Reduction in Endoglin Activity Limits Cardiac Fibrosis and Improves Survival in Heart Failure

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Abstract

Background—Heart failure is a major cause of morbidity and mortality worldwide. The ubiquitously expressed cytokine, transforming growth factor beta-1 (TGFβ1), promotes cardiac fibrosis, an important component of progressive heart failure. Membrane-associated endoglin is a co-receptor for TGFβ1 signaling and has been studied in vascular remodeling and preeclampsia. We hypothesized that reduced endoglin expression may limit cardiac fibrosis in heart failure.

Methods and Results—We first report that endoglin expression is increased in the left ventricle (LV) of human subjects with heart failure and determined that endoglin is required for TGFβ1 signaling and has been studied in vascular remodeling and preeclampsia. We hypothesized that reduced endoglin expression may limit cardiac fibrosis in heart failure.

We further identified that reduced endoglin expression attenuates cardiac fibrosis, preserves LV function, and improves survival in a mouse model of pressure-overload induced heart failure. Prior studies have shown that the extracellular domain of endoglin can be cleaved and released into the circulation as soluble endoglin (sEng), which disrupts TGFβ1 signaling in endothelium. We now demonstrate that sEng limits TGFβ1 signaling and Type I collagen synthesis in cardiac fibroblasts and further show that sEng treatment attenuates cardiac fibrosis in an in vivo model of heart failure.
Conclusions—Our results identify endoglin as a critical component of TGFβ1 signaling in the cardiac fibroblast and that targeting endoglin attenuates cardiac fibrosis, thereby providing a potentially novel therapeutic approach for individuals with heart failure.

Keywords
cardiac remodeling; fibrosis; heart failure

Heart failure is a major cause of morbidity and mortality that affects over 24 million individuals worldwide. Irrespective of the injurious mechanism, a decline in left ventricular (LV) function increases LV pressure and activates several signaling cascades that promote cardiomyocyte hypertrophy and cardiac fibrosis, a process known as cardiac remodeling. At each phase of cardiac remodeling from acute load to compensatory hypertrophy, various signaling cascades are implicated. Among these, transforming growth factor beta 1 (TGFβ1) is a pro-fibrogenic cytokine that contributes to multiple fibro-proliferative disorders including cardiac fibrosis associated with heart failure. In response to angiotensin-II (Ang-II), TGFβ1 expression is increased, converts fibroblasts into myofibroblasts, and generates extracellular matrix (ECM) proteins, such as Type I collagen. Excess collagen deposition exaggerates mechanical stiffness of the LV, impairs myocyte contractility, disrupts electrical coupling, and worsens tissue hypoxia (4), thereby promoting heart failure. Given its central role in stimulating fibrosis, TGFβ1 has been non-selectively targeted in heart failure models, using multiple approaches; none of which have produced clearly beneficial therapeutic effects.

TGFβ1 signals through a heteromeric receptor complex comprised of a Type II ligand-binding receptor in association with a Type I activin like kinase (ALK) signaling receptor. Once activated by TGFβ1, this receptor complex triggers phosphorylation of downstream effector proteins known as Smads (canonical pathway) or mitogen-activated protein kinases (non-canonical pathway). Specifically, TGFβ1-induced phosphorylation of Smad-2/3 promotes Type I collagen synthesis and fibrosis. Endoglin (Eng; CD105) is a 180-kDa homodimeric glycoprotein that serves as a co-receptor for TGFβ1 signaling. Over the past two decades, several lines of evidence suggest that endoglin plays a critical role in vascular remodeling. First, loss of function mutations in human endoglin result in the autosomal dominant vascular dysplastic syndrome, hereditary hemorrhagic telangiectasia type 1 (HHT1) characterized by endoglin haploinsufficiency and visceral arteriovenous malformations (AVMs). Second, endoglin null mice die at embryonic day 10.5 due to impaired cardiovascular development and extra-embryonic angiogenesis. However, endoglin heterozygous mice (Eng<sup>+/−</sup>) are viable, have reduced levels of endoglin, and a phenotype that recapitulates that of HHT1. The role of endoglin as a modulator of TGFβ1 signaling in heart failure, where fibrosis plays a major role, has not been explored to date.

The extracellular domain of endoglin can be proteolytically cleaved by MMP14 and circulates as soluble endoglin (sEng). We recently demonstrated that levels of sEng correlate with clinical measures of heart failure including LV end-diastolic pressure (LVEDP) and New York Heart Association Classification. Based on these observations, we hypothesized that impaired function of the TGFβ1 co-receptor, endoglin, limits TGFβ1-
induced collagen synthesis and cardiac fibrosis, thereby identifying endoglin as a potentially novel therapeutic target in heart failure. To explore this hypothesis we employed a model of pressure overload-induced heart failure in Eng\textsuperscript{+/−} mice.

**Methods**

**Reagents**

We purchased recombinant human sEng (RhsEng: 1-587 amino acids corresponding to the extracellular domain of endoglin; R&D Systems) and recombinant human TGFβ1 (Sigma). Mouse monoclonal antibodies to human endoglin (SC-73934), human Type I collagen (SC-80497), human DDR-2 (SC-81707), and fibroblast marker (ER-TR7) were purchased from Santa Cruz. A polyclonal antibody to the N-terminal region of human endoglin (SC-19790) was purchased from Santa Cruz. Goat polyclonal antibodies against mouse endoglin and Type I collagen were purchased from R&D Systems (BAF1320) and Santa Cruz (SC-25974) respectively. Polyclonal antibodies to human and mouse pSmad-2/3 (AB-3849), pSmad-1 (#06-702), and pERK-1/2 (05-797) were purchased from Millipore; polyclonal antibodies to human and mouse total Smad-2/3 (#3102), total Smad-1 (#9743), and total ERK (#9102) were purchased from Cell Signaling. Rabbit polyclonal antibodies to mouse calcineurin were purchased from Cell Signaling. A rat monoclonal antibody to mouse CD31 was purchased from Pharmingen (#01951A). Enzyme linked immunosorbent assay (ELISA) kits for human and mouse sEng and TGFβ1 were purchased from R&D Systems.

**Human LV tissue sampling**

Viable LV free wall tissue was obtained from human subjects with heart failure (n=20) referred for left ventricular assist device (LVAD) placement (HeartMate, Thoratec Corp, Pleasanton, CA). In seven subjects, an additional LV sample was obtained after LVAD support at the time of cardiac transplantation. Control LV tissue was obtained from the National Disease Research Interchange (NDRI). All tissue was immediately frozen in liquid nitrogen and stored at −80°C until further processing as described below. All surgical procedures and tissue harvesting were performed in concordance with the National Institutes of Health and Tufts University Institutional Review Board guidelines.

**Mouse model of pressure-overload induced heart failure**

Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science), and protocols were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. Adult, male, 14-16 week old C57BL/6 wild-type (WT) and Eng\textsuperscript{+/−} mice underwent thoracic aortic constriction (TAC) as previously described\textsuperscript{14,15}. At 2, 4, and 10 weeks after TAC, mice were sacrificed and tissue was obtained for further analysis by real-time polymerase chain reaction (RT-PCR), immunoblotting, histology, and ELISA according to the manufacturer’s instructions. Eng\textsuperscript{+/−} mice were generously provided by Dr Michelle Letarte, University of Toronto.

**Physiologic Characterization in vivo**

Transthoracic echocardiography and pressure volume loop analysis was performed on mice as previously described\textsuperscript{14-16}.
TGFβ-1 induced Type I collagen expression in cardiac fibroblasts

The Tufts Medical Center Institutional Review Board (IRB) approved collection of human tissue for cell culture. Human cardiac fibroblasts (hCF) were isolated from myocardial tissue harvested during cardiac surgery at Tufts Medical Center and mouse CF were isolated from WT and Eng<sup>+/−</sup> mice and stimulated with TGFβ1 for analysis as previously described.16,17

Loss of function studies in human cardiac fibroblasts

For neutralizing antibody studies, hCF were pretreated with 0.5 °g/mL of either an antibody to endoglin or control IgG isotype for 24 hours in fibroblast basal medium without supplementation prior to stimulation with TGFβ1 (10 ng/mL). After 24 hours, cells were harvested for RT-PCR and Western blot analysis. For endoglin silencing experiments, 50 °M siRNA stock (Ambion) was diluted to 1.0 nM in Optimem (Invitrogen) and combined with 2 °L Lipofectamine (Invitrogen) diluted in 98 uL Optimem. After 20 minutes incubation, cells were exposed to: i) human endoglin siRNA (Ambion #145527), ii) scrambled siRNA (negative control; Ambion # 4390844) or iii) GAPDH siRNA (positive control; Ambion # 4390850). At varying times between 24-48 hours after transfection, cells were harvested for analysis.

Recombinant soluble endoglin inhibition of type I collagen synthesis in vitro

Serum-starved hCF were treated for 24 hours with RhsEng, then stimulated with TGFβ1 and harvested for further analysis.

Over-expression of human soluble endoglin and full-length endoglin in vitro

For conditioned media studies, COS-1 (American Type Culture Collection) cells were transfected with adenovirus overexpressing human sEng (AdhsEng; generously provided by Dr. S.A. Karumanchi) or adenovirus with no transgene (AdNull) for 24 hours. Human sEng levels in conditioned media were confirmed by ELISA. Conditioned media was then transferred into 12-well dishes containing serum-starved hCF, then stimulated with TGFβ1. For over-expression studies, hCF were transfected with adenovirus expressing full-length endoglin (AdFL-Eng; generously provided by Dr. Calvin P. Vary), then stimulated with TGFβ1 and harvested for analysis.

Soluble Endoglin inhibits pressure overload induced cardiac fibrosis

Adult, male, 14-16 week old C57BL/6 mice received intravenous injections of AdNull or AdhsEng, one day prior to TAC. Serum levels of human and mouse sEng were quantified by ELISA. Four weeks after TAC, mice were sacrificed and tissue obtained for further analysis.

Real-time Quantitative Polymerase Chain Reaction (RT-PCR)

For all cell-based RT-PCR experiments, total RNA was extracted directly using Trizol (Invitrogen), converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For all RT-PCR experiments, samples were quantified in triplicate using 40 cycles performed at 94°C for 30 sec., 60°C for 45 sec, 72°C for 45 sec using an ABI Prism® 7900 Sequence Detection System using appropriate primers (Supplement 5) as described.14-16
Immunoblot Analysis (Western)
Total protein was extracted and quantified from tissue homogenates or cultured cells as described \cite{14-16}. Immunoblot analysis was then performed as previously described using antibodies for human and mouse targeted proteins.

Histologic Quantification of Cardiac Hypertrophy and Fibrosis
LV collagen abundance was quantified by picrosirius red staining as described \cite{18,19}. Cardiomyocyte cross-sectional area and capillary density were quantified as described \cite{14}.

Statistical Analysis
Results are presented as mean ± standard deviation. Intergroup comparisons were made with two-factor ANOVA. Two-way ANOVA was performed to examine the effects of cardiac unloading by a LVAD and time on endoglin expression. Repeated measures ANOVA were used as needed to account for time. All multiple comparisons versus a control group were performed using Dunnett's method. Kaplan-Meier analysis with log-rank testing was employed for survival analysis. All statistical analyses were performed using SigmaStat Version 3.1 (Systat Software, Inc). An alpha level of \(P<0.05\) was considered to indicate a significant effect or between-groups difference.

Results

Left Ventricular Endoglin Expression is Increased in Human Heart Failure
To determine whether endoglin expression is increased in patients with heart failure, LV samples were obtained from individuals with end-stage heart failure referred for surgical implantation of a LVAD. An additional LV sample was obtained in 7 subjects at the time of cardiac transplantation after LVAD support to examine the effect of hemodynamic unloading on endoglin expression. Compared to subjects without heart failure, endoglin expression was increased in the failing LV at the time of LVAD implantation (Figure 1A) and reduced back to control levels after LVAD support (Figure 1B). These findings indicate an association between cardiac pressure overload and LV endoglin expression in heart failure. To determine what cardiac cell types express endoglin, cardiomyocytes, fibroblasts and endothelial cells were next isolated from WT mouse LVs. Endoglin was expressed by cardiac fibroblasts and endothelium, but not by cardiac myocytes (Figure 1C-D).

Increased membrane-associated and circulating endoglin expression in heart failure
To explore the functional role of endoglin in heart failure, we studied \(\text{Eng}^{+/−}\) mice. Compared to WT, LV endoglin expression was lower in \(\text{Eng}^{+/−}\) mice (Figure 2A). We then employed the well-established mouse model of LV pressure overload induced by TAC, followed by tissue characterization at 2, 4, and 10 weeks. In WT mice, compared to sham-operated controls, LV endoglin mRNA was increased within 2 weeks and remained elevated at 4 and 10 weeks after TAC (Figure 2B). LV endoglin protein expression similarly increased after 2 and 4 weeks of heart failure and returned to normal levels by 10 weeks in WT mice (data not shown). No change in endoglin levels was observed in the aorta distal to the site of TAC ligature (Supplemental Figure 1A), suggesting a direct effect of cardiac

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pressure overload on endoglin expression. Serum levels of sEng were also elevated across all time points of pressure-overload induced heart failure (Figure 2C). TAC also increased LV endoglin mRNA (Figure 2B) and circulating sEng levels (Figure 2C) in the Eng+/− mice, but levels were significantly reduced compared to WT mice at each time point.

Reduced endoglin expression preserves LV function and promotes survival in heart failure

We next examined the functional impact of reduced endoglin expression in heart failure. Eng+/− mice demonstrated preserved cardiac function and improved survival (88% versus 50%, respectively, p=0.01) compared to WT mice after TAC (Figures 2D-2F and Table 1). Consistent with these observations, WT mice manifest reduced total body weight at both 4 and 10 weeks after TAC, while Eng+/− mice did not (Table 1). Compared to baseline values, lung weights were increased in both mouse groups after TAC, however lung weights were lower after 10 weeks of TAC in Eng+/− mice compared to WT (Table 1). After 4 weeks of TAC, LV systolic and LVEDP were increased in both WT and Eng+/− mice compared to respective sham-operated controls. While TAC-induced LVSP was higher in Eng+/− mice compared to WT at both 4 and 10 weeks, LVEDP was not different between WT and Eng+/− mice at any time point. LV contractility (dP/dt max) also decreased in a time-dependent manner in WT mice, but remained unchanged across all phases of cardiac pressure overload in Eng+/− mice (Table 1). Echocardiography demonstrated a significant time-dependent TAC-induced reduction in LV fractional shortening (FS%) in WT, but not in Eng+/− mice. After 10 weeks of TAC, WT mice demonstrated greater LV chamber dilatation and lower posterior wall thickness (PWT) compared to Eng+/− mice (Table 1). These findings suggest that despite identical degrees of LV pressure overload, reduced endoglin expression in the Eng+/− mice preserved LV function and improved survival.

Reduced endoglin expression preserves TAC-induced cardiomyocyte hypertrophy and promotes myocardial capillarity

To study the mechanism underlying improved survival in Eng+/− mice, we first examined changes in cardiac hypertrophy. Across all time points, fold-changes in LV mass normalized to tibia length were similar between Eng+/− and WT mice (Table 1). Cardiomyocyte cross-sectional area was also increased to a similar degree in both WT and Eng+/− mice after TAC (Figures 3A-B). Expression of fetal genes and proteins such as β-MHC, SERCa, and calcineurin demonstrated similar patterns in both WT and Eng+/− mice after 4 (Figures 3C-E) and 10 weeks of TAC (Supplemental Figures 2A-B). Next, we observed a significant increase in capillary to cardiomyocyte ratio and total capillary density in both WT and Eng+/− mice after 4 weeks of TAC. Both measures of myocardial capillarity were higher in Eng+/− compared to WT mice after TAC (Figures 3F-H). Taken together, these data support that TAC-induced LV hypertrophy is preserved, while myocardial capillarity is enhanced in Eng+/− mice compared to WT mice.

Reduced endoglin expression attenuates cardiac fibrosis

Compared to controls, TAC induced a time-dependent increase in LV collagen deposition in WT, but not Eng+/− mice (Figures 4A-B). Type I collagen mRNA (Figure 4C) and protein expression (Figure 4D) were similarly increased in WT mice after TAC. Eng+/− mice...
exhibited a small increase in Type I collagen mRNA after 4 (Figure 4C) and 10 weeks (Supplemental Figure 3A), and a modest increase in Type I collagen protein expression after 10 weeks of heart failure (Figure 4D). At all time points, Type I collagen mRNA and protein were lower in Eng^+/− mice compared to WT. Importantly, similar increases in TGFβ1 mRNA (Figure 4E) and active TGFβ1 protein (Supplemental Figure 3B) were observed in the LV of both WT and Eng^+/− mice after 4 weeks of TAC. Levels of the downstream target of TGFβ1 signaling, pSmad-2/3 were increased by 4 and 10 weeks of TAC (Figure 4F and Supplemental Figure 3C) in WT mice. However, despite similar increases in LV TGFβ1 levels in Eng^+/− mice, TAC did not induce a significant increase in pSmad-2/3. Consistent with this impairment of TGFβ1 signaling in Eng^+/− mice, expression of plasminogen activator inhibitor 1 (PAI-1) was increased in WT, but not Eng^+/− mice after 4 weeks of TAC (Supplemental Figure 3D). To confirm that reduced endoglin expression by Eng^+/− mice limits TGFβ1 induced cardiac fibrosis, mouse cardiac fibroblasts (mCF) were isolated from the LV of WT and Eng^+/−. TGFβ1 simulation induced Type I collagen mRNA expression in WT-mCF, but not Eng^+/−-mCF (Supplemental Figure 3E). These data identify that reduced endoglin expression attenuates TGFβ1 signal transduction and limits cardiac fibrosis. These in vivo observations led us to explore the dependence of TGFβ1 profibrotic signaling on endoglin expression in vitro.

**Canonical and Non-canonical signaling in endoglin-deficient mice**

We next studied expression of the TGFβ Type I receptors, ALK1 and ALK5 in human cardiac fibroblasts (hCF) from fresh tissue samples obtained during cardiac surgery. We observed lower ALK1 mRNA expression in hCF compared to human umbilical vein endothelial cells (HUVEC), while ALK5 expression was similar in both cell types (Supplemental Figure 4A). Next, TGFβ1 stimulation increased pSmad-2/3 expression with no change in phosphorylated Smad-1 (pSmad-1) expression (Supplemental Figure 4B). Compared to sham-operated controls, LV pSmad1 expression was increased in WT mice after 4 weeks of TAC, while no change was observed in Eng^+/− mice (Supplemental Figure 4C). Immunostaining confirmed increased nuclear accumulation of pSmad-2/3 and less accumulation of pSmad-1 in WT mice after TAC. In contrast, no significant increase in nuclear accumulation of pSmad-2/3 or pSmad-1 was observed in Eng^+/− mice after TAC (Supplemental Figure 4D-G).

Next, we studied phosphorylation of the TGFβ1 non-canonical signaling effector, extracellular regulated kinase (ERK). Using an siRNA approach, reduced endoglin expression attenuated TGFβ1 induced phosphorylation of ERK-1/2 in hCF in vitro (Supplemental Figure 4H). Consistent with this observation, pERK-1/2 expression was increased in WT mice, but not Eng^+/− mice after 4 weeks of TAC (Supplemental Figure 4I). These findings suggest that reduced endoglin expression limits TGFβ1 signaling via Smad-2/3 and ERK.

**Membrane-associated endoglin is required for TGFβ1-induced Type I collagen synthesis**

To explore this question, the role of endoglin was examined using a loss of function approach in hCF. Compared to an isotype control antibody, pre-treatment of hCF with an antibody to endoglin attenuated TGFβ1-induced Type I Collagen mRNA and protein
expression (Figure 5A). Similarly, silencing endoglin expression significantly reduced TGFβ1-induced Type I Collagen mRNA and protein expression, PAI-1 expression (Figures 5B-C), and connective tissue growth factor (CTGF) in hCF (Supplemental Figure 5A). These findings identified endoglin as a necessary component for TGFβ1 signaling in hCF.

**Soluble endoglin antagonizes TGFβ1 signaling in cardiac fibroblasts**

Previous studies have suggested that sEng attenuates TGFβ1 signaling in endothelium. We next explored whether sEng modulates cardiac fibroblast function. We first treated hCF with RhsEng and observed a dose-dependent decrease in TGFβ1-induced Type I collagen expression (Figures 5D-E). To confirm the role of sEng as a negative modulator of TGFβ1 activity, we transfected COS-1 cells with AdhsEng and confirmed a dose-dependent increase in the level of sEng in conditioned culture media (Supplemental Figure 5B). Similar to treatment with RhsEng, treatment of hCF with conditioned media from AdhsEng-transfected COS-1 cells also inhibited TGFβ1-induced Type I collagen and pSmad-2/3 expression (Figure 5F).

Next, we explored the effect of over-expressing full-length endoglin using an adenoviral mediated approach (AdFL-Eng) in hCF and paradoxically observed a reduction in TGFβ1-induced Type 1 collagen expression (Supplemental Figure 5C). To study this further, we measured increased levels of sEng in hCF transfected with AdFL-Eng (Supplemental Figure 5D). These findings implicated sEng as a negative feedback mechanism that down-regulates TGFβ1 activity in hCF.

**Soluble endoglin attenuates cardiac fibrosis in pressure overload induced heart failure**

To explore whether sEng limits cardiac fibrosis in vivo, WT mice received intravenous injections of AdhsEng, which increased circulating levels of human sEng (Figure 6A). Compared to controls, treatment with AdhsEng significantly reduced cardiac fibrosis (Figure 6B) and LV Type I collagen expression after 4 weeks of TAC (Figure 6C). No significant change in LV contractility (data not shown) was observed during this subacute phase of LV pressure-overload. These findings support that sEng blocks TGFβ1 signaling and limits cardiac fibrosis in pressure overload induced heart failure.

**Discussion**

Our central finding is that endoglin is required for TGFβ1 signaling in hCF and that selectively inhibiting TGFβ1 signaling by reducing endoglin activity attenuates cardiac fibrosis and improves survival in a mouse model of heart failure. In contrast to the functional role of endoglin in promoting TGFβ1 signaling, sEng limits TGFβ1 signaling, Type I collagen synthesis and ultimately cardiac fibrosis (Figure 7).

Our findings have several important clinical implications. First, previous studies of non-selective TGFβ1 blockade have produced mixed results in heart failure. The ability to selectively modulate TGFβ1 activity by limiting the TGFβ1 co-receptor, endoglin, offers a potentially novel approach to managing heart failure. Second, studies involving endoglin have focused on vascular remodeling with minimal data exploring endoglin’s role in heart function.
failure. However, endoglin has been shown to mediate collagen synthesis induced by Ang-II and TGFβ1 in rat cardiac fibroblasts. Our findings confirm that endoglin is an important component of cardiac remodeling in both human LV tissue and cardiac fibroblasts and in a mouse model. Third, recent publications have focused on the role of sEng in preeclampsia, vascular function, and renal fibrosis. We recently identified sEng as a biomarker of heart failure and now describe a functional role for sEng as an autocrine antagonist of TGFβ1 signaling in heart failure. These findings provide new insight into the pathophysiology of heart failure, regulation of TGFβ1 activity in heart failure, and further identify sEng as a potentially novel therapeutic approach to limit cardiac fibrosis in heart failure.

We first identified that cardiac levels of endoglin are increased in subjects with end-stage heart failure referred for mechanical LVAD support and that endoglin levels are reduced by mechanical LV unloading. Previous studies have shown increased cardiac expression of profibrogenic genes including TGFβ1 in patients with end-stage heart failure referred for LVAD support. In both studies, higher levels of TGFβ1 were observed in subjects requiring LVAD support until cardiac transplantation, suggesting that TGFβ1 may limit myocardial recovery by promoting tissue fibrosis. We now identify endoglin as a potentially important target to limit TGFβ1 activity in heart failure.

Next by confirming that endoglin is expressed by cardiac fibroblasts, we studied the effect of reduced endoglin expression on cardiac fibrosis in a murine model of heart failure. We first observed increased endoglin expression in the LV with no change in abdominal aortic expression in WT mice subjected to TAC. Importantly, two isoforms of membrane-associated endoglin exist, namely, the long (L-endoglin) and the less abundant short isoforms (S-endoglin). We confirmed that L-endoglin is the dominantly expressed isoform in the mouse LV (data not shown). Consistent with our human observations, we identified that cardiac pressure overload increases endoglin expression in heart failure. Though TAC induced a similar pattern of endoglin expression in Eng+/− and WT mice, levels of endoglin expression were significantly lower in the Eng+/− mice throughout. Reduced endoglin expression improved survival in this model of pressure overload-induced heart failure, while preserving cardiomyocyte hypertrophy, modestly increasing myocardial capillarity, and significantly reducing cardiac fibrosis. Collectively, these changes were more consistent with adaptive as opposed to maladaptive cardiac remodeling, however the most dramatic observation in this model was the nearly complete attenuation of cardiac fibrosis in the pressure-overloaded myocardium.

We then confirmed the dependence of TGFβ1 signaling on endoglin expression in human cardiac fibroblasts using loss of function approaches. Furthermore, treatment with sEng as either a recombinant protein or by adenoviral over-expression of either sEng or full-length endoglin in vitro mirrored the phenotype of the reduced endoglin levels in the Eng+/− mice, suggesting that sEng also limits TGFβ1 signaling and Type I collagen synthesis. Several prior studies have shown that over-expressing endoglin in rat myoblasts and mouse fibrosarcoma cell lines limits TGFβ1 induced collagen expression. In the context of our findings, these observations may highlight important differences between cell types and species with regards to endoglin’s biologic activity. Furthermore, these reports may be
consistent with our gain of function observations in human cardiac fibroblasts and could suggest that transfecting endoglin into stable cell lines also increases levels of soluble endoglin in vitro, thereby attenuating TGFβ1 activity. Finally, we studied a potential role of sEng in pressure overload induced heart failure and observed reduced cardiac fibrosis in mice treated with an adenovirus over-expressing human sEng.

Previous studies have highlighted the critical role that TGFβ1 signaling plays in cardiac remodeling and heart failure. Benefits of blocking TGFβ1 activity such as improving diastolic function in hypertensive heart disease have been reported, but in other models of heart failure, this increased mortality after induction of ischemic heart failure. Given the potential for adverse effects of non-selective TGFβ1 blockade, targeting specific aspects of the TGFβ1 signaling cascade may yield better outcomes. Our findings support this concept as Eng+/− mice demonstrated reduced phosphorylation of Smad-2/3 in association with LV hypertrophy and limited cardiac fibrosis after TAC. Since endoglin is highly expressed in cardiac fibroblasts, modulating endoglin expression in these cells may selectively influence fibrosis without affecting hypertrophy. This combination results in sustained LV contractility and improved survival despite chronic pressure-overload induced heart failure.

The role of sEng in heart failure also remains poorly understood. First, the mechanism underlying increased sEng expression in heart failure is unknown. Proteolytic cleavage of sEng from endoglin may occur both locally in cardiac tissue or systemically as levels of MMP14 are known to be elevated in heart failure. Second, the mechanism by which elevated sEng levels interrupt TGFβ1 signaling remains poorly characterized. Several possibilities exist (Figure 7). First, endoglin may modulate signaling via several TGFβ-family ligands and sEng could serve as a ligand trap for TGFβ1 or other ligands including BMPs. However, recent studies indicate that BMP9 and BMP10 may be the only ligands that bind to sEng with high affinity. Second, sEng may promote alternate signaling pathways that indirectly inhibit TGFβ1 signaling such as BMP-7. Finally, release of sEng by ectodomain shedding may render the receptor non-functional, thereby further limiting TGFβ1 activity in heart failure.

The present study has several limitations. First, we employed a mouse model with reduced total body expression of endoglin as opposed to changes in cardiac restricted expression. Second, due to the technical challenges of sustaining elevated levels of exogenous sEng using an adenoviral approach, we were unable to examine whether sEng improves cardiac function in a longer-term model of heart failure.

In conclusion, TGFβ1 is a powerful cytokine that governs the development of cardiomyocyte hypertrophy and cardiac fibrosis in heart failure. Therapies designed to non-selectively block TGFβ1 activity in heart failure have failed to demonstrate clear benefit. We now demonstrate the important functional role of endoglin in heart failure, by specifically showing that endoglin facilitates, whereas sEng attenuates, TGFβ1-mediated cardiac fibrosis, and further, that reduced endoglin expression can limit cardiac fibrosis, preserve cardiac function, and improve survival in pressure overload-induced heart failure.
studies support that targeting endoglin provides a potentially unique and novel therapeutic approach for individuals with heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Heart failure is a major cause of global mortality. Transforming growth factor beta-1 (TGFβ1) is a cytokine that promotes cardiac fibrosis in heart failure. *Endoglin* is a coreceptor that regulates TGFβ1 signaling via downstream effector proteins known as Smads (canonical pathway) or mitogen activated protein kinases (non-canonical pathway). The extracellular domain of endoglin can be cleaved into the circulation as soluble endoglin (sEng), which may serve as a natural antagonist to TGFβ1 activity. We now report that endoglin expression is increased in failing human left ventricular tissue and in a murine model of thoracic aortic constriction (TAC)-induced heart failure. Using the endoglin haploinsufficient mouse model, we observed improved survival, limited cardiac fibrosis, and enhanced myocardial capillarity after TAC. To study the role of endoglin *in vitro*, loss of function studies demonstrated the dependence of TGFβ1 activity on endoglin expression in human cardiac fibroblasts. Paradoxically, adenoviral-mediated over-expression of full-length endoglin also blocked TGFβ1 induced collagen synthesis. Further study showed that levels of sEng were elevated in the conditioned media after treatment with the adenovirus, thereby implicating sEng as a negative regulator of TGFβ1 activity. This observation was confirmed by adenoviral-mediated over-expression of human sEng (AdhsEng) or treatment with recombinant human sEng *in vitro*. To begin exploring the utility of sEng as an anti-fibrotic approach *in vivo*, treatment with AdhsEng attenuated cardiac fibrosis in wild-type mice after TAC. Together these data identify endoglin as an important component of cardiac remodeling and a potentially novel target of therapy in heart failure.
Figure 1.
Increased endoglin expression in the failing human LV. A) Endoglin expression in LV tissue from human subjects without heart failure (Non-HF) and with heart failure prior to insertion of a LV assist device (pre-LVAD; *, p<0.05 vs Non-HF). B) LV endoglin expression in 7 subjects before and after LVAD support (post-LVAD; *, p<0.05 vs pre-LVAD). Representative western blots are shown above quantification graphs. C) Representative western blot of endoglin expression (relative to GAPDH) in isolated mouse endothelium, cardiomyocytes, and cardiac fibroblasts. D) Endoglin mRNA expression by isolated mouse cardiomyocytes and cardiac fibroblasts (***, p<0.001 between groups).
Figure 2. Reduced endoglin expression preserves cardiac function and survival in heart failure. A) Endoglin expression in LV protein lysates from WT and Eng±/− mice (n=3/group). B) LV endoglin mRNA expression in WT mice after TAC (n=6/group). C) Levels of circulating sEng in Eng±/− mice compared to WT (n=6/group). D) M-mode echocardiography in WT mice compared to Eng±/− mice after TAC. E) Representative LV pressure-volume loops after 4 weeks of TAC in Eng±/− and WT mice. F) Kaplan-Meier survival curves in Eng±/− and WT mice after TAC (n=18/group). *, p<0.05 vs WT-Sham; †, p<0.05 vs Eng±/−-Sham; ‡, p<0.05 vs WT at the corresponding time point.
Figure 3.
Reduced endoglin expression does not affect cardiac hypertrophy and is associated with increased myocardial capillarity. A) Representative histologic staining and B) bar graph of LV cardiomyocyte cross-sectional area in both WT and Eng^{+/−} mice after 4 weeks of TAC. C-D) LV β-MHC and SERCa mRNA and E) calcineurin protein expression in WT and Eng^{+/−} mice after 4 weeks of TAC (n=6/group). F) Representative immunostaining of LV myocardial capillaries (CD31+) in Eng^{+/−} and WT mice after 4 weeks of TAC. G-H) Bar graphs quantifying LV myocardial capillarity after 4 weeks of TAC in WT and Eng^{+/−} mice (n= 6/group). *, p<0.05 vs Sham; †, p<0.05 vs WT TAC.
Figure 4.
Reduced cardiac fibrosis in Eng<sup>+/−</sup> mice after pressure overload induced heart failure. A) Representative immunostaining of LV Type I collagen in WT and Eng<sup>+/−</sup> mice after TAC. B) Quantification of LV fibrosis in Eng<sup>+/−</sup> and WT after TAC (n=6/group). C) Levels of LV Type I Collagen mRNA and D) protein expression in Eng<sup>+/−</sup> and WT mice after TAC (n=6/group). E) Levels of TGFβ1 mRNA and F) pSmad-2/3 protein expression in both WT and Eng<sup>+/−</sup> mice after 4 weeks of TAC. pSmad-2/3 is expressed relative to total Smad-3 in the bar graph (n=6/group) and representative Western blot. *, p<0.05 vs Sham; †, p<0.05 vs WT TAC at each corresponding time point.
Figure 5.
Endoglin and sEng modulate TGFβ1-activity. A) Type I collagen mRNA (bar graph) and protein expression (representative Western blot) in hCF after treatment with a neutralizing anti-endoglin antibody. B-C) Type I collagen mRNA (bar graph) and protein expression (representative Western blot) and plasminogen-activator inhibitor-1 (PAI-1) mRNA expression in hCF after silencing endoglin expression. D-E) Type I collagen protein (quantification of Western blot shown in bar graph) and mRNA expression after treatment with RhsEng. F) Type I collagen protein synthesis (bar graph and Western blot) and pSmad-2/3 expression (Western blot) after treatment with conditioned media from COS-1 cells transfected with AdhsEng. *, p<0.05 vs Control; †, p<0.05 vs TGFβ1-stimulated controls.
Figure 6.
Over-expressing human sEng limits cardiac fibrosis in vivo. A) Circulating serum levels of human and mouse sEng in WT mice after treatment with AdhsEng (n=3 mice/group). B) Representative histology of LV collagen expression and C) Type I collagen mRNA expression in WT mice treated with AdhsEng and AdNull.
Figure 7.
Reduced Endoglin activity limits cardiac fibrosis by disrupting TGFβ1 signaling. i) Endoglin expression is required for TGFβ1-induced Type I collagen synthesis and cardiac fibrosis. ii) Reduced endoglin expression in Eng+/− mice attenuates TGFβ1-induced pSmad-2/3, Type I collagen expression, and cardiac fibrosis. iii) Potential mechanisms by which sEng interrupts TGFβ1-signaling: 1) disrupted ligand-binding, 2) dimerizing with endoglin, and 3) stimulating inhibitory pathways.
Table 1
Characterization of heart failure in wild-type and Eng+/- mice.

<table>
<thead>
<tr>
<th>Mass</th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 weeks</td>
<td>10 weeks</td>
</tr>
<tr>
<td>Total Body Weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>35±3.9</td>
<td>29±3.1*</td>
</tr>
<tr>
<td>Eng+/-</td>
<td>34±1.2</td>
<td>34±4.6#</td>
</tr>
<tr>
<td>LV weight (mg) / Tibia length (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>7±1</td>
<td>11±1*</td>
</tr>
<tr>
<td>Eng+/-</td>
<td>5±0.7#</td>
<td>9±1*#</td>
</tr>
<tr>
<td>Total Lung Weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>9±0.7</td>
<td>240±10*</td>
</tr>
<tr>
<td>Eng+/-</td>
<td>8±0.3#</td>
<td>190±10*</td>
</tr>
</tbody>
</table>

**Hemodynamic Data**

Heart Rate (beats/min)

|                       |       |        |
|                       | WT    | Eng+/- |
|                       | 535±45 | 549±32 |
|                       | 553±40 | 522±49 |
| LV End-systolic Pressure (mmHg) |       |        |
| WT                    | 114±12 | 99±15  |
| Eng+/-                | 130±18 | 157±19*# |
| LV End-diastolic Pressure (mmHg) |       |        |
| WT                    | 11±4  | 11±4  |
| Eng+/-                | 9±5   | 24±12* |

**Echo Data**

|                       |       |        |
|                       | WT    | Eng+/- |
|                       | 2.4±0.4 | 1.8±0.1# |
|                       | 3.9±0.4* | 3.4±0.6* |

Fractional Shortening (%)

<p>| | | |
|                       |       |        |
|                       | WT    | Eng+/- |
|                       | 72±4  | 57±8#  |
|                       | 42±4* | 40±7  |
| Posterior Wall Thickness (mm) |       |        |
| WT                    | 0.9±0.2 | 1.4±0.3* |
|                       |        | 1.3±0.3* |</p>
<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
<th>4 weeks</th>
<th>10 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eng+/−</td>
<td>0.5±0.04</td>
<td>1.3±0.2</td>
<td>*</td>
<td>2.3±0.1</td>
</tr>
</tbody>
</table>

* p<0.05: 4wk vs Before TAC
** p<0.05: 10wk vs Before TAC
# p<0.05: Eng+/− vs WT