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

Navin K. Kapur, MD; Szuhuei Wilson, MS; Adil A. Yunis, BSc; Xiaoying Qiao, PhD; Emily Mackey, BA; Vikram Paruchuri, MD; Corey Baker, BSc; Mark J. Aronovitz, MS; S. Ananth Karumanchi, MD; Michelle Letarte, PhD; David A. Kass, MD; Michael E. Mendelsohn, MD; Richard H. Karas, MD, PhD

Background—Heart failure is a major cause of morbidity and mortality worldwide. The ubiquitously expressed cytokine transforming growth factor-β1 (TGFβ1) promotes cardiac fibrosis, an important component of progressive heart failure. Membrane-associated endoglin is a coreceptor 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 of human subjects with heart failure and determined that endoglin is required for TGFβ1 signaling in human cardiac fibroblasts using neutralizing antibodies and an siRNA approach. We further identified that reduced endoglin expression attenuates cardiac fibrosis, preserves left ventricular 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, which disrupts TGFβ1 signaling in endothelium. We now demonstrate that soluble endoglin limits TGFβ1 signaling and type I collagen synthesis in cardiac fibroblasts and further show that soluble endoglin treatment attenuates cardiac fibrosis in an in vivo model of heart failure.

Conclusion—Our results identify endoglin as a critical component of TGFβ1 signaling in the cardiac fibroblast and show that targeting endoglin attenuates cardiac fibrosis, thereby providing a potentially novel therapeutic approach for individuals with heart failure. (Circulation. 2012;125:2728-2738.)

Key Words: fibrosis ■ heart failure ■ ventricular remodeling

Heart failure is a major cause of morbidity and mortality that affects >24 million individuals worldwide.1–3 Regardless 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 to compensatory hypertrophy, various signaling cascades are implicated.4 Among these, transforming growth factor-β1 (TGFβ1) is a profibrogenic cytokine that contributes to multiple fibroproliferative disorders, including cardiac fibrosis associated with heart failure.5 In response to angiotensin II, TGFβ1 expression is increased, converts fibroblasts into myofibroblasts, and generates extracellular matrix proteins such as type I collagen.6 Excess collagen deposition exaggerates mechanical stiffness of the LV, impairs myocyte contractility, disrupts electric coupling, and worsens tissue hypoxia,4 thereby promoting heart failure. Given its central role in stimulating fibrosis, TGFβ1 has been nonselectively targeted in heart failure models using multiple approaches, none of which has produced clearly beneficial therapeutic effects.7,8

Editorial see p 2689
Clinical Perspective on p 2738

TGFβ1 signals through a heteromeric receptor complex comprising 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 (noncanonical pathway). Specifically, TGFβ1-induced phosphorylation of Smad-2/3 promotes type I collagen synthesis and fibrosis.5,6 Endoglin (CD105) is a 180-kDa homodimeric glycoprotein that serves as a coreceptor for TGFβ1 signaling. Over the past 2 decades, several lines of evidence have suggested 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, characterized by endoglin haploinsufficiency and visceral arteriovenous malformations.9 Second, endoglin-null mice die at embryonic day 10.5 as a result of impaired cardiovascular development and early embryonic angiogenesis.10 However, endoglin-heterozygous mice (Eng+/−) are viable, have reduced levels of endoglin, and have a phenotype that recapitulates that of hereditary hemorrhagic telangiectasia type 1.11 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 matrix metalloproteinase-14 and circulates as soluble endoglin (sEng).12 We recently demonstrated that levels of sEng correlate with clinical measures of heart failure, including LV end-diastolic pressure and New York Heart Association classification.13 From these observations, we hypothesized that impaired function of the TGFβ1 coreceptor 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 used a model of pressure-overload–induced heart failure in Eng+/− mice.

Methods

Reagents

We purchased recombinant human sEng (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 phosphorylated Smad (pSmad)-2/3 (AB-3849), pSmad-1 (06–702), and phosphorylated extracellular regulated kinase (ERK)-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 R&D Systems (05–797) and polyclonal antibodies to mouse calcineurin were purchased from Cell Signaling, respectively. Goat polyclonal antibodies against mouse endoglin and type I collagen were purchased from Santa Cruz. Goat polyclonal antibodies against mouse endoglin and type I collagen were purchased from R&D Systems (BASF1320) and Santa Cruz (SC-25974), respectively. Polyclonal antibodies to human and mouse phosphorylated Smad (pSmad)-2/3 (AB-3849), pSmad-1 (06–702), and phosphorylated extracellular regulated kinase (ERK)-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.

Human LV Tissue Sampling

Viable LV free wall tissue was obtained from human subjects with heart failure (n=20) referred for LV assist device (LVAD) placement (HeartMate; Thoratec Corp, Pleasanton, CA). In 7 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. 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 accordance 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- to 16-week-old C57BL/6 wild-type (WT) and Eng−/− mice underwent thoracic aortic constriction (TAC) as previously described.14,15 At 2, 4, and 10 weeks after TAC, mice were euthanized and tissue was obtained for further analysis by real-time polymerase chain reaction, immunoblotting, histology, and ELISA according to the manufacturer’s instructions. Eng−/− mice were generously provided by Dr Michelle Letarte, University of Toronto.

Physiological Characterization In Vivo

Transthoracic echocardiography and pressure-volume loop analyses were performed on mice as previously described.14,15

TGFβ1-Induced Type I Collagen Expression in Cardiac Fibroblasts

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

Loss-of-Function Studies in hCFs

For neutralizing antibody studies, hCFs were pretreated with 0.5 μg/mL of either an antibody to endoglin or control IgG isotype for 24 hours in fibroblast basal medium without supplementation before stimulation with TGFβ1 (10 ng/mL). After 24 hours, cells were harvested for real-time polymerase chain reaction and Western blot analysis. For endoglin silencing experiments, 50 μmol/L siRNA stock (Ambion) was diluted to 1.0 nmol/L in OptiMEM (Invitrogen) and combined with 2 μL lipofectamine (Invitrogen) diluted in 98 μL OptiMEM. After 20 minutes of incubation, cells were exposed to human endoglin siRNA (Ambion; 145527), scrambled siRNA (negative control; Ambion; 4390844), or GAPDH siRNA (positive control; Ambion; 4390850). At various times between 24 and 48 hours after transfection, cells were harvested for analysis.

Recombinant sEng Inhibition of Type I Collagen Synthesis In Vitro

Serum-starved hCFs were treated for 24 hours with recombinant human sEng, stimulated with TGFβ1, and harvested for further analysis.

Overexpression of Human sEng 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. Karunan-chi) or adenovirus with no transgene 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 hCFs and then stimulated with TGFβ1. For overexpression studies, hCFs were transfected with adenovirus expressing full-length endoglin (generously provided by Dr Calvin P. Vary), stimulated with TGFβ1, and harvested for analysis.

sEng Inhibits Pressure-Overload–Induced Cardiac Fibrosis

Adult male 14- to 16-week old C57BL/6 mice received intravenous injections of adenovirus with no transgene or AdhsEng 1 day before TAC. Serum levels of human and mouse sEng were quantified by ELISA. Four weeks after TAC, mice were euthanized and tissue was obtained for further analysis.

Real-Time Quantitative Polymerase Chain Reaction

For all cell-based real-time polymerase chain reaction experiments, total RNA was extracted directly with Trizol (Invitrogen) and
converted to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For all real-time polymerase chain reaction experiments, samples were quantified in triplicate using 40 cycles performed at 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds with an ABI Prism 7900 Sequence Detection System using appropriate primers (see the online-only Data Supplement) as described.14 –16

Immunoblot Analysis (Western)
Total protein was extracted and quantified from tissue homogenates or cultured cells as described.14 –16 Immunoblot analysis was then performed as previously described using antibodies for human and mouse targeted proteins.

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

Statistical Analysis
Results are presented as mean±SD. Intergroup comparisons were made with 2-factor ANOVA. Two-way ANOVA was performed to examine the effects of cardiac unloading by an LVAD and time on endoglin expression. Repeated-measures ANOVA was used as needed to account for time. All multiple comparisons with a control group were performed with the Dunnett method. Kaplan-Meier analysis with log-rank testing was used for survival analysis. All statistical analyses were performed with SigmaStat version 3.1 (Systat Software, Inc). An α level of P<0.05 was considered to indicate a significant effect or between-group difference.

Results
LV 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 an 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 with 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 and 1D).

Increased Membrane-Associated and Circulating Endoglin Expression in Heart Failure
To explore the functional role of endoglin in heart failure, we studied Eng+/− mice. Compared with WT, LV endoglin expression was lower in Eng+/− mice (Figure 2A). We then used 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 with 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 was 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 (Figure IA in the online-only Data Supplement), suggesting a direct effect of cardiac 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 with 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; $P<0.05$) compared with WT mice after TAC (Figure 2D–2F and the Table). Consistent with these observations, WT mice manifest reduced total body weight at both 4 and 10 weeks after TAC (Figure 2D–2F and the Table). Echocardiography demonstrated a significant time-dependent TAC-induced reduction in LV fractional shortening (Table). 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 both WT and $Eng^{+/−}$ mice at any time point. LV hypertrophy is preserved whereas myocardial capillarity is enhanced in $Eng^{+/−}$ mice compared with WT mice.

**Reduced Endoglin Expression Attenuates Cardiac Fibrosis**

Comparing controls, TAC induced a time-dependent increase in LV collagen deposition in WT but not $Eng^{+/−}$ mice (Figure 4A and 4B). Type I collagen mRNA expression (Figure 4C) and protein expression (Figure 4D) were simi-
lower in \(\text{Eng}^{+/−}\) mice compared with WT mice. Importantly, similar increases in TGFβ1 mRNA (Figure 4E) and active TGFβ1 protein (Figure IIIB in the online-only Data Supplement) were observed in the LV of both WT and \(\text{Eng}^{+/−}\) 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 Figure IIIC in the online-only Data Supplement) in WT mice. However, despite similar increases in LV TGFβ1 levels in \(\text{Eng}^{+/−}\) mice, TAC did not induce a significant increase in pSmad-2/3. Consistent with this impairment of TGFβ1 signaling in \(\text{Eng}^{+/−}\) mice, expression of plasminogen activator inhibitor-1 was increased in WT but not \(\text{Eng}^{+/−}\) mice after 4 weeks of TAC (Figure IIID). To confirm that reduced endoglin expression by \(\text{Eng}^{+/−}\) mice limits TGFβ1-induced cardiac fibrosis, mCFs were isolated from the LV of WT and \(\text{Eng}^{+/−}\). TGFβ1 stimulation increased type I collagen mRNA expression in WT-mCF but not \(\text{Eng}^{+/−}\)-mCF (Figure IIIE in the online-only Data Supplement). 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 Noncanonical Signaling in Endoglin-Deficient Mice**

We next studied the expression of the TGFβ type I receptors ALK1 and ALK5 in hCFs from fresh tissue samples obtained during cardiac surgery. We observed lower ALK1 mRNA expression in hCFs compared with human umbilical vein endothelial cells, whereas ALK5 expression was similar in both cell types (Figure IVA in the online-only Data Supplement). Next, TGFβ1 stimulation increased pSmad-2/3 expression with no change in pSmad-1 expression (Figure IVB in the online-only Data Supplement). Compared with sham-operated controls, LV pSmad-1 expression was increased in WT mice after 4 weeks of TAC, whereas no change was observed in \(\text{Eng}^{+/−}\) mice (Figure IVC in the online-only Data Supplement). 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 \(\text{Eng}^{+/−}\) mice after TAC (Figure IVD–IVG in the online-only Data Supplement).

Next, we studied phosphorylation of the TGFβ1 noncanonical signaling effector ERK. With an siRNA approach, reduced endoglin expression attenuated TGFβ1-induced phosphorylation of ERK-1/2 in hCFs in vitro (Figure IVH in the online-only Data Supplement). Consistent with this observation, phosphorylated ERK-1/2 expression was increased in WT mice but not \(\text{Eng}^{+/−}\) mice after 4 weeks of TAC (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 topic, the role of endoglin was examined by use of a loss-of-function approach in hCFs. Compared with an isotype control antibody, pretreatment of hCFs with an antibody to endoglin attenuated TGFβ1-induced type I collagen mRNA and protein expression (Figure 5A). Similarly, silencing en-
doglin expression significantly reduced TGFβ1-induced type I collagen mRNA and protein expression, plasminogen activator inhibitor-1 expression (Figure 5B and 5C), and connective tissue growth factor in hCFs (Figure VA in the online-only Data Supplement). These findings identified endoglin as a necessary component for TGFβ1 signaling in hCFs.

sEng 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 hCFs with recombinant human sEng and observed a dose-dependent decrease in TGFβ1-induced type I collagen expression (Figure 5D and 5E). 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 (Figure VB in the online-only Data Supplement). Similar to treatment with recombinant human sEng, treatment of hCFs 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 overexpressing full-length endoglin using an adenovirus-mediated approach (adenovirus expressing full-length endoglin) in hCFs and paradoxically observed a reduction in TGFβ1-induced type I collagen expression (Figure VC in the online-only Data Supplement). To study this further, we measured increased levels of sEng in hCFs transfected with adenovirus expressing full-length endoglin (FigureVD in the online-only Data Supplement). These findings implicated sEng as a negative feedback mechanism that downregulates TGFβ1 activity in hCFs.
sEng 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 with controls, treatment with AdhsEng significantly reduced cardiac fibrosis (Figure 6B) and LV type I collagen expression (Figure 6C) after 4 weeks of TAC. 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 hCFs 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 nonselective TGFβ1 blockade have produced mixed results in heart failure. The ability to selectively modulate TGFβ1 activity by limiting the TGFβ1 coreceptor endoglin offers a potentially novel approach to managing 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).

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, 2 isoforms of membrane-associated endoglin exist, namely the long and the less abundant short isoforms.25 We confirmed that long 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. Although TAC induced a similar

![Figure 5. Endoglin and soluble endoglin (sEng) modulate transforming growth factor-β1 (TGFβ1) activity.](http://circ.ahajournals.org/doi/10.1161/CIRCULATIONAHA.108.797478)
pattern of endoglin expression in $\text{Eng}^{+/+}$ and WT mice, levels of endoglin expression were significantly lower in the $\text{Eng}^{+/+}$ 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 hCFs using loss-of-function approaches. Furthermore, treatment with sEng either as a recombinant protein or by adenoviral overexpression of sEng or full-length endoglin in vitro mirrored the phenotype of the reduced endoglin levels in the $\text{Eng}^{+/+}$ mice, suggesting that sEng also limits TGFβ1 signaling and type I collagen synthesis. Several prior studies have shown that overexpressing endoglin in rat myoblasts and mouse fibrosarcoma cell lines limits TGFβ1-induced collagen expression.27–30 In the context of our findings, these observations may highlight important differences between cell types and species with regards to the biological activity of endoglin. Furthermore, these reports may be consistent with our gain-of-function observations in hCFs 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 overexpressing human sEng.

Previous studies have highlighted the critical role that TGFβ1 signaling plays in cardiac remodeling and heart failure.5,31,32 Benefits of blocking TGFβ1 activity such as improving diastolic function in hypertensive heart disease are likely to be shared by a range of interventions aimed at reducing cardiac fibrosis.33–35 Among these, therapies targeting endoglin and TGFβ1 signaling may be of particular interest due to the potential for improvement of both fibrosis and remodeling.

**Figure 6.** Overexpressing human soluble endoglin (AdhsEng) limits cardiac fibrosis in vivo. **A,** Circulating serum levels of human and mouse sEng in wild-type (WT) mice after treatment with AdhsEng ($n=3$ mice per group). **B,** Representative histology of left ventricular (LV) collagen expression and **C** type I collagen mRNA expression in WT mice treated with AdhsEng and adenovirus with no transgene (AdNull).

**Figure 7.** Reduced endoglin activity limits cardiac fibrosis by disrupting transforming growth factor-β1 (TGFβ1) signaling. Left, Endoglin expression is required for TGFβ1-induced type I collagen synthesis and cardiac fibrosis. Middle, Reduced endoglin expression in $\text{Eng}^{+/+}$ mice attenuates TGFβ1-induced pSmad2/3, type I collagen expression, and cardiac fibrosis. Right, Potential mechanisms by which soluble endoglin (sEng) interrupts TGFβ1-signaling: (1) disrupted ligand binding, (2) dimerizing with endoglin, and (3) stimulating inhibitory pathways.
ease 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 nonselective TGFβ1 blockade, targeting specific aspects of the TGFβ1 signaling cascade may yield better outcomes. Our findings support this concept in that Eng−/− mice demonstrated reduced phosphorylation of Smad-2/3 in association with LV hypertrophy and limited cardiac fibrosis after TAC. Because 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 because levels of matrix metalloproteinase-14 from endoglin may occur both locally in cardiac tissue or systemically because levels of matrix metalloproteinase-14 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 bone morphometric protein. However, recent studies indicate that bone morphometric protein-9 and -10 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 bone morphometric protein. Finally, release of sEng by ectodomain shedding may render the receptor nonfunctional, thereby further limiting TGFβ1 activity in heart failure.

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

Conclusions

TGFβ1 is a powerful cytokine that governs the development of cardiomyocyte hypertrophy and cardiac fibrosis in heart failure. Therapies designed to nonselectively 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. Our studies support that targeting endoglin provides a potentially unique and novel therapeutic approach for individuals with heart failure.

Source of Funding

Dr Kapur is supported by a grant from the National Institutes of Health (K08HL094909–03).

Disclosures

Dr Karumanchi and Dr Letarte are listed as co-inventors on patents held by the Beth Israel Deaconess Medical Center for the use of soluble endoglin in diagnosis and therapy of preeclampsia.

References


### CLINICAL PERSPECTIVE

Heart failure is a major cause of global mortality. Transforming growth factor-beta1 (TGFβ1) is a cytokine that promotes cardiac fibrosis in heart failure. Endoglin is a coreceptor that regulates TGFβ signaling via downstream effector proteins known as Smads (canonical pathway) or mitogen-activated protein kinases (noncanonical 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–induced heart failure. Using the endoglin haploinsufficient mouse model, we observed improved survival, limited cardiac fibrosis, and enhanced myocardial capillarity after thoracic aortic constriction. 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, adenovirus-mediated overexpression 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 adenovirus-mediated overexpression of human sEng or treatment with recombinant human sEng in vitro. To begin exploring the utility of sEng as an antifibrotic approach in vivo, treatment with adenovirus-mediated overexpression of human sEng attenuated cardiac fibrosis in wild-type mice after thoracic aortic constriction. Together, these data identify endoglin as an important component of cardiac remodeling and a potentially novel target of therapy in heart failure.
Reduced Endoglin Activity Limits Cardiac Fibrosis and Improves Survival in Heart Failure


Circulation. 2012;125:2728-2738; originally published online May 16, 2012;
doi: 10.1161/CIRCULATIONAHA.111.080002
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/125/22/2728

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/05/14/CIRCULATIONAHA.111.080002.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL
**Supplementary Figure Legend:**

Supplementary Figure 1. A) Increased Endoglin mRNA expression in the LV not distal aorta of WT mice after TAC. (*, p<0.05 vs Sham; n=6/group).

Supplementary Figure 2. Similar changes in A) β-MHC and B) SERCa in WT and Eng+/− mice after 10 weeks of TAC. (*, p<0.05 vs Sham; n=6/group).

Supplementary Figure 3. A) Type I collagen mRNA expression after 10 weeks of TAC. B) Levels of active TGFβ1 protein expression in LV lysates after 4 weeks of TAC (n=6/group). C) Western blot showing increased pSmad-2/3 expression in WT, not Eng+/− mice after 10 weeks of TAC. D) mRNA expression of PAI-1 in WT and Eng+/− mice (n=6/group). E) TGFβ1-induced Type I collagen mRNA expression in cultured cardiac fibroblasts from WT and Eng+/− mice. (*, p<0.05 vs Sham; †, p<0.05 vs WT TAC).

Supplementary Figure 4. A) Relative expression of ALK-1 and ALK-5 in hCF compared to HUVEC. B) Western blot showing TGFβ1 simulated pSmad-2/3 and pSmad-1 expression in hCF, relative to total Smad-2/3 or total Smad-1, respectively. C) Western blot showing LV pSmad-1 expression in WT and Eng+/− mice after 4 weeks of TAC. D) Percentage of LV nuclei staining positive for pSmad-2/3 or E) pSmad-1 in WT and Eng+/− mice after 4 weeks of TAC. F-G) Representative LV immunostaining for pSmad-2/3 or pSmad-1 in WT and Eng+/− mice after 4 weeks of TAC. H) Western blot showing pERK-1/2 relative to total ERK in hCF stimulated with TGFβ1 in the presence and absence of an endoglin siRNA. I) Western blot showing pERK-1/2 expression relative to total ERK in LV lysates from WT or Eng+/− mice 4 weeks after TAC. (*, p<0.05 vs control).

Supplementary Figure 5. A) Silencing endoglin expression attenuates TGFβ1-induced CTGF mRNA expression in hCF. B) COS1 cells transfected with AdhsEng demonstrate
a dose-dependent increase in levels of sEng detected in the culture media. C) Western blot showing TGFβ1-induced Type I collagen and endoglin expression relative to GAPDH in hCF after treatment with AdFL-Eng. D) Levels of sEng detected in culture media from hCF transfected with AdFL-Eng or AdNull. (*, p<0.05 vs Vehicle control; †, p<0.05 vs TGFβ1-stimulated controls).
Supplementary Figure 3

(A) (10 week) Type 1 Collagen mRNA

Fold-Change vs WT Sham

Sham  TAC  Sham  TAC

WT  Eng^{−/−}

(B) Active TGFβ Levels (Tissue Lysate) Fold Change vs Sham

Sham  TAC  Sham  TAC

WT  Eng^{−/−}

(C) Western Blot for pSMAD-2/3 and Total SMAD-3

WT  Eng^{−/−}

(D) MAI-1 mRNA

Fold-Change vs WT Sham

Sham  TAC  Sham  TAC

WT  Eng^{−/−}

(E) Type I Collagen mRNA Ratio

Vehicle  TGFβ1  Vehicle  TGFβ1

WT  Eng^{−/−}
Supplementary Figure 5

A

![Image of a graph showing CTGF/rRNA Ratio with bars for Vehicle, TGFβ1, Vehicle, and TGFβ1 with Scrambled siRNA and Endoglin siRNA.]

B

![Image of a bar graph showing Soluble Endoglin (pg/mL) with AdhSEng (μL/mL) at 0, 0.1, 0.5, 1, and 2.]

![Image of Western blot analysis for Type I Collagen, Endoglin, and GAPDH with AdFL-Eng and TGFβ1 conditions.]

![Image of another bar graph showing Soluble Endoglin (pg/mL) with AdNull and AdFL-Eng conditions.]

Legend:

- Vehicle
- TGFβ1
- Scrambled siRNA
- Endoglin siRNA

* and † indicate significant differences.
Supplementary Table of RT-PCR Primers:

<table>
<thead>
<tr>
<th>Mouse Primers</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mENG</td>
<td>forward</td>
<td>5’ - CTG CCA ATG CTG TGC GTG AA</td>
</tr>
<tr>
<td>mENG</td>
<td>reverse</td>
<td>3’ - GCT GGA GTC GTA GGC CAA GT</td>
</tr>
<tr>
<td>mCOL-1</td>
<td>forward</td>
<td>5’ - AAG GGT CCC TCT GGA GAA CC</td>
</tr>
<tr>
<td>mCOL-1</td>
<td>reverse</td>
<td>3’ - TCT AGA GCC AGG GAG ACC CA</td>
</tr>
<tr>
<td>mTGFβ1</td>
<td>forward</td>
<td>5’ - CAC CGG AGA GCC CTG GAT A</td>
</tr>
<tr>
<td>mTGFβ1</td>
<td>reverse</td>
<td>3’ - TGC CGC ACA CAG CAG TTC</td>
</tr>
<tr>
<td>m18S rRNA</td>
<td>forward</td>
<td>5’ - GGA CAG GAC TAG GCG GAA CA</td>
</tr>
<tr>
<td>m18S rRNA</td>
<td>reverse</td>
<td>3’ - AGG GGA GAG CGG GTA AGA GA</td>
</tr>
<tr>
<td>mPAI-1</td>
<td>forward</td>
<td>5’ - GAC ACC CTC AGC ATG TTC ATC</td>
</tr>
<tr>
<td>mPAI-1</td>
<td>reverse</td>
<td>3’ - AGG GTT GCA CTA AAC ATG TCA G</td>
</tr>
<tr>
<td>mSERCa</td>
<td>forward</td>
<td>5’ - CTG TGG AGA CCC TTG GTT GT</td>
</tr>
<tr>
<td>mSERCa</td>
<td>reverse</td>
<td>3’ - CAG AGC ACA GAT GGT GGC TA</td>
</tr>
<tr>
<td>mβMHC</td>
<td>forward</td>
<td>5’ - ATG TGC CGG ACC TTG GAA</td>
</tr>
<tr>
<td>mβMHC</td>
<td>reverse</td>
<td>3’ - CCT CGG GTT AGC TGA GAG ATC A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Primers</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>hENG</td>
<td>forward</td>
<td>5’ - GCG TGT GCG AGT AGA TGT ACC A</td>
</tr>
<tr>
<td>hENG</td>
<td>reverse</td>
<td>3’ - TCA TGC GCT TGA ACA TCA TCA</td>
</tr>
<tr>
<td>hCOL-1</td>
<td>forward</td>
<td>5’ - GTC GAG GGC CAA GAC GAA G</td>
</tr>
<tr>
<td>hCOL-1</td>
<td>reverse</td>
<td>3’ - CAG ATC ACG TCA TCG CAC AAC</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>forward</td>
<td>5’ - CGA GCC TGA GGC CGA CTA C</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>reverse</td>
<td>3’ - TCG GAG CTC TGA TGT GTT GAA</td>
</tr>
<tr>
<td>hPAI-1</td>
<td>forward</td>
<td>5’ - TGC TGG TGA ATG CCC TCT ACT</td>
</tr>
<tr>
<td>hPAI-1</td>
<td>reverse</td>
<td>3’ - CGG TCA TTC CCA GGT TCT CTA</td>
</tr>
<tr>
<td>18S-rRNA</td>
<td>forward</td>
<td>5’ - CTC AAC AGC GGA AAC CTC AC</td>
</tr>
<tr>
<td>18S-rRNA</td>
<td>reverse</td>
<td>3’ - AGC ATG CCA GAG TCT CGT TC</td>
</tr>
</tbody>
</table>