Genetic Analysis of Connective Tissue Growth Factor as an Effector of Transforming Growth Factor β Signaling and Cardiac Remodeling

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The matricellular secreted protein connective tissue growth factor (CTGF) is upregulated in response to cardiac injury or with transforming growth factor β (TGF-β) stimulation, where it has been suggested to function as a fibrotic effector. Here we generated transgenic mice with inducible heart-specific CTGF overexpression, mice with heart-specific expression of an activated TGF-β mutant protein, mice with heart-specific deletion of Ctgf, and mice in which Ctgf was also deleted from fibroblasts in the heart. Remarkably, neither gain nor loss of CTGF in the heart affected cardiac pathology and propensity toward early lethality due to TGF-β overactivation in the heart. Also, neither heart-specific Ctgf deletion nor CTGF overexpression altered cardiac remodeling and function with aging or after multiple acute stress stimuli. Cardiac fibrosis was also unchanged by modulation of CTGF levels in the heart with aging, pressure overload, agonist infusion, or TGF-β overexpression. However, CTGF mildly altered the overall cardiac response to TGF-β when pressure overload stimulation was applied. CTGF has been proposed to function as a critical TGF-β effector in underlying tissue remodeling and fibrosis throughout the body, although our results suggest that CTGF is of minimal importance and is an unlikely therapeutic vantage point for the heart.

Heart failure is associated with structural alterations of the ventricles that include hypertrophy and/or elongation of individual cardiac myocytes as well as myocardial fibrosis (1). Myocardial fibrosis involves an increase in extracellular matrix (ECM) deposition that adversely affects the function of the heart by disrupting electrical conductivity and mechanical performance (2). The release of growth factors and cytokines induces a negative profile of cardiac stress stimulation resulting in cardiac ventricular remodeling, cumulative myocyte loss, reduced cardiac contractility, and fibrosis (3).

One of the cytokines of major importance in the pathogenesis of myocardial fibrosis is transforming growth factor β (TGF-β) (4). TGF-β functions as a profibrotic cytokine as well as a growth factor involved in multiple pathophysiological processes (5). In the heart, TGF-β is largely thought to serve a maladaptive role leading to cardiac fibrosis (6). Inhibition of TGF-β signaling specifically in cardiomyocytes protected the heart under stress (7). However, global inhibition of TGF-β with neutralizing antibodies failed to suppress cardiac pathology and even worsened aspects of ventricular remodeling after stress, attesting to the complexity of TGF-β biology (7–9). Connective tissue growth factor (CTGF) (also known as CCN2) is a well-characterized downstream mediator of TGF-β action in connective tissue cells during the fibrotic response (10, 11), although a definitive genetic evaluation of this factor’s function in vivo is lacking.

CTGF is a matricellular protein of the CCN family of ECM-associated proteins (12). Mice lacking the Ctgf gene die at birth due to respiratory distress with severe chondrodysplasia, showing the importance of CTGF in skeletal development (13). In the adult heart, CTGF is not normally expressed unless induced by an injury event or by other stresses that activate TGF-β, which is a potent inducer of CTGF (14–16). Indeed, CTGF is strongly induced during cardiac fibrosis and is constitutively upregulated in experimental animal and human heart failure, where it is deposited in the ECM (14, 17, 18). However, the contribution of CTGF to cardiac disease or ventricular remodeling remains unclear.

MATERIALS AND METHODS

Mice. A tetracycline/doxycycline (Dox)-responsive binary α-myosin heavy chain (α-MHC) transgene system was used to temporally regulate the expression of a constitutively active mutant form of TGF-β1 carrying a switch in cysteines 223 and 225 to serine (TGF-β1Cys223,225Ser) in cardiomyocytes (19). These 2 mutations allow TGF-β to escape latency and to be constitutively active. TGF-β1Cys223,225Ser mice were then crossed with mice with heart-restricted α-MHC transgenic (TG) mice expressing the tetracycline transactivator (tTA) protein (all in the FVB/N background) to generate a Dox-regulated TG system. Heart-specific CTGF-overexpressing mice were generated by using the same tetracycline-responsive binary α-MHC TG system. Dox was provided in the food with a special diet formulated by Purina (625 mg/kg food) for the first 4 weeks of age to inhibit CTGF or TGF-β1Cys223,225Ser expression but removed thereafter, resulting in the expression of the respective proteins over the next several weeks. The generation of Ctgf LoxP-targeted (fl) mice (Ctgffl/fl) was previously described (C57BL/6 background) (20). Ctgffl/fl mice were crossed with mice expressing the cre recombinase gene under the control of the heart-specific β-MHC promoter to obtain heart-restricted deletion of Ctgf. All experiments involving animals were approved by the Institutional Animal


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Care and Use Committee at Cincinnati Children’s Hospital Medical Center.

AAV production and infusion in mice. Adeno-associated virus (AAV) serotypes 2, 4, 5, 6, 7, 8, and 9 that express cre recombinase were produced separately in HEK293 cells as previously reported (21) and then mixed at an equimolar ratio for injection into mice so that the most possible cell types in vivo could be infected. A total combined titer of 10^{12} viirions was injected via the tail vein of mice with this mixed-serotype cocktail of AAV, 10 days prior to the induction of pressure overload stimulation. The control AAV encoded green fluorescent protein, and it was infused at the same titer.

Echocardiography, pressure overload procedures, invasive hemodynamics, and agonist infusion. All mice were anesthetized with 2% isoflurane by inhalation. Echocardiography was performed in M mode by using a Sonos 5500 instrument equipped with a 15-MHz transducer (Hewlett-Packard Inc.). Cardiac hypertrophy was induced by transverse aortic constriction (TAC) to produce pressure overload, as previously described (22). In short, the transverse aortic arch was visualized through a median sternotomy, and a 7-0 silk ligature was tied around the aorta using a 27-gauge wire to obtain a defined degree of constriction between the right brachiocephalic and left common carotid arteries. Doppler echocardiography was performed on all mice subjected to TAC to ensure equal pressure gradients across the aortic constriction between groups. Assessment of invasive hemodynamics was performed by using the closed-chest technique with invasive hemodynamics revealed no change in systolic performance (rate of rise of left ventricular pressure \( \frac{dP}{dt} \)), but increased levels of active TGF-\( \beta \) (Fig. 1A). The ELISA detects TGF-\( \beta \) that is released from the latent complexes and therefore is bioavailable. TGF-\( \beta \) in TG mice showed high levels of cardiac fibrosis in the ventricles and atria, with a substantial reduction in life span and full lethality by 9 months of age (Fig. 1B to E). Gravimetric analysis revealed cardiac hypertrophy and lung congestion in these inducible TGF-\( \beta \) TG mice at 6 months of age (Fig. 1F and G).

RESULTS

Generation of heart-specific inducible TGF-\( \beta \) transgenic mice. To investigate the role of CTGF as a downstream mediator of TGF-\( \beta \) action in the heart, we first generated TG mice with enhanced TGF-\( \beta \) activity in the adult heart. To generate a form of TGF-\( \beta \) that would escape latency, we used the previously characterized TGF-\( \beta 1 \) cDNA in which Cys223 and Cys225 were mutated to serine (TGF-\( \beta 1 \)Cys223,225Ser) (25). This cDNA was conditionally expressed in the heart by using the Dox-regulated bi-TG system in which a modified \( \alpha \)-myosin heavy chain (\( \alpha \)-MHC) promoter serves as the responder transgene to drive TGF-\( \beta 1 \)Cys223,225Ser expression when crossed with TG mice containing the \( \alpha \)-MHC promoter-driven tetracycline transactivator (tTA) protein. All mice were bred on Dox to suppress transgene expression during embryonic and postnatal development, and thereafter, Dox was then removed at weaning (age of 4 weeks) to subsequently induce protein expression. TGF-\( \beta 1 \)Cys223,225Ser TG mice showed increased levels of active TGF-\( \beta \) in the heart by 2 months of age (4 weeks after Dox removal) by an ELISA, which was further increased at 6 months of age (Fig. 1A). The levels achieved in the heart at 6 months of age were comparable to those with the induction of endogenous TGF-\( \beta \) in the heart after 6 weeks of pressure overload stimulation with TAC (Fig. 1A). The ELISA detects TGF-\( \beta \) that is released from the latent complexes and therefore is bioavailable. TGF-\( \beta \) TG mice showed high levels of cardiac fibrosis in the ventricles and atria, with a substantial reduction in life span and full lethality by 9 months of age (Fig. 1B to E). The ELISA detects TGF-\( \beta \) that is released from the latent complexes and therefore is bioavailable. TGF-\( \beta \) TG mice showed high levels of cardiac fibrosis in the ventricles and atria, with a substantial reduction in life span and full lethality by 9 months of age (Fig. 1B to E). Gravimetric analysis revealed cardiac hypertrophy and lung congestion in these inducible TGF-\( \beta \) TG mice at 6 months of age (Fig. 1F and G).

TGF-\( \beta \) TG mice showed increased left ventricular chamber dimension at 6 months of age and thickening of the ventricular walls (Fig. 1H and I). However, TGF-\( \beta \) TG mice showed increased cardiac fractional shortening by echocardiography at 4 and 6 months of age compared with control tTA TG mice (Fig. 1J). This increase in fractional shortening may reflect an increase in ventricular stiffness. Indeed, assessment of cardiac function by invasive hemodynamics revealed no change in systolic performance (rate of rise of left ventricular pressure \( \frac{+dP}{dt} \); mm Hg/s), but there was an increase in diastolic left ventricular pressure, indicative of increased ventricular stiffness due to a fibrotic response with aging (Fig. 1K and L).

Analysis of \( \text{Ctgf} \) gene-targeted mice. In agreement with previously reported data showing that TGF-\( \beta \) is a potent inducer of...
CTGF expression, we observed induction of CTGF expression in the hearts of TGF-β TG mice by Western blotting and immunohistochemistry (Fig. 2A and B). Similarly, pressure overload stimulation in the heart that was localized predominantly to the ECM was achieved only within cardiac myocytes of the heart and that relevant CTGF protein might still be produced from resident non-myocytes of the heart, especially the fibroblasts, to achieve an even greater total deletion of CTGF protein from purified cardiomyocytes than that with just the Ctgf gene alone (Fig. 3B). Importantly, the combined disruption of the Ctgf gene in the nonmyocytes of the heart, especially the fibroblasts, to achieve an even greater total deletion of this protein from the entire organ (Fig. 3B). The data show an ~90% deletion of CTGF protein from fibroblasts isolated from AAV-cre-treated hearts, 2 weeks after TAC stimulation (Fig. 3B). Infusion of AAV-cre also appeared to produce an even greater total deletion of CTGF protein from purified cardiomyocytes than that with just the β-MHC–cre transgene alone (Fig. 3B). Importantly, the combined disruption of the Ctgf gene from the myocytes and fibroblasts of the heart did not further alter the cardiac phenotype after TAC stimulation compared to control mice (Fig. 3C to J). Indeed, the combined deletion strategy did not alter cardiac structure or function (Fig. 3C to F), fibrosis (Fig. 3G and H), or expression of fibrotic markers (Fig. 3I and J). Thus, loss of CTGF from the heart at baseline or after

FIG 1 Generation of a murine model with inducible TGF-β expression in the heart. (A) ELISA for TGF-β activity in hearts from TTA control and TGF-β TG mice at 2 and 6 months of age versus the induction of endogenous TGF-β after 1 and 6 weeks of pressure overload stimulation by TAC. (B) Representative Masson’s trichrome-stained histological sections for fibrosis (blue) in tTA control and TGF-β TG mouse hearts at 6 months of age. (C) Survival rate of tTA control and TGF-β TG mice in the first 12 months of life. (D and E) Hydroxyproline biochemical assay for fibrosis in tTA control and TGF-β TG mouse ventricles and atria at 6 months of age. (F and G) Ratios of lung weight to body weight (LW/BW) (F) and ventricular weight to body weight (VW/BW) (G) for tTA control and TGF-β TG mice at 6 months of age. (H to J) Echocardiographic analysis of left ventricle end diastolic dimension (LVEDD) (H), fractional shortening (FS) (I), and left ventricular pressure overload stimulation by TAC (Fig. 3B). The data show an ~90% deletion of CTGF protein from fibroblasts isolated from AAV-cre-treated hearts, 2 weeks after TAC stimulation (Fig. 3B). Infusion of AAV-cre also appeared to produce an even greater total deletion of CTGF protein from purified cardiomyocytes than that with just the β-MHC–cre transgene alone (Fig. 3B). Importantly, the combined disruption of the Ctgf gene from the myocytes and fibroblasts of the heart did not further alter the cardiac phenotype after TAC stimulation compared to control mice (Fig. 3C to J). Indeed, the combined deletion strategy did not alter cardiac structure or function (Fig. 3C to F), fibrosis (Fig. 3G and H), or expression of fibrotic markers (Fig. 3I and J). Thus, loss of CTGF from the heart at baseline or after

CTGF expression, we observed induction of CTGF expression in the hearts of TGF-β TG mice by Western blotting and immunohistochemistry (Fig. 2A and B). Similarly, pressure overload stimulation in the heart that was localized predominantly to the ECM (Fig. 2B and C). Generation of purified cardiomyocytes from TAC hearts showed that CTGF is most prominently produced within these cells (Fig. 2D). Indeed, heart-restricted Ctgf deletion was obtained by crossing Ctgf<sup>β-cre</sup> mice with mice expressing cre recombinase under the control of the heart-specific β-MHC promoter, resulting in Ctgf<sup>β-cre</sup> mice that carry the β-MHC–cre transgene (Ctgf<sup>β-cre</sup>-MHC-cre mice), which showed a ~80% loss of total CTGF protein that is normally induced within the entire heart after TAC stimulation (Fig. 2E). Hence, the myocytes appear to be the primary source of CTGF production in diseased hearts, although a previous analysis of total mRNA levels from isolated myocytes and fibroblasts suggested that cardiac fibroblasts are also a major source for induced expression of CTGF in the heart (26).

To assess the importance of CTGF induction during cardiac stress, we subjected mice with myocyte-specific deletion of Ctgf to pressure overload stimulation by TAC for 2 and 8 weeks but observed no difference in heart weight, cardiac function, or lung weight and the transition to heart failure compared with control mice (Fig. 2F to H). These results indicate that although strongly

induced in the stressed heart, deletion of Ctgf from myocytes appears to have no discernible effect on cardiac hypertrophy, ventricular remodeling, or ventricular performance after 2 or 8 weeks of pressure overload stimulation.

One concern with the data presented above is that Ctgf depletion was achieved only within cardiac myocytes of the heart and that relevant CTGF protein might still be produced from resident non-myocytes in the heart. To address this concern, we adopted a protocol for combined deletion of Ctgf from the heart using both the β-MHC–cre transgene and an infusion of AAV-cre, followed by TAC stimulation (Fig. 3A). Infusion of AAV-cre (see Materials and Methods) would promote the deletion of the Ctgf gene in the nonmyocytes of the heart, especially the fibroblasts, to achieve an even greater total deletion of this protein from the entire organ (Fig. 3B). The data show an ~90% deletion of CTGF protein from fibroblasts isolated from AAV-cre-treated hearts, 2 weeks after TAC stimulation (Fig. 3B). Infusion of AAV-cre also appeared to produce an even greater total deletion of CTGF protein from purified cardiomyocytes than that with just the β-MHC–cre transgene alone (Fig. 3B). Importantly, the combined disruption of the Ctgf gene from the myocytes and fibroblasts of the heart did not further alter the cardiac phenotype after TAC stimulation compared to control mice (Fig. 3C to J). Indeed, the combined deletion strategy did not alter cardiac structure or function (Fig. 3C to F), fibrosis (Fig. 3G and H), or expression of fibrotic markers (Fig. 3I and J). Thus, loss of CTGF from the heart at baseline or after

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"Survival rate of tTA control and TGF-β TG mice in the first 12 months of life. (D and E) Hydroxyproline biochemical assay for fibrosis in tTA control and TGF-β TG mouse ventricles and atria at 6 months of age. (F and G) Ratios of lung weight to body weight (LW/BW) (F) and ventricular weight to body weight (VW/BW) (G) for tTA control and TGF-β TG mice at 6 months of age. (H to J) Echocardiographic analysis of left ventricle end diastolic dimension (LVEDD) (H), fractional shortening (FS) (I), and left ventricular pressure overload stimulation by TAC (Fig. 3B). The data show an ~90% deletion of CTGF protein from fibroblasts isolated from AAV-cre-treated hearts, 2 weeks after TAC stimulation (Fig. 3B). Infusion of AAV-cre also appeared to produce an even greater total deletion of CTGF protein from purified cardiomyocytes than that with just the β-MHC–cre transgene alone (Fig. 3B). Importantly, the combined disruption of the Ctgf gene from the myocytes and fibroblasts of the heart did not further alter the cardiac phenotype after TAC stimulation compared to control mice (Fig. 3C to J). Indeed, the combined deletion strategy did not alter cardiac structure or function (Fig. 3C to F), fibrosis (Fig. 3G and H), or expression of fibrotic markers (Fig. 3I and J). Thus, loss of CTGF from the heart at baseline or after

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Role of CTGF in Cardiac Remodeling

TAC stimulation, by deletion either in myocytes alone or in myocytes with fibroblasts, did not significantly alter cardiac compensation after TAC stimulation.

CTGF does not synergize with TGF-β. CTGF is reported to synergize with TGF-β in a wide array of tissues and cell types in mediating the fibrotic response (27–29), although such an effect has not been previously examined in the heart. To directly examine if CTGF synergizes with TGF-β in the heart, we crossed heart-specific and inducible CTGF TG mice with TGF-β TG mice to generate double-TG (DTG) mice. We confirmed CTGF overexpression and greater TGF-β activity in both CTGF TG mice as well as DTG mice by Western blotting, using the ELISA activity assay and immunostaining (Fig. 4A to C). However, overexpression of CTGF in the heart did not increase TGF-β activity, nor did it modify TGF-β activation when both TGF-β and CTGF were simultaneously overexpressed (Fig. 4B). Moreover, the levels of CTGF overexpression achieved in the heart with the inducible transgenic strategy were in a range similar to the levels of CTGF induction after TAC stimulation (Fig. 5A and B). Downstream TGF-β signaling was also unaffected by CTGF overexpression, as assessed by Western blotting for phosphorylated SMAD2 (Fig. 4A) or by analysis of markers of the fibrotic response at baseline or after TAC stimulation (Fig. 5C to F). The phenotype induced by TGF-β activation in the heart was also unaltered by CTGF overexpression, directly demonstrating that these factors do not synergize in this tissue (Fig. 4D to I). For example, survival rates of TGF-β TG and DTG mice were similar with aging (Fig. 4D), and cardiac fibrosis was not significantly increased, as measured by Masson’s trichrome histology and hydroxyproline content (Fig. 4E to G). The propensity toward heart failure as assessed by lung weight and cardiac hypertrophy was also unchanged in DTG compared to TGF-β TG mice (Fig. 4H and I).

To evaluate if a phenotypic interaction between CTGF and TGF-β could be revealed during cardiac stress, we also subjected these mice to 1 week of pressure overload stimulation. Simultaneous overexpression of CTGF and TGF-β resulted in a response to pressure overload (TAC) that was similar to that with overexpression of TGF-β alone (Fig. 4J to L). Specifically, we observed similarly exacerbated fibrotic responses to TAC in TGF-β TG and DTG mice (Fig. 4J) and similar degrees of lung congestion and ventricular hypertrophy (Fig. 4K and L). We also evaluated mice after 6 weeks of TAC stimulation and again observed no greater cardiac phenotypic effect in the DTG mice than in either the CTGF TG or the TGF-β TG mice separately, when analyzed for changes in cardiac hypertrophy, lung congestion, or the extent of fibrosis (Fig. 5G to I). In addition to TAC stimulation, we also infused the profibrotic agonists angiotensin II (Ang) and phenylephrine (PE) simultaneously with osmotic minipumps in each of the groups of mice and again observed no significant differences in the cardiac phenotypic responses to these agonists in DTG mice versus either the TGF-β TG or CTGF TG mice (Fig. 6). Finally, we did not observe a cardiac phenotype in mice overexpressing CTGF alone in the heart with aging or pressure overload stimulation compared with tTA control TG mice (Fig. 4D to L). These results suggest that CTGF does not activate or synergize with TGF-β in the heart, nor does its overexpression alter cardiac structure-function in a readily obvious manner at baseline or with multiple stress stimuli.
Deletion of Ctgf does not rescue TGF-β-driven cardiac pathology. To assess if CTGF acts as a downstream effector of TGF-β in the heart, we crossed TGF-β-overexpressing mice to heart-restricted Ctgf gene-deleted (Ctgffl/fl-MHC-cre) TGF-β TG mice. CTGF protein was again successfully deleted by the β-MHC-cre transgene in Ctgffl/fl-MHC-cre TGF-β TG mice, as assessed by Western blotting (Fig. 7A). Deletion of Ctgf did not affect TGF-β activation at baseline or otherwise affect the activity of the TGF-β mutant protein (Fig. 7B). Downstream TGF-β signaling was also equally activated in TGF-β TG mice with and without Ctgf deletion, as assessed by SMAD2 phosphorylation levels in the heart (Fig. 7A). Deletion of Ctgf from the heart also did not reduce mortality in TGF-β TG mice with aging compared with controls (Fig. 7C). Analysis of cardiac fibrosis showed no differences between Ctgffl/fl-MHC-cre TGF-β TG controls and Ctgffl/fl-MHC-cre TGF-β TG mice by both Masson’s trichrome staining and hydroxyproline biochemical assays (Fig. 7D to F). The cardiac hypertrophic response and lung edema propensity were also not altered in TGF-β TG mice versus the same mice with the loss of Ctgf (Fig. 7G and H), nor was the expression of fibrotic markers altered (Fig. 7I and J). The finding that Ctgffl/fl-MHC-cre TGF-β TG (FVBN/C57BL/6 hybrid) mice did not show some degree of cardiac hypertrophy, as observed for TGF-β TG mice (Fig. 1F), can be attributed to the different murine background, as the latter mice were purely FVBN. Regardless of the genetic background issues, the data uniformly show that CTGF appears to play no substantial role in mediating TGF-β actions in the heart at baseline.

Ctgf deletion with TAC alters TGF-β-mediated cardiac remodeling. Since Ctgf deletion appeared to be ineffective in influencing the cardiac response to TGF-β or pressure overload, we went one step further and combined both stimuli. Here we subjected heart-restricted Ctgf-deleted mice with the activated TGF-β transgene to pressure overload stimulation. Analysis of cardiac function by echocardiography revealed increased fractional shortening in Ctgffl/fl-MHC-cre TGF-β TG mice after 1 week of pressure overload, which was absent in Ctgffl/fl-MHC-cre TGF-β TG mice (Fig. 8A). After 5 weeks of pressure overload, Ctgffl/fl-MHC-cre TGF-β TG mice maintained cardiac function, while this compensated phenotype was lost in Ctgffl/fl-MHC-cre TGF-β TG mice (Fig. 8A). Also, overexpression of TGF-β conferred some protection against cardiac hypertrophy at both 1 and 5 weeks of pressure overload, which was not observed for Ctgf-deleted mice with the TGF-β transgene (Fig. 8B). Analysis of pulmonary congestion showed increases in lung weights in only Ctgffl/fl-MHC-cre TGF-β TG mice at the 1-week time point, but by 5 weeks of pressure overload, both Ctgffl/fl-MHC-cre TGF-β TG and Ctgffl/fl-MHC-cre TGF-β TG mice showed similar increases (Fig. 8C). Quantification of cardiac fibrosis revealed that deletion of Ctgf blunted the exacerbated induction of fibrosis due to the TGF-β transgene after

FIG 3 Analysis of myocyte and nonmyocyte Ctgf-deleted mice. (A) Diagram of the experimental approach in which Ctgffl/fl-β-MHC-cre mice were infused with AAV-cre, followed by TAC and then tissue harvesting. (B) Western blot analysis for CTGF and GAPDH from isolated fibroblasts and cardiomyocytes from the hearts of Ctgffl/fl-β-MHC-cre mice infused previously with control (Ctrl.) AAV or AAV-cre, both after 2 weeks of TAC stimulation. Ctgffl/fl mice were used as a control without TAC stimulation. (C and D) Ventricular weights (C) and lung weights (D) normalized to body weights in the groups of mice shown. (E and F) Echocardiographic assessment of fractional shortening (FS) (E) and left ventricular chamber dimension in diastole (LVED) (F) in the groups of mice shown. (G and H) Histological assessment of fibrosis by Masson’s trichrome (G) and hydroxyproline content (H) in hearts of the indicated groups of mice. (I and J) mRNA analysis for Col1a1 (I) and Postn (J) gene expression in the indicated groups of mice. The number of mice used is shown within the bars in each graph. *, P < 0.05 versus Ctgffl/fl mice without TAC.
FIG 4 CTGF does not synergize with TGF-β in the heart. (A) Western blot analysis of CTGF, phospho-SMAD2, total SMAD2, and GAPDH from heart homogenates of TG mice of the indicated genotypes. (B) ELISA for TGF-β activity in hearts from tTA control, CTGF TG, TGF-β TG, and CTGF/TGF-β double-transgenic (DTG) mice at 2 months of age. (C) Immunohistochemistry for CTGF protein (green) localization in hearts of tTA control versus inducible CTGF TG mice. (D) Survival rate of mice of the indicated genotypes in the first 12 months of life. (E) Representative Masson’s trichrome-stained cardiac histological sections for fibrosis (blue) in mice of the indicated genotypes at 6 months of age. (F and G) Hydroxyproline biochemical assay for fibrosis in cardiac ventricles (F) and atria (G) of mice of the indicated genotypes at 6 months of age. (H and I) Ratios of lung weight to body weight (LW/BW) (H) and ventricular weight to body weight (VW/BW) (I) in mice of the indicated genotypes at 6 months of age. *, P < 0.05 versus the tTA control. (J) Hydroxyproline biochemical assay for fibrosis in mouse hearts of the indicated genotypes subjected to sham treatment or 1 week of TAC. (K and L) Ratios of lung weight to body weight (K) and ventricular weight to body weight (L) from mice of the indicated genotypes subjected to sham treatment or 1 week of TAC. The number of mice used is shown within each graph. *, P < 0.05 versus sham; #, P < 0.05 versus tTA TAC.

1 week of pressure overload, yet this effect was lost after 5 weeks of pressure overload (Fig. 8D). Despite these mild phenotypic changes, the amount of active TGF-β in these pressure-overloaded hearts was not affected by the absence of CTGF protein, suggesting a minimal role for CTGF as an effector of TGF-β activation or expression (Fig. 8E). Similar to our observations described above (Fig. 2F to H), deletion of Ctgf by itself (Ctgffl/fl;cre tTA mice) did not alter cardiac function, hypertrophy, or fibrosis (Fig. 8A, B, and D). Moreover, deletion of Ctgf from the heart with angiotensin II and phenylephrine infusion using osmotic mini-pumps did not significantly alter the cardiac phenotype compared with that of TGF-β TG mice alone (Fig. 9). Taken together, the results presented here demonstrate that CTGF can have a very subtle modulatory effect on TGF-β responsiveness in the heart and disease manifestations that is probably indirect but only when combined with pressure overload stimulation and not with neuroendocrine agonist infusion. However, we are not certain why deletion of Ctgf from the heart has an effect on TGF-β-associated remodeling and fibrosis only when combined with TAC stimulation, especially at the 1-week time point (some of the effect is lost by 5 weeks).

**DISCUSSION**

CTGF has been implicated in a variety of biological functions, each of which appears to depend on the tissue examined and/or the pathological insult used (30). In general, Ctgf gene induction appears to be a uniform response following disease or injury events that is associated with tissue fibrosis across the organism (31). In vivo evidence for CTGF as an effector of bleomycin-induced skin fibrosis was recently reported (20, 32). The fibrotic response is also highly correlated with TGF-β induction of both gene expression and activation of latent protein within the ECM (33). TGF-β is a potent inducer of CTGF expression, which is partly due to a transcriptional mechanism at the level of the Ctgf promoter (34). Despite the strong link between TGF-β activation and CTGF expression, in vivo assessment of CTGF as a mediator of TGF-β-directed tissue fibrosis or remodeling is largely uncertain, in part because the field previously lacked an appropriate loss-of-function animal model. Global deletion of Ctgf induces perinatal lethality accompanied by major skeletal defects in mice, implicating CTGF in the regulation of skeletal development (13).

Remarkably, almost all of our in vivo evidence strongly suggests that CTGF is not an important effector of TGF-β in the heart, nor is CTGF otherwise involved in cardiac remodeling, hypertrophy, or fibrosis at baseline, with aging, after pressure overload, with TGF-β stimulation, or with neuroendocrine agonist stimulation. Our observations are in agreement with data from previous studies showing that overexpression of CTGF in the heart had either a subtle effect or no effect whatsoever on stimulating myocardial fibrosis (35, 36). However, transgenic mice overexpressing CTGF in the heart generated by another group were shown to have attenuated left ventricular remodeling after myocardial infarction.
injury as well as improved cardiac function in association with reduced inflammation and apoptosis in the heart (37). This same group also showed that CTGF TG mice were partially protected from cardiac hypertrophy induced by abdominal aortic banding (38). In contrast to the latter report, CTGF was previously shown to actually induce hypertrophy in neonatal cultured cardiomyocytes and not to protect from it (39). More perplexing, CTGF TG mice showed either cardiac dysfunction by 7 months of age with no protection from ischemia/reperfusion (I/R) injury (36) or accelerated deterioration of cardiac function with pressure overload stimulation (40). The latter two studies are in dramatic contrast to the protective effects with CTGF overexpression reported by Gravning et al. (37,38). Thus, these four papers in the literature reporting CTGF overexpression in the heart, spanning three independently generated mouse models, reached dramatically different conclusions, with one group claiming that CTGF is protective to the heart and the other two groups claiming that it has maladaptive effects.

In our hands, CTGF overexpression in the adult heart produced no cardiac effect whatsoever, including no induction of cardiac hypertrophy with aging or an increase in cardiac hypertrophy following cardiac pressure overload or with angiotensin II/phenylephrine infusion. Thus, our results suggest that CTGF is not a prohypertrophic factor for the adult heart when overexpressed, nor does it protect or otherwise compromise the adult heart when overexpressed. We are not certain why the results of Panek et al. (36) and Yoon et al. (40) mostly contradict the two reports of Gravning et al. (37,38) or why our data are largely

**FIG 5** TGF-β overexpression with CTGF does not produce greater cardiac disease with pressure overload stimulation. (A) Western blot analysis of CTGF and GAPDH expression in the hearts of mice of the indicated genotypes and with the indicated treatments. tTA TG is a control expressing only the Tet transactivator protein in the heart. (B) Protein quantification of CTGF expression normalized to GAPDH expression from panel A (CTGF/GAPDH). *, *P < 0.05 versus TGF-β TG mice; #, *P < 0.05 versus tTA TAC. (C to F) mRNA expression levels for Col1a1 and Postn in the indicated groups of mice at baseline (C and D) or with 1 week of TAC stimulation (E and F). (G) Ratios of ventricular weight to body weight (VW/BW) in mice of the indicated genotypes after 6 weeks of TAC or sham surgery. (H) Ratios of lung weight to body weight (LW/BW) in mice of the indicated genotypes and with the indicated treatments. (I) Hydroxyproline biochemical assay for fibrosis in hearts of mice of the indicated genotypes and with the indicated treatments. For each experiment, the number of mice is given within the graph. *, *P < 0.05 versus sham; #, *P < 0.05 versus tTA TAC (for panels C to I).

**FIG 6** CTGF overexpression, with or without TGF-β, does not predispose to greater cardiac disease with Ang/PE infusion. (A) Ratios of ventricular weight to body weight (VW/BW) in mice of the indicated genotypes after 2 weeks of Ang/PE infusion or vehicle infusion. (B) Ratios of lung weight to body weight (LW/BW) in mice of the indicated genotypes and with the indicated treatments. (C) Hydroxyproline biochemical assay for fibrosis in hearts of mice of the indicated genotypes and with the indicated treatments. For each experiment, the number of mice is given within the graph. *, *P < 0.05 versus vehicle.
disparate from those of Gravning et al. One possibility is that different spliced forms of the CTGF cDNAs were used among the four groups or that the absolute levels of CTGF overexpression varied dramatically. Another important variable is that the CTGF-overexpressing model that we employed was specific to the adult heart, such that CTGF levels were not elevated during postnatal development and juvenile maturation, as was the case for the three other models generated by Panek et al., Yoon et al., and Gravning et al. Hence, our inducible transgenic approach more uniformly isolates the effect of CTGF on the adult heart.

Heart-restricted deletion of Ctgf also did not modulate the cardiac pathological response to TGF-β. Inducible activation of TGF-β in the heart results in dramatic ventricular remodeling and fibrosis and subsequent death of the mice by 6 to 9 months of age. Loss of Ctgf from the heart did not affect TGF-β-dependent lethality, cardiac remodeling, or fibrosis, suggesting that Ctgf is not an important effector of TGF-β in this tissue. We also used a mixed-serotype AAV-cre infusion approach to further delete Ctgf from nonmyocytes of the heart, especially fibroblasts, which indeed produced an even greater loss of total CTGF protein from the heart and a 90% loss from cardiac fibroblasts, yet this approach also failed to uncover a phenotypic effect. However, it is possible that in the absence of CTGF (CCN2), another CCN family member fully compensates. Indeed, of the 6 known CCN proteins, CCN1 and CCN4 are reported to be expressed in the heart, and each of these proteins share similar domain structures, such as 4 conserved cysteine-rich domains, a thrombospondin (TSP) type 1 repeat domain, an insulin-like growth factor-binding domain, and a Von Willebrand factor type C domain (41).

Deletion of Ctgf from the heart also did not negatively or positively impact cardiac hypertrophy, propensity for heart failure, remodeling, or cardiac fibrosis after short-term or long-term TAC stimulation, nor did it have an impact after angiotensin II/phenylephrine infusion. These observations are in contrast to a recent report showing that systemic administration of a monoclonal antibody (MAb) against CTGF in mice over 8 weeks of TAC stimulation preserved cardiac function better and reduced left ventricular dilation but without reducing cardiac fibrosis (42).

While the preponderance of our results suggested that CTGF had no effect on the fibrotic response or TGF-β-driven pathology, when we instituted a more contrived situation in which TGF-β overexpression was superimposed with pressure overload stimulation, the loss of Ctgf now appeared to impact the cardiac remodeling profile of the heart albeit in a minor way. A similar result was previously obtained in a study showing that only in the presence of mechanical stress was CTGF necessary for TGF-β stimulation of myofibroblast differentiation and subsequent collagen matrix contraction in vitro (43). Another intriguing result was that increasing TGF-β activity in the heart with the transgene leads to a reduced hypertrophic response and preservation of heart function.

FIG 7 CTGF expression has essentially no effect on TGF-β-driven cardiac pathology. (A) Western blot analysis of CTGF, phospho-SMAD2, total SMAD2, and GAPDH from heart homogenates of Ctgf-fl/fl TTA, Ctgf-fl/fl TGF-β TG, and Ctgf-fl/fl-cre/TGF-β TG mice. (B) ELISA for TGF-β activity in hearts from mice of the indicated genotypes at 6 months of age. (C) Survival rates of mice of the indicated genotypes in the first 12 months of life. (D) Representative Masson’s trichrome-stained cardiac histological sections for fibrosis (blue) in mice of the indicated genotypes at 6 months of age. (E and F) Hydroxyproline biochemical assay for fibrosis in mouse ventricle (E) and atrium (F) of mice of the indicated genotypes at 6 months of age. (G and H) Ratios of ventricular weight to body weight (VW/BW) (G) and lung weight to body weight (LW/BW) (H) of mice of the indicated genotypes at 6 months of age. (I and J) mRNA analysis for Col1a1 and Postn gene expression in the indicated groups of mice. The number of mice used is shown in each graph. *, P < 0.05 versus the Ctgf-fl/fl TTA control.
after pressure overload, and these effects were at least partially abolished when Ctgf was deleted. Although the protection conferred by TGF-β activation during pressure overload stimulation might appear counterintuitive, especially since these mice eventually die by 9 months of age, previous reports suggested that treatment of mice with a neutralizing antibody against TGF-β adversely affected cardiac remodeling following pressure overload (7–9), suggesting that activation of TGF-β might have some beneficial effects in a temporally controlled manner or with acute stress stimulation.

CTGF is thought to function as a matricellular protein, where it affects cellular interactions through integrins, heparin sulfate proteoglycans (44,45), and possibly other uncharacterized binding partners. CTGF was previously implicated as a secreted growth factor, where it can function when applied directly to the medium in cultured cells (46,47). However, a CTGF receptor has not been identified, so it is unclear how CTGF might otherwise modify cellular responses to injury as a traditional paracrine factor. Thus, it appears unlikely that CTGF functions as a traditional secreted signaling factor.

We also observed that while the induction of CTGF in the heart by pressure overload stimulation or due to the TGF-β transgene led to abundant CTGF localized within the ECM, a fair amount was also localized intracellularly, within the vesicular network of the cardiomyocyte. CTGF also contains a TSP type 1 domain that could have an intracellular function in chaperoning ECM proteins as they are being secreted, not unlike what was proposed previously for thrombospondin proteins (48, 49), which also contain TSP functional domains. Thus, CTGF could function as a secondary effector of TGF-β responsiveness by altering the content of other ECM proteins that are deposited after an injury response, which could further affect how TGF-β is activated by stretch. Changing the composition of the ECM due to altered CTGF activity as an intracellular chaperone might also change the activity or even the direct content of known TGF-β-binding proteins such as the latent TGF-β-binding proteins, fibrillin, and fibronectins (50, 51), therefore creating a more favorable environment for TGF-β action. Thus, while we did not observe a direct effect of CTGF on cardiac remodeling or TGF-β responsiveness in the

![FIG 8](image_url)

**FIG 8** Ctgf gene deletion mildly alters TGF-β effects during cardiac stress. (A) Echocardiographic assessment of fractional shortening (FS) in mice of the indicated genotypes after 1 week (TAC-1w) and 5 weeks (TAC-5w) of TAC or sham surgery. (B and C) Ratios of ventricular weight to body weight (VW/BW) (B) and lung weight to body weight (LW/BW) (C) in mice of the indicated genotypes and with the indicated treatments. (D) Hydroxyproline biochemical assay for fibrosis in hearts of mice of the indicated genotypes and with the indicated treatments. (E) ELISA for TGF-β activity in hearts of mice of the indicated genotypes after 1 week of TAC. For each experiment, the number of mice is given within the graph. *, *P < 0.05 versus sham-treated Ctgffl/fl tTA mice; #, *P < 0.05 versus TAC Ctgffl/fl tTA mice.

![FIG 9](image_url)

**FIG 9** Deletion of Ctgf from the heart does not affect Ang/PE infusion-induced disease with TGF-β overexpression. (A) Ratios of ventricular weight to body weight (VW/BW) in mice of the indicated genotypes after 2 weeks of treatment with Ang/PE or the vehicle control. (B) Ratios of lung weight to body weight (LW/BW) in mice of the indicated genotypes and with the indicated treatments. (C) Hydroxyproline biochemical assay for fibrosis in hearts of mice of the indicated genotypes and with the indicated treatments. For each experiment, the number of mice is given within the graph. *, *P < 0.05 versus vehicle.
heart, the context of other ECM proteins or the manner in which latent TGF-β is “stored” in the ECM and activated by stretch could differ greatly from that in other tissues. However, in the myocardium, CTGF appears to be only a minor effector which latent TGF-

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We declare that we have no conflicts of interest.

references


