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Intercellular transfer of SerpinE2 activates PI3K-AKT and β-catenin signaling to promote cardiac hypertrophy

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Abstract

Background: Effective inhibition of pathological cardiac hypertrophy is critical for managing various cardiovascular diseases, especially in cold environments. The communication between cardiomyocytes and fibroblasts, mediated by secreted proteins, plays a significant role in the development and progression of pathological cardiac hypertrophy. Serpin Family E Member 2 (serpinE2), secreted by fibroblasts into the extracellular space, has been implicated in this process. However, whether serpinE2 can be internalized by cardiomyocytes and whether cold exposure influences this process remains unclear. **Materials and methods:** Mice were subjected to cold exposure (4 °C, 12 h/day for 8 weeks), and cardiac hypertrophy was induced by transverse aortic constriction (TAC). SerpinE2 expression was silenced by short interfering RNA (siRNA). Cardiac fibroblasts were stimulated with angiotensin II (Ang II) to induce serpinE2 secretion. Exogenous recombinant serpinE2, labeled with DyLight 488 or His-tag, was used to evaluate its internalization and functional role in cardiomyocytes. Internalization was inhibited by using antibodies against serpinE2, heparin, or endocytosis inhibitors (β-cyclodextrin, nystatin, dynasore, and chlorpromazine). Chromatin immunoprecipitation followed by quantitative polymerase chain reaction (PCR) was used to assess the binding of the transcription factor CDX1 to the serpinE2 promoter. **Results:** Cold exposure significantly increased serpinE2 mRNA and protein expression in mouse hearts. SerpinE2 levels were also upregulated in plasma and cardiac tissue following TAC. Knockdown of serpinE2 attenuated TAC-induced hypertrophy, restored left ventricular function, and reduced atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain fragment levels. Exogenous serpinE2 promoted cardiomyocyte hypertrophy, an effect that was reversed by serpinE2 knockdown. Co-culture with conditioned medium from Ang II-stimulated fibroblasts increased serpinE2 expression in cardiomyocytes. Exogenous serpinE2 was internalized *via* endocytosis, which was inhibited by antibodies, heparin, and endocytosis blockers. Internalized serpinE2 activated the protein kinase B (AKT)/β-catenin pathway in cardiomyocytes. CDX1 bound to the serpinE2 promoter and promoted its transcription in fibroblasts. CDX1 overexpression increased serpinE2 and collagen expression, while its suppression had the opposite effect. Administration of exogenous fibroblast growth factor 4 (FGF4) or overexpression of FGF4 plasmid upregulated CDX1, serpinE2, and collagen expression in fibroblasts. **Conclusions:** SerpinE2 expression is responsive to cold stress and mediates intercellular communication between fibroblasts and cardiomyocytes. Fibroblast-secreted serpinE2 is internalized by cardiomyocytes *via* endocytosis, promoting hypertrophy through activation of the phosphatidylinositol 3-kinase (PI3K)-AKT/β-catenin pathway. The FGF4-CDX1 axis regulates serpinE2 expression and secretion in cardiac fibroblasts.

Keywords

cold exposure; serpinE2; cardiac hypertrophy; cellular communication; endocytosis

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1 Introduction

Prolonged exposure to cold environments has been associated with a heightened risk of cardiovascular disease (CVD), posing a serious threat to human health[1]. A 2017 meta-analysis reported that cold exposure increased the risk of CVD-related mortality by approximately 5%[2]. This effect is primarily attributed to increased sympathetic nervous system activity and vasoconstriction, which adversely affect cardiac mechanics and contribute to the development of cardiac hypertrophy[3]. Pathological cardiac hypertrophy is a major precursor to heart failure and remains a leading cause of cardiovascular morbidity and mortality worldwide[4]. Heilongjiang, one of the northernmost provinces in China, is characterized by harsh winters and substantial seasonal temperature variation, contributing to a higher burden of CVDs in its population[3]. Since cardiac hypertrophy is a common pathological feature across various CVDs, its effective inhibition is especially important for reducing disease incidence in cold climates.

Cardiomyocytes and cardiac fibroblasts are central to maintaining the structural and functional integrity of the heart[5]. Communication between these two cell types is crucial for homeostasis and plays a significant role in the pathogenesis of cardiac diseases[6]. In pathological cardiac hypertrophy, this intercellular communication becomes dysregulated, resulting in myocardial fibrosis, altered extracellular matrix (ECM) composition, and hypertrophic growth of cardiomyocytes[5]. This crosstalk is mediated by a range of secreted signaling molecules, including microRNAs and exosome proteins[7]. Identifying the key molecules that mediate cardiomyocyte-fibroblast communication is therefore essential for elucidating the mechanisms of cardiac hypertrophy and discovering novel therapeutic targets.

Serine protease inhibitors (serpins) are important regulators of cellular and tissue responses to environmental stressors[8]. SerpinE2, also known as protease nexin-1 (PN-1), is secreted serpin with anti-serine protease activity[9], widely distributed in the extracellular space and plasma membrane. Previous research has demonstrated that serpinE2 is involved in tumor metastasis[10-11] and in the pathophysiology of certain CVDs[8]. Additionally, serpinE2 expression has been found to be temperature-sensitive[12]; however, its potential role in cold-induced cardiac hypertrophy remains unclear.

Our previous research demonstrated that serpinE2 was primarily produced by cardiac fibroblasts, with its expression upregulated by angiotensin II and transforming growth factor-β (TGF-β), and increased in both *in vivo* and *in vitro* models of cardiac fibrosis[13]. Furthermore, exogenous serpinE2 can be internalized into fibroblasts *via* endocytosis, leading to enhanced collagen synthesis and promotion of cardiac fibrosis[14]. These findings suggest that fibroblast-secreted serpinE2, after being released into the ECM, can be taken up by fibroblasts to reinforce fibrotic remodeling. Given the close anatomical relationship between fibroblasts and cardiomyocytes, we hypothesized that serpinE2 secreted by fibroblasts might also be internalized into cardiomyocytes, potentially inducing hypertrophy through intercellular signaling.

The objectives of this study were to determine whether serpinE2 expression in myocardial tissue is influenced by cold exposure, whether serpinE2 participates in the intercellular communication between fibroblasts and cardiomyocytes, and whether it contributes to the development of pathological cardiac hypertrophy—particularly through exosomal pathways and specific signaling mechanisms.

2 Materials and methods

2.1 Animal treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (approval ID: DEC6121) and performed in accordance with the National Institutes of Health (NIH) Guidelines for the Protection and Use of Laboratory Animals. Eight-week-old C57BL/6 mice (20-25 g) were used. For cold exposure, mice were subjected to 4 °C for 12 h per day over 8 weeks using a ventilated cold air circulation system[15]. To induce cardiac hypertrophy, transverse aortic constriction (TAC) surgery was performed. Mice were anesthetized with intraperitoneal injection of 2% avertin (0.1 mL/10 g body weight), and a left thoracotomy was performed to expose the aorta. The transverse aorta was ligated using a 7-0 nylon suture and a 27-gauge needle. After ligation, the needle was removed, and the chest was closed. Sham-operated mice underwent the same procedure without ligation. Cardiac function was evaluated using echocardiography.

2.2 Knockdown of serpinE2 in mice

SerpinE2-RNAi lentivirus and negative control lentivirus were purchased from the GeneChem (Shanghai, China). The serpinE2-RNAi sequence was 5'-TTGGCATTACTGAGATGTT-3' and the negative control (NC) sequence was 5'-TTCTCCGAACGTGTCACGT-3'. Mice were injected with 1×10^7 TU of lentivirus *via* tail vein. One week later, TAC surgery was performed, and mice were maintained for 4 additional weeks.

2.3 Transfection of siRNAs and overexpression plasmids in cardiomyocytes

SerpinE2 siRNAs were obtained from Invitrogen (Carlsbad, CA,

USA). Mouse FGF4 and CDX1 expression plasmids (Myc-DDK-tagged) were acquired from Origene (Northampton, MA, USA). Cells were cultured in serum-free DMEM for 6 h before transfection. Transfection was carried out using X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturers' protocols.

2.4 Exosome extraction

Cell culture media were collected after achieving the desired cell density. The Total Exosome Isolation Kit (4478359, Thermo Fisher Scientific, Waltham, MA, USA) was used per the manufacturer's protocol. Briefly, supernatant was mixed with extraction reagent (1 : 0.5 volume ratio), incubated overnight at 4 °C, and centrifuged at 10,000 × g for 1 h. The pellet was resuspended in PBS and used for protein quantification.

2.5 Quantitative real-time polymerase chain reaction

Total RNA from heart tissue and neonatal mouse cardiomyocytes (NMCMs) was extracted using TRIZOL reagent (Invitrogen Carlsbad, CA, USA). cDNA was synthesized using the First-Strand cDNA Synthesis Kit (AU341-02-V2, TransGen, Beijing, China). qRT-PCR was performed with SYBR Green Master Mix (TG-AQ601-04, TransGen, Beijing, China) using an ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA). Gene expression was normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. Primers were synthesized by Invitrogen (see Table 1).

2.6 Enzyme-linked immunosorbent assay

Protein concentrations in plasma and fibroblast culture supernatants were measured using ELISA kits (Elabscience, Wuhan, China) according to manufacturer instructions and previous protocols [16].

2.7 Isolation and culture of mouse neonatal cardiomyocytes and fibroblasts

Hearts from 1- to 3-day-old mice were harvested and incubated in pancreatin (Beyotime, Shanghai, China) at 4 °C for 12 h. After washing, tissue was digested with type II collagenase, and cells were centrifuged at 1500 × g for 5 min. Cells were resuspended in DMEM with 5% FBS (Biological Industries, Kiryat Malakhi, Israel) and 1% penicillin/streptomycin (Solarbio, Beijing, China) and plated for 2 h at 37 °C in 5% CO_2. Cardiomyocytes and fibroblasts were separated and cultured for 48 h.

2.8 Cell treatment

Cardiomyocytes were treated with 10 nmol/L Ang II or 10-40 ng/mL recombinant serpinE2 for 24-48 h to induce hypertrophy. To inhibit PI3K signaling, 10 μmol/L LY294002 was added for 24 h. FGF4 was added to fibroblasts at 10-80 ng/mL for 24 h to assess its effects.

Table 1 Primers used for the Real-Time PCR (qRT-PCR) analysis

RNA name	Primers from 5' to 3'
ANP-F	ACCTGCTAGACCACCTGGAG
ANP-R	CCTTGGCTGTTATCTTCGGTACCGG
BNP-F	GAGGTCACTCCTATCCTCTGG
BNP-R	GCCATTTCCTCCGACTTTTCTC
β-MHC-F	CCGAGTCCCAGGTCAACAA
β-MHC-R	CTTCACGGGCACCCTTGGA
GAPDH-F	GGGGCTCTCTGCTCCTCCCTG
GAPDH-R	CGGCCAAATCCGTTCACACCG
FGF4-F	GACCAAGAAGGGGAACCGAG
FGF4-R	CGGAGGGTCACAGTCTAGGA
Cdx1-F	TCTACACAGACCACCAACGC
Cdx1-R	TTTACCTGCCGCTCTGTGAG
SerpinE2-F	CGATCTCCCTCCCGGTTTC
SerpinE2-R	TCCCTGTGTTGGAGCCTAGT

2.9 Echocardiography

Mice were anesthetized with 2% avertin (0.1 mL/10 g) and evaluated using a Vevo2100 Imaging System (VisualSonics, Toronto, Canada). M-mode images from a short-axis view were used to measure ejection fraction (EF), fractional shortening (FS), LV volume, and LV mass.

2.10 Western blot analysis

Proteins from LV tissue or cultured cells were extracted using RIPA buffer (N8030, Solarbio, Beijing, China) containing protease inhibitors. Protein concentrations were measured with a BCA assay (Beyotime, Shanghai, China). Proteins were separated by SDS-PAGE, transferred to NC membranes (Millipore, Bedford, MA, USA), and blocked with 5% milk for 2 h. Membranes were incubated with primary antibodies against β-MHC, β-catenin, serpinE2, phospho-AKT (S473), total AKT, CDX1, and GAPDH (sources as specified). Exosomal proteins were prepared using TransExo™ Kit (FE101-02, TransGen, Beijing, China).

2.11 Cell size measurement

NMCMs were fixed in 4% formaldehyde for 25 min, permeabilized with 0.2% Triton X-100 (Sigma St. Louis, Missouri, USA), and blocked with 8% goat serum (Abbkine, Wuhan, China) for 1 h. Cells were stained with anti-α-actinin and Alexa Fluor-conjugated secondary antibodies (Invitrogen Carlsbad, CA, USA). Nuclei were stained with DAPI. Fluorescence images

were captured using a Nikon 80i microscope.

2.12 Immunofluorescence

Cardiomyocytes were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 8% goat serum. Cells were incubated overnight at 4 °C with antibodies against serpinE2 or α-actinin, followed by Alexa Fluor 594-conjugated secondary antibodies. Nuclei were stained with DAPI. Imaging was performed using a Nikon 80i microscope.

2.13 Validation of endocytosis of serpinE2 in cardiomyocytes

Recombinant mouse serpinE2 (ab92712, Abcam, Cambridge, UK) was labeled with DyLight® 488 using a Fast Conjugation Kit (Abcam, Cambridge, UK). The conjugate, formed *via* primary amines (*e.g.*, lysine, arginine), was applied to cardiomyocytes. Endocytosis was assessed using inhibitors including β-cyclodextrin (non-selective), dynasore and chlorpromazine (clathrin-mediated), and nystatin (clathrin-independent). SerpinE2 antibody and heparin were also used as blockers.

2.14 Chromatin immunoprecipitation-PCR assay

ChIP was performed using a commercial kit (#26156, Pierce, Thermo Fisher, Waltham, MA, USA). Binding of CDX1 to the rat serpinE2 promoter (NM_019197.1) was analyzed by qPCR using primer sets targeting five predicted CDX1-binding sites. RT-PCR was performed in triplicate, and a 181 bp distal region served as a negative control.

The PCR primer pairs used were shown in Table 2. They were designed to amplify CDX1 site1 (273 bp), CDX1 site2 (213 bp), CDX1 site3 (265 bp), CDX1 site2 (151 bp), and CDX1 site2 (305 bp) fragments, respectively, from selected genomic regions. RT-PCR of genomic regions containing putative CDX1-binding sites was performed in triplicate.

Table 2 Primers used for the Reverse Transcription PCR (RT-PCR) analysis

RNA name	Primers from 5' to 3'
CDX1-ChIPF1	GATCATTGGAAACACTGATG
CDX1-ChIPR1	GAAAATCTGAGAGCTGCTTTCC
CDX1-ChIPF2	TGGAGGCGTGCTCATTTCTGG
CDX1-ChIPR2	CAGGGCATCCCTGTGTCCAAAG
CDX1-ChIPF3	CTGTAGTCTCCATCTCTGTCTC
CDX1-ChIPR3	CCCTCTCAGGTCACAGTCAGAC
CDX1-ChIPF4	CAGGAGCATTTGGGACAGAGGG
CDX1-ChIPR4	GTAGGGACAAATGCTATGGAAG
CDX1-ChIPF5	GTATAGGTTCAAAACACCTGG
CDX1-ChIPR5	GGAGTGGGCGACACCGAAGCC
negative control-F	TTCCCTCAGAACAATAACGCAG
negative control-R	CCTTCCAAGTAGAAGCTTGGAATG

2.15 Statistics

Data are presented as mean ± SEM. Statistical significance between two groups was determined using unpaired Student's *t*-test. For comparisons among multiple groups, one-way ANOVA followed by post-hoc testing was used. A p-value < 0.05 was considered statistically significant. Analyses were performed using GraphPad Prism 9.5.1

3 Results

3.1 Chronic cold exposure increases serpinE2 expression in the heart

To investigate the effect of cold stress on cardiac hypertrophy, mice were exposed to 4 °C 12 h/ d for 8 w (Fig. 1A). Cold exposure led to a significant increase in cardiac volume and in the heart weight-to-body weight ratio (Fig. 1B). β-catenin, a protein associated with cold-induced myocardial hypertrophy[17], was also significantly upregulated under these conditions (Fig. 1C). Following confirmation of the cold-induced cardiac hypertrophy model, we assessed serpinE2 expression. Both mRNA and protein levels of serpinE2 were significantly elevated in cold-exposed mice compared to those housed at room temperature (Fig. 1D, E), suggesting that serpinE2 is responsive to cold stress and may play a regulatory role in cardiac function.

3.2 SerpinE2 secreted by fibroblasts is internalized by cardiomyocytes through endocytosis

To explore the origin and functional uptake of serpinE2, we first compared its expression levels between cardiac fibroblasts and cardiomyocytes. SerpinE2 was found to be significantly more abundant in fibroblasts (Fig. 2A). Additionally, exosome isolation from fibroblast-conditioned medium revealed elevated serpinE2 levels under pathological stimulation (Fig. 2B and Supplementary Fig. 1), indicating that fibroblasts release serpinE2-rich exosomes into the extracellular matrix.

When fibroblasts were pre-treated with angiotensin II and co-cultured with cardiomyocytes for 24 h, serpinE2 levels significantly increased in cardiomyocytes, as detected by ELISA (Fig. 2C). To visualize uptake, exogenous recombinant serpinE2 was labeled with DyLight® 488, which emits green fluorescence. The labeled protein was successfully internalized by cardiomyocytes, as indicated by increased green fluorescence (Fig. 2D). This uptake was significantly reduced by pre-treatment with serpinE2 antibody or heparin, which block serpinE2 binding

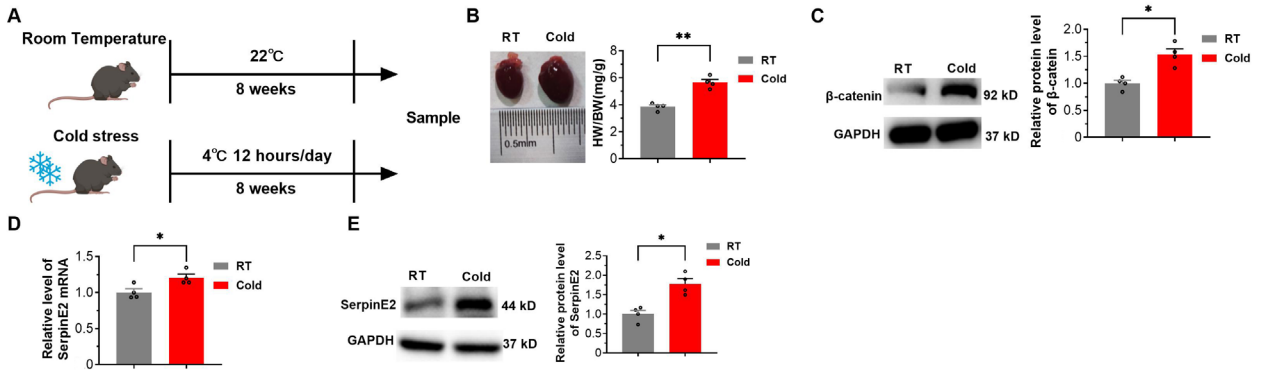

Fig. 1 SerpinE2 expression is upregulated during chronic cold exposure

(A) Schematic diagram illustrating the construction of the mouse mode of cold stimulation. (B) Representative image of heart sizes and statistical results of heart weight/body weight ratios ($N = 4$). (C) Relative protein levels of β-catenin ($N = 4$). (D) Relative mRNA levels of serpinE2 detected using real-time polymerase chain reaction (PCR) ($N = 4$). (E): Relative protein levels of serpinE2 in hearts detected by western blot analysis ($N = 4$). Data are presented as mean ± SEM. $^{*}P < 0.05$. RT, room temperature. Cold: cold stress (4 °C).

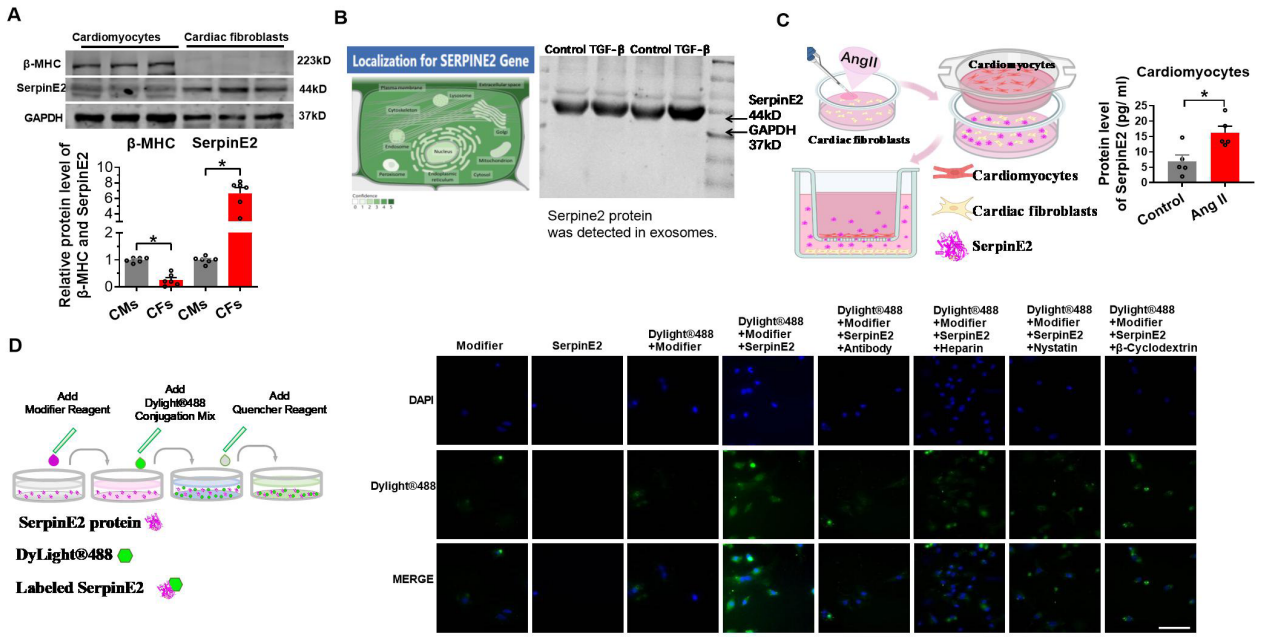

Fig. 2 SerpinE2 secreted by fibroblasts is internalized into cardiomyocytes

(A) SerpinE2 and β-myosin heavy chain (MHC) protein levels in cardiomyocytes and cardiac fibroblasts determined using western blot under normal culture conditions ($N = 6$). (B) Subcellular locations of serpinE2 obtained from the GENECARDs database (https://www.genecards.org/). Exosomes from the supernatant of fibroblasts were identified using Western blot analysis. (C) Cardiac fibroblasts treated with Ang II for 24 h and co-cultured with cardiomyocytes for another 24 h. SerpinE2 protein levels in cardiomyocytes measured using an enzyme-linked immunosorbent assay (ELISA) ($N = 5$). (D) Primary cultured rat cardiomyocytes treated with serpinE2 for 24 h, followed by incubation with serpinE2-antibody, heparin, β-cyclodextrin, and nystatin for 24 h. Results were examined under a fluorescence microscope (Scale bar 50 μm). Data are presented as mean ± SEM. $^{*}P < 0.05$.

to the cell membrane. Additionally, fluorescence intensity was decreased in the presence of endocytosis inhibitors (nystatin and β-cyclodextrin), confirming that serpinE2 enters cardiomyocytes through endocytosis.

3.3 SerpinE2 endocytosis occurs *via* both clathrin- and lipid raft-dependent pathways

Endocytosis of serpinE2 was further evaluated to determine

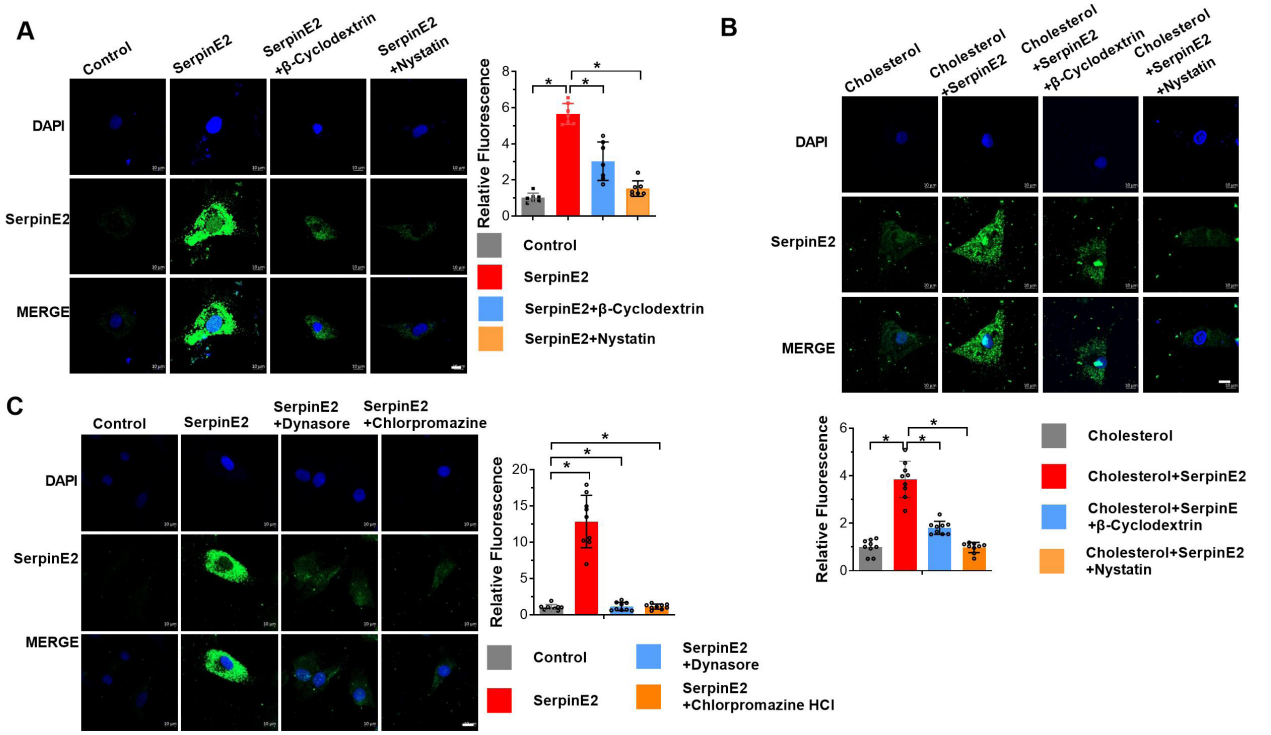

Fig. 3 SerpinE2 endocytosis is regulated by both clathrin-dependent and lipid-raft-dependent endocytic mechanisms

(A-B) Primary cultured rat cardiomyocytes treated with serpinE2, serpinE2 + β-cyclodextrin, or serpinE2 + nystatin for 24 h, with or without cholesterol (Scale bar 10 μm, *N* = 7-9). (C) Primary cultured rat cardiomyocytes treated with serpinE2, serpinE2 + dynasore, or serpinE2 + chlorpromazine for 24 h (Scale bar 10 μm, *N* = 9). Quantitative results of serpinE2 fluorescence density determined by Image J software v1.0. Data are presented as mean ± SEM. $^{*}P < 0.05$.

the underlying mechanisms. Two major pathways were assessed: clathrin-mediated endocytosis (CME) and clathrin-independent (lipid raft-mediated) endocytosis (CIE). Inhibitors of the CIE pathway (β-cyclodextrin, nystatin) and CME pathway (dynasore, chlorpromazine) were applied[18]. As shown in Fig. 3A-C, immunofluorescence revealed that His-tagged serpinE2 was internalized into cardiomyocytes under normal and cholesterol-enriched conditions. This internalization was blocked by all four inhibitors, demonstrating that both CME and lipid raft-dependent pathways are involved in serpinE2 uptake.

3.4 SerpinE2 induces cardiomyocyte hypertrophy *in vitro*

Public gene expression datasets (GSE4678, GSE3383, GSE5500, GSE1621) revealed elevated serpinE2 levels in cardiac hypertrophy (Fig. 4A). *In vitro*, administration of 40ng/mL exogenous serpinE2 significantly increased cardiomyocyte cross-sectional area, while co-treatment with siRNA targeting serpinE2 (si-serpinE2) abrogated this effect (Fig. 4B). Similarly, mRNA levels of hypertrophic markers ANP and BNP, and protein levels of β-MHC were elevated following serpinE2 treatment, whereas co-treatment with si-serpinE2 effectively attenuated serpinE2-induced upregulation of these markers (Fig. 4C-D). Under basal conditions, si-serpinE2 alone had no significant effect. Furthermore, serpinE2-induced β-MHC expression was reduced by co-treatment with serpinE2 antibodies, heparin, nystatin, or β-cyclodextrin (Fig. 4E), indicating that internalized serpinE2 promotes cardiomyocyte hypertrophy.

3.5 Inhibition of serpinE2 attenuates pressure overload-induced cardiac hypertrophy

ELISA analysis showed increased serpinE2 levels in the plasma and heart tissue of TAC-operated mice (Fig. 5A, B). To assess serpinE2's role *in vivo*, we employed a lentiviral vector expressing serpinE2-targeted RNAi (LV-serpinE2-RNAi). In mice subjected to TAC, serpinE2 knockdown significantly reduced heart size and weight (Fig. 5C). Echocardiographic analysis showed that TAC-induced reductions in EF and FS, and increases in LV systolic volume, were reversed by serpinE2 knockdown (Fig. 5D, E). Expression of hypertrophic markers (ANP, BNP, β-MHC) was similarly suppressed at both mRNA and protein levels (Fig. 5F, G).

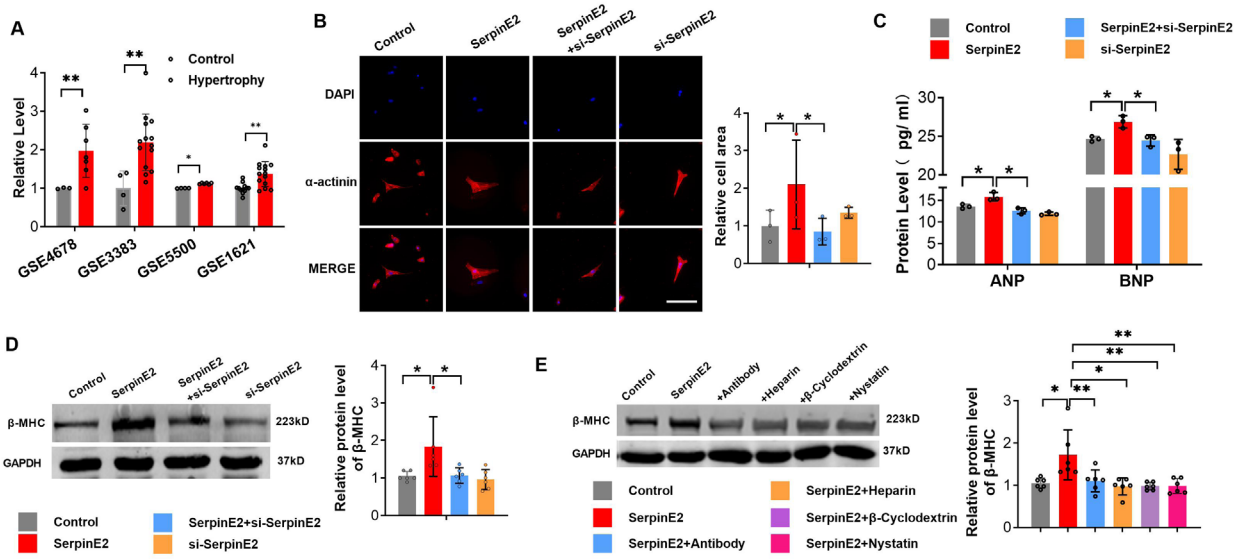

Fig. 4 SerpinE2 induces rat cardiomyocyte hypertrophy *in vitro*

(A) SerpinE2 mRNA levels in cardiac hypertrophy obtained from four mRNA microarray GEO databases (N = 3-14). (B) Primary cultured rat cardiomyocytes treated with serpinE2, serpinE2 + si-serpinE2, or si-serpinE2 for 24 h, and cell cross-sectional area determined using immunofluorescence assay (Scale bar 50 μm, N = 3); (C) Protein levels of ANP and BNP measured using ELISA (N = 3); (D) Relative protein levels of β-myosin heavy chain (β-MHC) determined using western blot analysis (N = 6). (E) Primary cultured rat cardiomyocytes treated with serpinE2, serpinE2 + antibody, serpinE2 + heparin, serpinE2 + β-cyclodextrin, or serpinE2 + nystatin for 24 h. Then the relative protein levels of β-MHC were detected using western blot (N = 6). Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.6 SerpinE2 promotes hypertrophy *via* β-catenin and AKT pathway activation

To decipher the signaling mechanisms, we assessed β-catenin and AKT activation. TAC surgery upregulated both β-catenin and phosphorylated AKT (p-AKT), which were significantly reduced following serpinE2 inhibition (Fig. 6A). Similarly, addition of exogenous serpinE2 to cardiomyocytes increased p-AKT and β-catenin levels (Fig. 6B). Immunofluorescence confirmed p-AKT activation by serpinE2, which was mitigated by antibodies and endocytosis inhibitors (Fig. 6C). SerpinE2-induced cardiomyocyte hypertrophy was also blocked by LY294002, a PI3K-AKT inhibitor (Fig. 6D), and β-MHC levels followed the same trend (Fig. 6E), demonstrating that serpinE2 induces hypertrophy through the PI3K-AKT/β-catenin pathway.

3.7 CDX1 transcription factor regulates serpinE2 expression in fibroblasts

To identify upstream regulators of serpinE2 expression, transcription factor binding sites were analyzed using TFSEARCH and TRANSFAC databases (Fig. 7A). Among the top candidates, CDX1 knockdown led to a marked reduction in serpinE2 mRNA levels (Fig. 7B). ChIP-PCR confirmed CDX1 binding to a response element (GGAATAAG) between -567 and -559 bp in the serpinE2 promoter (Fig. 7C, D). Although CDX1 is primarily known for intestinal development[19], it was shown here to modulate serpinE2 expression in cardiac fibroblasts. CDX1 knockdown decreased serpinE2 expression at both protein and mRNA levels(Fig. 7E-G) and reduced collagen content in fibroblasts and their supernatants (Fig. 7H). CDX1 overexpression significantly increased serpinE2 protein expression and collagen secretion compared to vector controls (Fig. 7I, J), confirming CDX1 as a transcriptional regulator of serpinE2.

3.8 FGF4 promotes CDX1 and serpinE2 expression in fibroblasts

Given CDX1's role in serpinE2 regulation and previous reports linking FGF4 to fibrosis[20-22], we investigated whether FGF4 modulates CDX1 expression. FGF4 treatment increased serpinE2 levels as assessed by ELISA (Fig. 8A), and elevated both CDX1 and serpinE2 protein expression (Fig. 8B). Similar effects were observed with FGF4 overexpression plasmids (Fig. 8C). Collagen content in fibroblast supernatants was also upregulated by both treatments (Fig. 8D, E). Immunofluorescence imaging revealed increased serpinE2 signal following FGF4 treatment (Fig. 8F).

Moreover, cold exposure significantly upregulated FGF4 and CDX1 mRNA and increased CDX1 protein expression (Supplementary Fig. 2), suggesting a cold-sensitive FGF4-CDX1-serpinE2 axis that regulates intercellular communication under environmental stress.

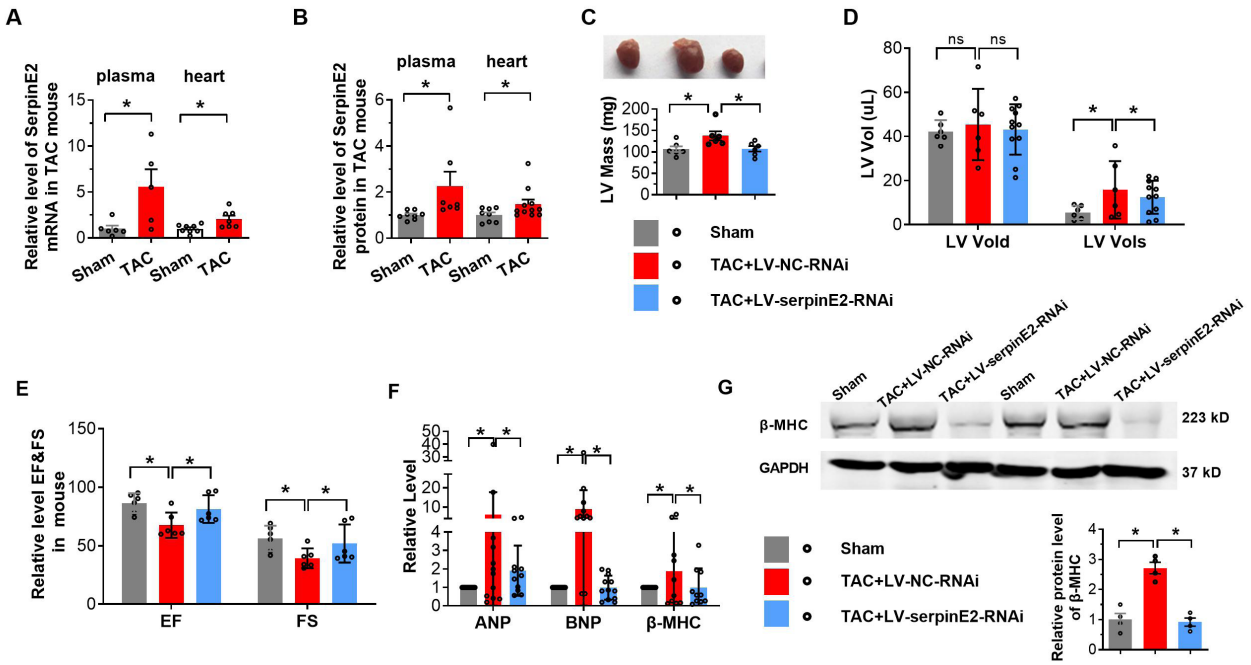

Fig. 5 Inhibition of SerpinE2 alleviates pressure overload-induced cardiac hypertrophy

(A-B) The cardiac hypertrophy mouse model was developed using transverse aortic constriction (TAC) methods and relative serpinE2 mRNA levels (N = 5-8) (A) and protein activities (B) in plasma and hearts were determined using real-time polymerase chain reaction (PCR) and ELISA, respectively (N = 7-11). (C) Two weeks after the injection of LV-NC or LV-RNAi-SerpinE2, TAC surgery was performed on mice to induce an *in vivo* cardiac hypertrophy model for 4 weeks. Representative images of the ventral side and LV mass of the heart (N = 6). (D) Assessments of echocardiographic parameters of end-diastolic volume (Vold) and end-systolic volume (Vols) (N = 6-11). (E) Ejection fraction (EF) and fraction shortening (FS) (N = 6). (F) Relative mRNA levels of ANP, BNP, and β-MHC were detected by real-time PCR (N = 10-11). (G) Relative protein levels of β-MHC in hearts were detected by western blot (N = 4). Data are presented as mean ± SEM. $^{*}P < 0.05$.

4 Discussion

In this study, we demonstrated that serpinE2 expression is significantly upregulated in response to cold exposure, implying that serpinE2 may function as a cold-responsive secreted protein involved in regulating pathological cardiac hypertrophy. We further identified the FGF4-CDX1 axis as a key regulator of serpinE2 synthesis and secretion in cardiac fibroblasts. Once secreted in the form of exosomes, serpinE2 mediates intercellular communication between cardiac fibroblasts and cardiomyocytes, participating in cardiac remodeling. Using a TAC-induced hypertrophy model, we confirmed that serpinE2 expression is markedly increased during cardiac hypertrophy, primarily due to activation of fibroblasts. Importantly, serpinE2 is endocytosed by cardiomyocytes, where it activates the AKT/β-catenin signaling pathway and promotes hypertrophy (Fig. 9).

Cardiac cell populations—including cardiomyocytes, endothelial cells, and fibroblasts—interact extensively through paracrine signaling to regulate cardiac structure and function[23]. Previous studies have shown that fibroblasts promote cardiac hypertrophy *via* the secretion of signaling molecules such as TGF-β[24], angiotensin II[25], insulin-like growth factor-1[26], and cardiotrophin-1[27]. Our prior work and others have also revealed that serpinE2 can act in an autocrine manner in fibroblasts to promote cardiac fibrosis[13,28-29]. In the present study, we extend those findings by demonstrating, for the first time, that fibroblast-secreted serpinE2 can be endocytosed by cardiomyocytes, thereby exerting a pro-hypertrophic effect.

Three lines of evidence support this conclusion: (1) Fibroblasts are the primary source of cardiac serpinE2. Baseline expression of serpinE2 is low in cardiomyocytes but high in fibroblasts, consistent with their secretory role. Supporting this, ExoCarta database entries (Experiment ID: 207) confirm serpinE2 as a component of fibroblast-derived exosomes. (2) Cardiomyocytes internalize extracellular serpinE2 *via* endocytosis. Our experiments using fluorescent labeling and inhibitors demonstrate that serpinE2 is taken up by cardiomyocytes through both clathrin-mediated and lipid raft-associated pathways. (3) Blocking serpinE2 endocytosis attenuates cardiomyocyte hypertrophy, further confirming its functional role in promoting hypertrophy.

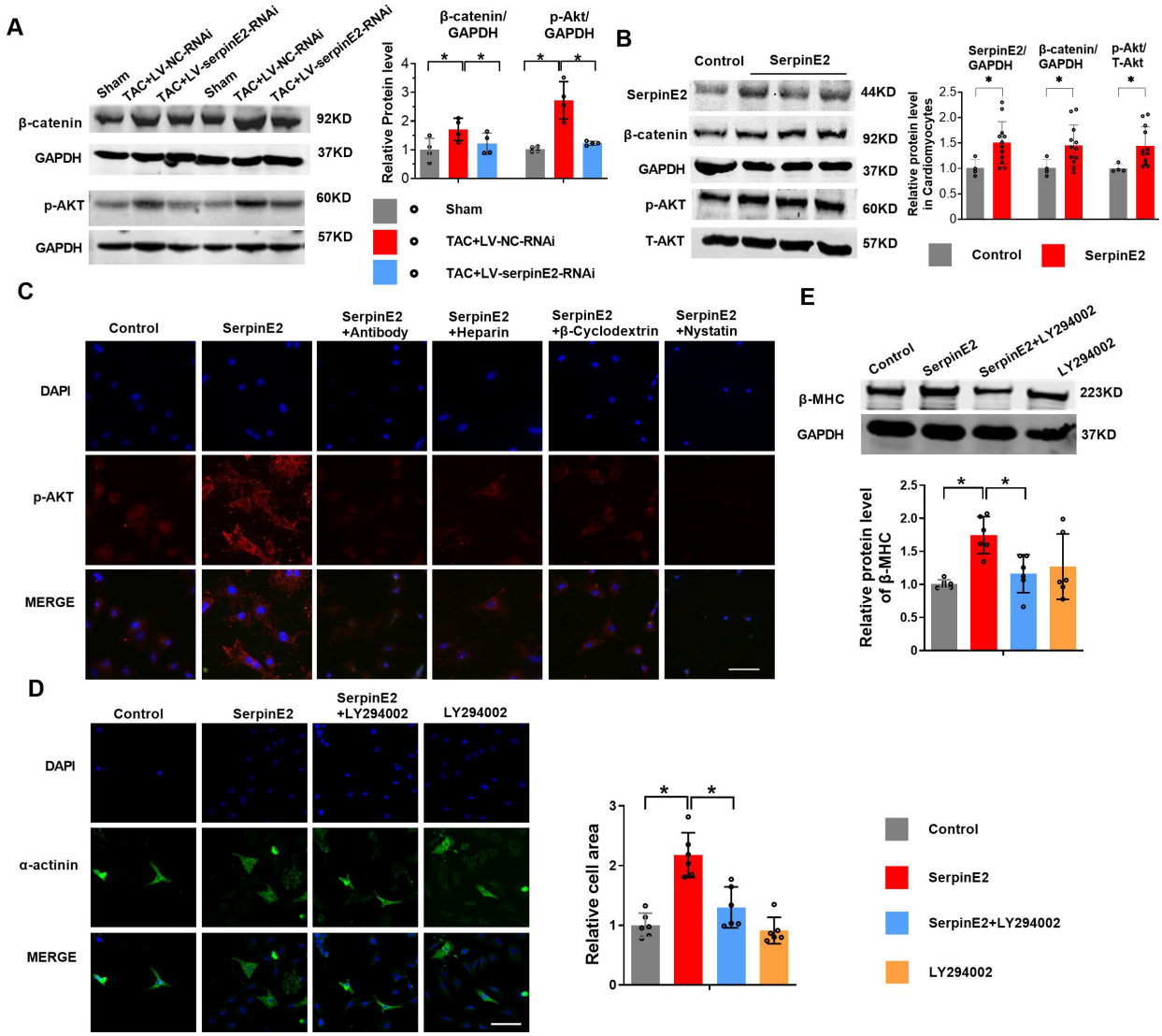

Fig. 6 SerpinE2 promotes cardiac hypertrophy by activating the β-catenin and protein kinase B (AKT) pathway

(A) Two weeks after the injection of LV-NC or LV-RNAi-SerpinE2, TAC surgery was performed for 4 weeks to establish a mouse model of cardiac hypertrophy. Relative protein levels of β-catenin, p-AKT detected by western blot (N = 4). (B) Primary cultured rat cardiomyocytes treated with serpinE2 for 24 h. Relative protein levels of serpinE2, β-catenin, p-AKT, and T-AKT detected by western blot (N = 4-12). (C) Primary cultured rat cardiomyocytes treated with serpinE2, serpinE2 + antibody, serpinE2 + heparin, serpinE2 + β-cyclodextrin, or serpinE2 + nystatin for 24 h, and cellular serpinE2 detected by immunofluorescence assay (Scale bar 50 μm). (D) Cardiomyocyte area measured using α-actinin immunofluorescence assay, following the administration of SerpinE2, SerpinE2 + LY294002, or LY294002 for 24 h (N = 6, Scale bar 50 μm). (E) β-MHC detected by western blot, following the administration of SerpinE2, SerpinE2 + LY294002, or LY294002 for 24 h (N = 6). Data are presented as mean ± SEM. $^{*}P < 0.05$.

Mechanistically, serpinE2 is internalized *via* endocytosis, which includes both clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) pathways[18, 30-31]. Inhibition with β-cyclodextrin or nystatin, which block lipid raft-dependent uptake, as well as with CME inhibitors dynasore and chlorpromazine, effectively suppressed serpinE2 uptake and hypertrophic responses. This indicates that serpinE2 employs multiple routes of entry into cardiomyocytes, enabling its robust intracellular signaling.

We also identified CDX1 as a novel transcriptional regulator of serpinE2. CDX1 was previously known to regulate intestinal and colorectal epithelial differentiation[32-34] and has been implicated in embryonic epicardial development[35]. Our study is the first to demonstrate its expression in myocardial fibroblasts, its binding to

A

Transcription Factor	Binding Sites		
	Homo sapiens	Rattus norvegicus	Mus musculus
HSF	112	179	131
SRY	35	48	39
CDX1	107	44	77
AP-1	3	35	25
GATA	30	27	11

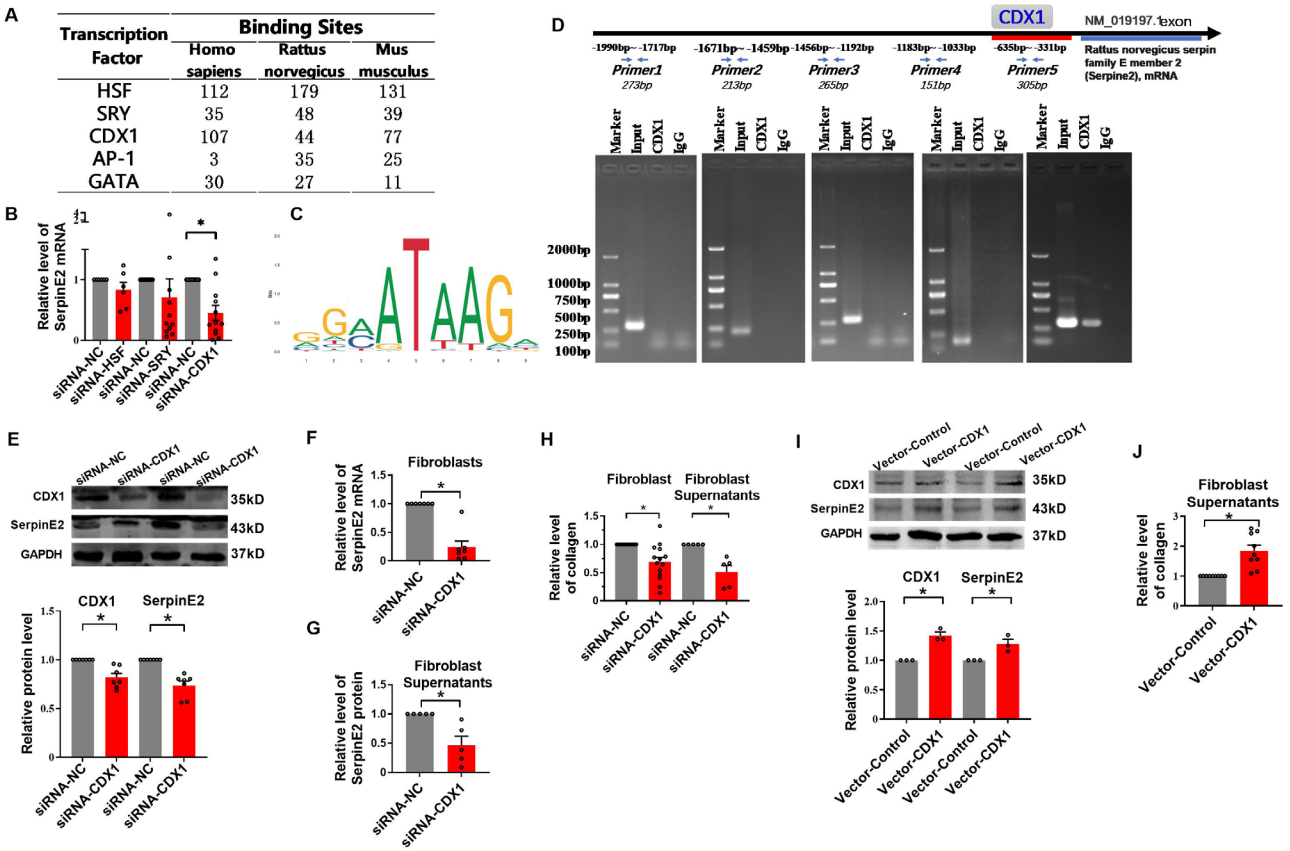

Fig. 7 CDX1 upregulates serpinE2 and collagen in rat cardiac fibroblasts

(A) The predicted transcript factors of SerpinE2. (B) SerpinE2 mRNA level in fibroblasts measured by real-time PCR ($N = 7$). (C) The predicted binding site of CDX1 on the genome of SerpinE2. (D) Schematic diagram of binding sites of CDX1 and primers used for CHIP-PCR using rat SerpinE2 gene sequences and agarose gel electrophoresis from ChIP-PCR showing CDX1 bound to the SerpinE2 promoter in rat cardiac fibroblasts. The promoter regions of SerpinE2 (-1990bp to -331bp) were amplified using the input and immunoprecipitated DNA as templates. (E) Protein levels of SerpinE2 and CDX1 ($N = 7$). (F) The mRNA level of SerpinE2 after inhibition of CDX1 in fibroblast ($N = 7$). (G) Protein levels of SerpinE2 in fibroblast supernatants detected using ELISA after inhibition of CDX1 ($N = 5$). (H) After inhibition of CDX1, collagen contents in fibroblasts or supernatants were measured by Sircol collagen assay ($N = 5$-14). (I) Protein levels of SerpinE2 and CDX1 in rat cardiac fibroblasts. (J) Collagen contents after overexpression of CDX1 ($N = 9$). Data are presented as mean ± SEM. $^{*}P < 0.05$.

the serpinE2 promoter, and its capacity to modulate serpinE2 and collagen expression. These findings reveal a new dimension of CDX1 function in cardiac pathophysiology.

Although the role of FGF4 in cardiac hypertrophy and fibrosis is not well characterized, prior studies suggest that FGF receptor inhibition can prevent left ventricular remodeling in renal and dietary disease models[36-37]. FGF4 has also been implicated in skeletal muscle hypertrophy under mechanical stress[38]. Our data indicate that FGF4 promotes serpinE2 expression through CDX1 activation, resulting in increased collagen secretion and fibroblast-derived exosomal signaling. This supports the hypothesis that excessive FGF4 levels may drive cardiac fibrosis and hypertrophy through the FGF4-CDX1-serpinE2 axis.

FGF4 has previously been shown to enhance the expression of collagens and ECM proteins in various tissues. In mice, FGF4 upregulates collagen type I in developing cranial sutures[20], and in chick limb development, it stimulates tendon-associated collagens such as Col1a1, Col3a1, Col5a1, Col12a1, and Col14a1[21]. Similarly, in embryonic valve precursor cells, FGF4 enhances the expression of scleraxis and tenascin[22]. These ECM-promoting functions are consistent with its known roles in cardiogenesis[39], heart maturation[40], and myocardial repair[41]. However, our study shows that excessive FGF4 activation can lead to pathological outcomes, including serpinE2 overproduction, collagen overexpression, and fibrotic remodeling, supporting its dual role in both cardiac repair and pathological hypertrophy.

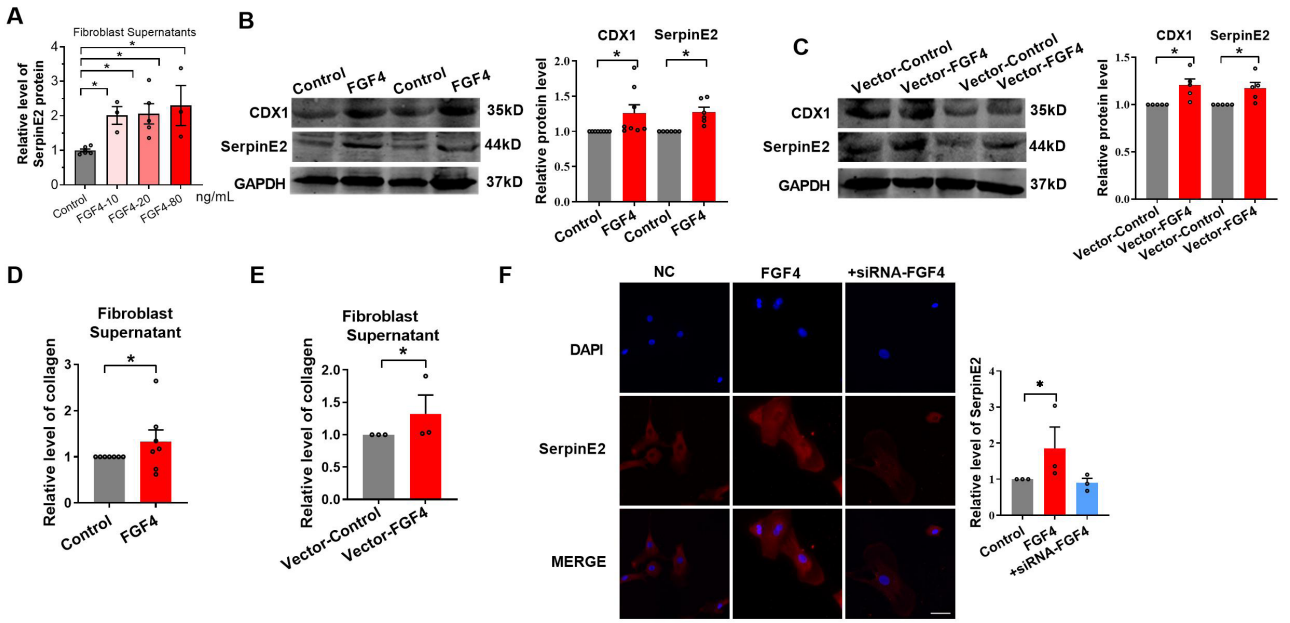

Fig. 8 FGF4 upregulates CDX1, serpinE2 and collagen expression in cardiac fibroblasts

(A) SerpinE2 in fibroblast supernatants were detected using an Enzyme-linked Immunosorbent Assay (ELISA) following the administration of 10, 20, or 80 ng/mL FGF4 (N = 3-6). (B-C) Relative protein levels of SerpinE2 and CDX1 in fibroblasts detected by western blot following the treatment with FGF4 (N = 6-8) (B) or FGF4 plasmid transfection (N = 5) (C). (D-E) Collagen contents in fibroblast supernatants determined by Sircol collagen assay following the treatment with FGF4 (N = 7) (D) or FGF4 plasmid transfection (N = 3) (E). (F) SerpinE2 expression in fibroblasts examined using immunofluorescence assay following the treatment with FGF4 or FGF4 + siRNA-FGF (Scale bar 50 μm, N = 3). Data are presented as mean ± SEM. $^{*}P < 0.05$.

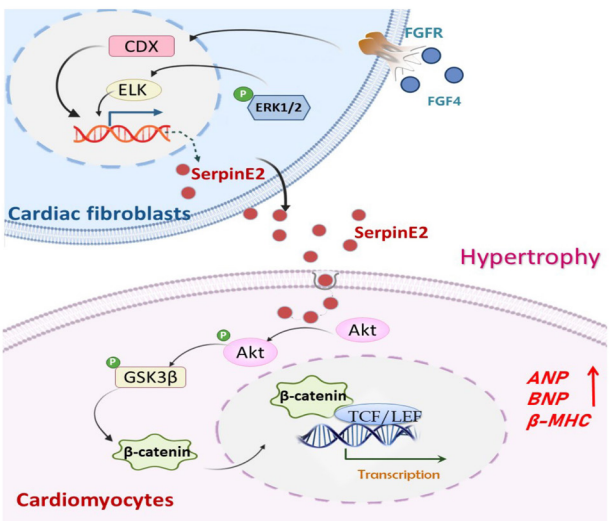

Fig. 9 Schematic diagram illustrating the proposed hypothesis on the mechanism of serpinE2 action

; the figure was drawn by Power Point 2021 (Microsoft, Redmond, Washington)

FGF4-CDX1 signaling promotes serpinE2 secretion from cardiac fibroblasts. Upon endocytosed into cardiomyocytes, serpinE2 promotes cardiomyocyte hypertrophy by activating the PI3K-AKT/β-catenin pathway; the figure was drawn by Power Point 2021 (Microsoft, Redmond, Washington).

In conclusion, our findings demonstrate that serpinE2 is a cold-responsive secreted factor from fibroblasts that enters cardiomyocytes *via* endocytosis and drives hypertrophy through the AKT/β-catenin pathway. The upstream FGF4-CDX1 signaling axis governs serpinE2 production in fibroblasts. These results identify serpinE2 as a promising therapeutic target for the treatment of pathological and cold-induced cardiac hypertrophy, and they open new avenues for addressing temperature-sensitive cardiovascular remodeling.

Acknowledgements

Not applicable.

Research ethics

All animal experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (approval ID: DEC6121).

Informed consent

Not applicable.

Author Contributions

Li X L, Shan H Land Lv L F designed the experiments and wrote the manuscript. Liu X and Wang X N performed the experiments and analyzed the data. Zhang H Z, Yang R N, Zhang M X performed the cell culture and participated in the statistical analysis. Li C and Liu Y performed molecular biology experiments. Zheng L and Xuan L N performed the animal experiment. Wei G Z , Gao Q, Li T Y cultured the cells, E X Q and Yu T revised and edited the manuscript.

Use of large language models, AI and machine learning tools

Not applicable.

Conflict of interests

All authors declare that they have no conflicts of interest or financial conflicts to disclose.

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Data availability

All data generated in this study are available on reasonable request from the corresponding author.

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