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Endoplasmic Reticulum Protein TXNDC5 Augments Myocardial Fibrosis by Facilitating Extracellular Matrix Protein Folding and Redox-Sensitive Cardiac Fibroblast Activation

Ying-Chun Shih[1], **Chao-Ling Chen**[1], **Yan Zhang**[3], **Rebecca L. Mellor**[3], **Evelyn M. Kanter**[3], **Yun Fang**[4], **Hua-Chi Wang**[1], **Chen-Ting Hung**[1], **Jing-Yi Nong**[1], **Hui-Ju Chen**[1], **Tzu-Han Lee**[1], **Yi-Shuan Tseng**[1], **Chiung-Nien Chen**[5], **Chau-Chung Wu**[6,9], **Shuei-Liong Lin**[7,8], **Kathryn A. Yamada**[3], **Jeanne M. Nerbonne**[2,3], and **Kai-Chien Yang**[1,9,*]

[1]Department and Graduate Institute of Pharmacology, National Taiwan University College of Medicine, Taipei, Taiwan

[2]Department of Developmental Biology, Washington University School of Medicine, St Louis, Missouri, USA

[3]Center for Cardiovascular Research, Department of Medicine, Cardiovascular Division, Washington University School of Medicine, St Louis, Missouri, USA

[4]Department of Medicine, University of Chicago, Illinois, USA

[5]Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan

[6]Department and Graduate Institute of Medical Education & Bioethics, National Taiwan University College of Medicine, Taipei, Taiwan

[7]Department and Graduate Institute of Physiology, National Taiwan University College of Medicine

[8]Division of Nephrology, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

[9]Division of Cardiology, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

Abstract

Rationale—Cardiac fibrosis plays a critical role in the pathogenesis of heart failure (HF). Excessive accumulation of extracellular matrix (ECM) resulting from cardiac fibrosis impairs cardiac contractile function and increases arrhythmogenicity. Current treatment options for cardiac fibrosis, however, are limited and there is a clear need to identify novel mediators of cardiac fibrosis to facilitate the development of better therapeutics. Exploiting co-expression gene network analysis on RNA sequencing data from failing human heart, we identified thioredoxin domain

Address correspondence to: Dr. Kai-Chien Yang, Department and Graduate Institute of Pharmacology, Division of Cardiology, Department of Internal Medicine, National Taiwan University College of Medicine, National Taiwan University Hospital, No. 1, Sec. 1, Ren-Ai Rd, 1150R, Taipei, Taiwan 100, Tel: +886-2-23123456 ext 88327, Fax: +886-2-23210976; kcyang@ntu.edu.tw.

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containing 5 (TXNDC5), a cardiac fibroblast (CF)-enriched endoplasmic reticulum (ER) protein, as a potential novel mediator of cardiac fibrosis and we completed experiments to test this hypothesis directly.

Objective—To determine the functional role of TXNDC5 in the pathogenesis of cardiac fibrosis.

Methods and Results—RNASeq and Western blot analyses revealed that TXNDC5 mRNA and protein were highly upregulated in failing human left ventricles (LV) and in hypertrophied/failing mouse LV. In addition, cardiac TXNDC5 mRNA expression levels were positively correlated with those of transcripts encoding transforming growth factor β1 (TGFβ1) and ECM proteins in vivo. TXNDC5 mRNA and protein were increased in human CF (hCF) under TGFβ1 stimulation in vitro. Knockdown of *TXNDC5* attenuated TGFβ1-induced hCF activation and ECM protein upregulation independent of SMAD3, whereas increasing expression of *TXNDC5* triggered hCF activation and proliferation and increased ECM protein production. Further experiments showed that TXNDC5, a protein disulfide isomerase, facilitated ECM protein folding and that depletion of TXNDC5 led to ECM protein misfolding and degradation in CF. In addition, TXNDC5 promotes hCF activation and proliferation by enhancing JNK activity via increased reactive oxygen species, derived from NAD(P)H oxidase 4. TGFβ1-induced TXNDC5 upregulation in hCF was dependent on ER stress and activating transcription factor 6-mediated transcriptional control. Targeted disruption of *Txndc5* in mice (*Txndc5*$^{-/-}$) revealed protective effects against isoproterenol (ISO)-induced cardiac hypertrophy, reduced fibrosis (by ~70%) and markedly improved LV function; post-ISO LV ejection fraction was 59.1±1.5 vs 40.1±2.5 (P<0.001) in *Txndc5*$^{-/-}$ vs wild-type mice, respectively.

Conclusions—The ER protein TXNDC5 promotes cardiac fibrosis by facilitating ECM protein folding and CF activation via redox-sensitive JNK signaling. Loss of TXNDC5 protects against β agonist-induced cardiac fibrosis and contractile dysfunction. Targeting TXNDC5, therefore, could be a powerful new therapeutic approach to mitigate excessive cardiac fibrosis, thereby improving cardiac function and outcomes in HF patients.

Keywords

TXNDC5; cardiac fibrosis; heart failure; ER; JNK; oxidative stress. Subject Terms: Basic Science Research; Fibrosis; Heart Failure; Mechanisms

Introduction

Heart failure (HF) is a major and growing public health problem, afflicting more than 23 million individuals worldwide.[1] While advances in care and treatment options over the past few decades have reduced dramatically the mortality rates associated with cardiovascular diseases such as hypertensive heart disease, acute coronary syndrome, congenital and valvular heart diseases,[2, 3] the incidence of HF and mortality rates in HF patients have improved only marginally.[4-6] The prevalence of HF is on the rise, and the 5-year mortality remains at ~50% for symptomatic HF, worse than that for many cancers.[7] A complex blend of molecular, structural and neurohumoral alterations may account for the progressive and recalcitrant nature of HF,[8, 9] and there is a clear and immediate need for novel therapies to improve the outcomes of HF patients.

In addition to cardiac muscle abnormalities, cardiac fibrosis is a key contributor to structural and functional remodeling in HF.[10, 11] Excessive accumulation of extracellular matrix (ECM) resulting from cardiac fibrosis can increase wall stiffness, leading to diastolic dysfunction.[12] Replacement of diseased/dying cardiac muscle cells with fibrotic tissue also contributes to worsened ventricular systolic function.[10, 11] Beyond mechanical dysfunction, cardiac fibrosis also contributes to electrical abnormalities by slowing conduction through the myocardium.[13] In addition, increased cardiac fibrosis promotes enhanced automaticity and early after depolarizations causing triggered activity.[14] These electrical abnormalities associated with cardiac fibrosis can foster life-threatening arrhythmias. Cardiac fibrosis, therefore, represents an important therapeutic target for the management of contractile dysfunction and arrhythmias in HF.

The pathogenesis of cardiac fibrosis involves alterations in the cellular and neurohumoral environments that lead to changes in fibroblast activity and ECM turnover.[15-17] The expression levels of local and circulating hormones of the renin-angiotensin-aldosterone system (RAAS), endothelin 1 and transforming growth factor-β1 (TGFβ1) increase in response to mechanical and metabolic stresses, triggering increased activation and proliferation of fibroblasts.[15-17] Activated fibroblasts transition into α-smooth muscle actin (α-SMA)-expressing myofibroblasts, which are responsible for producing an excessive amount of ECM that characterizes fibrotic tissue.[18] Myofibroblasts also modify ECM turnover by modulating the balance between matrix metalloproteinases (MMPs) and their natural inhibitors (tissue inhibitor of MMP; TIMPs) to promote fibrosis.[19] The changes in the ECM also alter the signals that cardiomyocytes receive from their scaffolding environment, triggering the activation of gene programs that are associated with cardiac hypertrophy and contractile dysfunction.[17]

Currently, there are no clinical therapies directly targeting cardiac fibrosis. Antagonists of RAAS, such as angiotensin converting enzyme inhibitor, angiotensin II receptor and aldosterone receptor blockers, have been shown to improve ventricular function and slow progression of myocardial fibrosis.[20-22] The use of these treatments, however, is limited by their hypotensive effects and their inability to stop fibrosis progression. Broad inhibitors of TGFβ such as pirfenidone and tranilast, on the other hand, have been shown to attenuate fibrosis by inhibiting fibroblast activation and ECM deposition without affecting blood pressure in experimental models.[23-25] The widespread clinical use of these agents, however, is limited due to undesirable side effects, including liver toxicity.[26] More recently, several groups have made significant progress in preclinical studies to mitigate cardiac fibrosis by targeting various fibrogenic molecules including TGFβ-activated kinase 1,[27] p38,[28] endothelin receptors,[29], G protein-coupled receptor kinase 2,[30] and miR-21.[31] The clinical efficacies of these anti-fibrotic strategies, however, remain to be demonstrated. There is, therefore, a clear and pressing need to identify additional novel mediators of cardiac fibrosis presentation and progression in response to pathological stimuli to facilitate the development of alternative therapeutic strategies targeting cardiac fibrosis.

In the studies presented here, we identified thioredoxin domain containing 5 (TXNDC5), a cardiac fibroblast (CF)-enriched endoplasmic reticulum (ER) protein, as a novel mediator of cardiac fibrosis. TXNDC5 is upregulated in failing human and mouse hearts and TXNDC5

and fibrogenic protein expression levels are strongly correlated. Results presented here suggest that TXNDC5 promotes cardiac fibrosis by redox-dependent CF activation, as well as by enhancing ECM production by facilitating ECM protein folding. In addition, we show here that targeted deletion of *Txndc5* protects against β agonist-induced cardiac fibrosis and LV dysfunction in mice. Taken together, these results indicate that modulating TXNDC5 could be a powerful new approach to reduce fibrosis and ECM accumulation, thereby improving cardiac function and outcomes in individuals suffering from HF.

Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

A detailed Methods section can be found in the Online Data Supplement.

Results

RNA sequencing and gene co-expression network analysis links TXNDC5 to cardiac fibrosis

To identify novel mediators of cardiac fibrosis, we performed gene co-expression network analysis on an RNA sequencing (RNASeq) dataset, obtained from human failing (HF, n=16) and non-failing (NF, n=8) left ventricles (LV) reported by our group previously (GSE46224).[32] In line with advanced HF status, the expression levels of markers of HF, including atrial natriuretic factor (ANF/*NPPA*) and brain-type natriuretic peptide (BNP/*NPPB*), were significantly increased in the HF, compared to the NF, LV samples (Figure 1A). The expression levels of genes encoding ECM proteins, such as type I collagens α1 (*COL1A1*) and α2 (*COL1A2*), type III collagen α1 (*COL3A1*), elastin (*ELN*), and fibronectin (*FN1*), as well as CCN family member 2 (*CCN2*), profibrotic cytokines (TGFβ1/*TGFB1*, TGFβ2/*TGFB2*) and transcription factors (*SMAD2* and *SMAD7*), were also significantly (P<0.05) upregulated in the HF LV samples (Figure 1A), consistent with the presence of extensive myocardial fibrosis in failing human hearts.

Pairwise correlations encompassing all detectable cardiac mRNA species in human LV samples were computed and a gene co-expression network was constructed using the Weighted Gene Co-expression Network Analysis (WGCNA).[33] A total of 15 gene co-expression modules were identified and color-coded (Figure 1B). Gene ontology analysis revealed that module Turquoise, a clustered gene module containing 1071 genes and assigned with the turquoise color label (Supplemental Table I), was significantly enriched in genes that are involved in collagen fibril organization (7.5-fold enrichment, $P=3.0 \times 10^{-5}$) and extracellular matrix organization (2.8-fold enrichment, $P=2.9 \times 10^{-5}$) (Figure 1C), two biological processes that are critical for the development of cardiac fibrosis. Among the genes in module Turquoise, the expression level of the gene encoding the ER protein, thioredoxin domain containing 5 (TXNDC5), showed a strong positive correlation with those of genes encoding TGFβ1 (r=0.83) and various ECM proteins, including *COL1A1* (r=0.80), *ELN* (r=0.77) and *FN1* (r=0.81) (Figure 1D). In addition, *TXNDC5* transcripts were significantly (P<0.05, Figure 1E) upregulated in human HF, compared with NF, LV

samples. The protein expression levels of TXNDC5, as well as of periostin (POSTN), a matricellular protein secreted by activated CF,[34, 35] were also strongly upregulated in failing human LV (Figure 1F). Analysis of an RNASeq dataset (GSE35350) obtained from a mouse model of cardiac hypertrophy, induced by transverse aortic constriction (TAC),[36] also revealed significant ($P<0.01$) upregulation of *Txndc5* with cardiac hypertrophy and a strong positive correlation between the transcript expression levels of *Txndc5* and fibrogenic factors, including *Tgfb1*, *Postn*, *Acta2* (gene encoding α-SMA) and of genes encoding various ECM proteins, such as *Col1a1*, *Fn1* and *Eln* (Supplemental Figure I). Taken together, the observed upregulation of *TXNDC5/Txndc5* expression in fibrotic human and mouse hearts and the strong positive correlations with genes involved in cardiac fibrosis suggest a potential role for TXNDC5 in the pathogenesis of myocardial fibrosis during cardiac hypertrophy and heart failure.

TXNDC5 is enriched in cardiac fibroblasts and upregulated in response to TGFβ1

Expression analysis demonstrated that TXNDC5 was selectively enriched in primary human cardiac fibroblasts (hCF): mRNA and protein expression levels of TXNDC5 in hCF were significantly higher than those in human LV cardiomyocytes (hCM) by 5.9-and 11.1-fold, respectively (Figure 2A, B). Consistent with the observed enrichment of TXNDC5 in hCF, immunohistochemical staining of sections from a mouse model of isoproterenol (ISO)-induced HF and fibrosis (see below) revealed strong expression of TXNDC5 in myocardial interstitium, but not in myocytes, a pattern similar to that observed for other fibrogenic proteins, such as COL1A1 (Figure 2C), CCN2 and POSTN (Supplemental Figure IIA). Using a *Col1a1-GFP*^{Tg} (GFP driven by *Col1a1* enhancer/promoter) transgenic mouse line that allows visualizing active cardiac fibroblasts with GFP,[37] immunofluorescence staining showed that TXNDC5 staining is highly co-localized with GFP-positive, collagen-producing CF (Supplemental Figure IIB) in fibrotic mouse myocardium induced by ISO treatment.

TGFβ1 expression is strongly upregulated in failing myocardium and contributes to fibrosis by promoting CF activation, proliferation and ECM production.[38] As the expression levels of *TXNDC5* and TGFβ1 are highly correlated in the myocardium, we hypothesized that *TXNDC5* expression in hCF could be regulated by TGFβ signaling. Consistent with this hypothesis, TGFβ1 treatment (4 ng/ml for 48 hours) of hCF induced significant upregulation of the TXNDC5 mRNA (2.0-fold, $P<0.001$, Figure 2D) and protein (2.2-fold, $P<0.001$, Figure 2E) expression levels, compared to vehicle-treated control cells (Ctrl). TGFβ1-induced upregulation of TXNDC5 in hCF was accompanied by increased expression of several fibrogenic gene transcripts and proteins including *COL1A1, ELN, CCN2* and *ACTA2*/α-SMA (Figure 2D,E). In addition, immunocytochemical staining revealed that TXNDC5 is strongly expressed in TGFβ1-activated/α-SMA expressing, but not in quiescent/α-SMA non-expressing, hCF (Supplemental Figure IIC).

TXNDC5 depletion attenuates TGFβ1-induced CF activation and proliferation, and the upregulation of ECM proteins

To determine whether TXNDC5 is requisite in the biological functions of CF, lentiviral vectors carrying *TXNDC5/Txndc5*-targeted shRNAs were utilized to knockdown *TXNDC5/Txndc5* in human (hCF) and mouse (mCF) cardiac fibroblasts. Consistent with the pro-

fibrotic effects of TGFβ signaling, TGFβ1 treatment increased the proliferative activity in non-targeted shRNA (shScr)-treated hCF (Figure 3A), as well as the transcript (Supplemental Figure IIIA) and protein expression levels of the ECM proteins COL1A1 and CCN2 and the myofibroblast marker αSMA (Figure 3B). Knockdown of *TXNDC5* in hCF, however, eliminated TGFβ1-induced fibroblast activation and transformation into myofibroblasts, as evidenced by inhibited cellular proliferation (Figure 3A) and reduced αSMA expression (Figure 3B) in spite of TGFβ1 stimulation. Knockdown of *TXNDC5* in hCF reduced expression of the ECM proteins COL1A1 and CCN2 at baseline and in response to TGFβ1 stimulation (Figure 3B), but had no effect on *COL1A1* and *CCN2* transcript expression levels (Supplemental Figure IIIA). Similar to the results in hCF, reduced ECM (COL1A1, ELN, CCN2) and α-SMA protein expression levels, as well as impaired responsiveness to TGFβ1 stimulation, were observed in mCF (Figure 3C) and in NIH-3T3 mouse fibroblasts (Supplemental Figure IIIB, C) with *Txndc5* knockdown.

TXNDC5 is essential for maintaining ECM protein stability in CF

To determine how TXNDC5 contributes to the regulation of ECM protein expression in CF, a cycloheximide protein chase assay was conducted to evaluate the stability of ECM proteins in hCF with and without *TXNDC5* knockdown. As shown in Figure 4A and Supplemental Figure IVA, *TXNDC5* knockdown in hCF led to accelerated degradation of ECM proteins COL1A1, ELN and CCN2, compared to shScr-treated cells. This observation suggests a critical role for TXNDC5 in maintaining the stability of ECM proteins in CF.

TXNDC5 is an ER resident protein with thioredoxin activity known to catalyze protein disulfide bond isomeration,[39] suggesting that TXNDC5 might facilitate protein folding in the ER. It has previously been demonstrated that nascent polypeptides destined for the secretory pathway fold in the ER, facilitated by ER chaperons and protein disulfideisomerases,[40] whereas misfolded proteins are subjected to ER-associated protein degradation (ERAD) via the ubiquitin-proteasome system.[41] We hypothesized that TXNDC5 functions to facilitate the folding of ECM proteins in the ER, allowing ECM proteins to mature and subsequently travel through the secretory pathway. Depletion of TXDNC5, therefore, may cause ECM protein misfolding and subsequent degradation by ERAD. To test this hypothesis directly, we first treated hCF with an ERAD inhibitor eeyarestatin I (Eey I, which blocks ERAD by inhibiting valosin containing protein [VCP], an essential component of the protein complex mediating the retrotranslocation of misfolded substrates from ER to cytosol, 0.5 μmol/L) or shRNA targeting VCP (shVCP) to block ERAD. As shown in Figure 4B, both Eey I treatment and *VCP* knockdown by shRNA (Supplemental Figure IVB) reversed the reduction of ELN and CCN2 protein caused by TXNDC5 depletion in hCF. These results suggest that ECM protein degradation resulting from TXNDC5 depletion in hCF is mediated through ERAD.

Additional co-immunoprecipitation experiments demonstrated that TXNDC5 associates with several ECM proteins, including COL1A1, CCN2 and ELN in hCF (Supplemental Figure IVC). In addition, the extent of ECM protein folding in control and TXNDC5-depleted hCF was examined using a fluorescence resonance energy transfer (FRET)-based protein folding assay that has been shown to detect protein folding and unfolding in cells successfuly.[42]

Human COL1A1 fused with cyan fluorescent protein (CFP) at the N terminus and yellow fluorescent protein (YFP) at the C terminus (CFP-COL1A1-YFP) was expressed in control and TXNDC5-depleted hCF; efficient FRET (from CFP to YFP) would only be detected when the COL1A1 ternary fusion proteins fold properly and bring CFP and YFP into close proximity (illustrated in Supplemental Figure IVD, bottom). Application of a standard acceptor photobleaching FRET protocol in hCF expressing CFP-COL1A1-YFP revealed that *TXNDC5* knockdown significantly ($P<0.01$) reduced FRET efficiency (Figure 4C), suggesting decreased COL1A1 protein folding in hCF with TXNDC5 depletion. A similar reduction in the folding of mouse ELN protein was observed in NIH-3T3 mouse fibroblasts with TXNDC5 depletion (Supplemental Figure V). Taken together, these results suggest that TXNDC5 functions to maintain the proper folding of ECM proteins, such as collagen and elastin, in CF. When TXNDC5 expression is reduced, ECM protein unfolding and breakdown are increased via an ER-associated degradation pathway.

TXNDC5 activates fibroblasts through SMAD-independent, redox-sensitive regulation of JNK signaling

The observations that TXNDC5 depletion in mCF and hCF attenuated αSMA expression and cellular proliferation in response to TGFβ1 stimulation suggest that TXNDC5 is required for TGFβ1-induced CF activation and transdifferentiation into myofibroblasts. To determine whether elevated TXNDC5 expression is sufficient to trigger CF activation, proliferation and ECM production, we overexpressed *TXNDC5* in hCF. These experiments revealed that ectopic overexpression of *TXNDC5* resulted in increased protein expression levels of COL1A1, αSMA and POSTN (Figure 5A), secretion of type 1 collagen (Figure 5B), as well as cellular proliferation in hCF (Figure 6B).

Because TXNDC5 is required for TGFβ1-induced CF activation (Figure 3), we hypothesized that TXNDC5 may regulate CF activity through effectors downstream of the TGFβ signaling pathway. Consistent with this hypothesis, we observed that knockdown of *TXNDC5* in hCF reduced TGFβ1-induced phosphorylation/activation of c-Jun N-terminal kinase (JNK, Figure 5C), a critical TGFβ downstream mediator that promotes fibroblast activity.[43, 44] In contrast, TGFβ1-induced phosphorylation of SMAD3 and ERK was not affected by knockdown of *TXNDC5* (Figure 5C). Also consistent with the results of *TXNDC5* knockdown, *TXNDC5* overexpression in hCF was sufficient to trigger the activation of JNK, but not SMAD3 (Figure 5A). Moreover, pharmacological inhibition of JNK with SP600125 (10 μmol/L) abolished the upregulation of COL1A1, αSMA and POSTN (Figure 6A), as well as hCF proliferation (Figure 6B), observed with ectopic *TXNDC5* expression. These data collectively demonstrate that TXNDC5 promotes the activity and proliferation of hCF through JNK signaling.

As JNK is redox-sensitive and TGFβ1 is known to promote ROS formation in fibroblasts through increased NAD(P)H oxidase (NOX) activity,[45] we hypothesized that TXNDC5 modulates JNK activity by regulating intracellular ROS levels. Consistent with this hypothesis, knockdown of *TXNDC5* reduced TGFβ1-induced ROS production, whereas overexpression of *TXNDC5* increased ROS levels in hCF (Figure 6C, D). Notably, the increased JNK activity, α-SMA/POSTN expression (Figure 6A) and cellular proliferation

(Figure 6B) in hCF following ectopic *TXNDC5* expression were abolished by the treatment with ROS scavenger N-acetylcysteine (NAC, 15 mmol/L). In addition, ROS production induced by *TXNDC5* overexpression was suppressed by the NOX inhibitor apocynin (2 mmol/L, Figure 6C, D), demonstrating that TXNDC5-induced ROS production is derived from NOX. Because NOX4 and 5 are the predominant NOX enzymes in CFs and only NOX4 is upregulated by TGFβ1,[45] we hypothesized that NOX4 is responsible for TXNDC5-induced ROS production in CFs. Consistent with this hypothesis, treatment with NOX1/4 inhibitor GSK137831 (5μmol/L) or siRNA targeting *NOX4* (siNOX4) abolished *TXNDC5* overexpression-induced ROS production, cellular proliferation (Supplemental Figure VI A-C) and ECM protein upregulation in hCF (Supplemental Figure VID). Taken together, these data suggest that NOX4-derived ROS productionn is essential for TXNDC5-induced JNK activation, cellular proliferation and ECM expression in hCF.

The activity of NOX is known to be modulated by protein disulfide isomerases (PDIs),[46, 47] suggesting that TXNDC5 may induce NOX4-derived ROS generation via its PDI activity. In agreement with this hypothesis, treatment of hCF with a PDI inhibitor 16F16 (20 μmol/L) prevented the increase in ROS produced by *TXNDC5* overexpression (Figure 6C, D). Activation of hCF resulting from *TXNDC5* overexpression was also significantly reduced by 16F16, as shown by reduced αSMA and POSTN protein expression (Figure 6A) and decreased proliferation (Figure 6B). To determine the requirement for the PDI activity of TXNDC5 in its pro-oxidant and fibrogenic functions directly, we generated a *TXNDC5* mutant that lacks PDI activity by introducing cysteine-to-alanine mutations in both ends (i.e. CGHC to AGHA) of each of its 3 thioredoxin domains, which are required for its PDI enzyme activity (*TXNDC5* AAA mutant; the mutation scheme is illustrated in Supplemental Figure VIIA). In contrast to WT *TXNDC5*, the *TXNDC5* AAA mutant failed to induce ROS production, cell proliferation (Supplemental Figure VIA, B), fibroblast activation (reflected by αSMA and POSTN expression levels) and/or ECM protein expression (Supplemental Figure VIIB) in hCF, suggesting that the PDI activity of TXNDC5 mediates the observed pro-oxidant and fibrogenic effects.

TGFβ1-induced TXNDC5 upregulation in CFs Is dependent on increased ER stress and activating transcription factor 6 (ATF6)

Additional experiments were focused on determining how TXNDC5 is regulated by TGFβ signaling. Because *TXNDC5* mRNA was upregulated in response to TGFβ1 stimulation, we hypothesized that the promoter activity of *TXNDC5* is regulated by the transcription factor(s) downstream of the TGFβ signaling pathway. Although SMAD proteins play critical roles in controlling the transcription of various TGFβ downstream genes, neither the human nor the mouse *TXNDC5/Txndc5* promoter harbors a SMAD binding element, indicating that *TXNDC5/Txndc5* could either be regulated indirectly by SMADs or by a SMAD-independent TGFβ pathway. TGFβ, for example, is known to trigger increased ER stress and unfolded protein response (UPR),[48, 49] which are mediated by multiple transcription factors including X-box binding protein 1 (XBP1), activating transcription factor 4 and 6 (ATF6). In addition, TXNDC5 is located in the ER and facilitates protein folding (Figure 4 and Supplemental Figure V), suggesting that TXNDC5 may function downstream of ER stress pathways. Consistent with this hypothesis, both the human and the mouse *TXNDC5/Txndc5*

promoters contain putative ATF6 and XBP1 binding sites.[39] In addition, increased ER stress level was observed in human failing heart and in fibrotic mouse myocardium, induced by ISO-treatment (Supplemental Figure VIIIA-C).

To test this hypothesis directly, we first examined the interrelationship between TGFβ signaling, ER stress and TXNDC5 expression in hCF. As shown in Supplemental Figure IX, TGFβ1 treatment markedly increased ER stress signaling activity, especially in the branches of ATF6 and inositol-requiring enzyme 1 α (IRE1α)-XBP1, and significantly upregulated the protein expression levels of TXNDC5 in hCF. Treating hCF with broad ER stress inhibitors 4-phenylbutyrate (4-PBA, 0.5 mmol/L) or tauroursodeoxycholic acid (TUDCA, 0.5 mmol/L) suppressed TGFβ1-induced ER stress, as evidenced by reduced expression level of activated ATF6 (ATF-p50) (Figure 7A), and abolished TGFβ1-induced upregulation of TXNDC5 protein (Figure 7A). These results suggest that TGFβ1-induced upregulation of TXNDC5 in hCF is mediated by ER stress.

To determine ifTGFβ signaling regulates TXNDC5 expression through ATF6 and/or XBP1, two ER stress pathway transcription factors with putative binding sites in the *TXNDC5/Txndc5* promoter, hCF with ATF6 or XBP1 knockdown were treated with TGFβ1, followed by assays to quantify *TXNDC5* mRNA expression. These experiments showed that knockdown of ATF6 (Figure 7B), but not XBP1 (Supplemental Figure XA), prevented the upregulation of *TXNDC5* mRNA with TGFβ1 stimulation. In further experiments, wild-type (WT *Txndc5*) and ATF6 binding site (TGACGTGG, +773~+780)-deleted (ΔATF6) mouse *Txndc5* promoter luciferase constructs (Figure 7C) were transfected into NIH-3T3 fibroblasts. Although TGFβ1 treatment significantly increased WT *Txndc5* promoter activity, deletion of the ATF6 binding site significantly reduced *Txndc5* promoter activity at baseline and in response to TGFβ1 stimulation (Figure 7C). A physical interaction between ATF6 and the *Txndc5* promoter was confirmed using an electrophoretic mobility shift assay (Figure 7D and Supplemental Figure XB). Taken together, these data demonstrate that TGFβ1-induced TXNDC5 upregulation in CF is dependent on increased ER stress and ATF6-mediated transcriptional regulation.

In vivo targeted deletion of Txndc5 protects against β agonist-induced cardiac fibrosis and myocardial dysfunction

To explore directly the physiological role of TXNDC5 in cardiac functioning in vivo, we generated a mouse line with targeted deletion of *Txndc5* (*Txndc5*$^{-/-}$) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) genome editing technology (details provided in the Supplemental Methods).[50] *Txndc5*$^{-/-}$ mice, which harbor a 6.8 kb deletion encompassing exons 2 & 3 of *Txndc5* (Supplemental Figure XIA), were born at the expected Mendelian frequency and, at baseline, showed no detectable developmental defects or structural anomalies. There were no discernable differences in *Txndc5*$^{-/-}$ and WT mouse heart sizes or contractile function at baseline (Figure 8 A,B and Supplemental Figure XIB).

To investigate the in vivo contribution of TXNDC5 to cardiac fibrosis, WT and *Txndc5*$^{-/-}$ mice were injected with isoproterenol (ISO, 30mg/kg/day subcutaneously for 10 days), a treatment paradigm shown previously to produce cardiac fibrosis, contractile dysfunction

and heart failure.[51, 52] As shown in Figure 8A, ISO-treatment induced significant increases in the mean ± SEM cardiac size and heart weight/body weight (HW/BW) ratio in WT mice (4.70±0.08 vs 5.75±0.07 mg/g in WT vs WT+ISO, respectively, $P<0.001$), but not in *Txndc5*$^{-/-}$ mice (4.60±0.31 vs 4.41±0.22 mg/g in *Txndc5*$^{-/-}$ vs *Txndc5*$^{-/-}$ +ISO, respectively, Figure 8A). In addition, Masson's trichrome staining of paraffin sections (Figure 8B) revealed that ISO-treatment was associated with extensive fibrosis in WT hearts (1.0±0.3% vs 8.1±1.5% of LV cross-sectional area in WT vs WT+ISO, respectively, $P<0.05$), whereas minimal fibrotic changes were observed in *Txndc5*$^{-/-}$ hearts in response to ISO (1.1±0.3% vs 2.5±0.8% of LV cross-sectional area in *Txndc5*$^{-/-}$ vs *Txndc5*$^{-/-}$ +ISO, respectively). Echocardiographic analyses (Supplemental Figure XIB) revealed significantly reduced LV contractility in post-ISO WT mice (LV ejection fraction [LVEF] 59.1±1.5 vs 40.1±2.5 % in WT vs WT+ISO, respectively, $P<0.001$), whereas LV contractile function was preserved in *Txndc5*$^{-/-}$ mice treated with ISO (LVEF 57.6±5.2 vs 59.0±4.1 % in *Txndc5*$^{-/-}$ vs *Txndc5*$^{-/-}$ +ISO, respectively). In addition, immunoblot analyses showed that ISO treatment significantly increased the expression levels of ECM proteins (COL1A1 and ELN), matricellular proteins CCN1 and CCN2 (but not CCN3),[53] markers for CF activation (αSMA and POSTN), as well as p-JNK in WT, but not in *Txndc5*$^{-/-}$, LV (Figure 8C and Supplemental Figure XII). Consistent with the notion that TXNDC5 plays a critical role in regulating CF activity, mCF isolated from *Txndc5*$^{-/-}$ mouse ventricles showed reduced proliferative activity compared with WT mCF, both at baseline and in response to TGFβ1 stimulation (Figure 8D). These results suggest that, in addition to preventing TGFβ1-induced CF activation and proliferation, loss of TXNDC5 protects the heart against ISO-induced cardiac hypertrophy, myocardial fibrosis and contractile dysfunction in vivo.

Discussion

Regulation of myocardial fibrosis by TXNDC5

The results presented here demonstrate that TXNDC5, a fibroblast-enriched ER protein, is upregulated in failing human LV and mouse heart with pressure overload-induced pathological cardiac hypertrophy, in parallel with advanced fibrosis and increased TGFβ signaling activity. The upregulation of TXNDC5 promotes cardiac fibrosis by facilitating ECM protein folding in CF, and by triggering CF activation and proliferation. TXNDC5-induced CF activation is mediated by SMAD3-independent activation of JNK via NOX4-derived ROS that requires the protein disulfide isomerase activity of TXNDC5. In addition, TGFβ1-induced upregulation of TXNDC5 in fibroblasts requires increased ER stress and ATF6-mediated transcriptional control. Consistent with the in vivo unbiased profiling results and in vitro mechanistic experiments, targeted deletion of *Txndc5* mitigated β-agonist-induced cardiac fibrosis, LV remodeling and contractile dysfunction in mice. Taken together, the results presented elucidate a novel pro-fibrotic mechanism mediated by the CF-enriched ER protein TXNDC5. A schematic illustrating the regulation and function of TXNDC5 in modulating cardiac fibrosis is presented in Figure 8E.

TXNDC5 as a unique fibroblast-enriched PDI that regulates ECM turnover and CF activation

TXNDC5 is a recently discovered member of the PDI family, which facilitates the formation of disulfide bonds and correct folding of nascent polypeptides.[54, 55] Among the 21 members of human PDI family genes, 16 were identified by RNASeq in human LV and 3 of these were dysregulated in HF (Supplemental Figure XIIIA).[56] *TXNDC5* was the only one that is highly enriched in CF among these 3 HF-associated PDI genes (Supplemental Figure XIIIB), suggesting its fibroblast-specific function that distinguishes TXNDC5 from other human PDIs. TXNDC5 contains an N-terminal signal sequence, followed by 3 thioredoxin (TRX) domains and a C-terminal ER retention signal (KDEL). It has been reported that the molecular architecture of TXNDC5 is radically different from other PDIs, such that the three TRX domains of TXNDC5 are separated, act independently and engage in rapid but promiscuous disulfide bond formation during early oxidative protein folding.[57] The significance of such unique molecular properties of TXNDC5 in regulating fibroblast function, however, remains to be determined.

The observation that TXNDC5 could affect ECM protein expression through modulating protein folding suggests a previously unrecognized intracellular, post-translational mechanism that regulates ECM protein turnover, which is distinct from that of extracellular ECM regulators like MMPs, TIMPs and lysyl oxidase. As TXNDC5 expression is increased in CF with ER stress, a stimulus known to promote tissue fibrosis,[4858] it is likely that TXNDC5 functions as a component of the ER stress/UPR pathway and contributes to the profibrotic effects of elevated ER stress. The absence of TXNDC5 did not lead to developmental defects or pathological changes that are linked to excessive ER stress, which could be explained by the functional redundancy among the PDI family genes and the fact that TXNDC5 expression is restricted to certain cell types such as fibroblasts and endothelial cells. Consistent with this suggestion, TXNDC5 depletion in hCF did not lead to increased cellular apoptosis (Supplemental Figure XIV).

In addition to the canonical function of TXNDC5 as a PDI to facilitate ECM protein folding, the results presented here suggests that TXNDC5 also promotes the activation of CF, triggering fibroblast-to-myofibroblast differentiation and proliferation by increasing NOX4-derived ROS and activation of redox-sensitive JNK signaling. PDI has previously been demonstrated to promote ROS production in vascular smooth muscle cells (VSMC) by interacting with NOX enzymes including NOX1, 2, and 4, as well as by inducing the transcription of NOX1 and 4.[59, 60] In contrast to VSMC, our results suggest that this non-canonical, ROS producing effect of TXNDC5 in CF is mediated through NOX4. While NOX1 and NOX2 are localized predominantly in the plasma membrane and endosomes,[61] NOX4 has been identified in various intracellular compartments including mitochondria[62], focal adhesions[63] and ER.[64, 65] It is possible that increased TXNDC5 in the ER promotes NOX4 activity directly by stabilizing NOX4 conformation or by facilitating the intracellular trafficking of NOX4 via its function as a PDI.[66] Alternatively, TXNDC5 could raise NOX4-derived ROS levels indirectly by increasing NOX4 expression levels in the CF. ER ROS levels, therefore, are increased with overexpression of TXNDC5, which can further lead to elevated cytosolic ROS levels by triggering ROS production from mitochondria and/or

depletion of cytosolic antioxidant glutathione,[67] thereby resulting in the activation of redox-sensitive JNK signaling in the cytosol. Although the results presented cannot exclude the possibility that TXNDC5 could also promote ROS production via NOX1 or NOX2, the extremely low expression levels of NOX1/2 observed in CF[45] and the fact that NOX1/2 are absent in the ER[61] make this alternative hypothesis much less likely.

Targeted deletion of Txndc5 ameliorates β agonist-induced myocardial fibrosis

The results presented also demonstrate that knockout of *Txndc5* has protective effects against β agonist-induced myocardial fibrosis, hypertrophy and contractile dysfunction. As a global, rather than fibroblast-specific, targeted deletion strategy was used, we cannot exclude the possibility that deletion of *Txndc5* in cardiac cell types other than CF could have contributed to such cardiac protective effects. The protein expression levels of TXNDC5 in hCM, however, is less than 10% of that in hCF (Figure 2B), suggesting a minor, if any, role of TXNDC5 in modulating cardiac myocyte function directly. Although endocardial, epicardial and bone marrow-derived cells have been suggested as sources of activated cardiac myofibroblasts to varying degrees in mice subjected to cardiac injuries, pre-existing resident CF lineages are considered to be the predominant source of cardiac myofibroblasts that undergo proliferation and ECM production in the diseased heart.[68, 69] We would expect, therefore, to observe similar protective effects against agonist-induced myocardial fibrosis if a fibroblast-specific *Txndc5* targeted deletion mouse line were to be used.

Conclusions

In summary, the present study demonstrates a critical role for the CF-enriched ER protein TXNDC5 in promoting cardiac fibrosis by facilitating ECM protein folding as well as by triggering CF activation and proliferation via JNK signaling that is dependent on NOX4-derived ROS. Cardiac TXNDC5 expression increases in hypertrophic and failing hearts, likely under the control of a TGFβ-ER stress-ATF6 signaling axis, contributing to excessive accumulation of myofibroblasts and ECM proteins that lead to cardiac fibrosis. Our findings reveal a novel SMAD-independent mechanism that mediates a TGFβ-induced fibrogenic response in the heart. These results also suggest that targeting TXNDC5 could be a powerful novel approach to mitigate cardiac fibrosis and dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

4-PBA 4-phenylbutyrate

ANF	atrial natriuretic factor
α-SMA	α-smooth muscle actin
ATF6	activating transcription factor 6
BNP	brain-type natriuretic peptide
CCN 1	2,3, CCN (CYR61, FISP12/CTGF, NOV) family members 1, 2 and 3
CF	cardiac fibroblasts
CFP	cyan fluorescent protein
COL1A1	type I collagen α1
COL1A2	type I collagen α2
COL3A1	type III collagen α1
CRISPR	clustered regularly interspaced short palindromic repeats
ECM	extracellular matrix
Eey I	Eeyarestatin I
ELN	elastin
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
FN1	fibronectin
FRET	fluorescence resonance energy transfer
hCF	human cardiac fibroblasts
hCM	human left ventricular cardiomyocytes
HF	heart failure
HW/BW	heart weight/body weight ratio
IRE1α	inositol-requiring enzyme 1 α
ISO	isoproterenol
JNK	c-Jun N-terminal kinase
LV	left ventricle
mCF	mouse cardiac fibroblasts
MMPs	matrix metalloproteinases
NAC	N-acetylcysteine

NF	non-failing
NOX	NAD(P)H oxidase
PDI	protein disulfide isomerase
POSTN	periostin
RNASeq	RNA sequencing
shScr	non-targeted shRNA
siScr	non-targeted siRNA
TGFβ1	transforming growth factor β1
TIMPs	tissue inhibitors of MMP
TRX	thioredoxin
TUDCA	tauroursodeoxycholic acid
TXNDC5	thioredoxin domain containing 5
UP	unfolded protein response
VCP	valosin containing protein
VSMC	vascular smooth muscle cells
WGCNA	weighted gene co-expression network analysis
WT	wild-type
XBP1	X-box binding protein 1
YFP	yellow fluorescent protein

Novelty and Significance

What Is Known?

- Cardiac fibrosis, a process that results from the activation and proliferation of cardiac fibroblasts (CF) and the secretion of excessive amounts of extracellular matrix (ECM) proteins, including type I collagen and elastin, plays a critical role in the pathogenesis of heart failure (HF).
- Current therapeutic options for controlling cardiac fibrosis are limited and there is a clear and urgent need to identify novel mechanisms contributing to cardiac fibrosis to facilitate the development of potent, new therapeutics.

What New Information Does This Article Contribute?

- Using co-expression gene network analysis on RNA sequencing data obtained from failing human heart, we identified thioredoxin domain containing 5 (TXNDC5), an endoplasmic reticulum (ER) protein enriched in CF, as a potential novel mediator of cardiac fibrosis.
- In vitro experiments revealed that TXNDC5, downstream of TGFβ1 signaling and ER stress pathway, promotes cardiac fibrosis by facilitating the folding of ECM proteins, as well as by triggering the activation and proliferation of CF through enhancing JNK signaling via increased reactive oxygen species.
- Targeted deletion of *Txndc5* in mice showed protective effects against isoproterenol-induced cardiac fibrosis, hypertrophy and contractile dysfunction.

Cardiac fibrosis contributes significantly to the pathogenesis of HF by increasing the activation/proliferation of CF, resulting in excessive production/deposition of ECM proteins. Currently, there are no approved clinical therapies directly targeting cardiac fibrosis. It is essential to identify novel mediators of cardiac fibrosis to develop new therapeutic strategies targeting cardiac fibrosis. Exploiting co-expression gene network analysis on RNA sequencing data from failing human heart, we identified TXNDC5, a CF-enriched ER protein with the enzymatic activity of a protein disulfide isomerase (PDI), as a previously unrecognized critical mediator of cardiac fibrosis. Using multiple genetic, molecular and cellular methods in vitro and in vivo, we have demonstrated that TXNDC5 is a central player in a novel profibrotic pathway involving increased ECM protein folding and CF activation triggered by redox-sensitive JNK signaling, downstream of TGFβ1-induced ER stress and ATF6-mediated transcriptional control. We have shown here, for the first time, that TXNDC5, as a PDI, contributes to cardiac fibrosis by modulating the turnover of ECM proteins and CF activity. In addition to uncovering a novel molecular mechanism that is critical for cardiac fibrosis, the results presented here also demonstrate the potential of targeting TXNDC5 as a powerful new approach to treat cardiac fibrosis and heart failure.

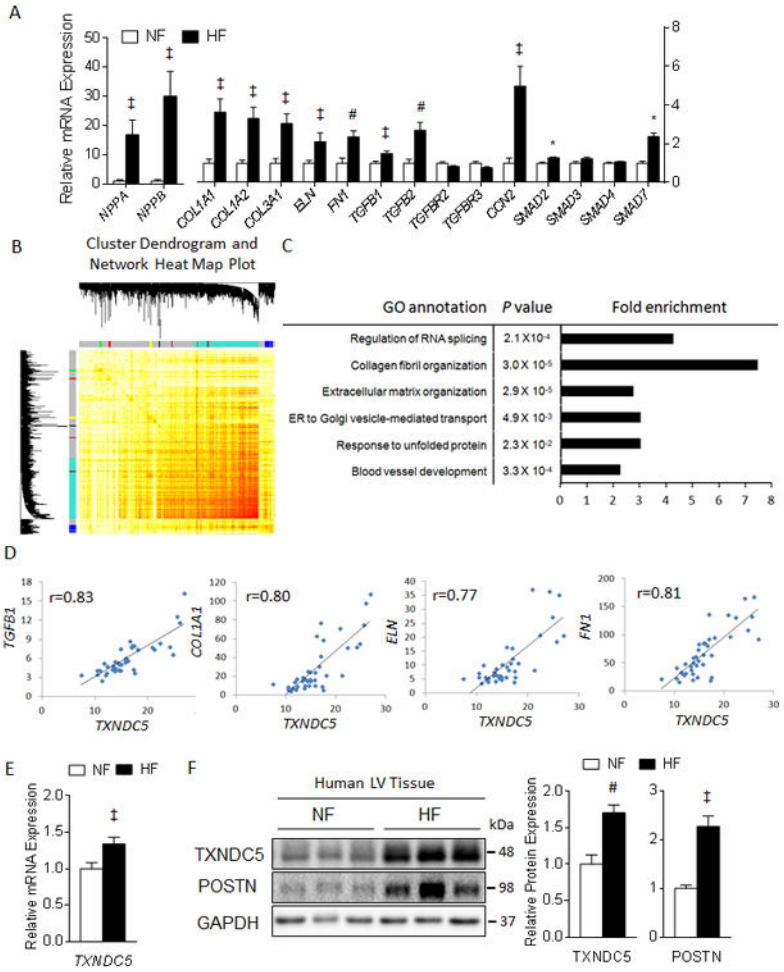

Figure 1. RNASeq and co-expression gene network analyses identified TXNDC5 as a potential novel mediator of cardiac fibrosis

(A) RNASeq analysis revealed upregulation of fibrogenic genes (including ECM protein genes *COL1A1, COL1A2, COL3A1, ELN* and *FN1,* matricellular protein CCN2 and genes involved in TGFβ signaling such as *TGFB1, TGFB2, SMAD2* and *SMAD7*) and HF markers (ANF/*NPPA* and BNP/*NPPB*) in human HF, compared to NF, LV. (B) Cluster dendrogram and network heat map plot of the 15 gene modules identified by Weighted Gene Co-expression Network Analysis (WGCNA) on the human LV RNASeq data. (C) Gene ontology analysis revealed that module Turquoise was enriched in genes that are involved in the pathogenesis of cardiac fibrosis. (D) *TXNDC5* expression in human LV showed strong positive correlations with TGFβ1 and ECM genes including *COL1A1, ELN* and *FN1.* The transcript (E) and protein (F) expression levels of TXNDC5 were significantly upregulated in human HF, compared to NF, LV samples ($^{‡}P<0.05$, $^{\#}P<0.01$, $^{*}P<0.001$).

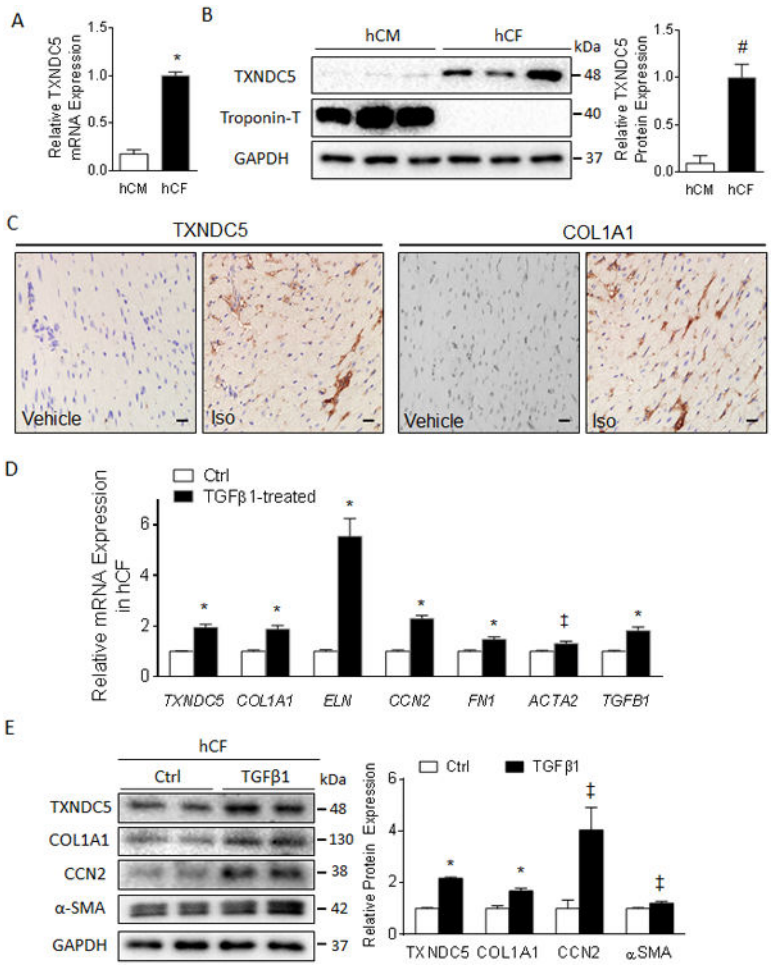

Figure 2. TXNDC5 is highly enriched in cardiac fibroblasts and upregulated in response to TGFβ1 stimulation Transcript

(A) and protein (B) expression analyses in isolated human cardiac fibroblasts (hCF) and cardiomyocytes (hCM) revealed strong enrichment of TXNDC5 in hCF compared to hCM. (C) Immunohistochemical staining of cardiac sections from a mouse model of isoproterenol (ISO)-induced heart failure showed strong staining of TXNDC5 in the myocardial intersitium but not in myocytes (left), similar to the distribution pattern of type 1 collagen (right) (Scale bar=20 μm). TGFβ1 treatment (4 ng/ml for 48 hours) in hCF induced significant upregulation of TXNDC5 mRNA (D) and protein (E) expression, as well as of various fibrogenic proteins including COL1A1, CCN2 and ACTA2/α-SMA, compared to vehicle-treated control cells (Ctrl) ($^{‡}P<0.05$, $^{\#}P<0.01$, $*P<0.001$).

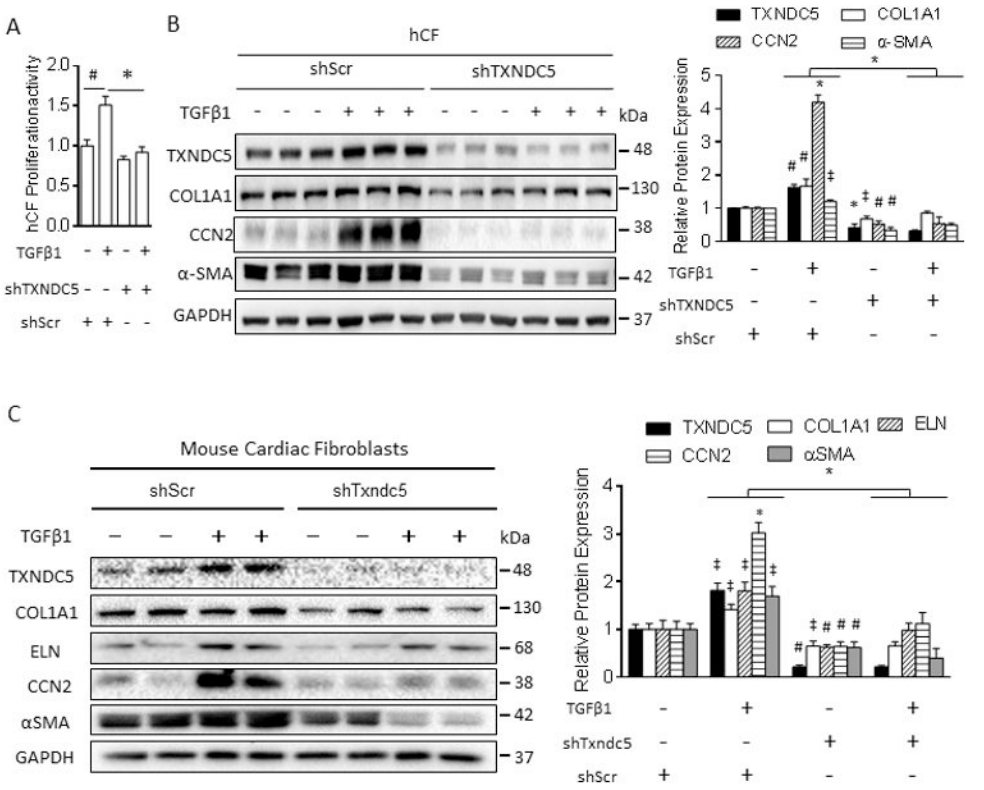

Figure 3. Knockdown of *TXNDC5* prevented TGFβ1-induced CF activation and ECM protein upregulation

(A) TGFβ1 treatment increased the proliferation rate of control hCF (transduced with shScr); knockdown of *TXNDC5* (sh*TXNDC5*) abolished TGFβ1-induced cellular proliferation in hCF. (B) Knockdown of *TXNDC5* in hCF prevented TGFβ1-induced upregulation of fibrogenic proteins including αSMA, COL1A1 and CCN2 (All significant symbols indicate comparisons to baseline shScr group without TGFβ1 treatment, except the symbol above the bars, which indicates the significant level of differences between groups of TGFβ1+shScr and TGFβ1+sh*TXNDC5*). (C) Knockdown of *Txndc5* in mouse cardiac fibroblasts (mCF) also diminished TGFβ1-induced upregulation of αSMA and ECM proteins COL1A1, ELN and CCN2 (n=6 in each group, $^{‡}P<0.05$, $^{\#}P<0.01$, $^{*}P<0.001$).

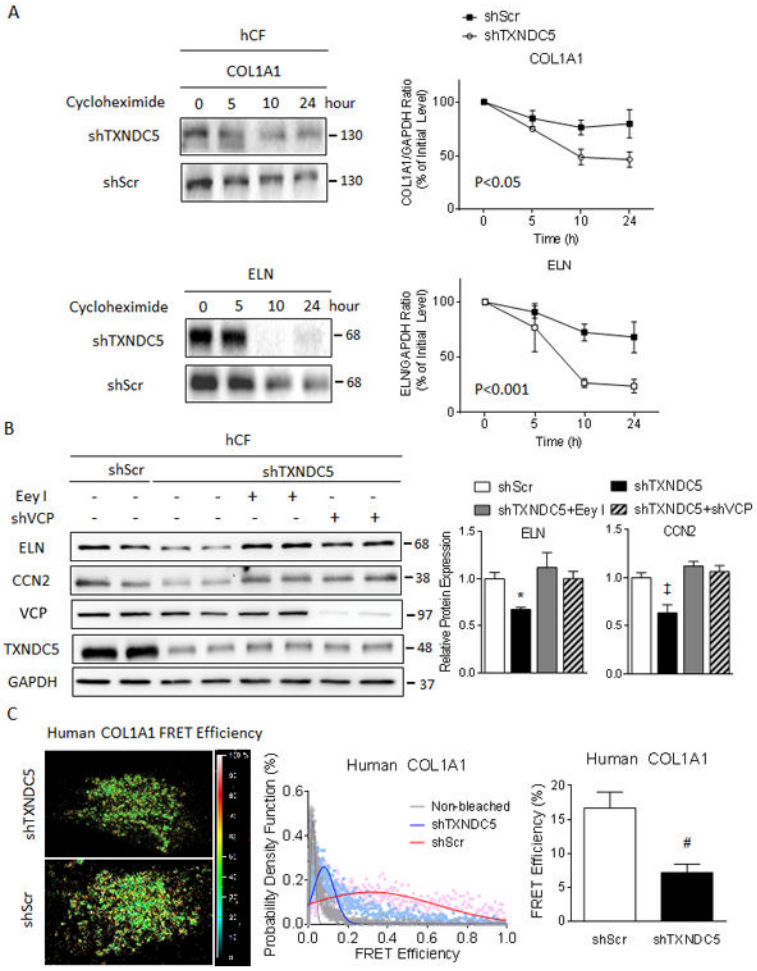

Figure 4. Knockdown of *TXNDC5* in hCF led to accelerated ECM protein degradation owing to ECM protein misfolding and subsequent removal through ER-associated protein degradation (ERAD)

(A) A cycloheximide protein chase assay revealed accelerated degradation of COL1A1 and ELN proteins in hCF with *TXNDC5* knockdown (sh*TXNDC5*-transduced), compared to control (shScr-transduced) cells. (B) ERAD inhibitor Eey I or shVCP treatment in hCF reversed the reduction in ELN and CCN2 protein expression resulting from knockdown of *TXNDC5*. (C) A fluorescence resonance energy transfer (FRET)-based protein folding assay using a dual fluorescence-labeled *COL1A1* construct in hCF showed significantly reduced COL1A1 FRET efficiency in cells with knockdown of *TXNDC5* (shTXNDC5, n=10), compared to scrambled control (shScr, n=10), indicating reduced COL1A1 folding with TXNDC5 depletion ($^{‡}P<0.05$, $^{\#}P<0.01$, $*P<0.001$).

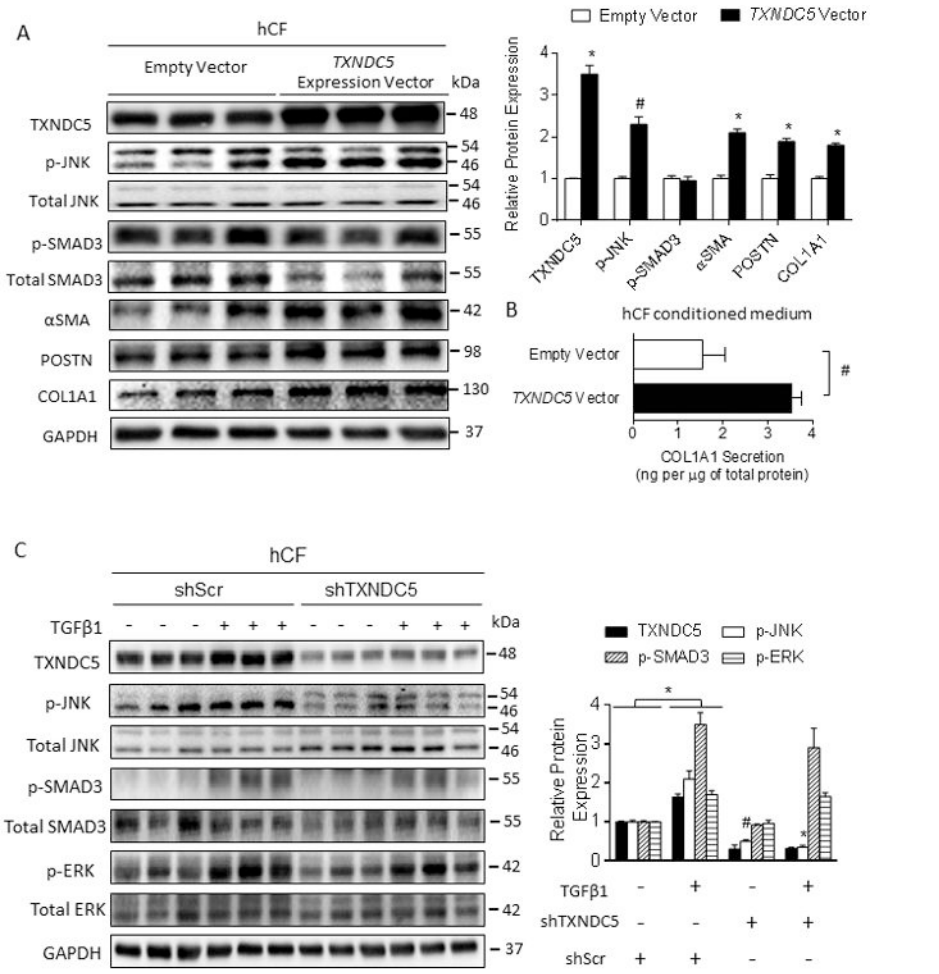

Figure 5. TXNDC5-dependent activation of hCF is associated with increased JNK, but not SMAD3 or ERK, activity

(A) Overexpression of *TXNDC5* in hCF was sufficient to trigger hCF activation (as reflected in increased αSMA and POSTN protein levels), and COL1A1 production. Phosphorylation of SMAD3 was not affected by ectopic *TXNDC5* expression. (B) Forced expression of *TXNDC5* led to significantly increased secretion of type 1 collagen (COL1A1) from hCF. (C) Knockdown of *TXNDC5* in hCF abrogated TGFβ1-induced phosphorylation of JNK, but not of SMAD3 or ERK. Phosphorylated JNK, SMAD3 and ERK were expressed relative to total JNK, SMAD3 and ERK, respectively ($^{\#}P<0.01$, $*P<0.001$).

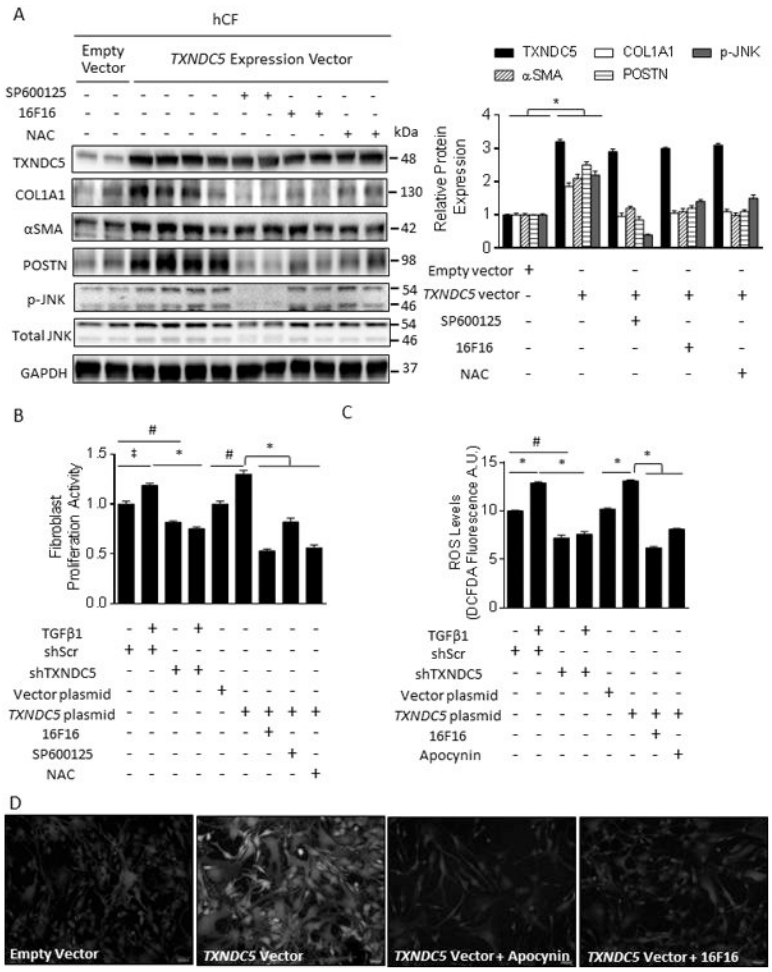

Figure 6. TXNDC5-mediated activation and proliferation of hCF require JNK activity triggered by NOX-derived ROS

(A) Treatment with a JNK inhibitor (SP600125, 10 μmol/L), PDI inhibitor (16F16, 20 μmol/L) or ROS scavenger N-acetylcysteine (NAC, 15 mmol/L) abolished upregulation of αSMA and POSTN (markers for hCF activation and myofibroblast formation) and COL1A1 induced by ectopic *TXNDC5* expression in hCF. Note that 16F16 and NAC treatment also reduced JNK phosphorylation resulting from *TXNDC5* overexpression. (B) Knockdown of *TXNDC5* abolished TGFβ1-induced hCF proliferation, whereas overexpression of *TXNDC5* was sufficient to induce hCF proliferation. Pharmacological inhibition of JNK (with SP600125), PDI (with 16F16) or ROS (with NAC) abrogated hCF proliferation induced by *TXNDC5* overexpression. (C) Knockdown of *TXNDC5* abrogated TGFβ1-induced ROS elevation in hCF, whereas *TXNDC5* overexpression was sufficient to increase ROS levels, which could be abolished by the PDI inhibitor, 16F16, or the NOX inhibitor, apocynin (2 mmol/L). (D) Representative photomicrographs illustrating increased ROS levels (measured using DCFDA fluorescence signal intensity) in hCF with ectopic *TXNDC5* expression, which is diminished by the NOX inhibitor apocynin and by the PDI inhibitor, 16F16 ($^{‡}P<0.05$, $^{\#}P<0.01$, $*P<0.001$).

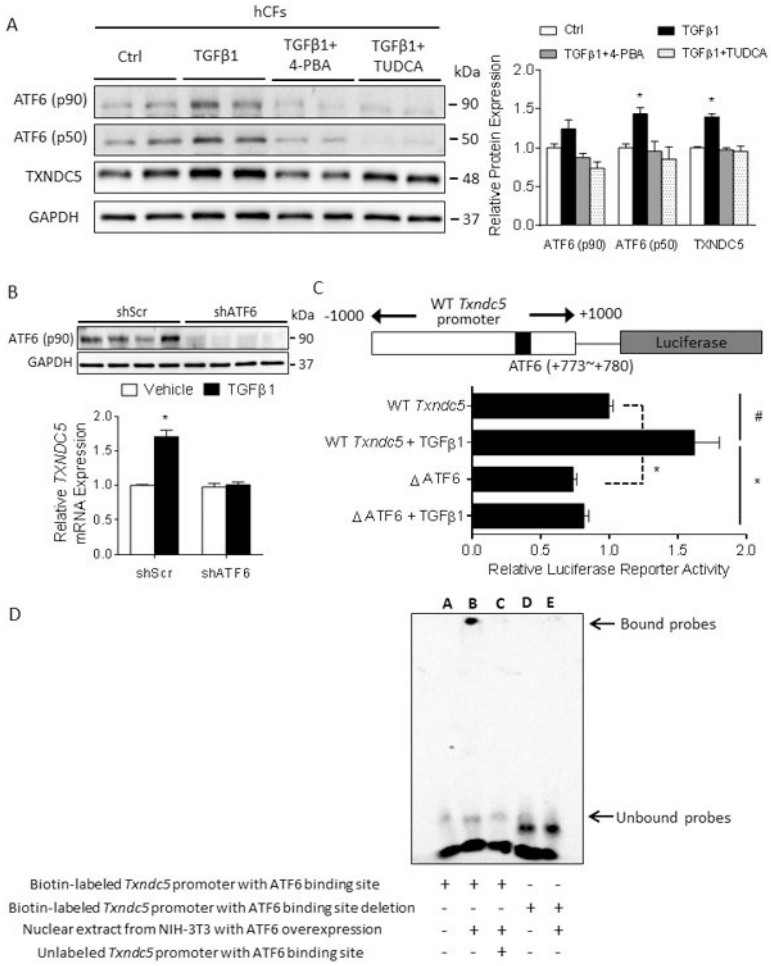

Figure 7. TGFβ1 induces TXNDC5 expression in CF through TGFβ1-ER stress-ATF6 signaling axis

(A) TGFβ1 treatment in hCF increased ER stress (as evidenced by the upregulated ER stress markers ATF6 p50 and p90) and TXNDC5 protein expression levels, which could be abrogated by the treatment with general ER stress inhibitors 4-phenylbutyrate (4-PBA, 0.5 mmol/L) or tauroursodeoxycholic acid (TUDCA, 0.5 mmol/L) (B) Knockdown of *ATF6* in hCF prevented the upregulation of *TXNDC5* mRNA in response to TGFβ1 stimulation. (C) Schematic illustration of the mouse *Txndc5* promoter luciferase reporter construct, which contains an ATF6 binding site (TGACGTGG, +773~+780). Deletion of the ATF6 binding site significantly reduced TGFβ1-induced transcriptional activity of the *Txndc5* promoter in the absence or presence of TGFβ1. (D) Electrophoretic mobility shift assay showed biotin-labeled *Txndc5* promoter probe containing ATF6 binding site was shifted (lane B) when treated with nuclear extract from NIH-3T3 fibroblasts with ectopic ATF6 expression. Unlabeled *Txndc5* promoter DNA was used as competitor (lane C) and revealed the specificity of ATF6 binding to *Txndc5* promoter. Biotin-labeled *Txndc5* promoter probe with ATF6 binding site deletion failed to interact with ATF6 (lane D, E).($^{\#}P<0.01$, $^{*}P<0.001$).

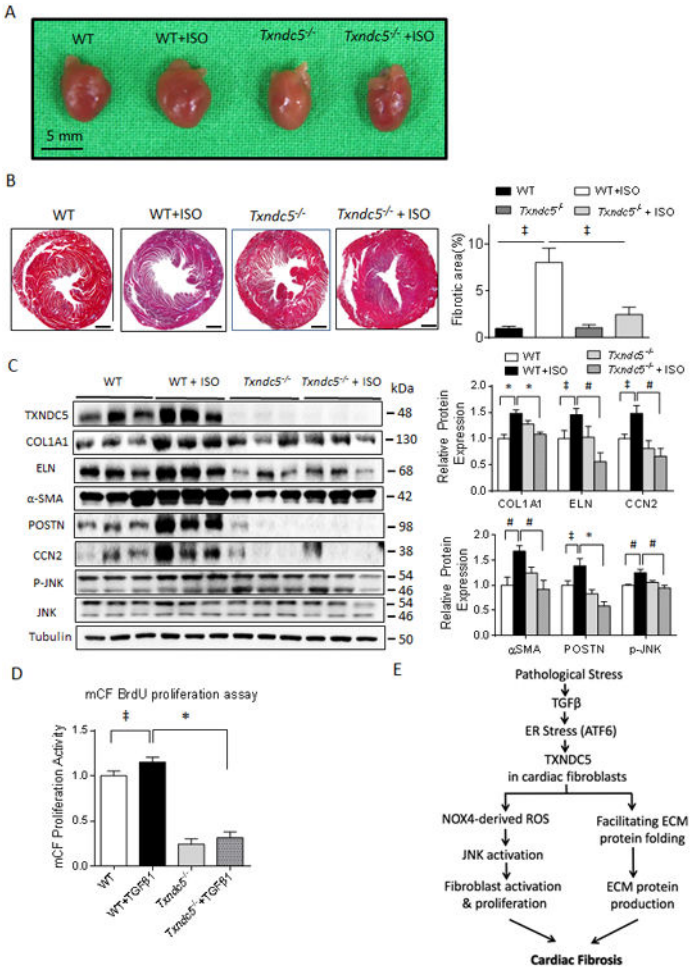

Figure 8. Targeted deletion of *Txndc5* protects against isoproterenol-induced cardiac hypertrophy, fibrosis and contractile dysfunction

(A) Isoproterenol (ISO, 30 mg/kg/day subcutaneously for 10 days) injection led to marked increase in HW/BW ratio, an indicator of cardiac hypertrophy, in WT, but not in *Txndc5*$^{-/-}$, mice. (B) Knockout of *Txndc5* attenuated the extent of myocardial fibrosis induced by ISO injection. (C) ISO treatment led to significantly increased fibrogenic proteins (including COL1A1, ELN, CCN2, αSMA, POSTN) and p-JNK expression in WT, but not in *Txndc5*$^{-/-}$, mouse LV. (D) Loss of *Txndc5* also significantly reduced the proliferation capacity of mCF at baseline and in response to TGFβ1 stimulation. (E) Schematic summary of the proposed profibrotic mechanisms by which TXNDC5 contributes to cardiac fibrosis.($^{‡}P<0.05$, $^{\#}P<0.001$, $*P<0.001$).