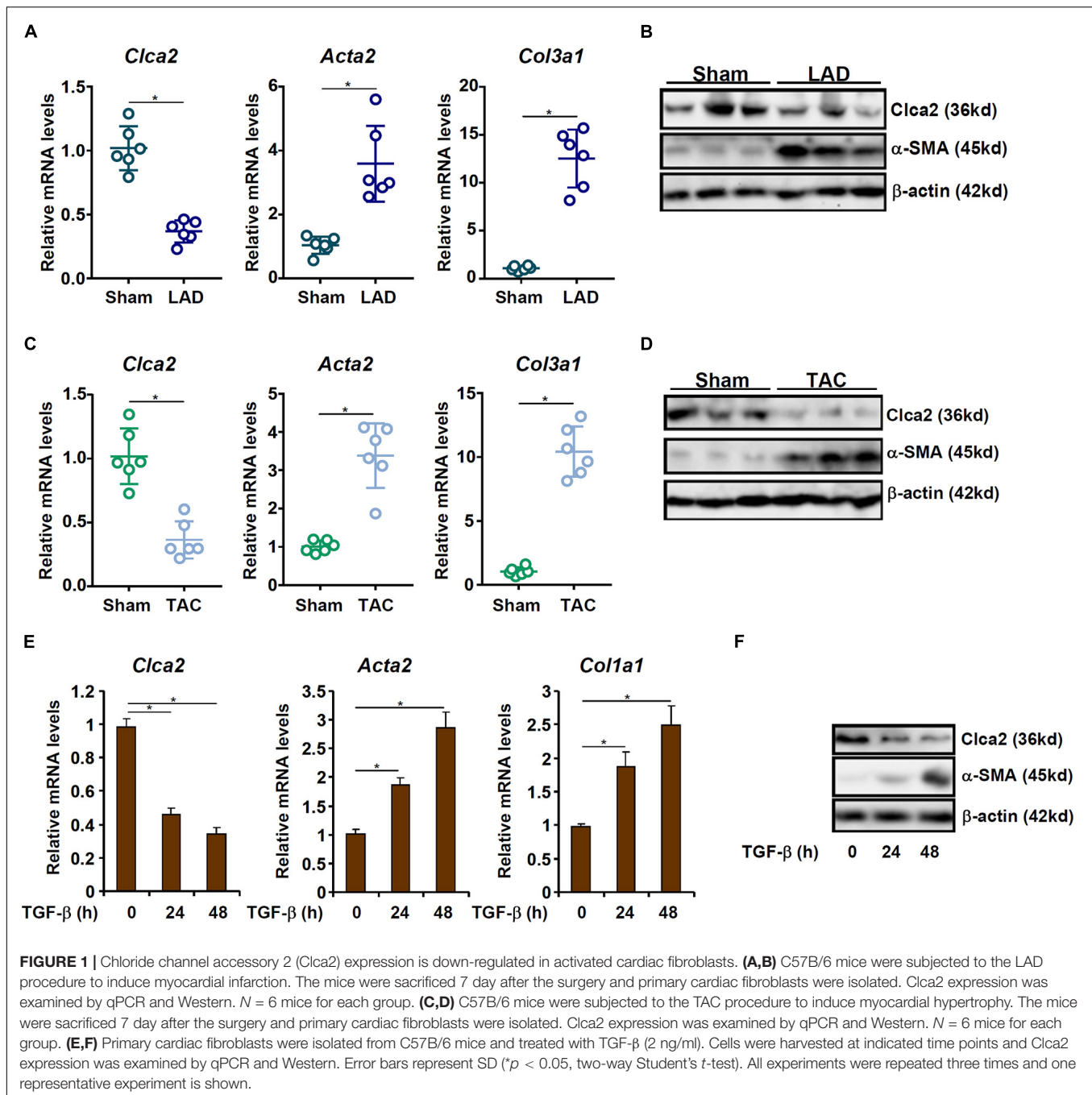
Chloride Channel Accessory 2 Expression Is Down-Regulated in Activated Cardiac Fibroblasts

When exposed to injurious stimuli, cardiac resident fibroblasts undergo *trans*-differentiation and become mature myofibroblasts to mediate the fibrogenic response. In order to compare Clca2 expression in quiescent cardiac fibroblasts and activated cardiac fibroblasts, C57B/6 mice were subjected to the LAD procedure to induce myocardial infarction; previous investigations have shown that FMyT peaks at 7 day after the surgery (Kanisicak et al., 2016). It was observed that compared to the sham-operated mice, expression levels of Acta2 (encoding α -SMA) and Col1a1 (encoding collagen type I), two typical myofibroblast markers, were significantly up-regulated in the primary cardiac fibroblasts isolated from the LAD-operated mice; on the contrary, Clca2 expression was down-regulated in the activated cardiac fibroblasts compared to the quiescent cardiac fibroblasts (Figure 1A). Western blotting confirmed that Clca2 protein levels were down-regulated as well (Figure 1B). In the second model of myocardial fibrosis, C57B/6 mice were subjected to the TAC procedure; FMyT typically peaks at 7 day after the surgery (Bursac, 2014). QPCR (Figure 1C) and Western blotting (Figure 1D) showed that Clca2 expression was lower in the activated cardiac fibroblasts isolated from the TAC mice than in the quiescent cardiac fibroblasts isolated from the sham mice, opposite to the changes in Acta2 expression and Col1a1 expression.

TGF- β is one of most potent inducer of FMyT and myocardial fibrosis (Davis and Molkenin, 2014). When quiescent cardiac fibroblasts were treated with TGF- β , it was found that both Acta2 and Col1a1 were progressively up-regulated whereas Clca2 expression was concomitantly down-regulated (Figures 1E,F).

TWIST1 Mediates Chloride Channel Accessory 2 *Trans*-Repression in Cardiac Fibroblasts

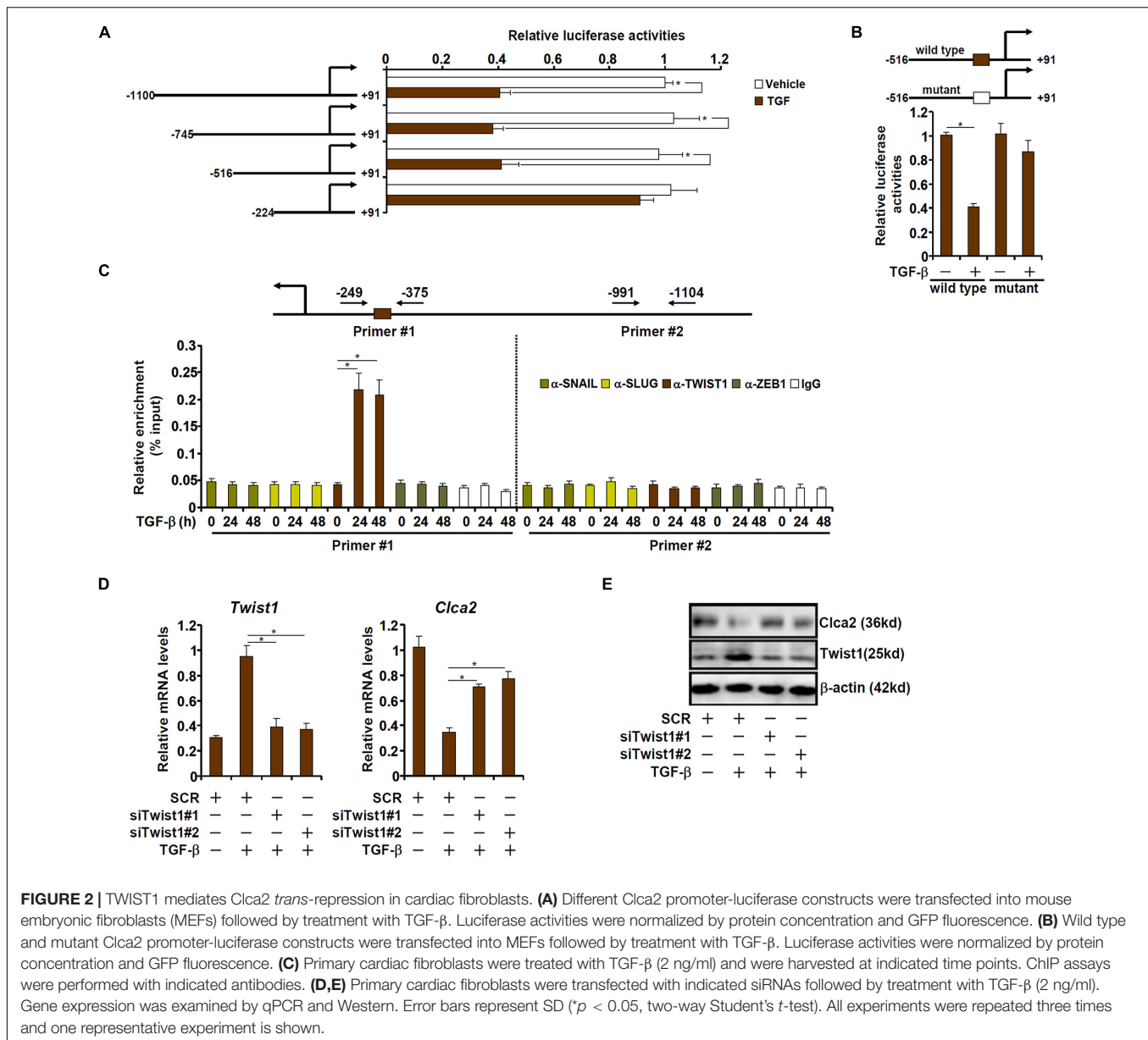
We next determined whether down-regulation of Clca2 expression by TGF- β occurred at the transcriptional level.



A series of *Clca2* promoter-luciferase reporter constructs were transfected into mouse embryonic fibroblasts (MEFs) followed by TGF- β treatment. As shown in **Figure 2A**, TGF- β treatment decreased the activity of the full-length *Clca2* promoter (-1100/+91) suggesting that TGF- β could indeed repress *Clca2* transcription. However, when deletions introduced to the full-length *Clca2* promoter extended beyond -516, TGF- β treatment could no longer repress the *Clca2* promoter activity. A closer examination revealed a conserved E-box (CAGGTG) located between -516 and -224 of the *Clca2* promoter; mutation

of the E-box completely abrogated the response to TGF- β treatment (**Figure 2B**).

The E-box binding family of zinc finger transcription repressors include Snail, Slug, Twist1, and Zeb1 (Kalluri and Weinberg, 2009). ChIP assay was performed to determine which one of these transcription factors (TFs). As shown in **Figure 2C**, Twist1, but not Snail, Slug, or Zeb1, occupied the proximal *Clca2* promoter containing the E-box in response to TGF- β treatment; none of the TFs were detected on the distal *Clca2* promoter. To further validate the role of Twist1 in *Clca2* *trans*-repression,



endogenous Twist1 was depleted with two independent pairs of siRNAs. Twist1 knockdown partially restored *Clca2* expression in the presence of TGF- β in cardiac fibroblasts (Figures 2D,E).

TWIST1 Represses Chloride Channel Accessory 2 Transcription by Promoting Histone Deacetylation

Transcriptional repression is usually associated with erasure of histone acetylation surrounding the promoter region (Jenuwein and Allis, 2001). As shown in Figure 3A, TGF- β treatment led to disappearance of acetylated histone H3 and acetylated histone H4 from the proximal, but not the distal, *Clca2* promoter; Twist1 knockdown normalized histone acetylation, suggesting that histone deacetylases (HDACs) might be involved in Twist1

mediated *Clca2* trans-repression. HDACs can be categorized into three classes: class I and class II HDACs primarily catalyze histone deacetylation whereas class III HDACs (the sirtuins) primarily catalyze non-histone lysine deacetylation (Yang and Seto, 2008). Pre-treatment with a pan-class I HDAC inhibitor (MS-275), but not a pan-class II HDAC inhibitor (MC-1568), blocked TGF- β induced *Clca2* repression (Figures 3B,C), indicating that class I HDAC might be involved in *Clca2* trans-repression.

Class I HDACs include HDAC1, HDAC2, HDAC3, and HDAC8. When individual class I HDACs were depleted with siRNAs, it was discovered that only HDAC1 knockdown significantly attenuated *Clca2* repression by TGF- β treatment (Figures 3D,E). Consistently, HDAC1 knockdown largely normalized histone acetylation levels surrounding the *Clca2* promoter (Figure 3F).

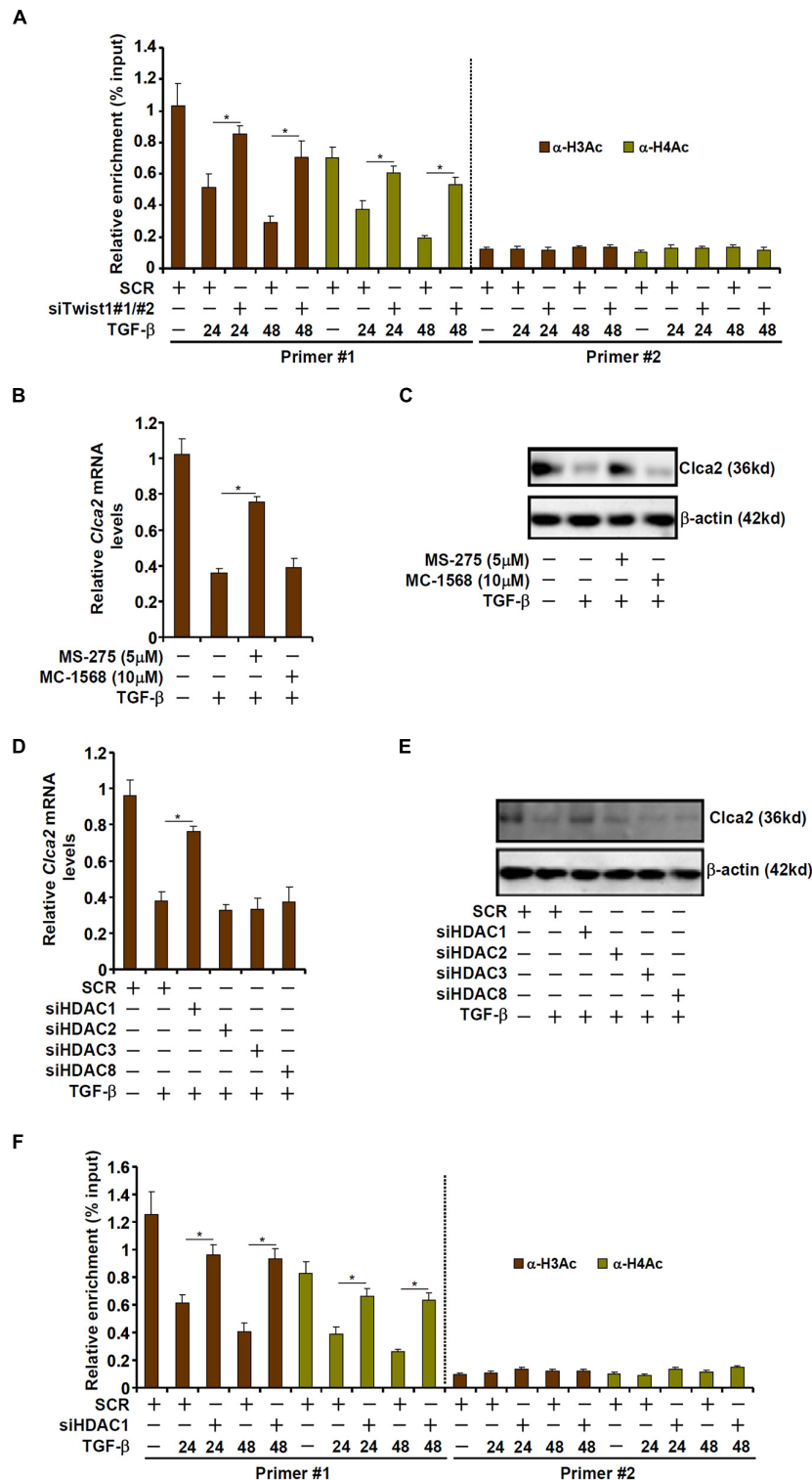


FIGURE 3 | TWIST1 represses Clca2 transcription by promoting histone deacetylation. (A) Primary murine cardiac fibroblasts were transfected with indicated siRNAs by treatment with TGF-β (2 ng/ml). ChIP assays were performed with indicated antibodies. **(B,C)** Primary murine cardiac fibroblasts were treated with TGF-β (2 ng/ml) in the presence or absence of different HDAC inhibitors. Clca2 expression was examined by qPCR and Western. **(D,E)** Primary murine cardiac fibroblasts were transfected with indicated siRNAs by treatment with TGF-β (2 ng/ml). Clca2 expression was examined by qPCR and Western. **(F)** Primary cardiac murine fibroblasts were transfected with indicated siRNAs by treatment with TGF-β (2 ng/ml). ChIP assays were performed with indicated antibodies. Error bars represent SD (**p* < 0.05, two-way Student's *t*-test). All experiments were repeated three times and one representative experiment is shown.

TWIST1 Interacts With and Recruits HDAC1 to Repress Chloride Channel Accessory 2 Transcription

We next investigated the possibility that Twist1 recruits HDAC1 to repress Clca2 transcription. ChIP assay showed that occupancies of HDAC1 on the Clca2 promoter were enhanced following TGF- β treatment with a kinetics similar to Twist1; Twist1 depletion blocked HDAC1 binding to the Clca2 promoter (Figure 4A). Co-immunoprecipitation confirmed that Twist1 and HDAC1 could interact with each other in cardiac fibroblasts (Figure 4B). Importantly, Re-ChIP assay showed that the Twist1-HDAC1 interaction was significantly cemented by TGF- β treatment on the Clca2 promoter (Figure 4C). In addition, whereas HDAC1 over-expression dose-dependently repressed the Clca2 promoter activity in reporter assay the mutant Clca2 promoter without the intact E-box was completely refractory to HDAC1 over-expression (Figure 4D).

Chloride Channel Accessory 2 Regulates Activation of Cardiac Fibroblasts

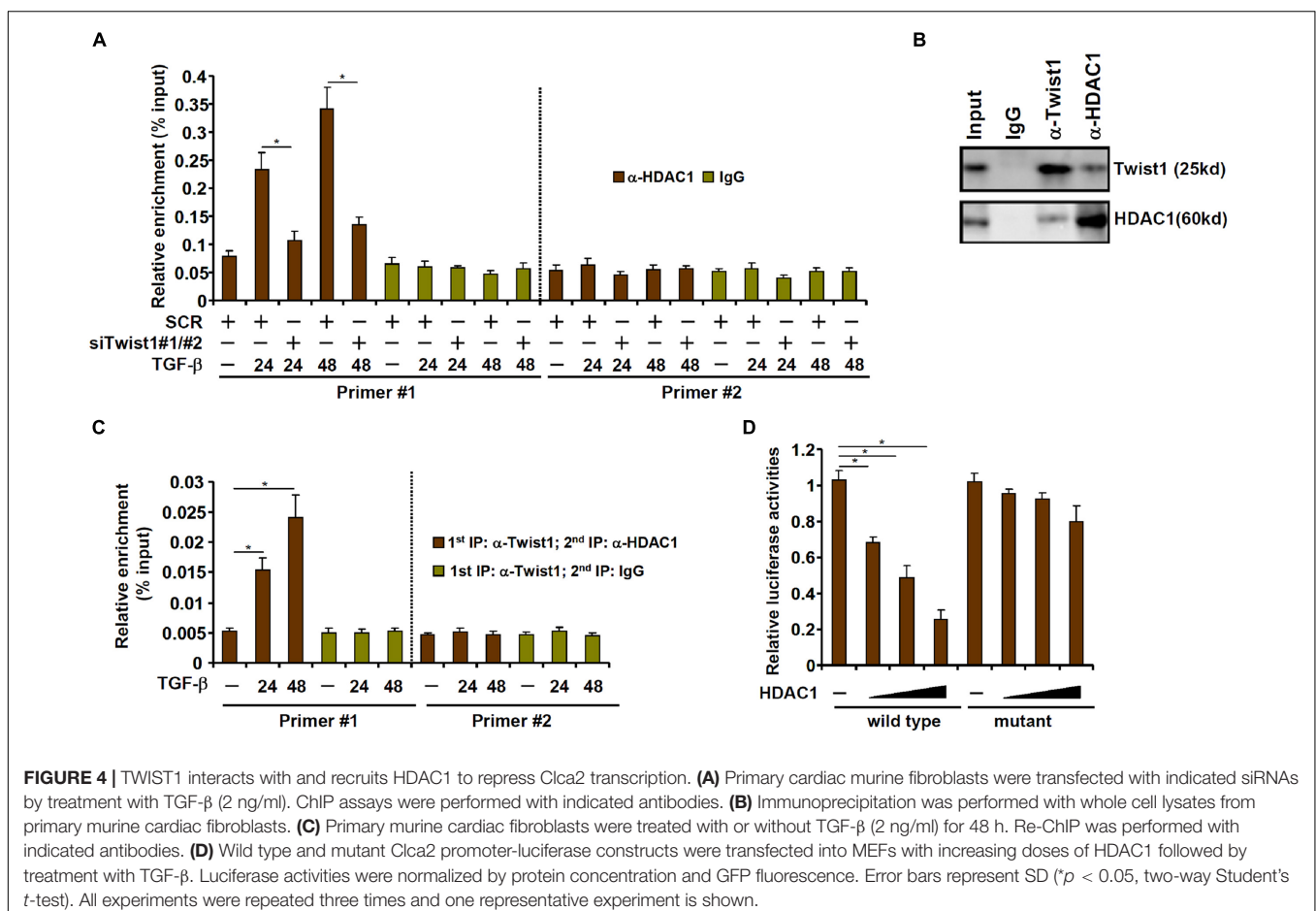
Finally, an attempt was made to place the finding that Clca2 transcription was epigenetically repressed during cardiac fibroblast activation in a pathophysiological perspective. To

this end, primary murine cardiac fibroblasts were transduced with adenovirus carrying a Clca2 expression vector (Ad-FLAG-Clca2) or an empty vector (Ad-EV). Ad-FLAG-Clca2 transduction significantly boosted Clca2 expression in cardiac fibroblasts (Figures 5A,B). More important, Clca2 over-expression significantly down-regulated the expression of myofibroblast marker genes at both mRNA (Figure 5C) and protein (Figure 5D) levels. In addition, Clca2 over-expression attenuated proliferation of cardiac fibroblasts as measured by EdU incorporation (Figure 5E).

Alternatively, Clca2 expression was depleted with two separate pairs of siRNAs (Figures 5F,G). Clca2 knockdown further enhanced TGF- β induced expression of myofibroblast marker genes (Figures 5H,I) and augmented cell proliferation (Figure 5J). Together, these data suggest that Clca2 might regulate FMyT *in vitro*.

DISCUSSION

Recent investigations have provided irrefutable evidence to support resident fibroblasts as the primary source of myofibroblasts contributing to cardiac fibrosis (Travers et al., 2016). Dynamic transcriptomic changes highlight the transition from quiescent cardiac fibroblasts to mature myofibroblasts



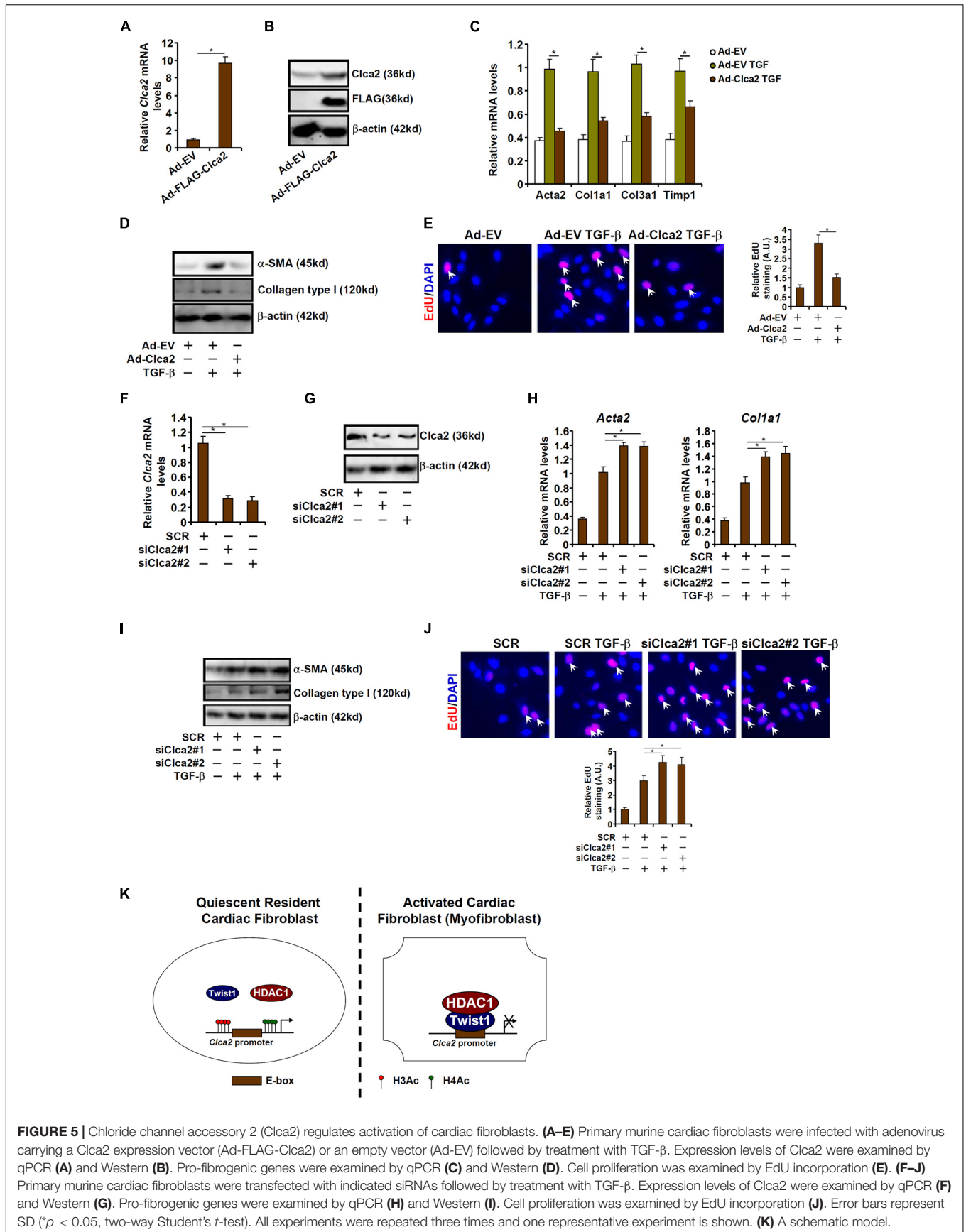


FIGURE 5 | Chloride channel accessory 2 (Clca2) regulates activation of cardiac fibroblasts. **(A–E)** Primary murine cardiac fibroblasts were infected with adenovirus carrying a Clca2 expression vector (Ad-FLAG-Clca2) or an empty vector (Ad-EV) followed by treatment with TGF- β . Expression levels of Clca2 were examined by qPCR **(A)** and Western **(B)**. Pro-fibrogenic genes were examined by qPCR **(C)** and Western **(D)**. Cell proliferation was examined by EdU incorporation **(E)**. **(F–J)** Primary murine cardiac fibroblasts were transfected with indicated siRNAs followed by treatment with TGF- β . Expression levels of Clca2 were examined by qPCR **(F)** and Western **(G)**. Pro-fibrogenic genes were examined by qPCR **(H)** and Western **(I)**. Cell proliferation was examined by EdU incorporation **(J)**. Error bars represent SD (* $p < 0.05$, two-way Student's t -test). All experiments were repeated three times and one representative experiment is shown. **(K)** A schematic model.

(Krstevski et al., 2020). We show here that Twist1 is responsible for Clca2 *trans*-repression in activated cardiac fibroblasts by directly binding to the E-box element located on the Clca2 promoter (Figure 5K). Consistent with our observation, Al-Hattab et al. (2018) have previously reported that Twist1 transcription can be activated by TGF- β in cardiac fibroblasts, which is mediated by scleraxis. Of note, several studies have found that Twist1 can be placed among signature markers for cardiac fibroblasts (Zhou et al., 2010; Liu et al., 2016; Han et al., 2021). Whether or not Twist1 can directly regulate cardiac fibrosis remains to be determined. On the one hand, pharmaceutical inhibition and fibroblast-specific deletion of Twist1 have been shown to mitigate liver fibrosis (Dong et al., 2020) and skin fibrosis (Palumbo-Zerr et al., 2017), respectively, supporting Twist1 as a promoter of tissue fibrosis. On the other hand, Twist1 residing in the parenchymal cells, the mesenchymal cells, or infiltrating immune cells is able to rein in tissue injury and antagonize tissue fibrosis (Tan et al., 2017; Ren et al., 2019, 2021). Therefore, more studies should be conducted to test the feasibility of targeting Twist1 as a therapeutic strategy against aberrant cardiac fibrosis.

Our data indicate that Twist1 represses Clca2 transcription *via*, at least in part, by recruiting the histone deacetylase HDAC1. Curiously, our observation is in contrast to a previous study by Xu et al. (2006) where it was demonstrated that HDAC1, recruited by the RFX1, directly binds to the collagen type I promoter (Col1a2) and represses Col1a2 transcription in lung fibroblasts in response to IFN- γ treatment. It is likely that HDAC1 may exert differential effects on tissue fibrogenesis depending on the stimuli and the cell type. Global deletion of HDAC1 in mice results in early developmental arrest and embryonic lethality precluding the analysis of cardiac fibrosis in adult animals (Montgomery et al., 2007). More recently, Renaud et al. (2015) have shown that administration of a pan-HDAC inhibitor (HDACi) attenuates cardiac fibrosis in mice subjected to pressure overload although the mechanism is less clear but possibly can be attributable to HDAC1-mediated repression of miR-133a, an anti-fibrotic non-regulatory RNA. Of note, HDAC1-null MEFs display weakened proliferation compared to wild type MEFs (Yamaguchi et al., 2010), suggesting that HDAC1 deficiency may prevent cardiac fibrosis by limiting the expansion of myofibroblasts *in vivo* (Yamaguchi et al., 2010). Future studies employing fibroblast/myofibroblast conditional transgenic animals should clarify the role of HDAC1 in cardiac fibrosis.

We present data to show that manipulating Clca2 expression in cardiac fibroblasts influences FMyT *in vitro*. The underlying

mechanism, however, awaits further investigation. Previous studies have shown that a variety of chloride channels may contribute to myofibroblast maturation *via* the MAPK-p38 signaling pathway (Shukla et al., 2014) or the PI3K-Akt signaling pathway (Sun et al., 2016) or the PKC signaling pathway (El Chemaly et al., 2014). Alternatively, chloride intracellular channel 4 (CLIC4) has been shown to promote TGF- β induced FMyT by inducing a dominant negative SMAD7 splicing isoform (Shukla et al., 2016). Despite the fact that several studies have provided evidence to show that chloride channel inhibitors/blockers can potentially attenuate the activation of cardiac fibroblasts (El Chemaly et al., 2014; Tian et al., 2018; Chen P. H. et al., 2021), no consensus seems to exist regarding the underlying mechanisms. It is therefore imperative for future investigators to focus on delineating the mode of action for Clca2 in the process of FMyT so that the plethora of data, including the ones presented here, can be exploited in the development of novel therapeutic solutions to treat adverse cardiac remodeling and heart failure.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Humane Treatment of Laboratory Animals of Jiangsu Health Vocational College.

AUTHOR CONTRIBUTIONS

MF conceived the project and secured funding and provided supervision. TS, YX, and MF designed the experiments. TS and YX performed the experiments, collected the data, and analyzed the data. All authors contributed to the drafting and editing of manuscript.

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