Appendix 1

Methods

Cell extraction

The surgical area was initially sterilized by wiping the precordial region of the mice with a cotton ball moistened with 70% ethanol. The heart was excised by creating an incision above the breast with surgical scissors, rapidly exposing the heart and gently compressing the chest cavity. The isolated heart was immediately immersed in ice-cold sterile phosphate-buffered saline buffer (PBS) (G4250, Servicebio) and gently squeezed with forceps, carefully removing all attached tissues to drain excess blood. Subsequently, the heart was divided into approximately 1 mm^3 pieces using a scalpel, and these pieces were placed in a digestive enzyme mixture. The digestive mixture consisted of 0.25% trypsin-EDTA (25200056, Gibco, USA) and 4 mg collagenase type II (17101015, Gibco, USA), diluted to a final concentration of 0.4 mg/kg in 1× PBS. The resulting solution was diluted to a final concentration of 0.4 mg/kg by the addition of 1× PBS. The final concentration was 0.4 mg/mL. The tubes were then digested in a water bath shaker at 37 ℃ for 30 minutes, with three digestions required before and after. At the end of digestion, the heart tissue was allowed to settle naturally, and the supernatant was carefully transferred to a 50 mL sterile centrifuge tube. The collected supernatant, which contained the primary cells, was then mixed with a medium consisting of DMEM/F12k (11320033, Gibco) and 10% fetal bovine serum (FBS) (10091148, Gibco). Finally, the cell-containing medium was added to a 10 cm sterile Petri dish and incubated for two hours at 37 ℃ and 5% CO_2 in a thermostat to allow fibroblasts to adhere to the wall. The supernatant (containing unadhered primary cardiomyocytes) was then removed and the medium was replaced.

Transfection

The STK38L gene was amplified in HEK293T cDNA and inserted into pcDNA3.1(+) vector, and finally the STK38L-siRNA and negative control siRNA were successfully constructed, and the above technology was supported by Jiangsu Gengfei Biotechnology Co. The siRNA sequences used in this experiment were: mouse STK38L siRNA sequence (si-40): 5'-GGACUGAAUUCUACAGAAATA-3'; mouse STK38L siRNA sequence (si-38): 5'-GGGUGUG AAGAGGUUCUATT-3'. Transfection reagents were selected from Poly plus jetPRIME Transfection Kit (101000015, Polyplus). Firstly, CFs cells were cultured in 6-well plates, and when the cell density was about 60–70%, the fresh medium was replaced to be proposed for siRNA transfection. We added 4 μL siRNA into 200 μL jetPRIME buffer in a sterile 1.5 mL tube, vortexed for 10 seconds, then added 4 μL jetPRIME reagent, vortexed for 1 second to form a transfection mixture, and left it at room temperature for 10 minutes on an ultra-clean bench, then added the transfection mixture into the mixed medium of the experimental group. After 24 hours of transfection, the medium can be replaced with fresh medium, and the transfection efficiency can be analyzed by PCR after 48 hours of transfection.

PCR

Cellular RNA was extracted using the RNA simple Total RNA Extraction Kit (TIANGEN, DP419) and converted to cDNA using the Prime RT Master Mix Kit (Takara Japan). PCR reactions were performed using the ABI7500 System (Applied Biosystems, Inc.). The PCR reaction was performed using the ABI7500 system (Applied Biosystems, Inc.) and consisted of denaturation at 94 ℃ for 30 seconds, annealing at 55 ℃ for 30 seconds, and extension at 68 ℃ for 10 minutes, with 30 to 35 cycles of repetition. Specific details of the gene primer sequences have been added (*Table S2*).

Western-blotting

Total cellular protein was extracted using RIPA buffer supplemented with protease inhibitor + phosphatase inhibitor. Extracted protein concentration was measured using a bicinchoninic acid (BCA) kit (P0012s, Beyotime) and then diluted with RIPA to adjust the amount of protein to approximately 10–20 μg per lane of uploaded sample. 10% sodium dodecyl sulfate (SDS) separation and concentration gels were prepared. The samples were mixed with 5 sampling buffer (P0015, Beyotime), heated at 100 ℃ for 10 minutes, and then loaded evenly onto each lane for electrophoresis. The electrophoresis conditions

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were set at a voltage of 80 V for 30 minutes, and then changed to 200 V for 50 minutes after the proteins ran out of the concentrated gel. The proteins in the gel were then transferred to polyvinylidene difluoride (PVDF) membranes by wet transfer. the PVDF membranes were soaked in 5% skimmed milk for 2 h, and then immersed in 1× Tris buffered saline with Tween-20 (TBST) solution and eluted on a shaker for three times for 5 min each time. Then, immersion in primary antibody dilutions was implanted overnight at 4 ℃ on a shaker. Antibody information detailed in the *Table S3*. At the end of the primary antibody incubation, the antibody was eluted three times with 1× TBST at room temperature for 5 minutes each time. Subsequently, the membrane was immersed in a dilution of secondary antibody and incubated at room temperature on a slow shaker for 1 h. The membrane was rinsed three times (5 min each) with 1× TBST. Subsequently, the membrane was immersed in Enhanced Chemiluminescence Reaction Solution (ECL Millipore) for 1 minute and the results were observed under a chemiluminescence instrument (Shanghai Tannen Technology Co. Ltd.). The relative expression of the protein was measured by the ratio of the gray value of the target band to the gray value of the internal reference band, using GAPDH as the internal reference.

CCK8 assay

Mouse primary CFs were transfected with STK38L siRNA, in 96-well plates were uniformly inoculated with CCK8 blank control group, negative control (CON) group, and si-STK38L group cell suspensions (100 μL/about 2,000 cells/well), each group of 6 replicate wells, PBS was added around the periphery of the 96-wells to reduce evaporation, incubated in a 37 ℃ temperature chamber, and then replaced with medium after the cells have adhered to the wall, and then incubated for 1 hour. Add 10 μL CCK-8 solution to each well and shake gently, continue incubation in the cell culture incubator for 1 hour, measure the absorbance value at 450 nm with an enzyme marker, and mark it as the 0 points; After that, repeat the CCK8 assay according to the above steps at 24, 48 and 72 hours of cell culture.

Cell scratching

Three interval lines were drawn at 5mm intervals on the back of a six-well plate using a marker pen. Cells were allowed to grow to 50% confluency before transfection. Forty-eight hours after transfection, when cells reached full confluency, they were scratched precisely with a yellow pipette tip held perpendicular to the plate. The movements were rapid and accurate. The original medium was discarded, and the cells underwent 2–3 washes with PBS, aligning with the scratch lines. New serum-free medium was added, and photographs of the scratch were taken at 0 and 24 hours.

Masson stain

The removed mouse heart was placed in 1× PBS solution to drain the residual blood, and then placed in tissue fixative for 48 hours. After tissue dehydration, paraffin embedding, sectioning and dewaxing, it was placed in hematoxylin for 10 minutes, then rinsed with distilled water, and then soaked in Masson stain for 8 minutes, then soaked in 2% glacial acetic acid for an appropriate time, differentiated in differentiation solution for 5 minutes, and then directly stained with aniline blue stain for 3 minutes. Finally, the slides were sealed with neutral gum. The slides were scanned with a Leica high-throughput scanner and analyzed with Image J software to observe the degree of myocardial fibrosis in mice.

Single-cell RNA-seq data analysis

The Seurat package in R (https://satijalab.org/seurat/, version 4.0.4) was used to analyze single-cell RNA-seq data from the GSE120064 dataset. Cell subtype annotations were based on descriptions from the original study and used highly expressed marker genes or known functional genes. Cell filtering parameters included nFeature_RNA >200, nFeature_RNA <5000 and percentage. FindClusters and FindNeighbors, with a resolution of 0.8, were used for cell binning in Seurat. We performed weighted kernel density calculations for gene expression using the plot_density function in Nebulosa package (v1.6.0).

Gene set enrichment analysis (GSEA)

The GSEA was performed using the GSEA software (http://software.broadinstitute.org/gsea/). GSEA performed according to the subgroups of AF and SR in a database based on Molecular Signatures Database (MsigDB) based on the AF and SR subgroups, GSEA was performed on the KEGG gene set based on MsigDB, and the top 10 gene sets were selected for display, with the screening conditions of NES >1.3 and FDR <0.25.

https://dx.doi.org/10.21037/cdt-24-164

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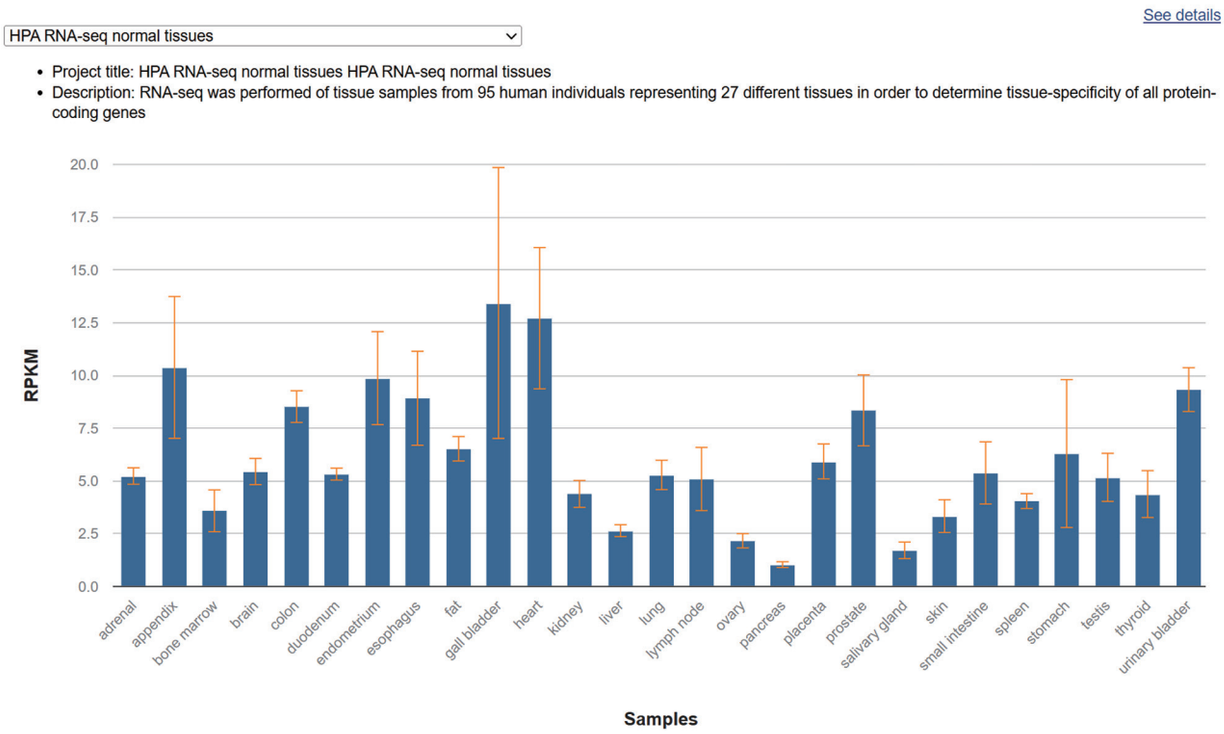

Figure S1 HPA RNA-seq of STK38L in different human normal tissues (https://www.ncbi.nlm.nih.gov/gene/23012). RPKM, reads per kilobase per million mapped reads; HPA, Human Protein Atlas; RNA-seq, RNA sequencing.

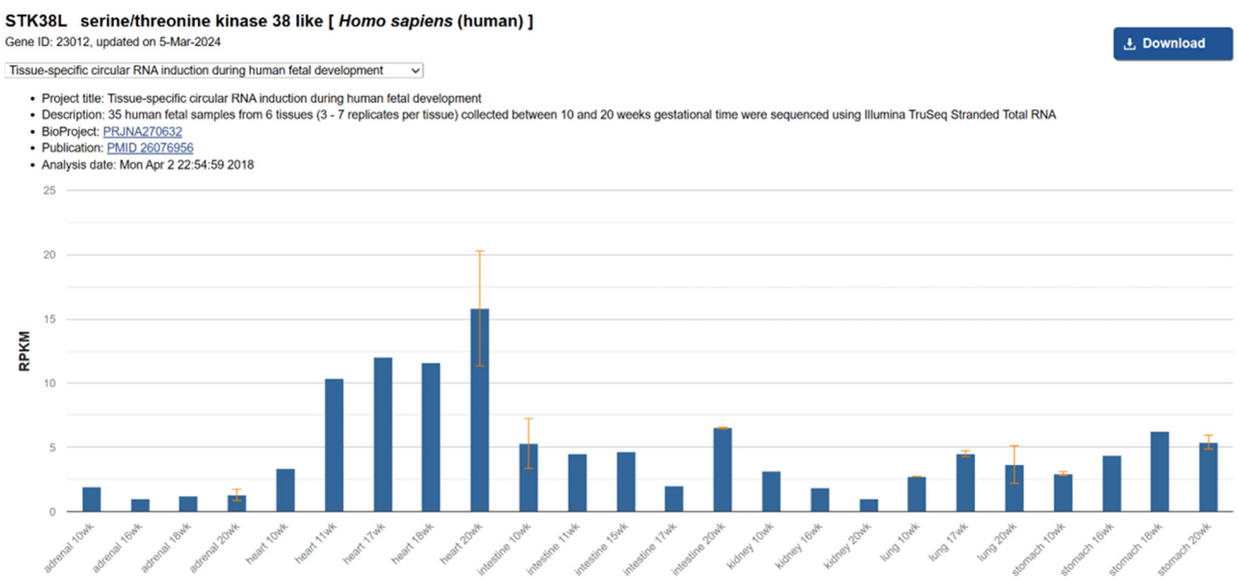

Figure S2 Tissue-specific circular RNA introduction of STK38L during human embryonic development (https://www.ncbi.nlm.nih.gov/gene/23012). RPKM, reads per kilobase per million mapped reads.

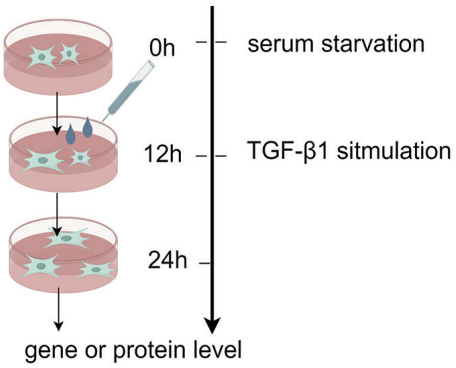

Figure S3 Flow chart of TGF-β1 stimulated activation of primary mouse CFs. TGF-β1, transforming growth factor β1; CFs, cardiac fibroblasts.

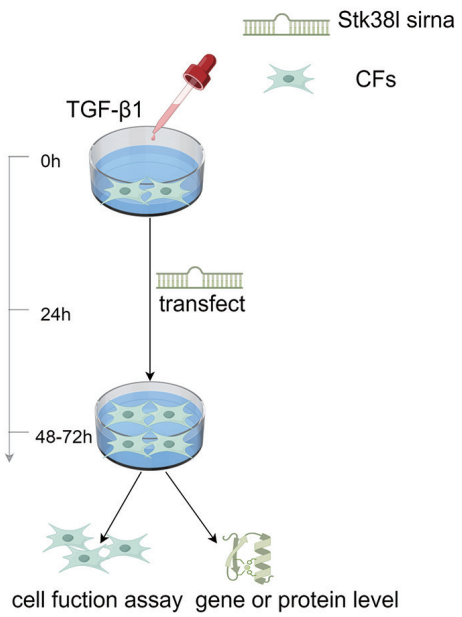

Figure S4 Flow diagram of experimental design for inhibiting STK38L in TGF-β1 stimulated primary mouse CFs using si-RNA. TGF-β1, transforming growth factor β1; CFs, cardiac fibroblasts; si-RNA, small interfering RNA.

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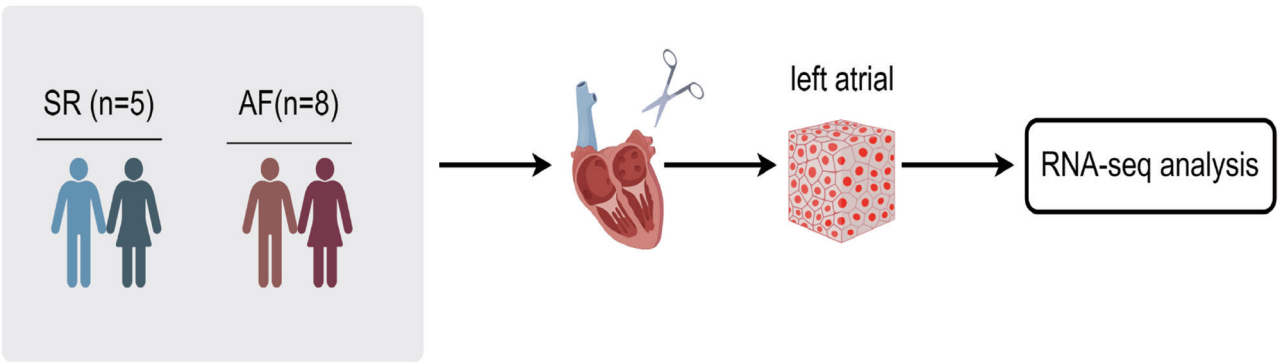

Figure S5 Flowchart of patient recruitment and sample collection. SR, sinus rhythm; AF, atrial fibrillation; RNA-seq, RNA sequencing.

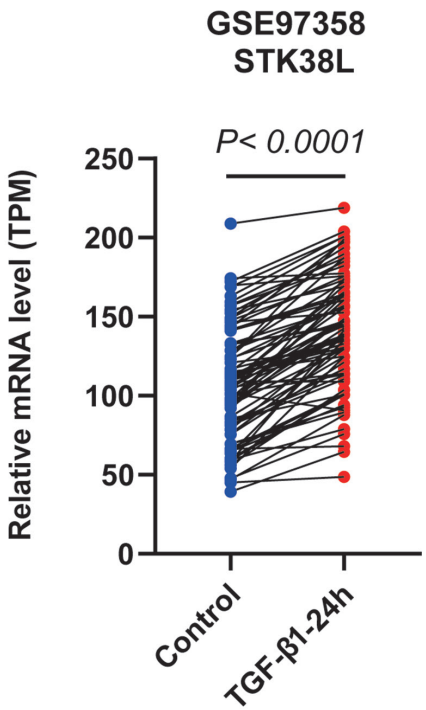

Figure S6 Results of STK38L gene expression levels in GSE97358 RNA-seq data, compared with the control group, paired t-test. TGF-β1, transforming growth factor β1; TPM, transcript per million; RNA-seq, RNA sequencing.

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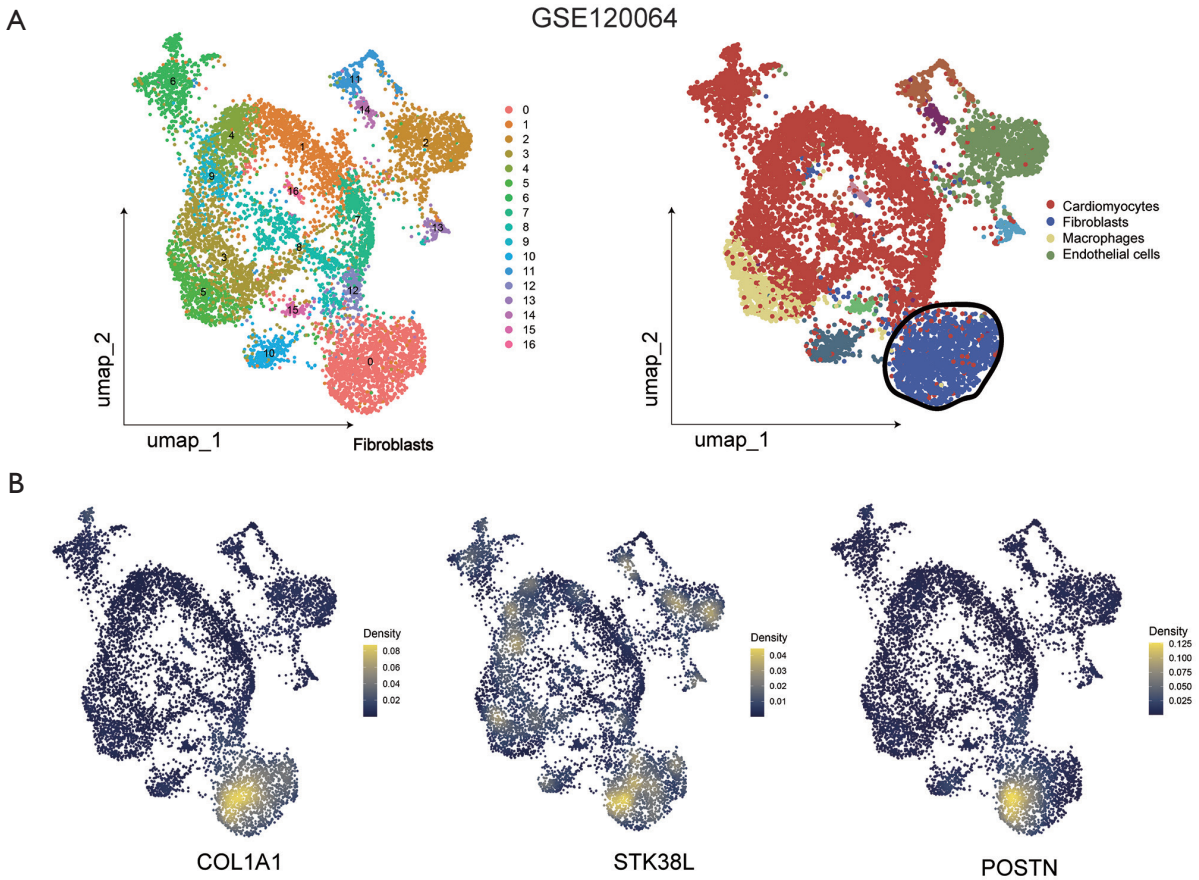

Figure S7 Analysis of GEO single-cell RNA-seq data reveals the expression distribution of COL1A1, STK38L, and POSTN across different cell types in the hearts of TAC model mice. (A) Using tSNE from GSE120064 single-cell RNA-seq data, the 2D visualization of single-cell clusters from the heart of a TAC model mouse was achieved. Fibroblasts were highlighted by circling them. (B) Density plot demonstrating the expression and distribution of COL1A1, STK38L, and POSTN in GSE120064 single-cell RNA-seq data of TAC model mice based on the E plot of umap. GEO, Gene Expression Omnibus; RNA-seq, RNA sequencing; TAC, transverse aortic constriction; tSNE, t-distributed stochastic neighbor embedding.

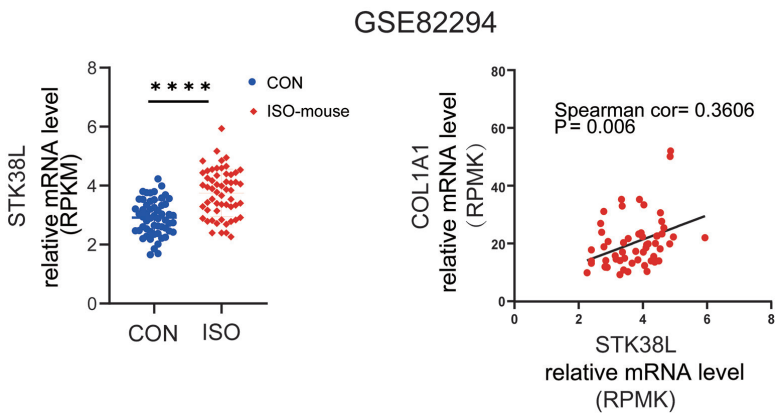

Figure S8 Comparison of STK38L relative mRNA levels between the CON and ISO groups from the GSE82294 RNA-Seq dataset. STK38L expression correlates with COL1A1 in the above dataset (spearman cor =0.3606, P=0.006). ****, P<0.0001. RPKM, reads per kilobase per million mapped reads; CON, control group; ISO, isoprenaline group; RNA-seq, RNA sequencing.

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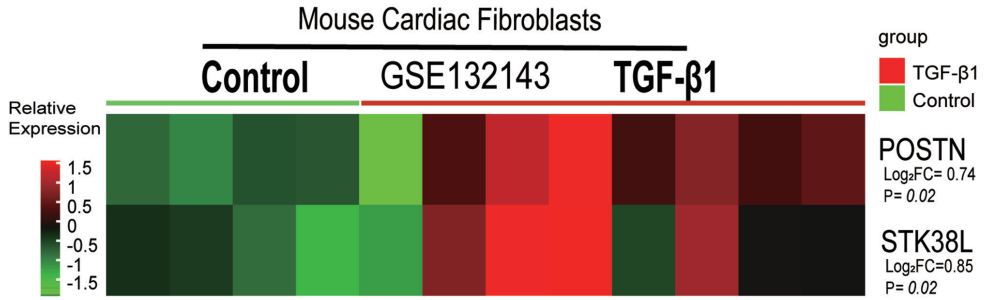

Figure S9 Elevated STK38L expression in mouse models of TGF-β1 stimulation-induced myocardial fibrosis as analyzed by the GEO database. Heatmap showing increased expression of POSTN (log_2FC =0.74; P=0.02) and STK38L (log_2FC =0.85, P=0.02) mRNA levels in the control and TGF-β1 groups of GSE 123143 RNA-seq data. TGF-β1, transforming growth factor β1; GEO, Gene Expression Omnibus; FC, fold change; RNA-seq, RNA sequencing.

Table S1 Baseline patient characteristics

Variables	Overall (n=13)	AF (n=8, 62%)	SR (n=5, 38%)	P value
Age (years)	63.15 (6.59)	64.00 (6.50)	61.80 (7.26)	0.596†
History of AF (years)	1.00 [0.00, 3.00]	2.50 [1.75, 5.25]	0.00 [0.00, 0.00]	0.003‡
BMI (kg/m^2)	23.85 (2.19)	23.84 (2.24)	23.87 (2.37)	0.981†
NT-proBNP (pg/mL)	401.00 [82.00, 492.00]	486.00 [437.00, 611.25]	66.00 [50.00, 82.00]	0.002‡
LAD (mm)	42.69 (12.74)	49.38 (11.55)	32.00 (4.64)	0.011†
LADI	29.16 [18.19, 50.07]	44.42 (11.72)	16.88(1.23)	<0.001¶
LVEF (%)	63.31 (4.01)	63.63 (4.90)	62.80 (2.39)	0.693†
Sex				>0.999§
Male	9 (69.23)	5 (62.50)	4 (80.00)	
Smoke	2 (15.38)	1 (12.50)	1 (20.00)	>0.999§
Hypertension	2 (15.38)	1 (12.50)	1 (20.00)	>0.999§
Furosemide	7 (53.85)	5 (62.50)	2 (40.00)	0.592§
Anti-arrhythmia_agent				0.736§
No	6 (46.15)	3 (37.50)	3 (60.00)	
Digoxin	2 (15.38)	2 (25.00)	0 (0.00)	
Metoprolol	4 (30.77)	2 (25.00)	2 (40.00)	
Amiodarone	1 (7.69)	1 (12.50)	0 (0.00)	
RAAS__inhibitors	3 (23.08)	2 (25.00)	1 (20.00)	>0.999§
Statins	4 (30.77)	2 (25.00)	2 (40.00)	>0.999§

The data were described by mean (SD), median [IQR], and n (%). A comparison of clinical data between patients with AF and SR was presented. †, unpaired *t*-test; ‡, Mann-Whitney test; §, Fisher test; ¶, Welch's test. AF, atrial fibrillation; SR, sinus rhythm; BMI, body mass index; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; LAD, left atrial diameter; LADI, left atrial volume index; LVEF, left ventricular ejection fractions; RAAS, renin-angiotensin-aldosterone system; SD, standard deviation; IQR, interquartile range.

https://dx.doi.org/10.21037/cdt-24-164

Table S2 The primer sequences for the genes

Gene name	Forward primer	Reverse primer
STK38L-mouse	5'-CACATAACCCGCCAAGCGACTT-3'	5'-GTCTGGTGTTCCAACTGTGGAG-3'
STK38L-human	5'-GTTACGTCGATCACAACACGCTC-3'	5'-CTTTCTTCTGGACCAACCGCAC-3'
CLO1A1-mouse	5'-CCTCAGGGTATTGCTGGACAAC-3'	5'-CAGAAGGACCTTGTTTGCCAGG-3'
CLO1A1-human	5'-GATTCCCTGGGACCTAAAGGTGC-3'	5'-AGCCTCTCCATCTTTGCCAGCA-3'
GAPDH-mouse	5'-CATCACTGCCACCCAGAAGACTG-3'	5'-ATGCCAGTGAGCTTCCCGTTCAG-3'
GAPDH-human	5'-GTCTCCTCTGACTTCAACAGCG-3'	5'-ACCACCCTGTTGCTGTAGCCAA-3'
POSTN-mouse	5'-CAGCAAACCACTTTCACCGACC-3'	5'-AGAAGGCGTTGGTCCATGCTCA-3'

The names, species, and specific sequences of the siRNAs constructed in the article were summarized. STK38L, serine/threonine kinase 38-like; CLO1A1, collagen type I alpha 1 chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POSTN, periostin.

Table S3 Antibody type

Antibodies name	Type & Company
STK38L	1:2,000, ta505176, Origene
Collagen I	1:1,000, ab260043, Abcam
POSTN	1:1,000, 66491-1-Ig, Proteintech
GAPDH	1:1,000, Abcam
HRP-labeled goat anti-rabbit IgG antibodies	1:10,000, ab6721 Abcam
HRP-labeled goat anti-mouse IgG antibodies	1:5,000, GB23301, Servicebio

STK38L, serine/threonine kinase 38-like; POSTN, periostin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase.

Table S4 GEO database public data list

GSE No.	Descriptions (overall design)	Citation(s)
GSE97358	Human cardiac fibroblasts (total 168), 84 control, 84 TGF-β1 induced. Cardiac fibroblasts were cultured in serum-free media for at least 16 hours prior to treatment with TGF-β1	(34)
GSE132143	This dataset contains TGF-β1-treated primary mouse cardiac fibroblasts with control sequencing data	(35)
GSE120064	Extract cells from left ventricle of TAC mouse heart and conduct single-cell sequencing	(36)
GSE82294	Cardiac mRNA expression profiles of the various inbred mouse strains were examined either under baseline condition (control) or in response to chronic administration of isoproterenol or atenolol at 10 mg/kg per day for 2 weeks. Expression data were produced by RNA-sequencing, in triplicates, using the HiSeq 2000 Illumina platform	(37)
GSE225336	This dataset uses multiple time points (6, 24, 48, 72 h) to detect differential expression and chromatin accessibility in primary human cardiac fibroblasts treated with human TGF-β1 and controls	(38)

GEO, Gene Expression Omnibus; GSE, GEO series; TGF-β1, transforming growth factor β1; TAC, transverse aortic constriction.

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