Reducing Endoglin Activity Limits Calcineurin and TRPC-6 Expression and Improves Survival in a Mouse Model of Right Ventricular Pressure Overload

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Background—Right ventricular (RV) failure is a major cause of mortality worldwide and is often a consequence of RV pressure overload (RVPO). Endoglin is a coreceptor for the profibrogenic cytokine, transforming growth factor beta 1 (TGF-β1). TGF-β1 signaling by the canonical transient receptor protein channel 6 (TRPC-6) was recently reported to stimulate calcineurin-mediated myofibroblast transformation, a critical component of cardiac fibrosis. We hypothesized that reduced activity of the TGF-β1 coreceptor, endoglin, limits RV calcineurin expression and improves survival in RVPO.

Methods and Results—We first demonstrate that endoglin is required for TGF-β1-mediated calcineurin/TRPC-6 expression and up-regulation of alpha-smooth muscle antigen (α-SMA), a marker of myofibroblast transformation, in human RV fibroblasts. Using endoglin haploinsufficient mice (Eng−/−) we show that reduced endoglin activity preserves RV function, limits RV fibrosis, and attenuates activation of the calcineurin/TRPC-6/α-SMA pathway in a model of angio-obliterrative pulmonary hypertension. Next, using Eng−/− mice or a neutralizing antibody (Ab) against endoglin (N-Eng) in wild-type mice, we show that reduced endoglin activity improves survival and attenuates RV fibrosis in models of RVPO induced by pulmonary artery constriction. To explore the utility of targeting endoglin, we observed a reversal of RV fibrosis and calcineurin levels in wild-type mice treated with a N-Eng Ab, compared to an immunoglobulin G control.

Conclusion—These data establish endoglin as a regulator of TGF-β1 signaling by calcineurin and TRPC-6 in the RV and identify it as a potential therapeutic target to limit RV fibrosis and improve survival in RVPO, a common cause of death in cardiac and pulmonary disease. (J Am Heart Assoc. 2014;3:e000965 doi: 10.1161/JAHA.114.000965)

Key Words: heart failure • pulmonary hypertension • fibrosis • right ventricle • cardiac remodeling
(ALK-5) promotes type I collagen synthesis and cardiac fibroblast proliferation. Endoglin has been shown to mediate TGF-β1 signaling through both ALK-1/Smad1/5/8 and ALK-5/Smad2/3 pathways. In contrast to membrane-bound endoglin, the extracellular domain of endoglin can be cleaved by matrix metalloproteinase-14 to form soluble endoglin (sol-Eng), which antagonizes TGF-β1 signaling. Levels of soluble endoglin are elevated in heart failure (HF) and pulmonary hypertension. Endoglin-null mice die at embryonic day 10.5 as the result of impaired cardiovascular development and extraembryonic angiogenesis. In contrast, endoglin heterozygous mice (Eng+/−) are viable and have reduced total body levels of endoglin. A functional role for endoglin in the RV has never been studied.

The calcium-dependent serine/threonine phosphatase, calcineurin, is another critical mediator of maladaptive cardiac remodeling, defined by excessive fibrosis and hypertrophy. Recent studies have shown that calcineurin increases expression of the canonical transient receptor protein channel 6 (TRPC-6), which triggers calcium influx and subsequent calcineurin activation, thereby setting up a self-propagating mechanism for pathologic hypertrophy, fibrosis, and increased mortality in HF. Very recently, noncanonical TGF-β1 signaling through TRPC-6 was reported to be an important stimulus for calcineurin-mediated alpha-smooth muscle cell actin (α-SMA) expression, a marker of myofibroblast transformation and a critical component of cardiac fibrosis. We also recently reported that RVPO increases TGF-β1 and calcineurin expression and is associated with increased RV fibrosis, hypertrophy, and reduced RV stroke volume, despite preserved contractility. These findings suggest that targeting TGF-β1 and calcineurin/TRPC-6 signaling may be an important approach to limit adverse RV remodeling. Calcineurin and TRPC-6 activity in the RV remains largely unexplored, and endoglin-dependent regulation of calcineurin expression in RV remodeling has not been studied.

We hypothesized that RVPO promotes expression of the TGF-β1 coreceptor, endoglin, which mediates calcineurin-dependent myofibroblast transformation through TRPC-6 in the RV. To explore this hypothesis, we employed several models of RVPO in both Eng−/− mice and wild-type (WT) mice treated with a neutralizing antibody (Ab) against endoglin.

### Methods

#### Reagents

Polyclonal Abs against human calcineurin, α-SMA, phosphorylated (p)Smad3, and total Smad2/3 were purchased from Cell Signaling Technology (2614S; Danvers, MA), Sigma-Aldrich (A2547; St. Louis, MO), and Cell Signaling Technology (8769S and 3102S), respectively. Polyclonal Abs against mouse endoglin, type I collagen, and α-SMA were purchased from R&D Systems (BAF1320; Minneapolis, MN), Santa Cruz Biotechnology (SC-25974; Santa Cruz, CA), and Sigma-Aldrich (A2547), respectively. Rabbit polyclonal Abs to mouse calcineurin (2614S) were purchased from Cell Signaling Technology. Polyclonal Abs to mouse pSmad3 (9520), and phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK1/2; SC-134900) were purchased from Cell Signaling Technology and Santa Cruz Biotechnology. Polyclonal Abs to mouse total Smad3 (SC-101154) and total ERK (SC-135900) were purchased from Santa Cruz Biotechnology. Sugen (SU5416) was purchased from Sigma-Aldrich. A neutralizing immunoglobulin G (IgG)1 Ab that binds human and mouse endoglin (TRC105) was provided by Tracon Pharmaceuticals (San Diego, CA). An ELISA kit for the detection of active TGF-β1 levels in mice was purchased from R&D Systems.

#### Mouse Model of Pharmacologically Induced RVPO

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 (Boston, MA). Adult, male, 12- to 14-week-old C57BL/6 WT and congenic Eng−/− mice received once-weekly intraperitoneal injections of Sugen and were exposed to either normoxic conditions (room air) or chronic normobaric hypoxia (10% O2), as previously described. After 5 weeks of exposure to either Sugen+Normoxia (Su-Norm) or Sugen+Hypoxia (Su-Hypox), mice underwent hemodynamic analysis with a RV conductance catheter (Millar Instruments Inc., Houston, TX), as described below, and tissue was then obtained for further analysis. Eng−/− mice were generously provided by Dr Michelle Letarte, University of Toronto (Toronto, Ontario, Canada).

#### Mouse Model of Surgically Induced RVPO

Adult, male, 12- to 14-week-old C57BL/6 WT and congenic Eng−/− mice underwent pulmonary artery constriction (PAC), as previously described. Specifically, mice were intubated using a 24G angiocath and mechanically ventilated (Harvard Apparatus, Holliston, MA) at 95 breaths per minute with a tidal volume of 0.3 mL with 2.0% to 2.5% isoflurane and 100% flow-through oxygen. Depth of anesthesia was monitored by assessing palpebral reflex, toe pinch, respirations, and general response to touch. Using a sterile technique, a left thoracotomy was performed to isolate and encircle the main pulmonary artery using a 7-0 nylon suture that was then tied tightly around a presterilized, blunt-end needle. After deairing, the thorax was closed with layered 6-0 Dexon sutures to eliminate the risk of pneumothorax. Postoperative analgesia
was immediately provided with buprenorphine (0.1 mL), which was continued twice-daily and as needed for an additional 72 hours. Severe RVPO was induced by PAC with a 25G needle for 7 days in WT and Eng<sup>−/−</sup> mice. To investigate the role of endoglin in RVPO, WT mice received 15 mg/kg of either a neutralizing Ab to endoglin (N-Eng; TRC105; Tracon Pharmaceuticals) or an IgG1 control Ab (IgG Ab; R&D Systems) by single intraperitoneal injection 1 day before and 3 days after induction of severe RVPO. To study the effect of blocking endoglin activity after induction of RVPO, WT mice were randomized to receive biweekly intraperitoneal injections for 3 weeks of 15 mg/kg of N-Eng antibody or IgG control Ab beginning 3 weeks after induction of moderate RVPO using a 23G needle for PAC. The Ab dose was based on a previous clinical study demonstrating effective saturation of endoglin receptors. After 7 days of severe PAC or 3 to 6 weeks of moderate RVPO, mice underwent hemodynamic analysis with an RV conductance catheter (Millar Instruments), as described below, and tissue was then obtained for further analysis.

**Nuclear Factor of Activated T-Cell Activity In Vivo**

Nuclear factor of activated T-cell (NFAT)-luciferase (NFAT-Luc) mice with nine copies of an NFAT-binding site from the interleukin (IL)-4 promoter (5'-TGGAAAAAT-3') inserted upstream of the luciferase reporter gene, driven by the α-myosin heavy-chain promoter, were purchased from The Jackson Laboratory (Bar Harbor, ME). Eng<sup>−/−</sup>-NFAT luciferase reporter mice were generated by crossing Eng<sup>−/−</sup> mice with the NFAT-luciferase mice. Severe RVPO was induced by PAC in 10- to 12-week-old Eng<sup>−/−</sup>-NFAT-Luc and Eng<sup>−/−</sup>-NFAT-Luc. After 7 days of severe PAC, RV tissue was then obtained for quantification of luciferase activity using firefly luciferase assays that were carried out as follows: 20 μL of whole RV tissue lysate was added to 100 μL of firefly luciferase assay buffer (Promega, Madison, WI). Samples were placed in a luminometer (Luminoskan Ascent; Labsystems Oy, Helsinki, Finland), and luminescence was determined in triplicate per sample over a 10-second interval.

**Hemodynamic Assessment of RV Function**

All animals underwent terminal hemodynamic evaluation. Right heart catheterization was performed at the time of sacrifice in all animals. Mice were anesthetized with 2.0% isoflurane administered by a noninvasive nose cone. Body temperature was monitored by a rectal thermistor probe and maintained at 37.5°C with heating pads and a cycling heat lamp. In the supine position, the right common carotid and right external jugular vein were surgically isolated. Silk ties were placed at the distal ends of both vessels while overhand loops were placed at the proximal ends with 7-0 nylon. A Millar PVR-1035 (Millar Instruments) mouse conductance catheter was used for RV recordings. Before insertion, conductance catheter calibration was performed using the cuvette method with freshly heparinized warm blood, then zeroed in warm saline as previously described. A transverse venotomy was performed using iris scissors at the proximal end of the external jugular vein. The PVR-1035 catheter was advanced through the superior vena cava and right atrium into the RV, leaving the chest wall intact. Once hemodynamic stability was achieved, steady-state baseline conditions were recorded from the RV. Stroke volume was calculated as end-diastolic minus end-systolic volume. Arterial elastance was calculated under steady-state conditions as end-systolic pressure/stroke volume. Tau, a measure of instantaneous isovolumic relaxation, was calculated using the Glantz method as $P(t) = P_0 e^{-t/\tau_e} + P_x$, where $P$ is pressure at time $t$, $P_0$ is the amplitude constant, $\tau_e$ is the Glantz relaxation constant, and $P_x$ is the nonzero asymptote resulting from pleural and pericardial pressure. RV compliance was calculated as stroke volume divided by peak RV pressure. Pressure-volume loop acquisition and analysis was performed using IOX software (emka TECHNOLOGIES, Paris, France). After completion of the hemodynamic study, with the animal still under isoflurane anesthesia, the chest was rapidly opened, and the mouse was euthanized by arresting the heart in diastole with 0.3 mL of 1 N of KCL injected directly into the LV. The heart was then removed and processed for either biochemical or histologic analyses.

**Histologic Quantification of Cardiac Hypertrophy and Fibrosis**

RV collagen abundance was quantified by picrosirius red staining, as previously described. Cardiomyocyte cross-sectional area was quantified as previously described.

**Loss-of-Function Studies in Cardiac Fibroblasts**

Human RV (RVFB) and LV (LVFB) fibroblasts were isolated from myocardial tissue harvested during cardiac surgery at Tufts Medical Center, and mouse RVFB and LVFB were isolated from WT and Eng<sup>−/−</sup> mice. Fibroblasts were stimulated with TGF-β1 for analysis, as previously described. For calcineurin inhibition studies, human RVFB were pretreated with 5 nM of cyclosporine A (CsA) or vehicle control for 24 hours in fibroblast basal medium (FBM) without supplementation, followed by stimulation with TGF-β1 (10 ng/mL) for 24 hours. For TRPC-6 silencing experiments, 50 μmol/L of siRNA stock was diluted to 5 nmol/L in OptiMEM (Invitrogen, Carlsbad, CA) and combined with 2 μL of Lipofectamine (Invitrogen) diluted in 98 μL of OptiMEM. After
20 minutes of incubation, cells were exposed to human TRPC-6 siRNA (Catalog No.: 439420; Ambion, Austin, TX) or scrambled siRNA (negative control; Catalog No.: 4390844; Ambion). After 48 hours after transfection, cells were treated with TGF-β1 (10 ng/mL) for 16 to 24 hours, then harvested for analysis. For neutralizing Ab studies in vitro, human RVFB and LVFB were pretreated with 10, 50, or 100 μg/mL of either an N-Eng Ab or control IgG Ab for 24 hours in FBM before stimulation with TGF-β1 (10 ng/mL). After 24 hours, cells were harvested for analysis. The Ab dose was based on previous studies demonstrating effective neutralization of endoglin activity in endothelium. All RVFB and LVFB stimulation studies were conducted in triplicate with cells cultured to within three lineage passes only.

Real-Time Quantitative Polymerase Chain Reaction

For all cell-based reverse-transcription polymerase chain reaction (RT-PCR) experiments, total RNA was extracted directly using Trizol (Invitrogen), then converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For all RT-PCR 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 using an ABI Prism 7900 (Applied Biosystems Inc) Sequence Detection System with appropriate primers (Table 1), as previously described.

Immunoblot Analysis (Western)

Total protein was extracted and quantified from tissue homogenates or cultured cells, as previously described. Immunoblot analysis was then performed, as previously described, using Abs for mouse targeted proteins.

Statistical Analysis

Results are presented as mean±SD. Intergroup comparisons were made for each variable with a two-way ANOVA, followed by a post-hoc Bonferroni’s correction for multiple comparisons (Tables 2 through 5). An alpha level of P<0.01 was considered to indicate a significant effect or between-groups difference. Multiple comparisons versus a control group were performed using one-way ANOVA, followed by post-hoc analysis with Dunnett’s method (Figures 1 through 7). Kaplan-Meier’s analysis with log-rank testing was employed for survival analysis (Figures 4C and 7A). All statistical analyses were performed using SigmaStat software (Version 3.1; Systat Software Inc., Richmond, CA). An alpha level of P<0.05 was considered to indicate a significant effect or between-groups difference.

Results

Endoglin Promotes TGF-β1-Mediated Calcineurin Expression and Myofibroblast Conversion in RV Fibroblasts

We first explored a role for calcineurin as a mediator of myofibroblast transformation in the RV. Human RVFB were stimulated with TGF-β1 in the presence or absence of the calcineurin inhibitor, CsA. Pretreatment with cyclosporine

Table 1. Primers

| Mouse Primers | Type I collagen | Forward | AAG GGT CCC TCT GGA GAA CC |
|              | Reverse | TCT AGA GCC AGG GAC ACC CA |
| Calcineurin (CN-PP) | Forward | CCACAGGGATGTTGCTACTGG |
|              | Reverse | GTCCCAGGTTTCTGAGTGGTA |
| Endoglin | Forward | CTT CCA ATG CTG TGC GTG AA |
|            | Reverse | GCT GGA GTC GTA GGC CAA GT |
| α-SMA | Forward | GCATCAGCGAAACACCTA |
|            | Reverse | CAGAGTAAAGCAATCAAGC |
| MYH7 | Forward | ATG TGC CGG ACC TTC GAA |
|            | Reverse | CCT GGG GTT AGC TGA GAG ATC A |
| TRPC-6 | Forward | GGC GGC TCT CTA AAG GCT G |
|            | Reverse | TGG GGT AGT AGC CAT ACG GTG |

| Human primers | Type I collagen | Forward | GTC GAG GGC CAA GAC GAC GAG |
|              | Reverse | CAG ACG TCA TCG CAC AAC |
| Calcineurin (CN-PP) | Forward | TGGATCAATTCTCGACAGG |
|              | Reverse | AAGGCCCACAAAATACACCAC |
| α-SMA | Forward | CGGACGGAATGCGAAGGA |
|            | Reverse | ACAGATTTTTGGGCGCGA |
| TRPC-6 | Forward | GGGTCAAGGAGCATCTGGAAT |
|            | Reverse | TGGAGTCACATCATGGGAGA |

α-SMA indicates alpha-smooth muscle antigen; TRPC-6, transient receptor protein channel 6.
attenuated TGF-β1-mediated increases in protein and mRNA levels of calcineurin and α-SMA (Figure 1A through 1C). TGF-β1 stimulation also increased TRPC-6 mRNA expression in human RVFB, which was prevented by cyclosporine treatment (Figure 1D). To examine the role of TRPC-6 in RV myofibroblast transformation, we employed a siRNA against TRPC-6 (siTRPC-6), which achieved a greater than 75% knockdown of TRPC-6 protein expression in RVFB (Figure 1E). Silencing TRPC-6 attenuated TGF-β1-mediated up-regulation of calcineurin and α-SMA in human RVFB (Figure 1F). These data indicate that TGF-β1 increases expression of TRPC-6 and α-SMA in a calcineurin-dependent manner in human RV fibroblasts.

Next, to explore the dependence of calcineurin expression on endoglin in human RVFB and LVFB, cells were treated with TGF-β1 in the presence of increasing concentrations of an N-Eng Ab (Figure 2A through 2B). Neutralizing endoglin activity blocked TGF-β1-induced calcineurin and α-SMA protein expression in human RVFB, not LVFB. Neutralizing endoglin attenuated pSmad3 expression in both human RVFB

Table 2. Characterization of Right Ventricular Pressure Overload Induced by Sugen and Hypoxia in Wild-Type and Eng+/− Mice

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Su-Norm</td>
<td>Su-Hypox</td>
<td>Su-Norm</td>
<td>Su-Hypox</td>
</tr>
<tr>
<td>Total body weight, g</td>
<td>27±2</td>
<td>27±1</td>
<td>29±2</td>
<td>28±2</td>
</tr>
<tr>
<td>RV weight/tibial length, g/mm</td>
<td>1.2±0.4</td>
<td>1.4±0.4</td>
<td>1.4±0.1</td>
<td>1.5±0.1</td>
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<tr>
<td>LV weight/tibial length, g/mm</td>
<td>4.8±3</td>
<td>4.4±3</td>
<td>4.9±2</td>
<td>5.3±1</td>
</tr>
<tr>
<td>Hemodynamic variables</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV systolic pressure, mm Hg</td>
<td>23±2</td>
<td>36±2</td>
<td>24±4</td>
<td>34±3</td>
</tr>
<tr>
<td>RV end-diastolic pressure (mm Hg)</td>
<td>2±1</td>
<td>3±1</td>
<td>3±3</td>
<td>2±2</td>
</tr>
<tr>
<td>RV +dp/dt, mm Hg/sec</td>
<td>2259±217</td>
<td>3203±456*</td>
<td>2476±257</td>
<td>2924±156*</td>
</tr>
<tr>
<td>RV –dp/dt, mm Hg/sec</td>
<td>2162±149</td>
<td>3212±642</td>
<td>2333±418</td>
<td>3100±493</td>
</tr>
<tr>
<td>RV stroke volume, μL</td>
<td>7±3</td>
<td>8±3</td>
<td>8±1</td>
<td>8±2</td>
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<tr>
<td>Cardiac output, mL/min</td>
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<td>3898±1670</td>
<td>4150±1345</td>
<td>3995±1529</td>
</tr>
<tr>
<td>Heart rate, beats per min</td>
<td>507±37</td>
<td>504±28</td>
<td>514±52</td>
<td>506±53</td>
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</tbody>
</table>

LV indicates left ventricular; RV, right ventricular.

*P<0.01 versus Su-Norm (n=6/group).

Table 3. Characterization of Right Ventricular Pressure Overload Induced by Severe Pulmonary Artery Constriction in Wild-Type and Eng+/− Mice

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=6)</td>
<td>PAC (n=7)</td>
<td>Sham (n=6)</td>
<td>PAC (n=8)</td>
</tr>
<tr>
<td>Total body weight, g</td>
<td>35±2</td>
<td>24±2*</td>
<td>34±4</td>
<td>28±2</td>
</tr>
<tr>
<td>RV weight/tibial length, g/mm</td>
<td>1.4±0.1</td>
<td>3±0.1*</td>
<td>1.7±0.3</td>
<td>2.3±0.1</td>
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<tr>
<td>LV weight/tibial length, g/mm</td>
<td>6±0.4</td>
<td>4±0.3*</td>
<td>5±0.2</td>
<td>4±0.3*</td>
</tr>
<tr>
<td>Hemodynamic variables</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV systolic pressure, mm Hg</td>
<td>21±6</td>
<td>50±4*</td>
<td>24±3</td>
<td>46±9</td>
</tr>
<tr>
<td>RV end-diastolic pressure, mm Hg</td>
<td>4±2</td>
<td>8±4</td>
<td>2±1</td>
<td>4±2</td>
</tr>
<tr>
<td>RV +dp/dt, mm Hg/sec</td>
<td>2358±392</td>
<td>3328±1163*</td>
<td>2064±343</td>
<td>3517±1118*</td>
</tr>
<tr>
<td>RV –dp/dt, mm Hg/sec</td>
<td>2514±187</td>
<td>2613±849</td>
<td>2079±341</td>
<td>2715±622*</td>
</tr>
<tr>
<td>RV stroke volume, μL</td>
<td>9±3</td>
<td>4±1*</td>
<td>8±2</td>
<td>7±1†</td>
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<tr>
<td>Cardiac output, mL/min</td>
<td>5±1</td>
<td>2±1*</td>
<td>4±1</td>
<td>4±1†</td>
</tr>
<tr>
<td>Heart rate, beats per min</td>
<td>540±62</td>
<td>532±51</td>
<td>509±13</td>
<td>521±24</td>
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</tbody>
</table>

PAC indicates pulmonary artery constriction; LV, left ventricular; RV, right ventricular.

*P<0.01 versus sham; †P<0.01 versus wild-type PAC.
Table 4. Characterization of Right Ventricular Pressure Overload Induced by Severe Pulmonary Artery Constriction in Wild-Type Mice Treated With Either a Neutralizing Antibody Against Endoglin (N-Eng Ab) or IgG-Isotype Control Antibody (IgG)

<table>
<thead>
<tr>
<th></th>
<th>Wild Type Sham</th>
<th>Wild Type Sham</th>
<th>Wild Type N-Eng Ab Sham</th>
<th>Wild Type N-Eng Ab Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight, g</td>
<td>29±2</td>
<td>23±2*</td>
<td>28±1</td>
<td>24±2*</td>
</tr>
<tr>
<td>RV weight/tibial length, g/mm</td>
<td>1.5±0.01</td>
<td>2.5±0.01*</td>
<td>1.5±0.01</td>
<td>1.9±0.01*</td>
</tr>
<tr>
<td>LV weight/tibial length, g/mm</td>
<td>6±0.01</td>
<td>4±0.01*</td>
<td>6±0.01</td>
<td>5±0.02*</td>
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<tr>
<td>Hemodynamic variables</td>
<td></td>
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</tr>
<tr>
<td>RV systolic pressure, mm Hg</td>
<td>22±3</td>
<td>48±4*</td>
<td>24±3</td>
<td>53±9*</td>
</tr>
<tr>
<td>RV end-diastolic pressure, mm Hg</td>
<td>4±1</td>
<td>7±4</td>
<td>3±2</td>
<td>4±2</td>
</tr>
<tr>
<td>RV +dp/dt, mm Hg/sec</td>
<td>2374±429</td>
<td>3189±982</td>
<td>2171±283</td>
<td>4130±563*</td>
</tr>
<tr>
<td>RV –dp/dt, mm Hg/sec</td>
<td>2419±304</td>
<td>2810±891</td>
<td>1963±257</td>
<td>3287±350*</td>
</tr>
<tr>
<td>RV stroke volume, µL</td>
<td>8±3</td>
<td>4±1*</td>
<td>8±2</td>
<td>5±1*</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>4.3±1</td>
<td>1.8±1*</td>
<td>4.0±1</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Heart rate, beats per min</td>
<td>538±25</td>
<td>548±33</td>
<td>512±59</td>
<td>541±52</td>
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</table>

PAC indicates pulmonary artery constriction; LV, left ventricular; RV, right ventricular.
*P<0.01 versus sham; †P<0.01 versus wild-type PAC (n=6/group).

and LVFB. To further examine the role of endoglin as a positive regulator of calcineurin in RVFB and LVFB, cells were isolated from WT and Eng+/− mice (Figure 2C through 2D). In WT RVFB and LVFB, TGF-β1 stimulated calcineurin and α-SMA mRNA expression, which was prevented in Eng+/− RVFB, not LVFB (Figure 2C). TGF-β1 also up-regulated protein levels of calcineurin and α-SMA in WT RVFB and LVFB, which was attenuated in Eng+/− RVFB, but not in Eng+/− LVFB (Figure 2D). These findings support that endoglin is required for TGF-β1-induced calcineurin expression and myofibroblast transformation in RVFB, a critical component of cardiac fibrosis.

Reduced Endoglin Expression Preserves RV Function and Limits RV Fibrosis in a Model of Angio-Obliterative Pulmonary Hypertension

To begin exploring a functional role for endoglin in RV remodeling, we studied the well-established model of angio-obliterative pulmonary hypertension induced by exposure to...
hypoxia and the anti-vascular endothelial growth factor compound, Sugen, in WT, compared to Eng+/−/C0 mice. All mice survived treatment with Sugen and Hypoxia for 5 weeks, and no significant change in total body weight, RV or LV weights, RV stroke volume, or cardiac output was observed between groups (Table 2). We observed increased RV systolic pressure (RVSP) in both WT and Eng+/−/C0 mice after 5 weeks of exposure to Sugen and Hypoxia (Figure 3A). No difference in RV dP/dtmax was observed between WT and Eng+/−/C0 groups treated with Sugen and Hypoxia, demonstrating a similar response to RVPO in both types of mice. WT mice exposed to Sugen and Hypoxia developed evidence of abnormal diastolic RV function, including increased Tau (a measure of instantaneous isovolumic relaxation) and decreased RV compliance (Figure 3B and 3C), whereas Eng+/−/ mice demonstrated no change in Tau and relatively preserved RV compliance. To explore the mechanism for the differences in RV diastolic function, we examined RV fibrosis and calcineurin signaling. Exposure to Sugen and Hypoxia increased type I collagen mRNA expression and histologic levels of collagen abundance in WT, not Eng+/−/ mice (Figure 3D through 3F). Calcineurin, TRPC-6, and α-SMA mRNA levels were increased by Sugen and Hypoxia in WT, not Eng+/−/ mice (Figure 3G through 3I). These findings suggest that, despite identical degrees of RVPO, reduced endoglin expression in Eng+/−/ mice preserved indices of RV diastolic function, limited RV collagen accumulation, attenuated up-regulation of calcineurin and TRPC-6, and limited myofibroblast transformation in the RV.

Reduced Endoglin Expression Preserves RV Function and Improves Survival in RVPO

Given the limited degree of RVF observed in the Sugen and Hypoxia model, we next explored the functional role of endoglin in a surgical model of severe RVPO induced by surgical constriction of the pulmonary artery (PAC) for 7 days in WT and Eng+/−/ mice. Compared to WT, baseline RV endoglin expression was lower in Eng+/−/ mice (Figure 4A and 4B). Compared to sham controls, PAC increased endoglin levels in the RV in WT mice, suggesting a direct effect of RVPO on endoglin expression. RVPO also increased endoglin expression in Eng+/−/ mice, but levels were significantly lower, compared to WT mice (Figure 4A and 4B). We next examined the functional impact of reduced endoglin levels in RVPO. Despite equally increased RVSP in both WT and Eng+/−/ mice after PAC, RV stroke volume was decreased in WT, but
Figure 2. Reduced endoglin activity limits calcineurin expression and myofibroblast transformation in right ventricular fibroblasts. A and B, Western blots showing calcineurin, α-SMA, pSmad3, and GAPDH levels in fibroblasts from human right (RVFB) and left (LVFB) ventricular fibroblasts after TGF-β1 stimulation in the presence and absence of increasing concentrations of a neutralizing endoglin antibody (N-Eng Ab). Quantification of calcineurin and α-SMA levels in human RVFB and LVFB (n=3/group). C, mRNA levels of calcineurin and α-SMA in right (RVFB) and left (LVFB) ventricular fibroblasts derived from WT and Eng+/− mice after TGF-β1 stimulation (n=6/group). D, Western blots showing calcineurin and α-SMA levels after TGF-β1 stimulation in RVFB and LVFB from WT and Eng+/− mice. D and E, Quantification of calcineurin and α-SMA protein levels in RVFB and LVFB from WT and Eng+/− mice stimulated with TGF-β1. *P<0.05 versus vehicle; †P<0.05 versus TGF-β1 stimulation; ‡P<0.05 versus LVFB+TGF-β1 stimulation. α-SMA indicates α-smooth muscle antigen; TGF-β1, transforming growth factor beta 1; WT, wild type.
not Eng\textsuperscript{+/−}, mice (Figure 4C through 4D). WT mice also manifested reduced total body weight after RVPO, whereas Eng\textsuperscript{+/−} mice did not (Figure 4E). Eng\textsuperscript{+/−} mice demonstrated substantially improved survival (100% vs. 58%, respectively; \( P=0.01 \)), compared to WT mice after PAC (Figure 4F). These findings suggest that, despite identical degrees of RVPO, reduced endoglin expression in Eng\textsuperscript{+/−} mice preserved RV function and improved survival.

**Reduced Endoglin Expression Limits RV Fibrosis and Signaling by TGF-β1 and Calcineurin in RVPO**

To study the mechanism underlying improved survival in Eng\textsuperscript{+/−} mice, we first examined changes in RV structure. RVPO increased RV mass in WT, but not Eng\textsuperscript{+/−}, mice (Table 3). RVPO increased RV fibrosis in WT, but not Eng\textsuperscript{+/−}, mice (Figure 5A and 5B). RV cardiomyocyte cross-sectional area was also increased in both WT and Eng\textsuperscript{+/−} mice after RVPO, but the degree of hypertrophy was lower in Eng\textsuperscript{+/−} mice (Figure 5C and 5D). These findings suggest that endoglin regulates changes in RV structure in response to RVPO.

Next, we studied TGF-β1 signaling in RVPO. Despite equally increased active TGF-β1 protein levels in WT and Eng\textsuperscript{+/−} mice (Figure 6A), levels of type I collagen, pSmad3, and pERK1/2 were increased in WT mice, but not Eng\textsuperscript{+/−} mice (Figure 6B through 6D). We observed reduced levels of calcineurin protein expression in the RV from Eng\textsuperscript{+/−} mice,
compared to WT, after RVPO (Figure 6E). Levels of downstream targets of calcineurin activity, including MYH7 and TRPC-6, were also reduced in Eng+/− mice, compared to WT, after RVPO (Figure 6F and 6G). Levels of α-SMA mRNA were also increased in WT, but not Eng+/−, mice after RVPO, indicating reduced fibroblast-to-myofibroblast conversion in Eng+/− mice (Figure 6H). To further explore whether endoglin regulates calcineurin activity, RVPO was induced in Eng+/−-NFAT-Luc and Eng+/−-NFAT-Luc mice. RVPO increased luciferase activity in total RV lysates from Eng+/−-NFAT-Luc, not Eng+/−-NFAT-Luc, mice (Figure 7). These observations suggest that, in addition to regulating canonical and noncanonical TGF-β1 pathways that promote cardiac fibrosis, reduced endoglin levels in the RV limit calcineurin expression and activity, including myofibroblast transformation. These findings support an important role for endoglin-mediated regulation of TGF-β1 and calcineurin activity in RV remodeling.

Neutralizing Endoglin Activity Prevents RV Fibrosis and Improves Survival in RVPO

To further explore the role of endoglin in mediating calcineurin expression in RVPO, WT mice were pretreated with a N-Eng Ab or control IgG before induction of severe RVPO by PAC. Treatment with the N-Eng Ab improved survival after 7 days of severe RVPO, compared to treatment with the IgG control (Figure 8A). Despite equally increased RVSPs in both groups (Table 4), RV fibrosis was increased in WT mice treated with the IgG control, but not in mice treated with N-Eng Ab after severe RVPO (Figure 8B and 8C). RV cardiomyocyte cross-sectional area was also increased in both groups after PAC, but less cardiomyocyte hypertrophy was observed in WT mice receiving the N-Eng Ab (Figure 8D). RV mass was also increased in both groups, but the degree of hypertrophy was attenuated in N-Eng Ab-treated mice after RVPO (Table 4). Consistent with observations in Eng+/− mice, WT mice treated with the N-Eng Ab showed reduced protein levels of type I collagen, pSmad3, pERK1/2, and calcineurin levels in the RV, compared to the IgG group after RVPO (Figure 8E through 8H). Downstream targets of calcineurin, including mRNA levels of MYH7, TRPC-6, and α-SMA, were also reduced in the N-Eng Ab group, compared to the IgG group, after RVPO (Figure 8I through 8K).

Neutralizing Endoglin Activity Reverses RV Fibrosis in Established RVPO

To study the potential clinical utility of blocking endoglin activity as an approach to reduce RV fibrosis after established RVPO, WT mice subjected to moderate RVPO for 3 weeks were randomized to receive either the N-Eng Ab or IgG control for an additional 3 weeks (Figure 9A). After 3 weeks of
moderate RVPO, total body weight was reduced, whereas RV mass and systolic pressure were increased and RV stroke volume decreased, compared to sham controls (Table 5). RV fibrosis, type I collagen, and calcineurin expression were also increased, compared to sham controls (Figure 9B through 9F). After an additional 3 weeks (6 weeks total) of moderate RVPO, both IgG- and N-Eng Ab-treated groups had persistently increased RV mass and RVSP with reduced cardiac output (Table 5). No mortality was observed after moderate RVPO in either group at any time point. RV fibrosis progressively worsened in mice treated within the IgG group, but was significantly reduced in the N-Eng Ab-treated group (Figure 9B and 9C). Type I collagen and calcineurin protein expression also increased progressively in the IgG group, but were reduced in the N-Eng Ab group (Figure 9D through 9F). These findings support that blocking endoglin activity reverses established RV fibrosis in chronic RVPO in mice.

Discussion

This is the first report to establish a role for endoglin in RV remodeling. Our central finding is that endoglin modulates TGF-β1 signaling through canonical, noncanonical, and calcineurin-mediated pathways in the RV and could be a novel therapeutic target to limit RV fibrosis and improve survival in diseases characterized by RVPO (Figure 10). We report several novel findings: (1) Endoglin is necessary for TGF-β1-induced increase in expression of TRPC-6 and α-SMA by a calcineurin-dependent mechanism in human RV fibroblasts; (2) TRPC-6 mediates a feedback loop promoting calcineurin expression and myofibroblast transformation in human RV fibroblasts that is also dependent on endoglin; (3) in Eng−/− mice exposed to Sugen+Hypoxia, reduced endoglin activity improved RV diastolic function, limited fibrosis, and attenuated expression of calcineurin, TRPC-6, and α-SMA; (4) in the most severe model of surgical pressure overload, reduced endoglin activity, induced either by genetic means or by treatment with a neutralizing Ab, improved survival, reduced RV fibrosis, and limited TGF-β1 signaling through canonical, noncanonical, and calcineurin-mediated pathways in the RV; and (5) in mice with established RV fibrosis, neutralizing endoglin activity reversed RV fibrosis and attenuated expression of both type I collagen and calcineurin. Given the importance of calcineurin and TRPC-6 in adaptive and

Figure 5. Reduced endoglin expression limits RV fibrosis and hypertrophy after right ventricular pressure overload. A and B, Representative histologic staining for RV collagen abundance in WT and Eng−/− mice after PAC. Quantification of RV fibrosis after PAC is shown (n=6/group). C and D, Representative histological staining for RV cardiomyocyte hypertrophy in WT and Eng−/− mice after PAC. Quantification of cardiomyocyte cross-sectional area after PAC is shown (n=6/group). *P<0.05 versus sham; †P<0.05 versus WT-PAC. PAC indicates pulmonary artery constriction; RV, right ventricular; WT, wild type.
maladaptive cardiac remodeling, these findings identify endoglin as a regulator of TGF-β1-signaling cascades involved in RV remodeling and further show that targeting endoglin activity may improve RV function in HF or lung disease.

Previous studies of TGF-β1 activity in cardiac remodeling have focused on LVF; yet, TGF-β1 signaling in the RV remains largely unexplored. A majority of our understanding of the mechanisms governing RV remodeling stem primarily from data generated in models of LVF. However, substantial differences between the RV and LV exist that support the potential for the two ventricles to have distinct responses to injury, including: (1) the developmental origin of the RV from a heart field distinct from the LV; (2) a thin RV free wall with susceptibility to increased wall stress; (3) a greater dependence of the RV stroke volume on afterload; and (4) enhanced RV contractile resilience to pressure overload. We recently reported that endoglin expression is increased in the LV of patients with LHF and is restricted to LV fibroblasts and endothelial cells, as opposed to cardiomyocytes. We also showed that reduced endoglin expression attenuates TGF-β1 signaling through Smad2/3, limits LV fibrosis, and preserves cardiomyocyte hypertrophy in a murine model of LVF induced by thoracic aortic constriction. The net effect of this adaptive LV remodeling pattern was improved survival and preserved cardiac function in Eng+/−/C0, compared to Eng+/+, mice after chronic LHF. In this study, reduced endoglin expression had no effect on LV expression of calcineurin. Despite all that is known in the LV, regulation of profibrotic signaling in the RV remains poorly understood and the role of endoglin in the RV has never been studied.
These new studies exploring the role of endoglin in the RV response to pressure overload reveal that, although some similarities exist with the LV, there are also pathways unique to endoglin’s role in the RV. Indeed, endoglin limited TGF-β1 signaling by Smad3 and ERK1/2 in both ventricles; however, in contrast to our previous observations in the LV, we now report that endoglin regulates TGF-β1-induced calcineurin expression and activity in the RV. Irrespective of the mechanism for RVPO, we uniformly observed that reduced endoglin activity attenuated calcineurin expression and activity, as evidenced by reduced levels of downstream targets of calcineurin activity, including MYH7 and TRPC-6. These observations open an exciting avenue for exploration of endoglin-dependent signaling involving two potent cardiac remodeling pathways, namely, TGF-β1 and calcineurin.

The TRPC family of Ca^{2+} permeable channels includes 7 members and can increase intracellular calcium levels ([Ca^{2+}]_i), which activates calcineurin expression in fibroblasts and promotes myofibroblast transformation. \(^45^,\(^46\) Several previous reports have established that TRPC-6 amplifies pathological signaling by participating in a self-propagating feed-forward circuit mediated by calcineurin activity and is therefore a potentially important target of therapy in cardiac remodeling. \(^20^,\(^26^,\(^47\) No studies to date have examined the functional role of TRPC-6 in RVPO. We now introduce a novel signaling pathway involving endoglin-dependent regulation of calcineurin-mediated myofibroblast transformation in the pressure-overloaded RV (Figure 9).

In this report, we studied both pharmacologically and surgically induced models of RVPO. Existing models of pulmonary hypertension include treatment with Sugen, intratracheal bleomycin, monocrotaline, and hypoxia. Each of these models confirms that RVPO is associated with RV hypertrophy and fibrosis. \(^48^–\(^50\) More recent clinical data from
patients with pulmonary hypertension also confirm that RV dysfunction resulting from pressure overload is dominated by a loss of diastolic properties as a result of RV fibrosis and hypertrophy.\textsuperscript{3,51,52} Using a conductance catheter inserted into the RV of closed-chest mice after treatment with Sugen under hypoxic conditions, we observed abnormal diastolic

**Figure 9.** Neutralizing endoglin activity reverses cardiac fibrosis after chronic right ventricular pressure overload. A, Schematic of randomization to treatment with a N-Eng Ab or IgG control Ab after 3 weeks of PAC in WT mice. B and C, Quantification of RV fibrosis and representative histologic staining for RV collagen abundance in IgG- versus N-anti-Eng Ab-treated mice after moderate RVPO (n=6/group). D through F, Quantification of type I collagen and calcineurin protein levels in WT mice after moderate RVPO for 3 and 6 weeks in the presence and absence of either an IgG control Ab or N-Eng Ab. Representative western blots are shown. *P<0.05 versus sham; †P<0.05 versus 3 weeks RVPO; ‡P<0.05 versus 6 weeks RVPO-IgG. PAC indicates pulmonary artery constriction; RV, right ventricular; RVPO, RV pressure overload; WT, wild type.

**Figure 10.** Reduced endoglin activity limits TGF-\(\beta\)-induced calcineurin expression and myofibroblast transformation in right ventricular fibroblasts. Postulated mechanism by which endoglin promotes RV fibrosis by facilitating TGF-\(\beta\) signaling in response to pressure overload through canonical and noncanonical pathways, including calcineurin-mediated myofibroblast transformation in RVFB. In contrast, reduced endoglin activity attenuates TGF-\(\beta\) signaling through canonical, noncanonical, and calcineurin pathways and limits myofibroblast transformation and fibrosis, thereby improving survival. \(\alpha\)-SMA indicates alpha-smooth muscle antigen; RV, right ventricular; RVBF, right ventricular fibroblasts; TGF-\(\beta\), transforming growth factor-beta; TRPC-6, transient receptor protein channel 6.
properties with relative preserved instantaneous contractile force, as measured by dP/dtmax. In Eng<sup>−/−</sup> mice, these diastolic abnormalities were attenuated. To further explore the specific effects of pressure overload on RV remodeling, we then uncoupled the RV from the pulmonary vasculature by surgically banding the pulmonary artery and creating either severe or moderate RVPO. Severe RVPO led to early mortality, exuberant RV fibrosis and hypertrophy, which were all improved in Eng<sup>−/−</sup> mice or in WT mice treated with a neutralizing endoglin Ab.

To test the translational potential of targeting endoglin in RVF, we employed an N-Eng Ab, which binds human and mouse endoglin with high avidity and is being studied in phase II clinical trials in oncology. We now introduce data from numerous tissues, the off-target effects of blocking endoglin b1-signaling pathways affect various biologic processes in the RV and to target calcineurin and TRPC6 activity in models of RV injury. 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, because of the fact that mortality was not increased with moderate PA constriction or treatment with Sugen+Hypoxia, we were technically unable to evaluate the effect of reducing endoglin activity on survival in these models. Third, future studies are required to examine the source of calcineurin expression in the RV and to target calcineurin and TRPC6 activity in models of RV injury. In conclusion, RHF is a significant global health problem and a primary mode of death for millions of individuals suffering from HF or lung disease, yet therapies specifically targeting RV dysfunction do not exist. We have now identified a paradigm that implicates endoglin as a master regulator of TGF-β1 signaling through canonical, noncanonical, and now calcineurin-mediated pathways involving TRPC6 in the RV. These findings support that endoglin could potentially be a novel therapeutic target to limit RV fibrosis and improve survival in disease states characterized by RVPO. Further study is required to explore the translational potential of this paradigm involving endoglin-dependent signaling in the RV.

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**Disclosures**

None.

**References**


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ORIGINAL RESEARCH

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The authors regret this error.

The online version of the article has been updated and is available at http://jaha.ahajournals.org/content/3/4/e000965.
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