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Original Article

Increased expression of Sestrin2 in human and experimental heart failure

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Abstract: Background: Sestrins (Sesns) are originally identified as critical antioxidant proteins and involved in complex regulation of cell viability in response to diverse stress conditions. However, the exact expression and function of Sesns in heart failure (HF) remains unclear. Methods and results: Sesns expression was detected in heart samples from end-stage HF patients and unmatched donors by real-time polymerase chain reaction (RT-PCR), Western Blotting and Immunofluorescence. Mice myocardial ischemia/ reperfusion (I/R) and myocardial infarction (MI) models and doxorubicin induced rat HF model were established to confirm the heart expression of Sesns. Sesn2 was significantly increased, whereas, no alteration for Sesn1 or Sesn3 in failing hearts when comparing with control hearts. Unexpectedly, the mRNA expression of Sesn2 in ischemic cardiomyopathy was higher than that in dilated cardiomyopathy. Immunofluorescence staining revealed all Sesns were expressed in non-cardiomyocytes in human heart. Furthermore, Sesn2 was co-expressed with vimentin, a marker for fibroblasts. Correlation analysis demonstrated Sesn2 levels were significantly correlated with expression levels of natriuretic peptide B (NPPB) and connective tissue growth factor (CTGF), markers for severity of HF and cardiac fibrosis. Meanwhile, the increased Sesn2 was validated in mice I/R and MI models, and also in doxorubicin induced rat HF model. Conclusions: Sesn2, which is expressed in cardiac fibroblasts, could be a potential biomarker to reflect the severity of HF and might play an important role during cardiac remodeling.

Keywords: Heart failure, Sestrin, oxidative stress, fibrosis, biomarker

Introduction

Redox signaling plays important role not only in physiological processes, but also in pathological conditions in heart. At low concentrations, ROS modulate excitation-contraction coupling, cell proliferation and differentiation through changes in cellular signalling and gene expression [1-3]. However, overproduction of ROS may result adverse cardiac remodeling, leading to further worsening of cardiac function [1]. It is accepted that oxidative stress is at least one main cause of heart failure (HF) [4]. Targeting oxidative stress is becoming an important strategy to fight HF.

Sestrins (Sesns) are highly conserved proteins originally identified as critical antioxidant with sulfinic acid reductase activity, which is necessary for the recycling of peroxiredoxin [5-7]. Mammals express three Sesns (Sesn1, Sesn2, and Sesn3), while most invertebrate express only one Sesn. The Sesn family proteins can be induced by a variety of environmental stresses, including DNA damage, oxidative stress, and hypoxia [6, 8]. Once induced, Sesns can protect cells against oxidative stress [9-11].

It has been revealed that Sesn can maintain cardiac function against aging in Drosophila [12]. Sesn null mutants and heart-specific depletion of Sesn cause irregularity of heartbeat, cardiac dilation, ultimately leading to decreased cardiac function [12]. Recently, a protective function of Sesn2 against cardiac ischemia and reperfusion injury was demonstrated in mice. However, the expression and function of Sesns during HF remains unclear. In the present study, we aimed to examine the expression of Sesns in human and experimental failing hearts, and explore their correlation with cardiac function and fibrosis.

Table 1. Primer pairs used in the present study

Target name	Forward primer	Reverse primer
H-GAPDH	GGATTTGGTCGTATTGGGCG	TTCCCGTTCTCAGCCTTGAC
H-NPPB	TCAGCCTCGGACTTGGAAAC	AGACCCTTGCACCATCTTGG
H-CTGF	CGACTGGAAGACACGTTTGG	TGCAGGAGGCGTTGTCATT
H-Sesn1	GCATGTTCCAACATTTCGTG	TCCCACATCTGGATAAAGGC
H-Sesn2	GCAGGGCCATCTGGAACTAT	GTGCCTCCAGAAGAGGTTGT
H-Sesn3	ATGCTTTGGCAAGCTTTGTT	GCAAGATCACAAACGCAGAA
R-GAPDH	ACGGGAAACCCATCACCATC	TGGTGGTGCAGGATGCATTG
R-NPPB	TTCCTTAATCTGTCGCCGCTGG	CAGCAGCTTCTGCATCGTGGAT
R-Sesn1	ACCGAGTACCTTCGTTCTGC	ACAGCATAAGCAGATGGGCA
R-Sesn2	CCTTTGTGTTTGGCTGTGGG	AGAGCTTCTATGTCACGGGC
R-Sesn3	CAATGGCCACCCATGAGGAT	GCTGTCATACATGCGCTTCG
M-GAPDH	TGATGGGTGTGAACCACGAG	TCATGAGCCCTTCCACGATG
M-NPPB	GAGGTCACTCCTATCCTCTGG	GCCATTTCCTCCGACTTTTCTC
M-Sesn1	GGACGAGGAACTTGGAATCA	ATGCATCTGTGCGTCTTCAC
M-Sesn2	TAGCCTGCAGCCTCACCTAT	TATCTGATGCCAAAGACGCA
M-Sesn3	TTACTTGAACGGAGCCTGAAG	TCCATCAGAAGCAGATTCACG

Materials and methods

Human heart tissues

Human heart tissues were collected from transplanted hearts with end-stage heart failure, without diabetes mellitus. All samples were obtained from identical myocardial loci of left-ventricular to extremely ensure optimal uniformity. Control hearts were procured from organ donators who died of traffic accidents with no cardiac disease history. These hearts had originally been intended for transplantation, but failed to get suitable matching recipients. The hearts were immediately dissection into small portions, snap-frozen in liquid nitrogen, and then maintained at -80°C until using. Heart samples used for pathological study were fixed in 10% phosphate-buffered formalin and embedded in paraffin.

Fully informed consent was obtained from all patients or family members before participating in the study. This study protocol was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Mice myocardial ischemia/reperfusion and myocardial infarction models

Male C57BL/6 (8-10 weeks old) mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. Pentobarbital sodium was used to anesthetize mice through intraperitoneal injections (50 mg/kg, Sigma-Aldrich). To establish myocardial ischemia/reperfusion (I/R) model, a median sternotomy was performed. And then the left coronary artery (LCA) was ligated with a piece of tubing placed between the LCA and the suture to minimize coronary artery trauma induced by occlusion and to facilitate reperfusion. Reperfusion was initiated after 1 h of occlusion. The thoracic cavity was closed, and the animal was extubated after recovering consciousness. The hearts were harvested after reperfusion with 24 h. Same procedure was repeated to establish myocardial infarction (MI) model, and the thoracic cavity was closed after occlusion of the LCA. The hearts were harvested at 1 week and 4 weeks after operation. Sham-operated animals underwent the same surgical procedure without LCA occlusion.

Doxorubicin induced rat heart failure model

Male SD (6-8 weeks old) rats were purchased from Hunan SJA Laboratory Animal Co., Ltd. and were randomly divided into two groups. The doxorubicin (Dox) group received Dox (2.5 mg/kg/week) through intraperitoneal injection for 6 weeks. The control group received same volume saline as the same way. Two weeks after the last injection, the rats were sacrificed and heart samples were collected for research.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from heart tissue with TRIzol reagent (Roche, Indiana) as the manual instructed, and the concentration was assessed by Nanodrop 2000. A total of 2 μg RNA was used to synthesis cDNA using Transcriptor First Stand cDNA Synthesis Kit (Roche). Quantitative real-time PCR (qRT-PCR) was performed with the LightCycler 480 SYBR Green I Master (Roche) using the LightCycler 480 real-time PCR system according to the

Table 2. Characteristics for heart failure patients

	CTL (n=10)	Heart failure (n=28)	
		ICM (n=10)	DCM (n=18)
Gender male, n	10	8	15
Age (yr), mean ± SD	52.30 ± 4.42	54.00 ± 5.42#	52.93 ± 7.40#
LVEDD (mm), mean ± SD	-	65.60 ± 10.37	69.63 ± 9.72*
LVEF (%), mean ± SD	-	29.80 ± 5.09	25.58 ± 9.20*

CTL: Control hearts; ICM: Ischemic cardiomyopathy; DCM: Dilated cardiomyopathy; LVEDD: Left ventricular end diastolic diameter; LVEF: Left ventricular ejection fraction. #P>0.05 vs. CTL; *P>0.05 vs. ICM.

manufacturer's instructions (Roche). The primer pairs were designed online using Primer-Blast and were shown in **Table 1**. The PCR conditions used were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds (denaturation), 60°C for 10 seconds (annealing), and 72°C for 20 seconds (extension). The relative expression levels of mRNAs were normalized to the reference gene GAPDH. All reactions were conducted in triplicates and the data was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

Western blot was used to examine the protein expression of specific targets. Total proteins were extracted from heart tissues using RIPA lysis buffer (720 μL of RIPA, 20 μL of PMSF, 100 μL of complete protease inhibitor cocktail, 100 μL of Phosstop, 50 μL of NaF, and 10 μL of Na_3VO_4 in 1 mL of lysis buffer). The protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). 50 μg protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and then transferred to a PVDF membrane (Millipore, USA) that was blocked with 5% skim milk in Tris-buffered saline for 60 min at room temperature. The membrane was incubated overnight at 4°C with primary antibodies anti-sesn1 (1:1000, Santa Cruz Biotechnology, USA), anti-sesn2 (1:1000, Proteintech, China), anti-sesn3 (1:1000, Abcam, USA) and anti-Nrf2 (1:1000, Abcam, USA). Then the membrane was incubated with a secondary antibody. The blots were detected using a Bio-Rad imaging system. Specific protein expression levels were normalized to GAPDH protein.

Histological analysis

Paraffin-embedded hearts were cut transversely into 5-μm sections. Picrosirius red (PSR) staining was performed to detect collagen deposition. Immunofluorescence staining was used to further confirm the expression and the location of Sesns in heart. After a 5-min high-pressure antigen retrieval process (sodium citrate buffer, 100×, pH 6.0), the heart sections were incubated in PBS containing 10% fetal bovine serum for 60 min and subsequently incubated overnight at 4°C with the primary antibodies. The sections were then washed with PBS and incubated with the appropriate secondary antibodies for 1 h at 37°C. The secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 568 conjugate (Invitrogen, CA) and anti-mouse IgG Alexa Fluor 568 conjugate (Invitrogen, CA). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Images were all obtained at 400× magnification with a fluorescence microscope (Olympus Dx51) and DP2-BSW software (Version 2.2), and the images were analyzed with Image-Pro Plus (Version 6.0) in a blinded manner.

Statistical analysis

Data were presented as mean ± SD. The SPSS18.0 was used for statistical analysis. Multiple-group comparison was performed by one-way analysis of variance. Nonparametric test was applied to assess the enumeration data. Spearman' correlation was used to assess the relation of Sesn2 mRNA levels with heart failure maker and myocardial fibrosis marker. Significance was assumed as $P<0.05$.

Results

Clinical characteristics

The clinical characteristics for all patients were shown in **Table 2**. Briefly, heart samples from 10 patients with ischemic cardiomyopathy (ICM) and 18 patients with dilated cardiomyopathy (DCM) were included as the failing hearts in this study. 10 donor hearts were included as control (CTL). Of the 10 ICM patients, 8 were males and the average age were 54.00 ± 5.42 years. Among DCM patients, 15 were males and the average age were 52.93 ± 7.40 years. There was no difference for age between HF

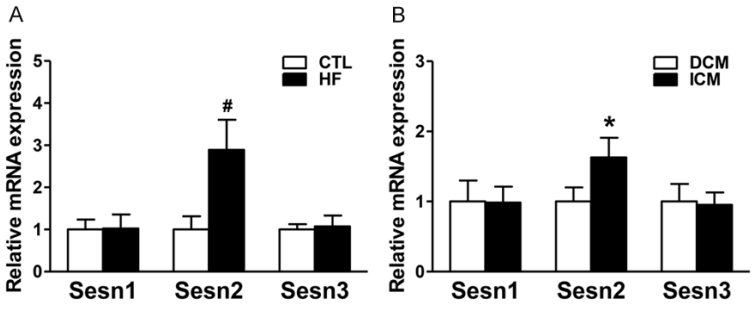

Figure 1. The mRNA expression of Sesns in control and HF patients. A: Compare of mRNA levels between heart failure patients and controls. B: Compare of mRNA levels between ICM and DCM patients. #P<0.05 vs. CTL; *P<0.05 vs. DCM.

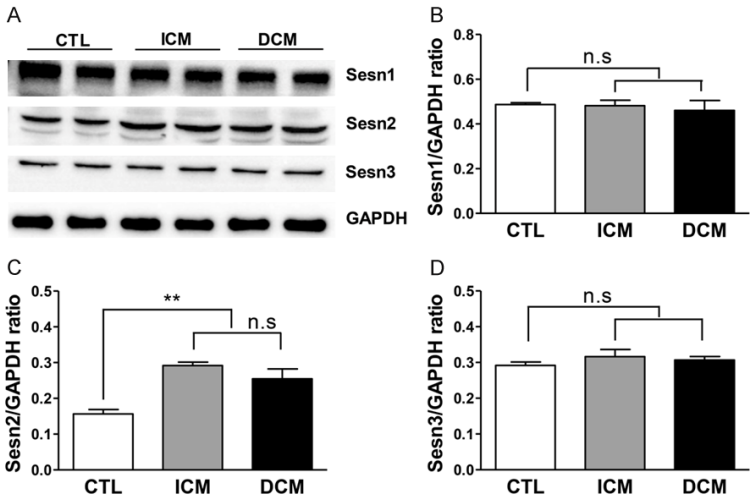

Figure 2. The protein expression of Sesns in control and HF patients. A: Representative bands showing the expression of Sesns in heart tissues. B-D: Quantitative analysis of Sesn1, Sesn2 and Sesn3 protein levels. **P<0.01 vs. CTL; n. s, no significance.

groups and CTL. These failing hearts remained severely decreased left ventricular function (ejection fraction: 29.80 ± 5.09 and 25.58 ± 9.20, respectively for ICM and DCM) as well as significantly enlarged left ventricular cavity size (left ventricular end-diastolic dimension: 65.60 ± 10.37 and 69.63 ± 9.72, respectively for ICM and DCM).

Expression of Sesns in human hearts

Comparing with control hearts, there was no difference for mRNA levels of Sesn1 or Sesn3 in failing hearts (**Figure 1A**). However, a significant increase of Sesn2 in failing hearts was found (**Figure 1A**). And unexpectedly, the expression of Sesn2 was higher in ICM than that in DCM (**Figure 1B**). Analysis of the protein levels by Western blot confirmed that Sesn2 protein was significantly higher in the cardiac tissues of heart failure patients (**Figure 2A-D**). However, for the etiology-specific difference in Sesn2 protein levels, there was just an up-trend in ICM than that in DCM (P= 0.054).

To further clarify the heart expression of Sesns, we performed immunofluorescence staining. As shown in **Figure 3A-C**, control hearts expressed all three members of Sesns. Meanwhile, Sesns were all expressed in the non-cardiomyocytes. To clarify the cell types that express Sesn2, we performed double immunofluorescence staining for Sesn2 and vimentin, a well-known marker for fibroblasts. The results revealed Sesn2 was localized in fibroblasts (**Figure 3D**).

Taken together, these results indicated that all three members of Sesns were expressed in the non-cardiomyocytes in human heart, and Sesn2, which expressed in fibroblasts, was remarkably increased in failing myocardium, including in ICM and DCM.

Expression of Sens2 and its correlation with severity of heart failure and myocardial fibrosis

Natriuretic peptide B (NPPB), a marker for heart failure, was up-regulated in failing hearts. To clarify the relationship between Sesn2 levels and severity of heart failure, Spearman' correlation was applied. The levels of Sesn2 were positively associated with that of NPPB (**Figure 4A**). PSR staining showed a relatively disperse distribution of collagen fibers in control hearts and an excessive accumulation of collagen in extracellular matrix of failing hearts (**Figure 4B**). Connective tissue growth factor (CTGF),

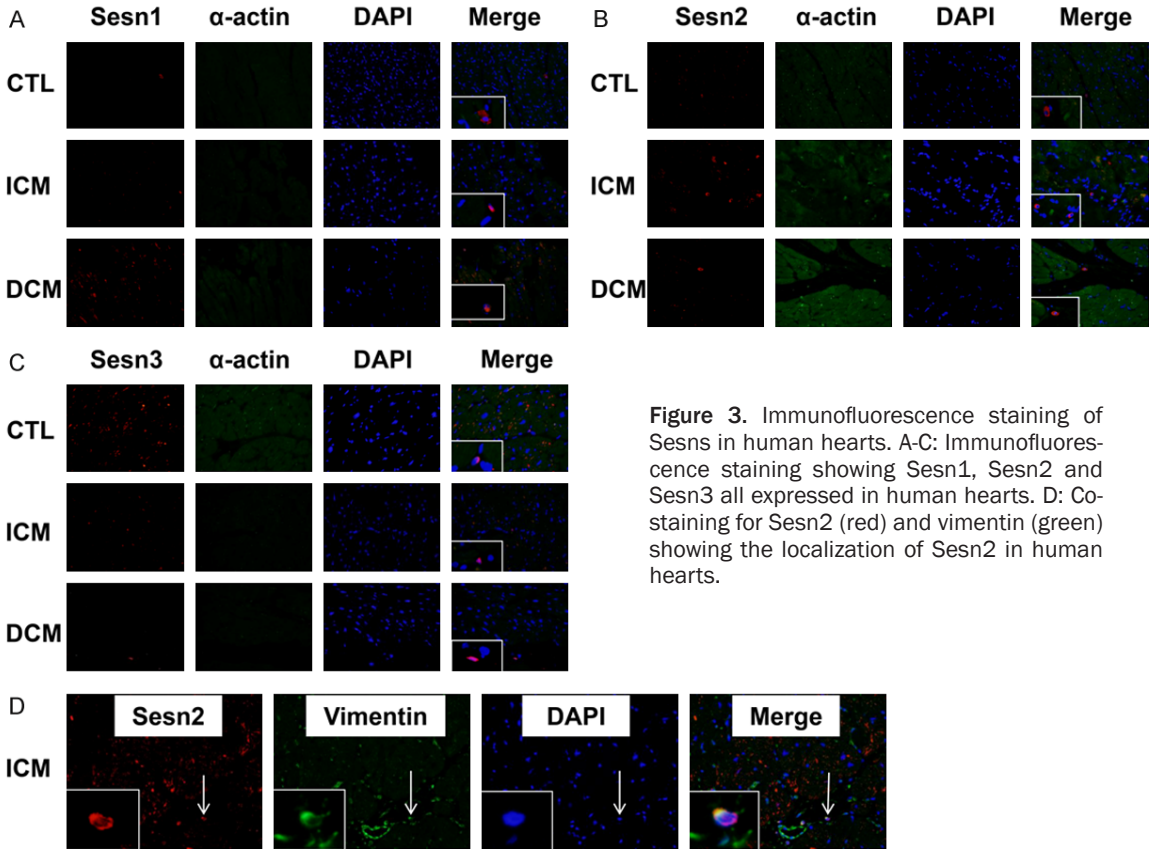

Figure 3. Immunofluorescence staining of Sesns in human hearts. A-C: Immunofluorescence staining showing Sesn1, Sesn2 and Sesn3 all expressed in human hearts. D: Co-staining for Sesn2 (red) and vimentin (green) showing the localization of Sesn2 in human hearts.

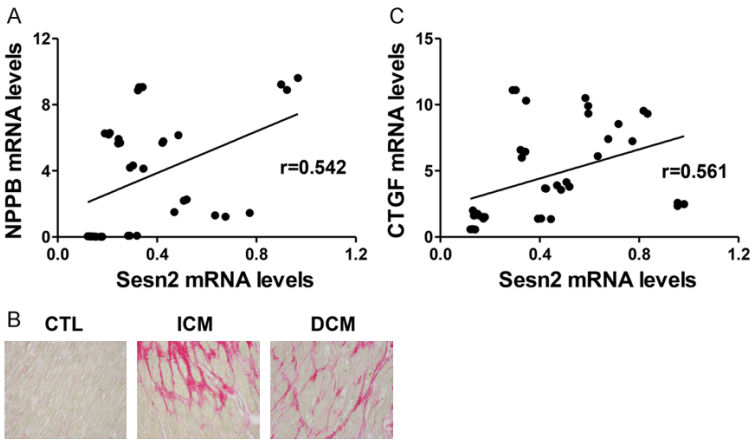

Figure 4. Sesn2 positively correlated with the severity of heart dysfunction and cardiac fibrosis in human hearts. A: Correlation between mRNA levels of Sesn2 and NPPB in human hearts (r=0.542, P<0.01). B: PSR staining showed a relatively disperse distribution of collagen fibers in CTL and an excessive accumulation of collagen in extracellular matrix of ICM and DCM (collagen was stained with red). C: Correlation between mRNA levels of Sesn2 and CTGF in human hearts (r=0.561, P<0.01). PSR, picrosirius red.

which can regulate fibrillar collagen gene transcription in the heart and often up-regulated in failing hearts, was also found significantly correlated with Sesn2 (**Figure 4C**).

Increased heart expression of Sesn2 in animal HF models

To further investigate the expression of Sesn2 in HF, we assessed the expression of Sesns in mice I/R, MI models and in doxorubicin induced rat HF model using RT-PCR and immunofluorescent staining. After construction of the models, NPPB was significantly increased in all three models (**Figure 5A** and **5C**). Sesns were found expressed in mice hearts, and as the same as the results in human ICM, mRNA levels of Sesn2, but not Sesn1 or Sesn3, were up-regulated in I/R and MI models (**Figure 5A** and **5B**). Meanwhile, Sesn2 levels were significantly higher in MI hearts after 4 weeks than that in 1 week (**P<0.01 vs. MI 1 w). Dox induced HF model is one model used to

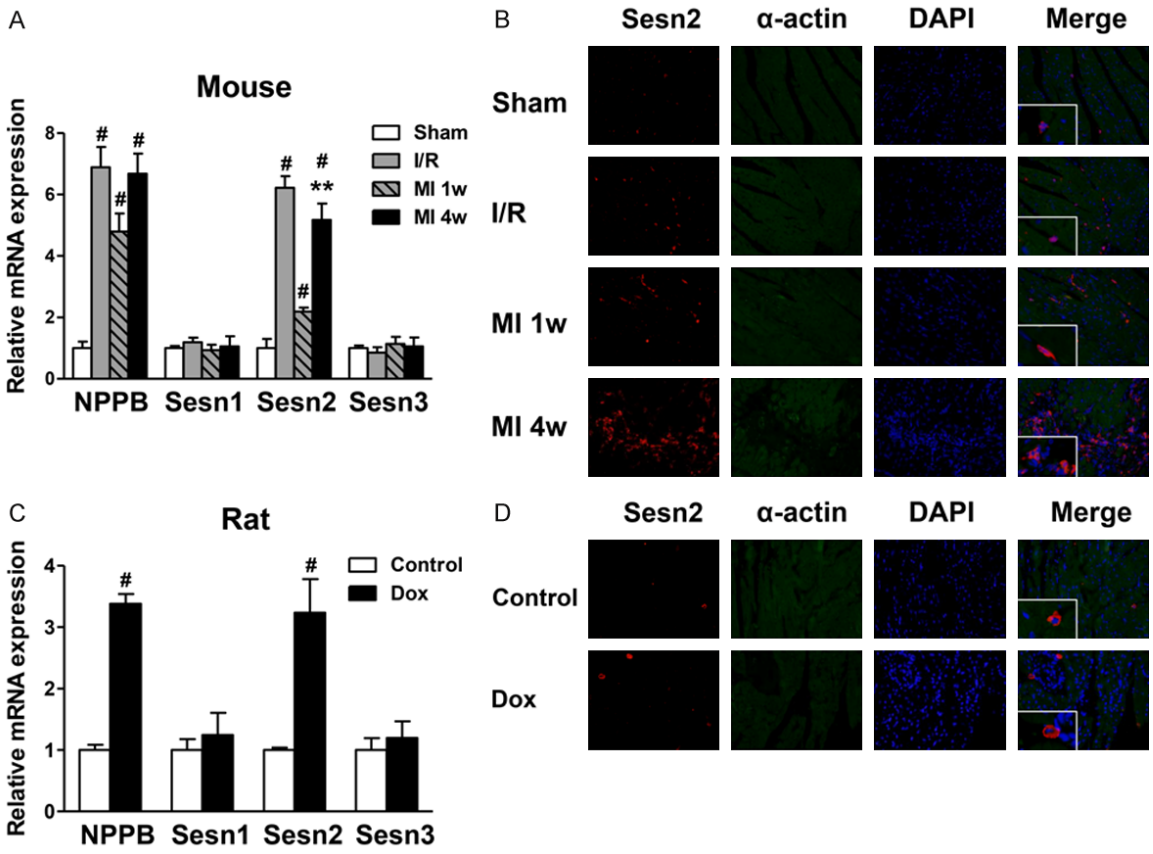

Figure 5. The expression of Sesns in mouse and rat heart failure models. A: The mRNA expression of NPPB and Sesns in mice I/R and MI models. B: Representative immunofluorescence images of Sesn2 in mice I/R and MI hearts. C: The mRNA expression of NPPB and Sesns in Dox induced rat HF model. D: Representative immunofluorescence staining images of Sesn2 in Dox induced rat HF model. I/R: ischemia/reperfusion; MI: myocardial infarction; Dox: doxorubicin. #P<0.01 vs. Sham or Control; **P<0.01 vs. MI 1 w.

simulation human DCM process [13]. As shown in **Figure 5C** and **5D**, increased expression of Sesn2 was found in rat failing heart, but no change for Sesn1 and Sesn3.

Discussion

In the present study, we investigated the expression and function of Sesns in human and experimental failing hearts. We discovered that Sesn1, Sesn2 and Sesn3 were all expressed in non-cardiomyocytes in human heart, and Sesn2 was up-regulated in failing hearts, including in ICM and DCM. Furthermore, Sesn2 levels were significantly correlated with expression levels of NPPB and CTGF, makers for severity of heart failure and cardiac fibrosis. The up-regulation of Sesn2 was validated in mouse I/R and MI models, and also in doxorubicin induced rat heart failure model.

Previous research has shown increased production of ROS and unbalanced relationship between oxidant and antioxidant proteins in human failing hearts [14, 15]. Increased ROS activates several signal pathways, such as mitogen activated protein kinases (MAPKs), NF-κB, calcium calmodulin-dependent kinase (CamKII) and transforming growth factor β (TGF-β) [1, 4], resulted in cell apoptosis and cardiac fibrosis. Recent research demonstrated Sesns could prevent apoptosis and fibrosis through its antioxidant ability [5, 6]. Silencing any of Sesn1, Sesn2 or Sesn3 by shRNA causes accumulation of ROS in various cell lines, leading to DNA damage or cell death [16-18]. In LNCaP cells, down-regulation of Sesn2 expression sensitized cells to energy stress induced apoptosis [19]. In glomerular mesangial cells, Sesn2 could inhibit high glucose induced fibronectin synthesis through blockade of Nox4-

dependent ROS generation, thus to prevent fibrotic injury in diabetes [20]. Also, studies in mouse models of chronic obstructive pulmonary disease revealed that inactivation of Sesn2 could induce TGF-β signaling and PDGFR β signaling [21, 22], two well-known pathways participated in fibrosis. All these studies indicated an important function of Sesn2 in regulating cell apoptosis and fibrosis.

More importantly, heart protective function of Sesns has been reported. Sesn played a significant role in maintaining basal cardiac integrity as Sesn null mutants and Sesn deficient hearts display irregularity of heartbeat, cardiac dilation, ultimately leading to decreased cardiac function [12]. Also, Alex Morrison et al. demonstrated increased myocardial infarct size after I/R challenge in Sesn2 knockout mice when compared with wild type mice [23]. Our results firstly illustrated the expression patterns of Sesns in human normal and failing hearts. Besides, increased expression of Sesn2 was revealed and the positive correlation between Sesn2 levels and cardiac fibrosis was illustrated. Similar to the increased antioxidant enzyme catalase in human end-stage HF [15], the up-regulation of Sesn2 in our study might be a compensatory reaction in response to cardiac stress. However, the increase might not enough to maintaining normal cardiac function. Furthermore, our results of increased Sesn2 in mice I/R model was consistent with the result of Alex Morrison et al [23], who reported increased Sesn2 in wild type mice after 5 min ischemia.

Study limitations

First, human heart samples in this study are not large enough and hearts from organ donators who died of traffic accidents are not really normal. More heart samples should be collected to identify the increased expression of Sesn2 in failing hearts. Second, although Sesn2 was found up-regulated in human failing hearts, and its levels were significantly correlated with heart dysfunction and cardiac fibrosis, these results cannot provide a causal relationship between the increased Sesn2 and the progress of HF. Intervention studies should be applied to investigate the function of Sesn2 during HF.

Conclusions

Our findings indicate that Sesns family members are expressed in human and rodent hearts and Sesn2, which is expressed in fibroblasts, might participate in the development and maintenance of cardiac fibrosis during HF. Further investigation should be done to investigate the exact mechanisms of Sesn2 during cardiac remodeling.

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Disclosure of conflict of interest

None.

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