Genetic analysis of CTGF as an effector of TGFβ signaling and cardiac remodeling

Federica Accornero, a Jop H. van Berlo, a Robert N. Correll, a John W. Elrod, b Michelle A. Sargent, a Allen York, a Joseph E. Rabinowitz, b Andrew Leask, c Jeffery D. Molkentin a, d

a Department of Pediatrics, University of Cincinnati, Cincinnati Children’s Hospital Medical Center, Cincinnati OH USA
b Temple University School of Medicine, Philadelphia, Pennsylvania, USA
c Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada.
d Howard Hughes Medical Institute.

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Correspondence: Jeffery D. Molkentin, email: jeff.molkentin@cchmc.org

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Non-standard abbreviations: AAV, adeno-associated virus; CTGF, connective tissue growth factor; DTG, double transgenic; Dox, doxycycline; ECM, extracellular matrix; fl, LoxP site; MHC, myosin heavy chain; TAC, transverse aortic constriction; TG, transgenic; TGFβ, transforming growth factor-β; TGFβ1 cys223,225ser transgenic, TGFβ TG; tTA, tetracycline transactivator; WT, wild-type.

Running title: Role of CTGF in cardiac remodeling
The matricellular secreted protein, connective tissue growth factor (CTGF), is upregulated in response to cardiac injury or with transforming growth factor β (TGFβ) stimulation, where it has been suggested to function as a fibrotic effector. Here we generated transgenic mice with inducible cardiac-specific CTGF overexpression, mice with cardiac-specific expression of an activated TGFβ mutant protein, mice with cardiac-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 TGFβ over activation 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 TGFβ overexpression. However, CTGF did mildly alter the overall cardiac response to TGFβ when pressure overload stimulation was applied. CTGF has been proposed to function as a critical TGFβ 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.
INTRODUCTION

Heart failure is associated with structural alterations of the ventricles that includes 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 (TGFβ) (4). TGFβ functions as a profibrotic cytokine as well as a growth factor involved in multiple pathophysiological processes (5). In the heart, TGFβ is largely thought to serve a maladaptive role leading to cardiac fibrosis (6). Inhibition of TGFβ signaling specifically in cardiomyocytes protected the heart under stress (7). However, global inhibition of TGFβ with neutralizing antibodies failed in suppressing cardiac pathology and even worsened aspects of ventricular remodeling after stress, attesting to the complexity of TGFβ biology (7-9). Connective tissue growth factor (CTGF, also known as CCN2) is a well characterized downstream mediator of TGFβ action in connective tissue cells during the fibrotic response (10, 11), although 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 TGFβ, 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 expression of a constitutively active mutant form of TGFβ1 carrying a switch in cysteine 223 and 225 to serine (TGFβ1cys223,225ser) in cardiomyocytes (19). These 2 mutations allow TGFβ to escape latency and to be constitutively active. TGFβ1cys223,225ser mice were then crossed with cardiac-restricted α-MHC transgenic (TG) mice expressing the tetracycline transactivator...
(tTA) protein (all in the FVBN background) to generate a Dox regulated TG system. Cardiac-specific CTGF overexpressing mice were generated using the same tetracycline-responsive binary α-myosin heavy chain (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\( \beta 1\) expression but removed thereafter, resulting in 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 cre recombinase under the control of the cardiac-specific β-MHC promoter to obtain heart-restricted deletion of Ctgf. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center.

AAV production and infusion in mice. Adeno-associated virus serotypes 2, 4, 5, 6, 7, 8 and 9 that express cre recombinase were produced separately in HEK293 cells as previously reported (21), 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 \(10^{12}\) virions was injected via the tail vein of mice with this mixed serotype cocktail of AAV, 10 days prior to induction of pressure overload stimulation. The control AAV encoded green fluorescent protein and it was infused at the same titer.

Echocardiography, pressure overload procedure, invasive hemodynamics and agonist infusion. All mice were anesthetized with 2% Isofluorane by inhalation. Echocardiography was performed in M-mode using a Hewlett Packard 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 7-0 silk ligature was tied around the aorta and a 27-gauge wire (which was removed) 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 the groups. Invasive hemodynamics was performed using the closed-chest approach by cannulating the right carotid artery with a Millar pressure transducing catheter placed through the aorta and into the left ventricle (Millar Inc). Recordings were made using a Millar MPVS-400 integrated system with AD-Instruments Powerlab technology that was further analyzed using Labchart software (Labchart Inc). Infusion of angiotensin II (432 \( \mu g/kg/d \)) and phenylephrine (100 mg/kg/d) was...
performed with implantation of Alzet minipumps for 2 weeks (Durect Inc).

Cardiomyocyte and fibroblast isolation and histological analysis. Adult cardiomyocytes were isolated as previously published (23). Briefly, whole hearts were mounted and perfused with Tyrode solution and gassed with 95% O₂ and 5% CO₂. Hearts were then perfused with buffer containing 1 mg/ml collagenase type II (Worthington) and 0.08 mg/ml protease type XIV (Sigma-Aldrich) at 37°C (10–14 minutes total). After perfusion, the ventricles were disassociated into individual myocytes. Following cardiomyocyte sedimentation the remaining suspension containing non-myocyte cells was plated in DMEM medium enriched with 10% bovine serum to obtain adherence and growth of cardiac fibroblasts. Masson’s trichrome staining for fibrosis (blue) was performed from histological sections generated from paraffin-embedded hearts. Immunohistochemistry was performed on cryosections using standard protocols. TRITC-conjugated wheat germ agglutinin (Sigma-Aldrich; L-5266) was used to outline cardiomyocytes. CTGF was visualized using antibodies from Santa Cruz Biotechnology (SC-14939).

Western blotting, ELISA, hydroxyproline assays and mRNA expression analysis. Standard western blot analysis was performed from isolated cardiomyocytes, fibroblasts or mouse heart homogenates. Antibodies used were CTGF (Santa Cruz Biotechnology; sc-14939), phospho-SMAD2 (Cell Signaling Technology; 31015), SMAD2 (Cell Signaling Technology; S3395) and GAPDH (Fitzgerald Industries; R-G109a). Densitometry of the western blots was performed using ImageJ software (NIH). TGFβ ELISA was performed on heart homogenates using a kit purchased from R&D Systems (SMB100B). Hydroxyproline content to assay for fibrosis was performed as previously described (24). Briefly, tissues were hydrolyzed in 6N HCl. Following neutralization, samples were vacuum-dried and resuspended in 5 mM HCl. After addition of chloramine T solution, sample absorbances were read at 558 nm. RNA was extracted from ventricles using the RNeasy kit according to manufacturer’s instructions (Qiagen). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Selected gene expression differences were analyzed by real-time qPCR using SYBR green (Applied Biosystems). Quantified mRNA expression was normalized to Rpl7 and expressed relative to tTA controls. Primers used were: Postn (5’-TGTATCAAGTGCTATCTGGG-3’; 5’-AATGCCCAGCGTGCCATAA-3’), Col1a1 (5’-GCTCTCTTAGGGGCCAT-3’; 5’-ATTGGGGACCCTTAGGCCAT-3’) and Rpl7 (5’-TGAAACCAGGAGGCTGTT-3’; 5’-CACAGCGGGAACCTTTTTC-3’).
Statistics. All results are presented as mean ± SEM. Statistical analysis was performed with unpaired 2-tailed t-test (for 2 groups) and 1-way ANOVA with Bonferroni correction (for groups of 3 or more). P values less than 0.05 were considered significant.

RESULTS

Generation of cardiac-specific inducible TGFβ transgenic mice

To investigate the role of CTGF as a downstream mediator of TGFβ action in the heart we first generated TG mice with enhanced TGFβ activity in the adult heart. To generate a form of TGFβ that would escape latency we used the previously characterized TGFβ1 cDNA in which Cys223 and Cys225 were mutated to serine (TGFβ1cys223,225ser) (25). This cDNA was conditionally expressed in the heart using the Dox-regulated bi-TG system in which a modified α-myosin heavy chain (α-MHC) promoter serves as the responder transgene to drive TGFβ1cys223,225ser expression when crossed with TG mice containing the α-MHC promoter-driven tetracycline transactivator (tTA) protein. All mice were bred on Dox to suppress transgene expression during embryonic and post-natal development and thereafter Dox was then removed at weaning (age of 4 weeks) to subsequently induce protein expression. TGFβ1cys223,225ser TG (TGFβ TG) mice showed increased levels of active TGFβ in the heart by 2-months of age (4 weeks after Dox removal) by 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 induction of endogenous TGFβ in the heart after 6 weeks of pressure overload stimulation with TAC (Fig. 1A). The ELISA assay detects TGFβ that is released from the latent complexes and therefore is bioavailable. TGFβ 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, C, D and E). Gravimetric analysis revealed cardiac hypertrophy and lung congestion in these inducible TGFβ TG mice at 6-months of age (Fig. 1F and G).

TGFβ TG mice showed increased left ventricular chamber dimension at 6-months of age and thickening of the ventricular walls (Fig. 1H and I). However, TGFβ 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 (+dP/dt) 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 Ctgf gene-targeted mice

In agreement with past data that TGFβ is a potent inducer of CTGF expression, we observed induction of CTGF expression in the hearts of TGfβ TG mice by western blotting and immunohistochemistry (Fig. 2A and B). Similarly, pressure overload stimulation (induced by TAC) in tTA control TG mice, which is known to induce TGFβ, also induced endogenous CTGF expression in the heart that was localized predominantly to the ECM (Fig. 2B and C). Generating 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 Ctgffl/fl mice with mice expressing cre recombinase under the control of the cardiac-specific β-MHC promoter (Ctgffl/fl+βcre), which showed a greater than 80% loss of total CTGF protein that is normally induced within the entire heart after TAC stimulation (Figure 2E). Hence, the myocytes appear to be the primary source of CTGF production in the diseased heart, although 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 Ctgf myocyte specific-deleted mice 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 that had an equal response (Fig. 2F, G, H). These results indicate that although strongly induced in the stressed heart, deletion of Ctgf from myocytes appears to have no discernable affect 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 deletion was only achieved 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 βMHC-cre transgene and infusion of an AAV-cre, followed by TAC stimulation (Fig. 3A). Infusion of AAV-cre (see methods) would promote deletion of the Ctgf gene in the non-myocytes 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 approximate 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 compared with just the βMHC-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, D, E, F, G, H, I and J). Indeed, the combined deletion strategy did not alter cardiac structure or function (Figure 3C, D and F), fibrosis (Fig. 3G and H) or expression of fibrotic markers (Figure 3I and J). Thus, loss of CTGF from the heart at baseline or after TAC stimulation, by deletion in either myocytes alone or in myocytes with fibroblasts, did not significantly alter cardiac compensation after TAC stimulation.

CTGF does not synergize with TGFβ

CTGF is reported to synergize with TGFβ 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 TGFβ in the heart, we crossed heart-specific and inducible CTGF TG mice with TGFβ TG mice to generate double TG mice (DTG). We confirmed CTGF overexpression and greater TGFβ activity in both CTGF TG mice, as well as in the DTG mice by western blotting, using the ELISA activity assay and immunostaining (Fig. 4A, B, and C). However, overexpressing CTGF in the heart did not increase TGFβ activity nor did it modify TGFβ activation when both TGFβ 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 similar range to levels of CTGF induction after TAC stimulation (Fig. 5A and B). Downstream TGFβ 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, D, E and F). The phenotype induced by TGFβ activation in the heart was also unaltered by CTGF overexpression, directly demonstrating that these factors do not synergize in this tissue (Figure 4D, E, F, G, H and I). For example, survival of TGFβ TG and DTG mice was similar with aging (Fig. 4D) and cardiac fibrosis was not significantly increased as measured with Masson’s trichrome histology and hydroxyproline content (Fig. 4E, F and G). The propensity towards heart failure as assessed by lung weight and cardiac hypertrophy were also unchanged in DTG compared to TGFβ TG mice (Fig. 4H and I).

To evaluate if a phenotypic interaction between CTGF and TGFβ could be revealed during cardiac stress, we also subjected these mice to 1 week of pressure overload stimulation. Simultaneous overexpression of CTGF and TGFβ resulted in a response to pressure overload (TAC) that was similar to overexpression of TGFβ alone (Fig. 4J, K and L). Specifically, we observed a similarly exacerbated fibrotic response to TAC in TGFβ TG and DTG mice (Fig. 4J) and a similar degree 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 versus either the CTGF TG or the TGFβ TG mice separately, when analyzed for changes in cardiac hypertrophy, lung congestion or extent of fibrosis (Fig. 5G, H and 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 response to these agonists in DTG mice versus either the TGFβ TG or CTGF TG mice (Fig. 6A, B and C). 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, E, F, G, H, I, J, K and L). These results suggest that CTGF does not activate, or synergize with TGFβ 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 TGFβ-driven cardiac pathology

To assess if CTGF acts as a downstream effector of TGFβ in the heart, we crossed TGFβ overexpressing mice to cardiac-restricted Ctgf gene-deleted mice (Ctgffl/fl-βcre TGFβ TG). CTGF protein was again successfully deleted by the β-MHC-cre transgene in Ctgffl/fl-βcre TGFβ TG mice as assessed by western blotting (Fig. 7A). Deletion of Ctgf did not affect TGFβ activation at baseline or otherwise affect the activity of the TGFβ mutant protein (Fig. 7B). Downstream TGFβ signaling was also equally activated in TGFβ TG mice with or 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 TGFβ TG mice compared with controls (Fig. 7C). Analysis of cardiac fibrosis showed no differences between Ctgffl/fl TGFβ TG controls and Ctgffl/fl-βcre TGFβ TG mice by both Masson’s trichrome staining and hydroxyproline biochemical assays (Fig. 7D, E and F). The cardiac hypertrophic response and lung edema propensity was also not altered in TGFβ 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). That Ctgffl/fl TGFβ TG (FVBN / C57BL/6 hybrid) did not show some degree of cardiac hypertrophy as observed in TGFβ TG mice (Fig. 1F) can be attributed to the different murine background, as the later mice were purely FVBN. Regardless of the genetic background issues, the data uniformly show that CTGF appears to play no substantial role in mediating TGFβ actions in the heart at baseline.

Ctgf deletion with TAC alters TGFβ-mediated cardiac remodeling
Since Ctgf deletion appeared to be ineffective in influencing the cardiac response to TGFβ or pressure overload, we went one step further and combined both stimuli. Here we subjected heart-restricted Ctgf-deleted mice with the activated TGFβ transgene to pressure overload stimulation. Analysis of cardiac function by echocardiography revealed increased fractional shortening in Ctgf<sup>fl/fl</sup> TGFβ TG mice after 1-week of pressure overload, which was absent in Ctgf<sup>fl/fl-βcre</sup> TGFβ TG mice (Fig. 8A). After 5-weeks of pressure overload Ctgf<sup>fl/fl</sup> TGFβ TG maintained cardiac function while this compensated phenotype was lost in Ctgf<sup>fl/fl-βcre</sup> TGFβ TG, similar to the control groups (Fig. 8A). Also, overexpression of TGFβ conferred some protection against cardiac hypertrophy at both 1- and 5-weeks of pressure overload that was not observed in Ctgf-deleted mice with the TGFβ transgene (Fig. 8B). Analysis of pulmonary congestion showed increases in lung weights in only Ctgf<sup>fl/fl-βcre</sup> TGFβ TG mice at the 1-week time point, but by 5-weeks of pressure overload both Ctgf<sup>fl/fl</sup> TGFβ TG and Ctgf<sup>fl/fl-βcre</sup> TGFβ TG mice showed a similar increase (Fig. 8C). Quantification of cardiac fibrosis revealed that deletion of Ctgf blunted the exacerbated induction of fibrosis due to the TGFβ transgene after 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 TGFβ in these pressure overloaded hearts was not affected by the absence of CTGF protein, suggesting a minimal role for CTGF as an effector of TGFβ activation or expression (Fig. 8E). Similar to our earlier observations shown in Figure 2F-H, deletion of Ctgf by itself (Ctgf<sup>fl/fl-βcre</sup> 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 minipumps did not significantly alter the cardiac phenotype compared with TGFβ TG mice alone (Fig 9A, B and C).

Taken together the results presented here demonstrate that CTGF can have a very subtle modulatory effect on TGFβ 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 TGFβ 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 that each 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 TGFβ induction, both gene
expression and activation of latent protein within the ECM (33). TGFβ 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 TGFβ activation and CTGF expression, in vivo assessment of CTGF as a
mediator of TGFβ-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 TGFβ in the heart, nor is CTGF otherwise involved in cardiac remodeling, hypertrophy or
fibrosis at baseline, with aging, after pressure overload, with TGFβ stimulation or neuroendocrine
agonist stimulation. Our observations are in agreement with previous studies showing that
overexpression of CTGF in the heart had either a subtle effect, or no effect whatsoever in 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 this later report, CTGF was
previously shown to actually induce hypertrophy in neonatal cultured cardiomyocytes, not protect from
it (39). More perplexing, CTGF TG mice showed either cardiac dysfunction by 7 months of age with no
protection from I/R injury (36), or accelerated deterioration of cardiac function with pressure overload
stimulation (40). These later 2 studies are in dramatic contrast to the reported protective effects with
CTGF overexpression by Gravning et al. (37, 38). Thus, these 4 papers in the literature with CTGF
overexpression in the heart, spanning 3 independently generated mouse models, reached dramatically
different conclusions, with one group claiming CTGF is protective to the heart and the other 2 groups
claiming 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 pro-hypertrophic 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 we have largely disparate data from Gravning et al. One possibility is that different spliced forms of the CTGF cDNAs were used between the 4 groups, or that the absolute levels of CTGF overexpression varied dramatically. Another important variable is that the CTGF overexpressing model 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 other 3 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.

Cardiac-restricted deletion of Ctgf also did not modulate the cardiac pathologic response to TGFβ. Inducible activation of TGFβ in the heart results in dramatic ventricular remodeling and fibrosis, and subsequent death of the mice by 6-9 months of age. Loss of Ctgf from the heart did not affect TGFβ-dependent lethality, cardiac remodeling or fibrosis, suggesting that Ctgf is not an important effector of TGFβ in this tissue. We also used a mixed serotype AAV-cre infusion approach to further delete Ctgf from the non-myocytes of the heart, especially the fibroblasts, which indeed produced 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 might fully compensate. 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 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 towards heart failure, remodeling or cardiac fibrosis after short-term or long-term TAC stimulation, nor after angiotensin II / phenylephrine infusion. These observations are in contrast to a recent report showing that systemic administration of a 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 TGFβ-driven pathology, when we instituted a more contrived situation in which TGFβ overexpression was superimposed with pressure overload stimulation, 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 TGFβ-stimulation of myofibroblast differentiation and subsequent collagen matrix contraction in vitro (43). Another intriguing result was that increasing TGFβ activity in the heart with the transgene lead to a reduced hypertrophic response and preservation of heart function after pressure overload, and these effects were at least partially abolished when Ctgf was deleted. Although the protection conferred by TGFβ activation during pressure overload stimulation might appear counterintuitive, especially since these mice eventually die by 9 months of age, previous reports have suggested that treatment of mice with a neutralizing antibody against TGFβ adversely affected cardiac remodeling following pressure overload (7-9), suggesting that activation of TGFβ 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 has been previously implicated as a secreted growth factor, where it can function when applied directly to the media 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 induction of CTGF in the heart by pressure overload stimulation or due to the TGFβ 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 thrombospondin (TSP) type-1 domain that could have an intracellular function in chaperoning ECM proteins as they are being secreted, not unlike what was proposed for thrombospondin proteins and COMP (48, 49), that also contain TSP functional domains. Thus, CTGF could function as a secondary effector of TGFβ responsiveness by altering the content of other ECM proteins that are deposited after an injury response, which could further affect how TGFβ 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 TGFβ binding proteins such as the latent TGFβ binding proteins, fibrillins and fibronectins (50, 51), therefore creating a more favorable environment for TGFβ action. Thus, while we did not observe a direct effect of CTGF on cardiac remodeling or TGFβ responsiveness in the heart, the context of other ECM proteins, or the manner in which latent TGFβ is “stored” in the ECM and activated by stretch could differ greatly from other tissues. However, in the
myocardium, CTGF appears to be only a minor effector of pathogenesis following disease stimulation.

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FIGURE LEGENDS

Figure 1 – Generation of a murine model with inducible TGFβ expression in the heart. (A) ELISA for TGFβ activity in hearts from tTA control and TGFβ TG mice at 2- and 6-months (2mo and 6mo) of age versus the induction of endogenous TGFβ 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 TGFβ TG mouse hearts at 6-months of age. (C) Survival rate of tTA control and TGFβ TG mice in the first 12 months of life. (D, E) Hydroxyproline biochemical assay for fibrosis in tTA control and TGFβ TG mouse ventricles and atria at 6-months of age. (F) Lung weight to body weight ratio (LW/BW) and (G) ventricular weight to body weight ratio (VW/BW) from tTA control and TGFβ TG mice at 6-months of age. (H, I, J) Echocardiographic analysis of left ventricle end-diastolic dimension (LVEDD), left ventricular posterior wall thickness in diastole (LVPWd) and fractional shortening (FS%) in tTA control and TGFβ TG mice at 2-, 4- and 6-months of age. (K, L) Invasive hemodynamic assessment of cardiac contractility (+dP/dt) and left ventricular pressure in diastole in tTA control and TGFβ TG mice. For each experiment, the number of mice is given within the graph. *P<0.05 versus tTA control. Statistical test for panels H, I and J was a 2-way ANOVA.

Figure 2 – Analysis of Ctgf heart-specific gene-deleted mice. (A) Western blot analysis of CTGF and GAPDH from tTA and TGFβ TG heart homogenates from 6 month-old mice. (B) Immunofluorescence staining for CTGF on the top row (green) and with wheat germ agglutinin (WGA)-TRITC (red) on the bottom row in heart histological sections from TGFβ TG, tTA control and tTA mice subjected to TAC. (C, D) Western blot analysis for CTGF and GAPDH from (C) heart homogenates and (D) isolated cardiomyocytes of tTA mice subjected to 1 week of TAC or sham surgery. (E) Western blot analysis of CTGF and GAPDH from hearts of Ctgf^{fl/fl} and Ctgf^{fl/fl}+βMHC-cre adult mice after 2 weeks of TAC. (F) Echocardiographic assessment of fractional shortening (FS%) in Ctgf^{fl/fl} and Ctgf^{fl/fl}+βMHC-cre mice after 2 and 8 weeks of TAC. (G) Ventricular weight to body weight ratio (VW/BW) and (H) lung weight to body weight ratio (LW/BW) in Ctgf^{fl/fl} and Ctgf^{fl/fl}+βMHC-cre mice after 2 and 8 weeks of TAC. The number of mice used is shown within the bars in each graph. *P<0.05 versus sham control.
Figure 3 – Analysis of myocyte and nonmyocyte Ctgf-deleted mice. (A) Diagram of the experimental approach in which Ctgf^{fl/fl}+βMHC-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 Ctgf^{fl/fl}+βMHC-cre mice infused previously with control (Ctrl.) AAV or AAV-cre, both after 2 weeks of TAC stimulation. Ctgf^{fl/fl} mice were used as a control without TAC stimulation. (C, D) Ventricular weights and lung weights normalized to body weights in the groups of mice shown. (E, F) Echocardiographic assessment of fractional shortening (FS%) and left ventricular chamber dimension in diastole (LVED) in the groups of mice shown. (G, H) Histological assessment of fibrosis by Masson’s trichrome and hydroxyproline content from hearts of the indicated groups of mice. (I, J) mRNA analysis for Col1a1 and Postn 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 Ctgf^{fl/fl} mice without TAC.

Figure 4 – CTGF does not synergize with TGFβ in the heart. (A) Western blot analysis of CTGF, phospho-SMAD2, total SMAD2 and GAPDH from heart homogenates of the indicated genotypes of TG mice. (B) ELISA for TGFβ activity in hearts from tTA control, CTGF TG, TGFβ TG and CTGF/TGFβ double transgenic (DTG) mice at 2-months of age. (C) Immunohistochemistry for CTGF protein (green) localization from 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, G) Hydroxyproline biochemical assay for fibrosis in cardiac ventricle and atria of the indicated genotypes of mice at 6-months of age. (H) Lung weight to body weight ratio (LW/BW) and (I) ventricular weight to body weight ratio (VW/BW) from mice of the indicated genotypes at 6-months of age. *P<0.05 versus tTA control. (J) Hydroxyproline biochemical assay for fibrosis in mouse hearts of the indicated genotypes subjected to sham or 1 week of TAC. (K) LW/BW and (L) VW/BW from mice of the indicated genotypes subjected to sham 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.

Figure 5 – TGFβ 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 the indicated genotypes of mice and treatments. tTA TG is a control only expressing 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 TGFβ TG; #P<0.05 versus tTA TAC. (C, D, E, F) mRNA expression levels for Col1a1 and Postn in the indicated groups of mice at baseline (C,D) or with 1 week of TAC stimulation (E,F). (G) Ventricular weight to body weight ratio (VW/BW) in mice of the indicated genotypes after 6 weeks of TAC or sham surgery. (H) Lung weight to body weight ratio (LW/BW) in mice of the indicated genotypes and treatments. (I) Hydroxyproline biochemical assay for fibrosis in mouse hearts of the indicated genotypes and treatments. For each experiment, number of mice is given within the graph. For C-I: *P<0.05 versus sham; #P<0.05 versus tTA TAC.

Figure 6 – CTGF overexpression, with or without TGFβ, does not predispose to greater cardiac disease with Ang/PE infusion. (A) Ventricular weight to body weight ratio (VW/BW) in mice of the indicated genotypes after 2 weeks of Ang/PE infusion or vehicle infusion. (B) Lung weight to body weight ratio (LW/BW) in mice of the indicated genotypes and treatments. (C) Hydroxyproline biochemical assay for fibrosis in mouse hearts of the indicated genotypes and treatments. For each experiment, number of mice is given within the graph. *P<0.05 versus vehicle.

Figure 7 – CTGF expression has essentially no effect on TGFβ-driven cardiac pathology. (A) Western blot analysis of CTGF, phospho-SMAD2, total SMAD2 and GAPDH from heart homogenates of Ctgffl/fl tTA, Ctgffl/fl TGFβ TG and Ctgffl/fl-βcre TGFβ TG mice. (B) ELISA for TGFβ activity in hearts from the indicated genotypes at 6-months of age. (C) Survival rate of mice of the indicated genotypes in the first 12 months of life. (D) Representative Masson’s trichrome-stained cardiac histological sections for fibrosis in mouse hearts of the indicated genotypes at 6-months of age. (E, F) Hydroxyproline biochemical assay for fibrosis in mouse ventricle and atrium of the indicated genotypes at 6-months of age. (G) Ventricular weight to body weight ratio (VW/BW) and (H) lung weight to body weight ratio (LW/BW) from mice of the indicated genotypes at 6-months of age. (I, 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 Ctgffl/fl tTA control.

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

Figure 9 – Deletion of Ctgf from the heart does not affect Ang/PE infusion-induced disease with TGFβ overexpression. (A) Ventricular weight to body weight ratio (VW/BW) in mice of the indicated genotypes after 2 weeks of Ang/PE treatment or vehicle control. (B) Lung weight to body weight ratio (LW/BW) in mice of the indicated genotypes and treatments. (C) Hydroxyproline biochemical assay for fibrosis in mouse hearts of the indicated genotypes and treatments. For each experiment, number of mice is given within the graph. *P<0.05 versus vehicle.
Fig 6

A

VW/BW (mg/g)

Vehicle  Ang/PE

3  3  4  4  3

B

LW/BW (mg/g)

Vehicle  Ang/PE

3  3  4  4  3

C

Hydroxyproline (µg/mg protein)

Vehicle  Ang/PE

3  3  4  4  3

Legend:

- tTA
- CTGF TG
- TGFβ TG
- DTG

* Indicates significant difference.