EXPERIMENTAL STUDY

Ex Vivo Hydrostatic Pressure Loading of Atrial Tissues Activates Profibrotic Transcription via TGF-β Signal Pathway

Xu Zhang,[1,2] Mhd, Yousuf Yassouf,[1,2], Kai Huang,[1,2], Yong Xu,[1,2], Zi-Sheng Huang,[1,2], Da Zhai,[1,2], Reiko Sekiya,[1,2], Ke-Xiang Liu,[3] MD and Tao-Sheng Li,[1,2] MD

Summary

Excessive mechanical stress causes fibrosis-related atrial arrhythmia. Herein, we tried to investigate the mechanism of atrial fibrogenesis in response to mechanical stress by *ex vivo* approach. We collected atrial tissues from mice and then cultured them as "explants" under atmospheric pressure (AP group) or 50 mmHg hydrostatic pressure loading (HP group) conditions. Pathway-specific PCR array analysis on the expression of fibrosis-related genes indicated that the loading of atrial tissues to 50 mmHg for 24 hours extensively upregulated a series of profibrotic genes. qRT-PCR data also showed that loading atrial tissues to 50 mmHg enhanced *Rhoa, Rock2*, and *Thbs1* expression at different time points. Interestingly, the enhanced expression of *Thbs1* at 1 hour declined at 6-24 hours and then increased again at 72 hours. In contrast, an enhanced expression of *Tgfb1* was observed at 72 hours. In contrast, daily loading to 50 mmHg for 3 hours significantly accelerated the outgrowth of mesenchymal stem-like stromal cells from atrial tissues; however, we did not observe significant phenotypic changes in these outgrowing cells. Our *ex vivo* experimental data clearly show the induction of profibrotic transcription of atrial tissues by HP loading, which confirms the common pathological feature of atrial fibrosis following pressure overload.

(Int Heart J 2022; 63: 367-374)

Key words: Mechanical stress, Mechanotransduction, Fibrogenesis, Arrhythmia

Atrial arrhythmia, such as atrial fibrillation (AF), whose treatment remains thorny, is the most common rhythm disturbance encountered in the clinical setting.[1)] The most common pathogenesis of AF is atrial fibrosis. Many studies already reported that excessive mechanical stresses play critical role in atrial fibrosis development.[2-4)] Although various profibrotic signals, especially the transforming growth factor-β (TGF-β), can induce interstitial fibrogenesis, the question remains to further clarify the molecular and cellular mechanisms of atrial fibrosis in response to mechanical stresses.

Mechanotransduction, the process of cells that sense the mechanical force from the microenvironment through mechanosensing receptors and then translate mechanical stress into biochemical signals, plays vital roles in tissue cell homeostasis and pathology, such as fibrogenesis.[5)] TGF-β signaling pathway is the core in mechanotransduction of cardiac fibrosis. Additionally, as an essential signal isoform, TGF-β_1 induces the expression of profibrotic genes through the canonical signaling pathway with activation of Smad2/3, or through noncanonical signaling pathways with activation of RhoA and its downstream kinase ROCK.[6)] As a multicomponent target, RhoA/ROCK signaling is known to involve in cardiac fibrosis.[7)] Thrombospondin-1 (TSP-1), another member of the TGF-β superfamily, can also be excessively induced in response to various stresses or tissue damage.[8)]

Mechanical stresses modulate many aspects of cell function, such as proliferation and differentiation, from single cells to pattern formation in whole organisms.[9)] Stromal cells within the defined tissues/organs are known to be the key player of fibrogenesis. Using a mouse unilateral ureteral obstruction model, we recently demonstrated that renal stromal cells shift to profibrogenic phenotype.[10)] Thus, we reasonably speculate the fibrotic phenotypic shifting of atrial stromal cells in response to excessive mechanical stresses, but direct experimental evidence is needed.

By *ex vivo* loading mouse atrial tissues to 50 mmHg HP, a kind of mechanical compressive stresses, we investigated the dynamic changes on the expression of fibrosis-related genes and the biological properties of stromal

From the [1]Department of Stem Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, [2]Department of Stem Cell Biology, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan and [3]Department of Cardiovascular Surgery, The Second Hospital of Jilin University, Jilin, China.

This study was mainly supported by a Grant-in-Aid for the Ministry of Education, Science, Sports, Culture and Technology of Japan and the Collaborative Research Program of the Atomic Bomb Disease Institute of Nagasaki University.

Address for correspondence: Tao-Sheng Li, MD, Department of Stem Cell Biology, Atomic Bomb Disease Institute, Nagasaki University, 1-12-4 Sakamoto, Nagasaki, Nagasaki 852-8523, Japan. E-mail: litaoshe@nagasaki-u.ac.jp

Received for publication August 10, 2021. Revised and accepted November 8, 2021.

Released in advance online on J-STAGE March 15, 2022.

doi: 10.1536/ihj.21-481

cells.

Methods

Experimental animals: C57BL/6 male mice (9-12 weeks old, CLEA, Japan) were used in this study. All experiments were approved by the Institutional Animal Care and Use Committee of Nagasaki University (No.1608251335-9), and animal procedures were performed following institutional and national guidelines.

***Ex vivo* culture of atrial tissues as "explants":** The *ex vivo* culture of atrial tissues as "explants" was performed as described previously,[11] with a few modifications. Briefly, mice were administered general anesthesia by an intraperitoneal injection of mixed anesthetic (0.75 mg/kg medetomidine, 4 mg/kg midazolam, 5 mg/kg butorphanol). Next, atrial tissues were collected into 6-cm dishes with 2 mL PBS and then minced into small fragments (almost 1 mm × 1 mm) using a surgical knife under zoom stereomicroscope (Olympus SZ61). Then, the minced tissue fragments were moved onto 6-cm culture dishes coated with 15 ug/mL fibronectin (CORNING) for culturing as "explants." We used Iscove's Modified Dulbecco's Medium with the supplement of 10% fetal bovine serum (HyClone, Thermo Scientific), 1% penicillin/streptomycin (Wako), and all culture was performed at 37°C in a humidified incubator under 5% CO_2 and 95% air.

Hydrostatic pressure loading: We loaded the atrial "explants" to 50 mmHg hydrostatic pressure (HP group), by using a set of commercial devices (STREX, Inc.). Of which, gas from the incubator flowed continuously into a closed container to keep the HP at 50 mmHg while maintaining the same temperature, humidity, and CO_2 level as the incubator. As a control, the atrial "explants" were kept in a common CO_2 incubator with AP.

Mouse fibrotic pathway-specific PCR array analysis: To compare the expression of fibrosis-related genes between HP and AP groups, we mixed an equal amount of RNA from three independent mice atrial tissue samples of each group for PCR array analysis. Briefly, total RNA was purified by using Quick-RNA™ Microprep Kit (ZYMO RESEARCH). The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and 1 ug of RNA was used to generate cDNA using the RT^2 First Strand Kit (Qiagen). The mouse fibrosis RT^2 Profiler PCR array was performed according to the manufacturer's instructions (#330231 PAMM-120ZA, Qiagen). This PCR array profiles 84 key genes involved in dysregulated tissue remodeling during the repair and healing of wounds. Roche®LightCycler®480 was used to performing the PCR procedure. Qiagen's online web analysis tool was utilized to analyze the data. *B2m* and *Hsp90ab1* were used as reference genes. The fold changes of gene expression were calculated by determining the ratio of HP group to AP group.

***qRT-PCR*:** To investigate the time-course dynamics of the transcription on *Rhoa, Rock1, Rock2*, *Tgfb1*, and *Thbs1* in atrial tissues following HP loading, we collected atrial "explants" at 1, 6, 24, and 72 hours (n = 3, at each time point for both groups) after the initiation of culture, and we purified total RNA as described above. For qRT-PCR, the first-strand cDNA was synthesized with SuperScript™ VILO™ MasterMix (Invitrogen) according to the manufacturer's recommendations, and the RT-PCR was performed using THUNDERBIRD®SYBR®qPCR MIX (TOYOBO) with the CFX96™ Real Time System (BIO-RAD). The gene expression was normalized by housekeeping gene *B2m*. Primers used for qPCR were the following: *Rhoa* (Forward: 5'- AGC TTG TGG TAA GAC ATG CTT G -3', Reverse:5'- GTG TCC CAT AAA GCC AAC TCT AC -3'); *Rock1* (Forward: 5'- AGC TTT TGT TGG CAA TCA GC -3', Reverse:5'- ACT TTC CTG CAA GCT TTT ATC CA -3'); *Rock2* (Forward: 5'- CAG TCC CTG GGT AGT TCA GC -3', Reverse:5'- GCC TGG CAT ATA CTC CAT C -3'); *Tgfb1* (Forward:5'-CTG CGC TTG CAG AGA TTA AA-3', Reverse:5'-GAA AGC CCT GTA TTC CGT CT-3'); *Thbs1* (Forward:5'-GGA ACG GAA AGA CAA CAC TG-3', Reverse:5'-AGT TGA GCC CGG TCC TCT TG-3').

Monitoring the outgrowth of stromal cells from atrial tissues: To further investigate the effect of HP loading on stromal cells, atrial tissues from mice were collected into 6-cm dish and minced into small fragments as described above. Then, the minced tissue fragments were randomly moved onto fibronectin-coated 6-cm dishes (a total of 40 pieces of minced tissue fragments/dish) and then cultured as "explants" with daily loading to 50 mmHg for 3 hours (HP group) or without pressure loading (AP group). The outgrowth of stromal cells from atrial "explants" was observed every other day, and we counted the number of "explants" with cell outgrowth at different time points (Supplemental Table I). The number of "explants" with cell outgrowth was divided by the total number of seeded "explants" at each time point, and then, the percentiles were used to draw the cell growth curves. Next, we collected the outgrowth cells at 12 days after the initiation of culture, and the total number of outgrowth cells was counted using a NucleoCounter cell-counting device (ChemoMetecA/S, Denmark). Three independent experiments were performed (n = 3 for both AP and HP groups).

Immunofluorescence staining: We also investigated the expression of CD105, CD90, and α-SMA in the outgrowth cells by immunofluorescence staining as described previously.[10] Briefly, the first-passaged outgrowth cells were cultured in 8-well chamber slides (1×10^4 /well, Lab-Tek, Thermo Scientific Nunc) coated with 15 ug/mL fibronectin (CORNING). Next, the cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako). After blocking, the cells were incubated with rabbit antimouse CD90 antibody (1:50 dilution, Abcam), rabbit antimouse CD105 antibody (1:50 dilution, Abcam), or rabbit antimouse α-SMA antibody (1:100 dilution, Abcam), respectively. Positive staining was detected using Alexa Fluorescent 546 goat antirabbit Ig (1:400 dilution, Dako) secondary antibody. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Scientific). The immunofluorescence was examined under a microscope (FV10CW3, Olympus). We counted the positively stained cells or measured mean intensity in ten images of each experiment. The percentage of positive cells or mean intensity from three independent experiments was used for

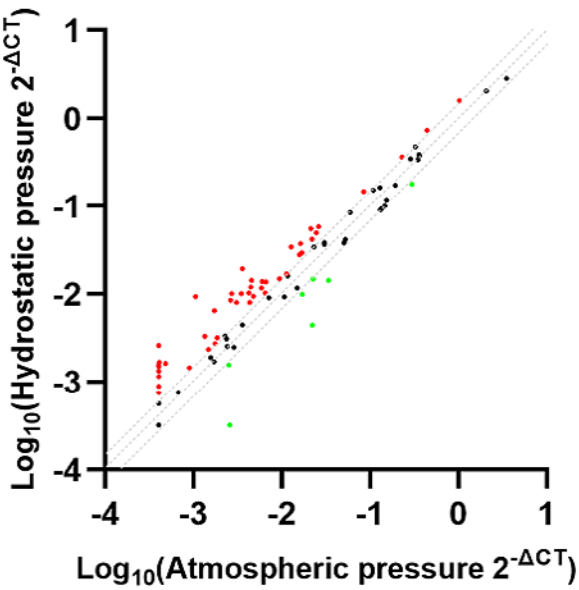

Figure 1. PCR array analysis on the expression of fibrosis-related genes in atrial tissues after 24 hours culture under 50 mmHg HP and AP. The scatter plots describe the relative expression between groups. Genes with more than 1.5-fold upregulation (red dots) and downregulation (green dots) are highlighted.

statistical analysis.

Statistical analysis: All the values were presented as the mean ± SD. For comparison of multiple sets of data, one-way analysis of variance followed by Tukey's test (Dr. SPSS II, Chicago, IL) was used for statistical analyses. For comparison of two sets of data, unpaired two-tailed t-test was used for statistical analyses. All analyses were carried out with the SPSS19.0 statistical software (IBM SPSS Co., USA). A *P*-value of less than 0.05 was accepted as significant.

Results

Loading the atrial tissues to 50 mmHg prevalently upregulated the expression of profibrotic genes: We first screened the overall changes in the expression of fibrosis-related genes in atrial "explants" loaded with 50 mmHg HP using the mouse RT^2 Profiler PCR array (Supplemental Table II). A scatter plot showed that 24 hours loading of atrial tissues to 50 mmHg upregulated many fibrosis-related genes by more than 1.5-fold (Figure 1), although a few genes showed a downregulation by more than 1.5-fold. We further categorized these genes according to their biological functions (Figure 2A) and noticed that these genes that functionally related to the ECM and cell adhesion molecules (Figure 2B), Inflammatory cytokines and chemokines (Figure 2C), TGF-β superfamily members (Figure 2D), Transcription factors (Figure 2E), and growth factors (Figure 2F) were widely upregulated.

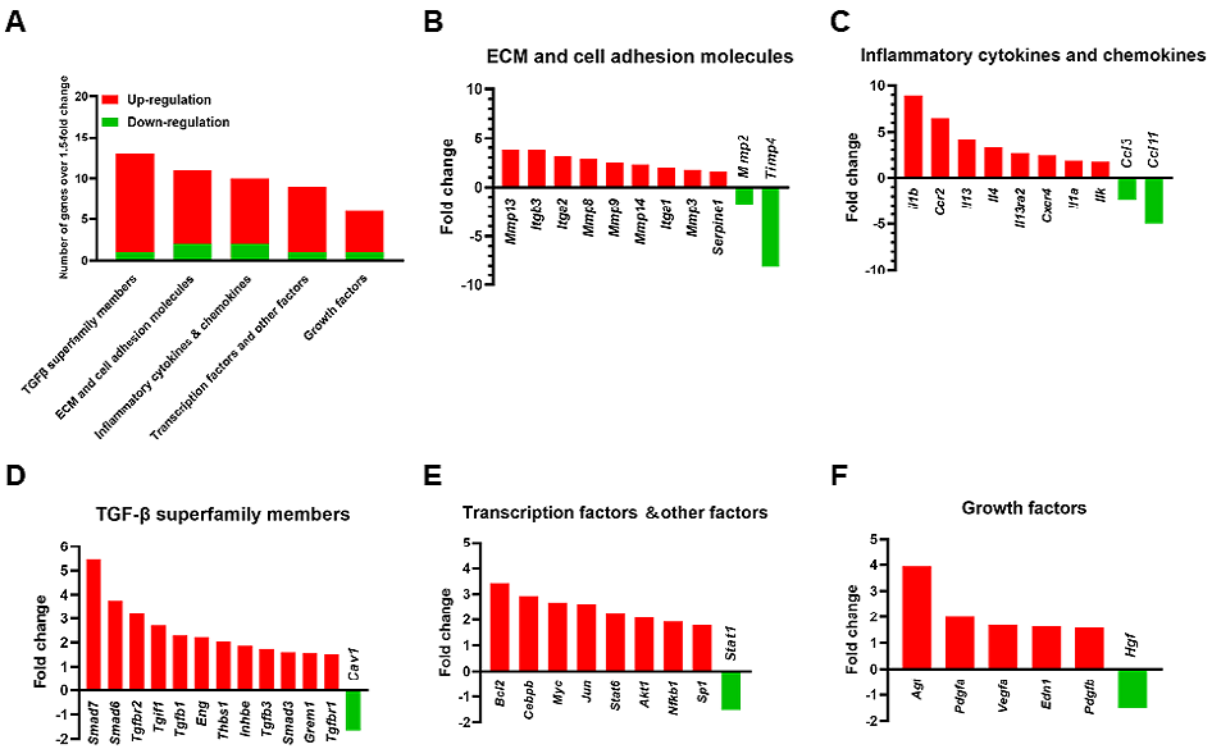

Figure 2. The functional categorization on the fibrosis-related genes included in PCR array analysis. **A:** The number of genes that upregulated and downregulated over 1.5-fold in atrial tissues after 24 hours culture under 50 mmHg HP. **B-F:** Data are also presented by dividing these genes into different categorizations according to their biological functions. Fold change of each gene functionally belongs to ECM and cell adhesion molecules (**B**), inflammatory cytokines and chemokines (**C**), TGF-β superfamily members (**D**), transcription factors (**E**), and growth factors (**F**).

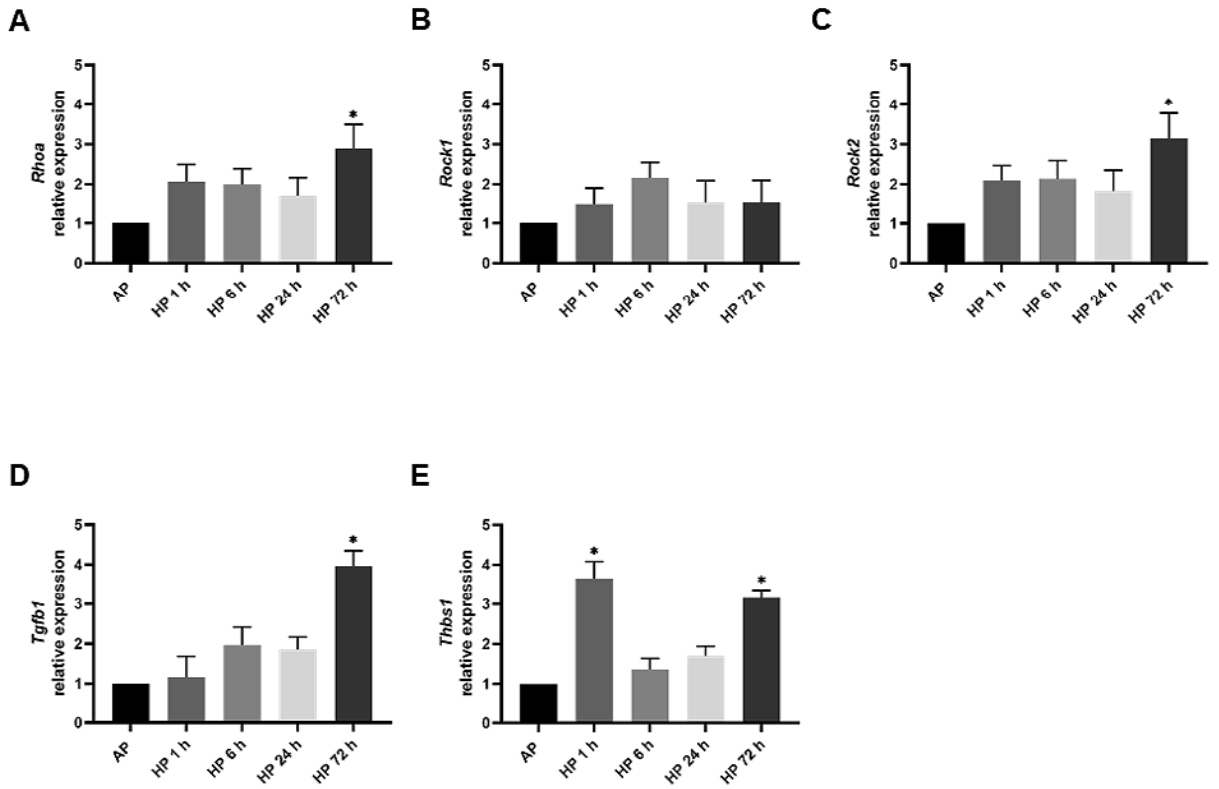

Figure 3. Time-course dynamics on the expression of *Rhoa*, *Rock1*, *Rock2*, *Tgfb1*, and *Thbs1* in atrial tissues followed by *ex vivo* loading to 50 mmHg HP. Quantitative RT-PCR data shows the relative expression of *Rhoa* (**A**), *Rock1* (**B**), *Rock2* (**C**), *Tgfb1* (**D**), and *Thbs1* (**E**) at 0, 1, 6, 24, 72 hours after culture. Data are represented by three independent experiments at each time point. *$P < 0.01$ versus AP group. AP indicates atmosphere pressure; and HP, hydrostatic pressure.

Loading the atrial tissues to 50 mmHg activated the TGF-β signaling pathway: Based on the changes of the PCR fibrosis array, we found that TGF-β is the most affected pathway; thus, we tried to check the dynamics of mechanotransduction in response to HP through this pathway. We loaded the atrial tissues with either 0 or 50 mmHg for 1, 6, 24, and 72 hours. Then, we evaluated the gene expression of *Rhoa*, *Rock1*, *Rock2*, *Tgfb1*, and *Thbs1* at each time point. Our qRT-PCR data showed that, as a mechanosensitive multicomponent target of TGF-β noncanonical signaling pathways, the expression of *Rhoa* and *Rock2* was significantly enhanced at 72 hours by 50 mmHg loading ($P < 0.01$, Figure 3A, C). However, as another downstream target of *Rhoa*, the expression of *Rock1* was not changed much at any time point following 50 mmHg loading (Figure 3B). Interestingly, the expression of *Thbs1* was quickly induced at 1 hour, but it declined at 6-24 hours, and then increased again at 72 hours after 50 mmHg loading (Figure 3E). In contrast, the expression of *Tgfb1* was not induced at the early time windows, but it was robustly enhanced at 72 hours after 50 mmHg loading ($P < 0.01$, Figure 3D).

Daily loading of the atrial tissues to 50 mmHg accelerated the outgrowth of stromal cells: The outgrowth of fibroblast-like stromal cells from atrial "explants" was observed mostly at 3-5 days after the initiation of culture, but the outgrowth cells from some "explants" in the HP group could be observed even at the second day of culture (Figure 4A). By plotting the percentiles of "explants" with cell outgrowth, we found that the cell outgrowth from "explants" was significantly earlier in HP group than AP group (Figure 4B). Next, we harvested all the outgrowth cells 12 days after the initiation of culture, and the total number of outgrowth cells was also significantly increased by daily loading the atrial tissue to 50 mmHg for 3 hours ($P = 0.03$, Figure 4C).

Further, we investigated the phenotypic characteristics of these cells that outgrew from atrial "explants" by immunostaining on the expression of CD90, CD105, and α-SMA (Figure 5). Extensive expression of CD90 and CD105 (almost 90%) was observed in these outgrowth cells (Figure 5A, B), suggesting a mesenchymal stem cell-like phenotype. Although the expression of α-SMA tended to slightly increase in these outgrowth cells from the HP group, no significant difference was found between groups ($P = 0.07$, Figure 5C).

Discussion

Clinical practice and animal experiments indicate that pressure overload-induced atrial fibrosis and remodeling play a vital role in the pathology of rhythm disturbance.[12] In addition to endothelial cells undergoing additional shear stress, cardiac tissue is mainly exposed to mechani-

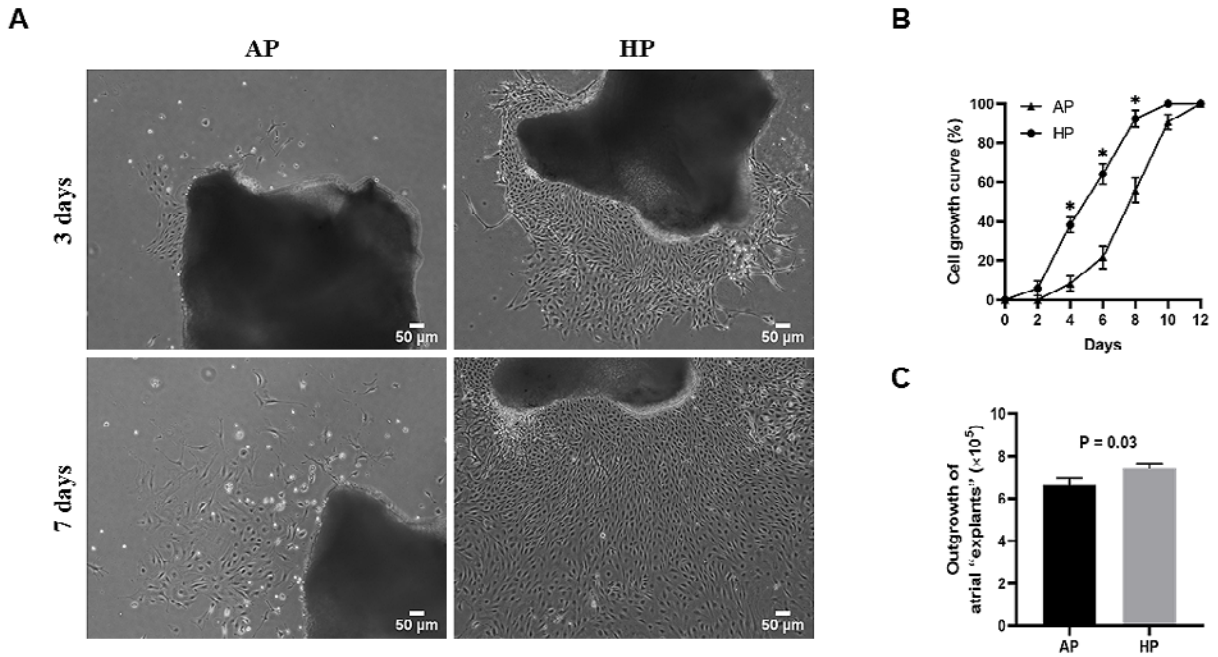

Figure 4. The outgrowth of stromal cells from atrial tissues with daily *ex vivo* loading to 50 mmHg HP for 3 hours. **A:** Representative images show the cells outgrowing from the atrial "explants" at 3 and 7 days after initiation of culture. **B:** The curve lines appear the percentiles of "explants" with cell outgrowing at different time points after the initiation of culture. **C:** The total number of outgrowth cells harvested at 12 days after the initiation of culture. Data are represented by three independent experiments. $*P < 0.05$ versus AP group. AP indicates atmospheric pressure; and HP, hydrostatic pressure.

cal compression stress. It is well known that mechanical stretch induces profibrotic fibroblast phenotypes, which is thought to promote the development of AF.[13,14] Recent studies also showed that HP involves in atrial remodeling.[15,16] However, the molecular and cellular mechanisms of atrial fibrosis in response to HP have not yet been well understood.

By *ex vivo* loading the atrial tissues from healthy mice to 50 mmHg HP, we confirmed the extensive enhancement on the expression of profibrotic genes. We further functionally classified these genes according to RT^2 Profiler PCR array manufacturer's instructions. Among these genes associated with ECM and cell adhesion molecules, the most upregulated genes were integrin and matrix metalloproteinase (MMP) family members. Indeed, previous studies showed that mechanical stress increases the mechanosensitive integrin subunit and induces the expression of *Mmp8, Mmp9*, and *Mmp13* in fibroblasts.[17-19] Mechanical stress can also induce the expression of transcription factors associated with inflammatory response and tissue remodeling, such as *Myc, Jun, Nfκb*.[20,21] Coincidentally, the loading of atrial tissues to HP also enhanced the expression of many transcription factors known as the master regulators of inflammatory cytokines and growth factors in our study. A computer biology model applied in a recent study demonstrated that the mechanosignaling network in the heart can be started from the mechanosensing step by several mechanosensors, such as integrin and angiotensin receptor, then induces the activation of multiple signaling cascades, such as TGF-β and RhoA/ROCK pathway, and finally alters the transcription of related genes to induce remodeling.[22]

TGF-β signal pathway is considered as the core in mechanotransduction of fibrogenesis in response to pressure overload to the heart. As an important signal isoform, TGF-β_1 can trigger the activation of cardiac fibroblasts, induce profibrotic genes through the canonical signaling pathway with activation of Smad2/3, or the noncanonical signaling pathways with activation of Rho/ROCK.[6] Recent studies further demonstrated that intermittent compressive force induces TGF-β_1 expression in human periodontal fibroblasts.[23,24] As a branch of TGF-β noncanonical signaling pathways, Rho/ROCK signaling plays a crucial role in various cellular processes, such as cell proliferation and migration. ROCK2 is known to be involved in angiotensin II-induced cardiac hypertrophy, and ROCK2-deficient mice are resistant to pressure overload-induced cardiac hypertrophy.[26,27] Moreover, a recent study further demonstrated that specific ROCK2 deficiency in cardiac fibroblasts protects the heart from angiotensin II-induced cardiac fibrosis.[28] Consistent with these previous studies, our data supported the mechanotransductive role of RhoA/ROCK2 in cardiac fibrosis. Although it has also been reported that the potential role of ROCK1 in the heart in response to mechanical stress,[29] we did not find the induction of ROCK1 in atrial tissues by *ex vivo* loading to 50 mmHg within 72 hours follow-up. We speculate that the mechanical stress threshold may be higher for inducing ROCK1 compared to ROCK2.

TSP-1, a mechanosensitive multi-functional matricellular protein of the TGF-β superfamily, has been identified to play a major role in cardiac fibrosis, mainly by ac-

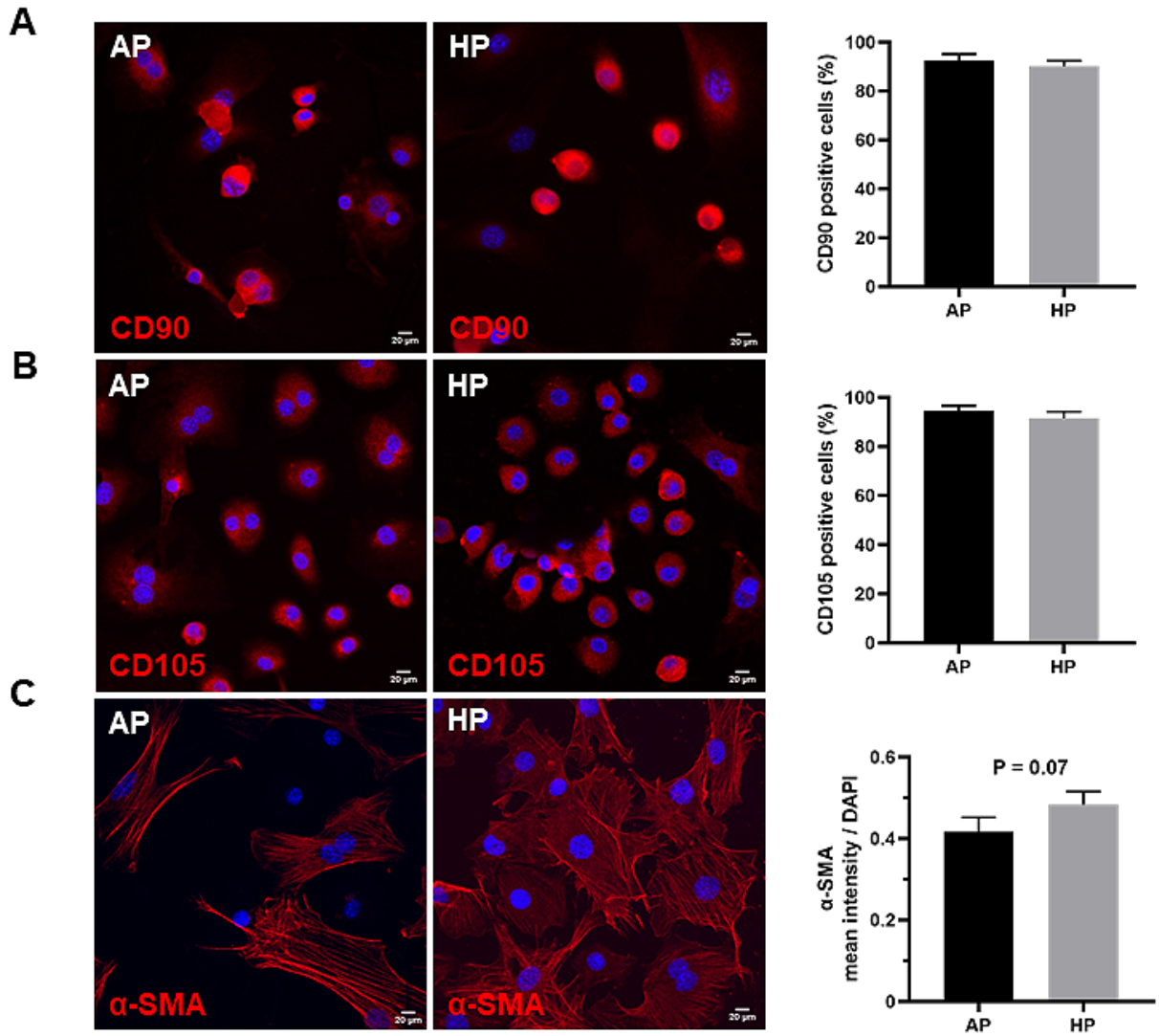

Figure 5. Immunocytochemical analysis on the expression of CD90, CD105, and α-SMA in the outgrowth cells. Representative images (left) and quantitative bar graph data (right) show the expression of CD90 (**A**), CD105 (**B**), and α-SMA (**C**) in the outgrowth cells from atrial "explants." Scale bar: 20 μm. AP indicates atmospheric pressure; and HP, hydrostatic pressure.

tivating the TGF-β isoforms, which in turn induces TSP-1 expression.[30-32] A recent study in mice demonstrated that TSP-1 deletion leads to the maladaptive remodeling of the aorta in response to pressure overload,[33] suggesting the protective role of TSP-1 in response to mechanical stress. Interestingly, our data showed that *ex vivo* loading atrial tissues to 50 mmHg quickly induced the expression of *Thbs1*; however, the expression of *Thbs1* increased again at 72 hours after the loading to 50 mmHg. The biphasic changes of TSP-1 in response to mechanical stress may represent dual roles of TSP-1 in the heart. In other words, instead of the cardioprotection about the increased TSP-1 soon after suffering from mechanical stresses, a delayed increase of TSP-1 may promote fibrogenesis in the heart with a persistent pressure overload. As we purified total RNA from the outgrowth cells together with the "explants" for the qRT-PCR analysis, data from our study reflected the comprehensive response of atrial tissues to HP.

Stromal cells, such as fibroblasts, within the defined tissues/organs, are known to be the key player of fibrogenesis. A recent study demonstrated that the increased stretch force or matrix stiffness promotes the proliferation and activation of cardiac fibroblasts.[14] Based on our observative data, HP loading accelerated the cell outgrow from "explants" (Figure 3B) and increased the yield of outgrowth cells (Figure 4C), suggesting the potential role of stromal cells in the atrial fibrosis in response to pressure overload. Although we did not further evaluate the regulatory role of RhoA, Rocks, and TSP-1 on outgrowth cells at a protein level, the upregulated mRNA expression suggests the probable role on the proliferation, migration, and other biological characteristics of resident stromal cells in response to HP.

We previously reported that cardiac-derived mesenchymal stem-like cells positively express CD90 and CD105, two markers commonly used for detecting mesen-

chymal stem stromal cells.[34] The extensive expression of CD105 and CD90 in these outgrowth cells suggests the mesenchymal stem-like cell characteristics. Although researchers previously reported the heterogeneity on the expression of CD90 in cardiac-derived stromal cells from human beings,[35] HP loading barely changed the expression of CD90 and CD105 in outgrowth cells in this study. As we did not evaluate the proliferative activity in this study, whether HP loading could change the proliferation of stromal cells is kept unknown. Researchers recently reported that the exposure of human cardiac fibroblasts to 200 mmHg for 8 hours significantly suppresses α-SMA expression.[36] Unexpectedly, as a typical marker of myofibroblasts, the expression of α-SMA was not significantly induced in response to loading of atrial tissues to 50 mmHg. The magnitude and duration of HP and the difference in experimental methods might affect the phenotypic shift of stromal cells.

This study exhibits some limitations. First, PCR array analysis was performed only once by using a mixture of RNA from three independent samples. Second, due to the tiny size of mouse atrial tissues, we could not collect enough protein to perform Western blotting analysis for further confirming the relevant molecular mechanism on our findings. Third, we only used 50 mmHg HP for all experiments, and optimizing the experimental conditions in future studies is necessary.

Conclusion

Our data showed the induction of profibrotic transcription of atrial tissues by HP loading, likely by activating TGF-β signal pathway and stromal cells. Data from the *ex vivo* experimental approach provided indirect evidence on the common pathological features of atrial fibrosis following pressure overload.

Disclosure

Conflicts of interest: The authors declare no competing financial interests.

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Supplemental Files

Supplemental Tables I, II

Please see supplemental files; https://doi.org/10.1536/ihj.21-481