 Syndecan-1 Amplifies Angiotensin II–Induced Cardiac Fibrosis

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Abstract—Syndecan-1 (Synd1) is a transmembrane heparan sulfate proteoglycan that functions as a coreceptor for various growth factors and modulates signal transduction. The present study investigated whether Synd1, by affecting growth factor signaling, may play a role in hypertension-induced cardiac fibrosis and dysfunction. Expression of Synd1 was increased significantly in mouse hearts with angiotensin II–induced hypertension, which was spatially related to cardiac fibrosis. Angiotensin II significantly impaired fractional shortening and induced cardiac fibrosis in wild-type mice, whereas these effects were blunted in Synd1-null mice. Angiotensin II significantly increased cardiac expression of connective tissue growth factor and collagen type I and III in wild-type mice, which was blunted in Synd1-null mice. These findings were confirmed in vitro, where angiotensin II induced the expression of both connective tissue growth factor and collagen I in fibroblasts. The absence of Synd1 in either Synd1-null fibroblasts, after knockdown of Synd1 by short hairpin RNA, or after inhibition of heparan sulfates by protamine attenuated this increase, which was associated with reduced phosphorylation of Smad2. In conclusion, loss of Synd1 reduces cardiac fibrosis and dysfunction during angiotensin II–induced hypertension. (Hypertension. 2010;55:249-256.)

Key Words: heart failure ■ cardiac fibrosis ■ syndecan-1 ■ angiotensin II ■ CTGF

Matrix accumulation, collagen deposition, and stiffening hallmark the adverse remodeling in chronically overloaded myocardium. The underlying mechanisms are not yet fully elucidated, but neurohormones and growth factors, such as angiotensin II (AngII), transforming growth factor-β1 (TGFβ1), and connective tissue growth factor (CTGF) are associated with enhanced synthesis of collagens and other matrix proteins and have been implicated in the development of such adverse structural changes. Apart from increasing blood pressure, AngII may also initiate adverse signaling in the heart, thereby causing cardiac hypertrophy and cardiac fibrosis. The profibrotic signaling caused by AngII is importantly mediated via activation of the TGFβ1 system and concomitant activation of CTGF expression. AngII induces the production of TGFβ1, CTGF, and the expression of endoglin, a TGFβ1 receptor, in cardiac fibroblasts (CFs), so that blockade of either TGFβ1 signaling or of CTGF effectively decreases AngII-induced fibrosis.

Syndecans are heparan sulfate proteoglycans, which are present at the cell membrane where they modulate cell-matrix interactions. They are able to bind to extracellular matrix proteins and mediate the signaling of growth factors, such as fibroblast growth factor and TGFβ1. Also, AngII-mediated signaling can be modulated by heparan sulfates. However, the precise role of syndecans in pathological remodeling of the hypertensive heart is unknown. Given its involvement in signal transduction modulation, we hypothesized that syndecan-1 (Synd1) is involved in AngII-induced cardiac remodeling by affecting growth factor–mediated signaling and secondary fibrosis.

Here, we show that the absence of Synd1 in mice protects against AngII-induced cardiac dysfunction and fibrosis. These findings were confirmed in vitro, where short-hairpin RNA (shRNA)–mediated reduction of Synd1 or protamine-mediated loss of Synd1 heparan sulfates significantly attenuates the AngII-induced increase of CTGF and collagen I (Col1) in CFs. Our data show that Synd1 is an important regulator in the development of cardiac fibrosis by mediating CTGF expression and, therefore, may be a new therapeutic target in pathological forms of cardiac fibrosis.

Materials and Methods

In Vivo Experiments

We used 14- to 20-week-old male inbred BALB/c Synd1-null mice and their wild-type (WT) littermates. Synd1-null mice were a gift from Mary-Ann Stepp (George Washington University Medical
AngII (1.5 mg/kg per day) was administered for 14 days via osmotic minipumps (ALZET osmotic minipumps; types 2ML4). At the end of the experiment, echocardiography was performed under 2% to 4% isofluorane anesthesia, as described. In a separate experiment, the BP response of Synd1-null and WT mice was investigated by applying increased dosages of AngII intravenously. Experiments were carried out according to the institutional guidelines of Maastricht University (Maastricht, The Netherlands) and Leuven University (Leuven, Belgium) for the experimental use of animals.

### Histology

After the study period, AngII-treated and sham-operated mice were anesthetized, and hearts were removed and prepared for further histological and molecular analyses. Immunohistochemistry for Synd1 was performed on paraffin sections using the 281-2 antibody (CD138; Pharmingen). Myocardial collagen was stained with picrosirius-red, as described. For the quantification of left ventricular collagen volume fraction, computerized planimetry was performed in ≥7 randomly selected fields per section per mouse.

### Cloning Lentiviral Synd1 Short Hairpin RNA-Expressing Constructs

Small-interfering RNA against Synd1 was designed by Eurogentec. From this design oligos with 1 hairpin loop (short hairpin RNA; shRNA) were created (Table 1). This vector (pLenti Lox 3.7) was modified from the pLenti Lox 3.7 by replacing the enhanced green fluorescent protein gene by puromycin. Next, the oligonucleotides were cloned into XhoI and HpaI sites of the vector pLenti Lox 3.7 puromycin. Constructs were verified by sequencing. Lentiviral production was performed by cotransfection of 3 μg of short hairpin Synd1/pLenti Lox 3.7 puromycin or empty pLenti Lox 3.7 puromycin packaging vectors into 293FT cells by Lipofectamine 2000 (Invitrogen), and virus-containing supernatant was harvested after 48 hours.

### Table 1. Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
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<tr>
<td>Synd1</td>
<td>CTTTTTGACAGGAAGGAAGTG</td>
<td>TCCTTCTCTCTACCCGGTACAG</td>
</tr>
<tr>
<td>Syndecan-2</td>
<td>ACAGAAGGTCTACGAGGCTTATT</td>
<td>CGGTGACCAATAGGAGATGAG</td>
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<tr>
<td>Syndecan-3</td>
<td>TCTCCTTGAGGCTTACCTTTTT</td>
<td>GGTTAGCTGCGTTCGCGTTTT</td>
</tr>
<tr>
<td>Syndecan-4</td>
<td>CCCAGGGACACATCTTT</td>
<td>AGGATACGAAAAACGAAA</td>
</tr>
<tr>
<td>CollI</td>
<td>TCTCCTTGAGGCTTACCTTTTT</td>
<td>GGTTAGCTGCGTTCGCGTTTT</td>
</tr>
<tr>
<td>CTGF</td>
<td>CAGAGGTGAGGGCGGGTTC</td>
<td>GATGAGCTTACCTCTCAGAAG</td>
</tr>
<tr>
<td>Tubulin</td>
<td>TGAGAAGATCTTGACACATC</td>
<td>ATCCTTATCCTCCCAAGAC</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>CAAAGGCTGACCAACACCAAA</td>
<td>GCCATACGACCCATACGCT</td>
</tr>
<tr>
<td>TGFβ</td>
<td>TCTGCTCACTACGGCAAGAGA</td>
<td>TGTTAGAGGGCAAGAAG</td>
</tr>
<tr>
<td>bFGF</td>
<td>GTATGCTGGCATGAAAGAAGCT</td>
<td>TCTGCTGACACCCATACGCT</td>
</tr>
<tr>
<td>ANF</td>
<td>ATGGACAGAGATTGAGGGCGAGT</td>
<td>TGACACCAACAGAAGCTAGAT</td>
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<tr>
<td>Endothelin 1</td>
<td>GCACCGGAGGCTGAGAATGG</td>
<td>GTGCAAGAAGTAGACCCCT</td>
</tr>
</tbody>
</table>

bFGF indicates basic fibroblast growth factor; ANF, atrial natriuretic factor; Fw, forward; Rev, reverse.

### Figure 1

A through E, Synd1 expression is increased in AngII-induced hypertension. A and B, Representative image of Synd1 expression in fibrotic regions of AngII-treated WT left ventricle. C and D, Synd1 protein detection was absent in Synd1-null hearts (C) and in the negative control (D). E, mRNA levels of Synd1 in sham and AngII-treated left ventricle (n=5; P<0.05). Bars represent 100 μm (A, C, and D) or 50 μm (B).
Adenoviral Overexpression of Synd1
Replciation-deficient adenoviruses containing rat Synd1 cDNA (AdSynd1) or the control R5 gene (AdR5), all under control of the cytomegalovirus promoter, were generated and produced as described.\textsuperscript{15} Adenoviral infection was performed with 10 infectious units per milliliter, as determined with the AdEasy viral titer kit (Stratagene).

CF Culture
DMEM and FBS were purchased from GIBCO BRL. Culture plates were obtained from Costar. CFs were isolated from 2-day-old neonatal Lewis rats. All of the experiments were performed on cells from the second passage. CFs were maintained in DMEM supplemented with 10% FBS along with 50 μg/mL of gentamicin and 100 U/mL of penicillin/streptomycin and were incubated at 37°C in a humidified chamber. CFs were allowed to attach and were then infected with lentivirus harboring shRNA against Synd1 or control lentivirus, facilitated by Sequabrene (Sigma).

After puromycin selection (3 μg/mL), cells were placed in low-serum medium (0.4% FBS) for 24 hours. CFs treated with Synd1 shRNA and WT CFs received AngII (1 μmol/L) or TGFβ1 (1 ng/mL) for 24 hours. For adenoviral transfection, cells were transfected with AdR5 (control virus) or AdSynd1 for 24 hours. Hereafter, CFs were placed on low-serum medium (0.4% FBS) for 24 hours. After synchronization, CF received AngII (1 μmol/L) or TGFβ1 (1 ng/mL) for 24 hours.

In another set of experiments, CFs were placed in low-serum medium (0.4% FBS) for 24 hours and were treated with protamine (100 ng/mL) for 1 hour before the addition of AngII (1 μmol/L) for 24 hours, after which the cells were harvested.

To investigate the effects of recombinant syndecan 4 and Synd1 (a kind gift of Pyong Park, Children’s Hospital Boston, Boston, MA), CFs were placed in low-serum medium (0.4% FBS) for 24 hours and were treated with protamine (100 ng/mL) for 1 hour before the addition of AngII (1 μmol/L) for 24 hours, after which the cells were harvested.

In a set of experiments, CFs were placed in low-serum medium (0.4% FBS) for 24 hours and were treated with protamine (100 ng/mL) for 1 hour before the addition of AngII (1 μmol/L) for 24 hours, after which the cells were harvested.

RNA Isolation and Expression
RNA was isolated from left ventricular tissue or CFs using the RNeasy mini kit (Qiagen) and stored in −80°C. RNA was reverse transcribed into cDNA using the iScript CDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was performed using iQ SYBR green supermix (BioRad) and primers designed with Primer Express Software (PE Applied Biosystems). Tubulin and cyclophilin were used as housekeeping genes. Primers used are shown in Table 1.

Western Blotting and Matrix Metalloproteinase Zymography
Western blotting was performed with the specific antibodies against CTGF (Genetex), Col1 (Rockland), total Smad2/3, and phospho-Smad2 (Cell Signaling Technology). To ensure equal loading, the same membranes were probed with GAPDH (RDI). Bands were visualized by enhanced chemiluminescence and quantified by the Quantity One Software (BioRad). Zymographic activity of matrix metalloproteinase (MMP)2 in sham and AngII-treated hearts was performed as described.\textsuperscript{15}

Statistical Analysis
The data are expressed as mean±SEM. In vitro data are derived from ≥2 different experiments performed in triplicate. Statistical analysis was performed using the Student t test. Differences between the groups were considered to be significant when P<0.05.

Results
Increased Expression of Synd1 After AngII Infusion in WT Mice
To investigate the involvement of Synd1 in AngII-induced cardiac dysfunction, we examined Synd1 expression in sham and AngII-treated WT mice. Immunohistochemistry showed more Synd1 protein in AngII-treated WT hearts (Figure 1A and 1B), predominantly in fibrotic regions, whereas no staining was present in fibrotic regions of Synd1-null mice (Figure 1C) or the negative control (Figure 1D). These results were confirmed by quantitative PCR, which revealed a significant increase of Synd1 mRNA in left ventricles of AngII-treated WT mice (Figure 1E).

Cardiac Fibrosis Is Attenuated in Synd1-Null Mice After AngII Infusion
Quantification of total collagen with a computer-assisted densitometric analysis showed a significantly increased collagen content after AngII treatment, in both Synd1-null and
WT mice, as compared with placebo-treated mice. However, Synd1-null mice display significantly less collagen in comparison with WT mice after AngII treatment (581.1 ± 92.2% versus 1018.3 ± 214.2%, respectively; P < 0.05; sirius red staining compared with WT sham (100%); Figure 2A through 2C). These findings were further confirmed by attenuated mRNA expression of Col1, collagen 3, and CTGF in the left ventricles of Synd1-null mice, as measured by quantitative PCR (Table 2), and were not attributable to an altered blood pressure response toward intravenous infusion of AngII in Synd1-null mice (Figure 2D). TGFβ1 and basic fibroblast growth factor mRNA levels did not significantly differ between Synd1-null and WT mice (Table 2). In addition, there were no significant differences in MMP2 activity between WT and Synd1-null mice (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Levels of left ventricular pSmad2 were slightly increased in AngII-treated WT mice in comparison with Synd1-null mice, although this did not reach statistical significance (Figure 3).

### Synd1-Null Mice Are Protected Against AngII-Induced Cardiac Dysfunction

The function of Synd1 during hypertension was examined by treating Synd1-null and WT mice with AngII. AngII treatment resulted in cardiac hypertrophy in both WT and Synd1-null mice (Table 3). Echocardiographic analysis at the end of the study revealed a significantly better systolic function in the Synd1-null mice treated with AngII, as compared with the AngII-treated WT mice (fractional shortening; Synd1-null 29.6 ± 2.8% versus WT 21.5 ± 2.8; n = 8 to 14; P < 0.05; Table 4).

### Synd1 Mediates CTGF and Col1 Expression After AngII Stimulation in CFs

The decrease in cardiac fibrosis in Synd1-null mice suggested that Synd1 may influence profibrotic signaling. Using lentiviral-mediated shRNA against Synd1, we reduced Synd1 mRNA in CFs (80% decrease in Synd1 mRNA levels) and examined the effect of this reduction on the expression of CTGF and Col1

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### Table 2. Transcript Levels of Growth Factor and Extracellular Matrix mRNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Synd1 WT Sham (n=6)</th>
<th>Synd1 KO Sham (n=4)</th>
<th>Synd1 WT + AngII (n=6)</th>
<th>Synd1 KO + AngII (n=10)</th>
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</thead>
<tbody>
<tr>
<td>TGFβ</td>
<td>1.0 ± 0.16</td>
<td>0.72 ± 0.11</td>
<td>1.42 ± 0.19</td>
<td>1.09 ± 0.16</td>
</tr>
<tr>
<td>bFGF</td>
<td>1.0 ± 0.15</td>
<td>1.2 ± 0.16</td>
<td>1.67 ± 0.13*</td>
<td>1.37 ± 0.13</td>
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<tr>
<td>Collagen 1</td>
<td>1.0 ± 0.08</td>
<td>0.77 ± 0.18</td>
<td>1.13 ± 2.98*</td>
<td>4.15 ± 1.27†</td>
</tr>
<tr>
<td>Collagen 3</td>
<td>1.0 ± 0.30</td>
<td>0.95 ± 0.09</td>
<td>10.7 ± 2.27*</td>
<td>4.99 ± 1.18†</td>
</tr>
<tr>
<td>CTGF</td>
<td>1.0 ± 0.12</td>
<td>1.19 ± 0.11</td>
<td>4.61 ± 0.46*</td>
<td>2.83 ± 0.29†</td>
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<tr>
<td>ANF</td>
<td>1.0 ± 0.21</td>
<td>1.24 ± 0.09</td>
<td>7.42 ± 1.1*</td>
<td>7.74 ± 1.83*</td>
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<tr>
<td>ET-1</td>
<td>1.0 ± 0.23</td>
<td>1.11 ± 0.23</td>
<td>2.16 ± 0.25*</td>
<td>1.64 ± 0.39</td>
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<td>Syn2</td>
<td>1.0 ± 0.07</td>
<td>1.08 ± 0.17</td>
<td>1.69 ± 0.24*</td>
<td>1.4 ± 0.17</td>
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<tr>
<td>Syn3</td>
<td>1.0 ± 0.18</td>
<td>1.05 ± 0.17</td>
<td>1.82 ± 0.28*</td>
<td>1.3 ± 0.24</td>
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<tr>
<td>Syn4</td>
<td>1.0 ± 0.07</td>
<td>1.08 ± 0.1</td>
<td>1.34 ± 0.2*</td>
<td>1.28 ± 0.29</td>
</tr>
</tbody>
</table>

Data are mean ± SD. KO indicates knockout; bFGF, basic fibroblast growth factor; ANF, atrial natriuretic factor; ET, endothelin; Syn, syndecan.

*P < 0.05 vs corresponding sham or WT sham.
†P < 0.05 vs WT AngII.

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### Table 3. General Parameters of WT and Synd1-Null Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=4)</th>
<th>WT AngII (n=14)</th>
<th>Synd1-Null (n=5)</th>
<th>Synd1-Null AngII (n=14)</th>
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</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>31 ± 0.4</td>
<td>23.2 ± 1.0*</td>
<td>24.4 ± 0.9*</td>
<td>20.9 ± 0.6*†</td>
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<tr>
<td>LW/BW</td>
<td>4.1 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>5.4 ± 0.2*</td>
<td>5.1 ± 0.2*</td>
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<tr>
<td>LW/BW</td>
<td>6.0 ± 0.2</td>
<td>6.9 ± 0.5</td>
<td>7.7 ± 0.8</td>
<td>8.0 ± 0.5</td>
</tr>
</tbody>
</table>

BW indicates body weight; LW, left ventricular weight; LW, lung weight.

*P < 0.05 vs corresponding sham or WT sham.
†P < 0.05 vs WT AngII.

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### Table 4. Functional Analysis of WT and Synd1-Null Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=4)</th>
<th>WT AngII (n=14)</th>
<th>Synd1-Null (n=5)</th>
<th>Synd1-Null AngII (n=14)</th>
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</thead>
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<tr>
<td>FS, %</td>
<td>30.3 ± 2.3</td>
<td>29.7 ± 1.2</td>
<td>21.5 ± 2.8*</td>
<td>29.6 ± 2.6†</td>
</tr>
<tr>
<td>LVIDd, cm</td>
<td>4.1 ± 0.1</td>
<td>3.7 ± 0.1*</td>
<td>4.1 ± 0.1</td>
<td>3.5 ± 0.1†</td>
</tr>
<tr>
<td>LVIDs, cm</td>
<td>2.8 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>2.5 ± 0.2†</td>
</tr>
<tr>
<td>IVSd, cm</td>
<td>0.93 ± 0.05</td>
<td>0.9 ± 0.04</td>
<td>1.09 ± 0.1</td>
<td>1.11 ± 0.05*</td>
</tr>
<tr>
<td>LVPWd, cm</td>
<td>0.92 ± 0.06</td>
<td>0.9 ± 0.04</td>
<td>1.1 ± 0.02*</td>
<td>1.12 ± 0.06*</td>
</tr>
</tbody>
</table>

Echocardiographic data were obtained at the end of the study. FS indicates fractional shortening; LVIDd, left ventricular diameter diastole; LVIDs, left ventricular diameter systole; IVSd, intraventricular septum diastole; LVPWd, left ventricular posterior wall diastole.

*P < 0.05 vs corresponding sham or WT sham.
†P < 0.05 vs WT AngII.

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### Figure 3. In vivo pSmad2 levels.

A. Representative Western blot indicating increased pSmad2 levels in AngII-treated WT and Synd1-null mice. B. Quantification of pSmad2 corrected for Smad2; n = 6 per group.
after AngII stimulation. As expected, CTGF and Col1 expressions in CF increased after 24 hours of AngII in control cells, as shown by Western blotting. This increase was significantly attenuated by Synd1 reduction (Figure 4A through 4C). We examined how Synd1 modulates profibrotic signaling in CFs. AngII is known to induce profibrotic signaling by recruiting Smad signaling.3,17 Interestingly, reduction in Synd1 significantly attenuated the increase in Smad2 phosphorylation in CFs treated with Synd1 shRNA after 24 hours of AngII treatment (Figure 4D and 4E).

TGFβ1 is an important mediator of AngII-induced profibrotic signaling. Therefore, we also examined the effect of Synd1 reduction on TGFβ1-induced profibrotic signaling. Interestingly, in concordance with the AngII stimulation experiments, Synd1 knockdown attenuated both CTGF and pSmad2 levels after TGFβ1 stimulation in CFs (Figure 5A through 5D). In contrast to the reduction of Synd1, adenoviral overexpression of Synd1 exaggerated the increase in CTGF and pSmad2 levels after 24 hours of AngII or TGFβ1 stimulation, indicating that Synd1 aug-

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**Figure 4.** Effects of AngII (1 μmol/L; 24 hours) treatment on Col1, CTGF, and pSMAD2 protein expression in WT CFs and CFs treated with Synd1 shRNA. A, Representative Western blots showing that Col1 and CTGF levels are significantly increased after AngII treatment in WT CFs, whereas reduction of Synd1 significantly attenuates this increase. B, Representative Western blots showing that pSMAD2 levels are significantly increased after AngII treatment in WT CFs, whereas reduction of Synd1 significantly attenuates this increase. C, Quantification of Col1 expression corrected for GAPDH. D, Quantification of CTGF expression corrected for GAPDH. E, Quantification of pSMAD2 expression corrected for SMAD2. *P<0.05; n=3 per experiment, quantification of 3 experiments.

**Figure 5.** Effects of TGFβ1 (1 ng/mL; 24 hours) treatment on CTGF and pSmad2 protein expression in WT CFs and CFs treated with Synd1 shRNA. A, Representative Western blots showing that CTGF levels are significantly increased after TGFβ1 treatment in WT CFs, whereas reduction of Synd1 significantly attenuates this increase. B, Representative Western blots showing that pSmad2 levels are significantly increased after TGFβ1 treatment in WT CFs, whereas reduction of Synd1 significantly attenuates this increase. C, Quantification of CTGF expression corrected for GAPDH. D, Quantification of pSmad2 expression corrected for Smad2. *P<0.05; n=3 per experiment, quantification of 3 experiments.
ments the fibrotic response after stimulation with profibrotic growth factors (Figure 6A through 6F).

**Synd1 Heparan Sulfates as Profibrotic Mediators**

To confirm the importance of Synd1 heparan sulfates in AngII-dependent Col1 and CTGF expression, we treated CFs with protamine, an agent that neutralizes heparin and heparan sulfate. The addition of protamine prevented the increase in CTGF and Col1 protein expression after AngII stimulation (Figure 7A through 7C). In another set of experiments, we treated CFs with recombinant mouse Synd1 ectodomain without heparan sulfate groups to examine whether the Synd1 ectodomain suffices to augment profibrotic signaling in CF. Interestingly, no differences in CTGF levels were observed after the addition of this recombinant Synd1, indicating that Synd1 heparan sulfates are importantly involved in profibrotic signaling (Figure 7D and 7E).

**Synd1-Null Dermal Fibroblast Display Decreased SMAD2 Phosphorylation, CTGF, and Coll Expression**

We confirmed the importance of Synd1 in mediating the expressions of CTGF and Coll in an independent in vitro model system using isolated Synd1-null and WT dermal fibroblasts; Synd1-null dermal fibroblasts expressed significantly less CTGF and Coll (Figure S2). Moreover, phosphorylated SMAD2 levels were significantly lower in Synd1-null fibroblasts. These results suggest that Synd1 is indeed important in the regulation of profibrotic protein expression.

**Discussion**

The main finding of this study is that Synd1, a heparan sulfate proteoglycan, amplifies AngII-mediated profibrotic signaling in vitro and aggravates cardiac dysfunction and cardiac fibrosis in vivo. This demonstrates for the first time that Synd1 is involved in pathological hypertensive cardiac remodeling. First, we dem-

**Figure 6.** Effects of AngII treatment (1 μmol/L; 24 hours) or TGFβ (1 ng/mL; 24 hours) on CTGF and pSmad2 expression in WT and AdSynd1-treated WT CFs. A, Representative Western blot showing that CTGF levels are significantly increased in WT CFs, whereas overexpressing of Synd1 significantly exaggerates this increase after AngII stimulation. B, Representative Western blot showing that CTGF levels are significantly increased in WT CFs, whereas overexpressing of Synd1 significantly exaggerates this increase after TGFβ stimulation. C, Representative Western blot showing that pSmad2 levels are significantly increased in WT CFs after AngII or TGFβ treatment, whereas overexpressing of Synd1 significantly exaggerates this increase. CTGF and pSmad2 expressions are compared with controls of the same group. D through F, Quantification of CTGF (D and E) and pSmad2 (F) levels, corrected for GAPDH and total Smad2, respectively. *P<0.05; n=3 per experiment, quantification of 2 experiments.
transmembrane functions. Synd1 has been shown to regulate signals intracellularly. They, therefore, seem to be able with a short cytoplasmic tail, which may serve to directly promote signaling molecules and guide them to receptors on the membrane. Second, they contain a transmembrane domain may trap signaling molecules and guide them to receptors on the membrane. Synd1 contains an extracellular ectodomain with attached glycosaminoglycan chains, mainly consisting of heparan sulfates. This extracellular domain can be regarded as a “sticky” part protruding into the ECM, where it may trap signaling molecules and guide them to receptors on the membrane. Synd1-mediated signaling by AngII and TGFβ1 in CFs, because a reduction of Synd1 reduced the extent to which growth factors induced phosphorylation of Smad2, a major downstream signaling molecule. In concordance with these results, inhibition of heparan sulfates, which are present on the Synd1 ectodomain with protamine, also decreased CTGF and Col1 protein expressions in CFs after AngII treatment. In addition, stimulation with recombinant Synd1 lacking heparan sulfates did not affect CTGF levels. Finally, Synd1-null dermal fibroblasts displayed decreased CTGF and Col1 expression in comparison with WT fibroblasts, accompanied by decreased Smad phosphorylation. Together, these data strongly suggest that Synd1 is important in the expression of the fibrotic proteins CTGF and Col1.

Syndecans are intriguing molecules, which seem to combine 2 major properties. First, they contain an extracellular ectodomain with attached glycosaminoglycan chains, mainly consisting of heparan sulfates. This extracellular domain can be regarded as a “sticky” part protruding into the ECM, where it may trap signaling molecules and guide them to receptors on the membrane. Second, they contain a transmembrane domain with a short cytoplasmic tail, which may serve to directly promote signals intracellularly. They, therefore, seem to be able to act both in the ECM, and yet at the same time may also have transmembrane functions. Synd1 has been shown to regulate basic fibroblast growth factor–dependent signaling, and, recently, Hayashida et al19 and Kato et al20 showed its involvement in TGFβ1-dependent signaling. Also, heparan sulfates are able to regulate AngII-mediated signaling.11 How Synd1 exactly modulates AngII signaling remains to be elucidated, but the notion that they enhance intracellular signaling through their ectodomain heparan sulfate groups seems to be favored by our findings. We showed that inhibition of the ectodomain heparan sulfates by protamine abolished the amplificatory effect of Synd1, whereas recombinant Synd1 ectodomain without heparan sulfates did not increase CTGF expression, indicating that heparan sulfates seem essential for these effects of Synd1. We speculate that the ectodomain heparan sulfates increase the efficiency for growth factor signaling by amplifying ligand recruitment by yet-unknown mediators, thereby enhancing signal transduction.

Recently, several reports have been published that point toward an important role for Synd1 in the development of pathological fibrosis in liver and lungs.21,22 Also, Synd1 expression is increased in fibrotic regions of bone marrow myeloma biopsies.23 In liver pathologies, Synd1 has evolved to a biomarker that predicts the amount of liver fibrosis, thereby contributing to early diagnosis and better treatment of patients suffering from liver pathologies. Kliment et al21 show that Synd1 enhances neutrophil chemotaxis, which contributes to pulmonary fibrosis. In addition, they show that the Synd1 ectodomain increases fibroblast proliferation and TGFβ release, which also contributes to the development of fibrosis. The influence of Synd1 on cellular TGFβ responses has also been shown with the use of Synd1-null keratinocytes.24 The lack of Synd1 in these cells resulted in elevated basal TGFβ signaling, however, Smad2 phosphorylation in response to TGFβ was impaired, and collagen accumulation was decreased in Synd1-null keratinocytes. Our experiments are in line with these findings, and we also found that loss of Synd1 is associated with decreased levels of phosphorylated Smad2. Although pSmad2 levels did not differ significantly between AngII-treated WT and Synd1-null hearts, in cultured CFs we indeed saw that Synd1 contributed to profibrotic SMAD2 phosphorylation and Col1 and CTGF protein expressions.
Recently, our group published a report that shows the function of Synd1 in cardiac wound healing after acute myocardial infarction. In this specific pathology, Synd1 seems to act as a "barrier" against the massive influx of inflammatory cells into the infarcted area. Mice lacking Synd1 displayed accelerated wound healing, associated with increased inflammation, MMP9 activity, and collagen deposition compared with WT mice at the same time after myocardial infarction. In AngII-induced LVH, this inflammatory and wound repair response seems less relevant, and we did not observe differences in MMP9 or MMP2 activity between WT and Synd1-null mice. We, therefore, hypothesize that Synd1 has different modes of action depending on the mechanisms that it is involved in: during acute inflammatory responses, it can regulate chemokine gradients and leukocyte-endothelial interactions, whereas during AngII-induced hypertension, with active TGFβ1 signaling, Synd1 seems to predominantly mediate intracellular signaling.

In conclusion, our data indicate that, in this model where inflammation is less prominent, Synd1 emerges as an important regulator of fibrotic signaling and thereby in the development of cardiac fibrosis.

**Perspectives**

In summary, this study indicates for the first time an amplificatory role for Synd1 in the development of cardiac fibrosis during AngII infusion. We report an increased expression of Synd1 in AngII-treated WT hearts, specifically located in the fibrotic regions. In the absence of Synd1, the development of cardiac fibrosis was significantly reduced, which prevented cardiac dysfunction. In vitro, we confirmed the amplificatory role of Synd1 in profibrotic signaling with the use of shRNA against Synd1, protamine treatment, and Synd1-null dermal fibroblasts. In addition to the anti-inflammatory function of Synd1 in infarcted hearts, we now show the involvement of Synd1 in profibrotic signaling during AngII-induced cardiac dysfunction. Although our results point toward a prominent role for the Synd1 ectodomain with its heparan sulfates, more research is needed to identify the precise mechanism. Our findings suggest that specific inhibition of Synd1 is a new therapeutic target for inhibiting cardiac fibrosis in the setting of hypertrophic cardiomyopathy.

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**Disclosures**

None.

**References**

18. Kato M, Wang H, Kainulainen V, Fitzgerald ML, Ledbetter S, Orntz DM, Bernfield M. Physiological degradation converts the soluble syndecan-1 ectodomain with its heparan sulfates, more research is needed to identify the precise mechanism. Our findings suggest that specific inhibition of Synd1 is a new therapeutic target for inhibiting cardiac fibrosis in the setting of hypertrophic cardiomyopathy.
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SYNDECAN-1 AMPLIFIES ANGIOTENSIN II-INDUCED CARDIAC FIBROSIS

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Short title: Syndecan-1 amplifies cardiac fibrosis
Supplementary figures

Figure S1

A. representative zymogram indicating increased MMP2 activity in Ang-II treated hearts. B. Quantification of MMP2 zymogram, *P<0.05, sham groups: n=4, AngII-treated groups: n=6.

Figure S1:
A. representative zymogram indicating increased MMP2 activity in Ang-II treated hearts. B. Quantification of MMP2 zymogram, *P<0.05, sham groups: n=4, AngII-treated groups: n=6.
Figure S2:
Absence of Synd1 significantly decreases Col1, CTGF, and pSMAD2 expression in dermal fibroblasts. A, representative Western blots showing that Col1 and CTGF levels are significantly decreased in Synd1-null dermal fibroblasts in comparison to WT dermal fibroblasts under basal conditions. B, representative Western blot showing that SMAD2 phosphorylation levels are significantly decreased in Synd1-null dermal fibroblasts under basal conditions in comparison to WT dermal fibroblasts. C, Quantification of Col1 expression corrected for GAPDH. D, Quantification of CTGF expression corrected for GAPDH. E, Quantification of pSMAD2 expression corrected for total SMAD2. *P<0.05, n=3 per experiment, quantification of 2 experiments.