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Original Article

Coxsackievirus B3 modulates expression of CCN5/Wisp2 *in vivo* and *in vitro*

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Abstract: Coxsackievirus B3 (CVB3) has been implicated as the common pathogenetic agent in myocarditis which is a major cause of sudden cardiac death in young people. However, the exact pathophysiology of CVB3-induced myocarditis is defectively understood. Insight in the molecular mechanisms is therefore essential. CCN5/Wisp2 has been shown to be involved in several important human diseases. However, to our knowledge, no prior studies have examined CCN5/Wisp2 in CVB3-induced myocarditis. Accordingly, we conducted the preliminary study on CCN5/Wisp2 expression in animals and cells. RT-PCR and immunohistochemistry were performed to detect the gene and protein expressions of CCN5/Wisp2 in mice heart. RT-PCR showed CCN5/Wisp2 mRNA expression increased markedly in the virus group. Similarly, immunohistochemical analysis revealed CCN5/Wisp2 protein expression in mice heart was up-regulated significantly in the virus group. In vitro studies showed that CCN5/Wisp2 mRNA was expressed in cardiomyocytes and cardiac microvascular endothelial cells. After infection with CVB3, CCN5/Wisp2 mRNA expression in cardiac microvascular endothelial cells was elevated in a time-dependent manner. In cardiomyocytes, CCN5/Wisp2 mRNA expression was also elevated in the virus group, although it was not significant. Our preliminary results suggested CCN5/Wisp2 might be involved in the pathogenesis of CVB3-induced acute viral myocarditis. The role of CCN5/Wisp2 in viral myocarditis deserves further investigation.

Keywords: Coxsackievirus B3, CCN5, wisp2, myocarditis, cardiac microvascular endothelial cell, cardiomyocytes

Introduction

Coxsackievirus B3 (CVB3) is a positive, single-strand RNA virus of the Picornavirus family [1]. It has been implicated as the common pathogenetic agent in myocarditis [2]. Myocarditis is a major cause of sudden cardiac death in young people [3]. However, the exact pathophysiology of CVB3-induced myocarditis is defectively understood [4]. Insight in the molecular mechanisms is therefore essential.

CCN5/Wisp2 is a member of the CCN family which was named after the first 3 members of the family identified: Cyr61 (cysteine-rich protein 61), CTGF (connective tissue growth factor) and NOV (nephroblastoma overexpressed gene) [5]. The CCN family contains six members: CCN1, CCN2, CCN3, CCN4, CCN5/Wisp2 and CCN6. All family members have three domains with sequence homology to insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C repeat (vWC), and thrombospondin type I repeat (TSP). Apart from CCN5/Wisp2, all CCN proteins have a fourth domain, a cysteine-rich carboxyl-terminal repeat (CT) [6]. Precisely because of the structural difference, CCN5/Wisp2 is becoming a research focus. In previous studies, CCN5/Wisp2 has been shown to be involved in several important human diseases [7-9]. Both *in vivo* and *in vitro* studies demonstrated a potential pathophysiologic role for CCN5/Wisp2 in regulating cell proliferation, differentiation, motility, invasiveness, and adhesion [5, 10, 11].

However, to our knowledge, no prior studies have examined CCN5/Wisp2 in CVB3-induced myocarditis. Accordingly, we conducted the preliminary study on CCN5/Wisp2 expression in mice and cells.

Materials and methods

Virus

CVB3 (Nancy strain) was preserved in our Laboratory. CVB3 was maintained by passage through Hela cells. Viral titer was measured prior to infection by a 50% tissue culture infectious dose (TCID50) assay of Hela cell monolayer.

Mice and virus infection

Male BALB/c mice (aged 3 to 4 weeks) were purchased from the Experimental Animal Center of Shanghai Medical College of Fudan University. The animals were randomly assigned to two groups, with 6 mice in each group, as follows: (1) Virus group: Each mouse was injected intraperitoneally (i.p.) with 0.2 mL of CVB3 (TCID50 = 10^3/mL, Nancy strain) diluted in virus dilution medium (Eagle's medium); (2) Control group: Each mouse was injected i.p. with 0.2 mL of Eagle's medium.

Tissue collection

Mice were sacrificed by cervical dislocation on 7th day post infection. Hearts were divided into two parts. One part was stored at -80°C for PCR and the other part was fixed in 10% neutral formalin for histopathological or immunohistochemical examination.

Tissue histopathology and myocarditis grading

Formaldehyde-fixed paraffin sections were stained with hematoxylin and eosin. The extent of inflammatory cell infiltration and myocardial necrosis were estimated using hematoxylin and eosin staining. The severity of myocarditis was quantified using a 0-4 scale as follows [12]: 0 indicates no inflammation; 1, one to five distinct mononuclear inflammatory foci with involvement of 5% or less of the cross-sectional area; 2, more than five distinct mononuclear inflammatory foci, or involvement of over 5% but not over 20% of the cross-sectional area; 3, diffuse mononuclear inflammation involving over 20% of the area, without necrosis; and 4, diffuse inflammation with secondary necrosis and acute inflammation. Slides were graded in dependently in a blinded manner by two observers, respectively.

Cell culture and virus infection

Neonatal rat cardiomyocytes were isolated and cultured as previously described with minor modifications [13]. In brief, the heart tissue of 1-4 day old Sprague-Dawley rats (provided by the experimental animal center of Fudan University) were quickly minced and digested in PBS containing 0.1% trypsin at 37°C for 5 minutes. An equal volume of Dulbecco modified Eagle media (DMEM) containing 10% fetal bovine serum (FBS) was added into the collected cell supernatant to offset the digestive effect of trypsin. The above steps were repeated for about 7 times. The cell suspension was then centrifuged at 300 g for 5 minutes. Supernatants were discarded, and the cell pellets were resuspended in DMEM cell culture medium with 10% FBS and placed in a humidified incubator gassed with 5% CO_2 at 37°C for 2 h. After pre-plating for 2 h, the suspended cells were cardiomyocytes. Cardiac microvascular endothelial cells were prepared as we previously reported [14].

For infection, cardiomyocytes and cardiac microvascular endothelial cells were seeded in 6-well-plate at 80% confluence and cultured for 36 h. Then the cells were infected with CVB3 or phospshate-buffered saline for 2 h in serum-free medium. After 2 h incubation with CVB3 at 37°C, cells were washed with PBS and replenished with DMEM containing 10% FBS.

Quantitative real-time PCR

All procedures were carried out according to the manufacturers' protocols. Total RNA was isolated from hearts or cells using Trizol reagent (Life Technologies-ambion, Catalog no: 15596026). One microgram of total RNA was reverse-transcribed using primeScript RT Reagent Kit (Takara Cat#RR037A). PCR was performed with an initial step of denaturation at 95°C 30 s, 40 cycles of 5 s at 95°C, 34 s at 60°C followed by a dissociation stage. Reactions were carried out on Applied Biosystems7500 using SYBR Premix Ex TaqII (Takara, Cat#RR820A). A melt-analysis was conducted for all products to access the specificity of the amplification. The primers used in real-time PCR amplification were shown in **Table 1**. Beta-actin was chosen as housekeeping gene for normalization. Relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

Table 1. Primer sequences used for real-time PCR

	Gene	Forward, 5'-3'	Reverse, 5'-3'
	CVB3	AATGCGGCTAATCCTAACTG	AAACACGGACACCCAAAG
Mouse	CCN5	GTACCTGGATGGGGAGACCT	GACCTTCCTGGCACCTGTAT
Mouse	Beta-actin	GCACCACACCTTCTACAATG	ACGACCAGAGGCATACAG
Rat	CCN5	TTAGCACTTGTGGTGGCTTG	CCATTGAGAGAAGGCAGAGG
Rat	Beta-actin	GTCCACCTTCCAGCAGAT	CTCAGTAACAGTCCGCCTA

Immunohistochemistry

The paraffin-embedded heart tissue was cut into 5-μm thick sections. Each tissue section was deparaffinized and rehydrated. Then, slides were incubated in 3% H_2O_2 to quench endogenous enzymes activity and subsequently boiled in citrate (10 mM, pH6.0) in a microwave oven to repair antigens. After blocking with BSA in PBS, the sections were stained with a rabbit anti-CCN5/Wisp2 polyclonal antibody (Santa Cruz Biotechnology, CA, USA) at 4°C overnight, followed by incubation with a goat anti-rabbit antibody at 37°C. After reaction with streptactividin-biotin complex working medium, sections were stained with diaminobenzidine and counterstained with hematoxylin. For the negative control the specific primary antibody was replaced by phosphate-buffered saline. Images were taken on a Leica microscope (Leica Micro systems, Wetzlar, Germany). An immunohistochemical semi-quantitative analysis of CCN5/Wisp2 expression was conducted by analyzing average optical density (integrated optical density/area) of positive reactions with Image-Pro Plus 6.0 software (Media Cybernetics, USA).

Immunofluorescence

Cultured cells were fixed in 4% paraformaldehyde at room temperature for 30 min and then incubated with 1% TritonX-100 for 5 min. After treated with 5% BSA for 1 h, cells were then incubated with rabbit anti-CCN5/Wisp2 polyclonal antibody (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Then FITC-conjugated Goat-anti-rabbit IgG secondary antibody was added for 1 h at 37°C. At last, the cells were observed under a fluorescence microscopy and photographed.

Statistical analysis

All statistical analyses were performed using Stata software (Version 12.0; STATA Corporation, College Station, Texas). The Student t-test was used to assess significance between two groups when data conformed to normal distribution and the homogeneity of variance. Otherwise, Wilcoxon Rank Sum Test was used. Spearman's correlation was applied to evaluate the correlation of CCN5 mRNA with CVB3 mRNA in cardiac microvascular endothelial cells. Statistical significance was defined at $P<0.05$.

Results

CVB3 infection induced inflammation and myocardial lesions

When the experiment was carried out to seventh day, there was no death in both control group and virus group. All mice were killed on the 7th day after CVB3 i.p. In the virus group, we detected CVB3 mRNA expression in mice heart by RT-PCR, which suggested virus infected mice heart successfully. Unsurprisingly, in the control group, we did not detect CVB3 mRNA expression in mice heart by RT-PCR (**Figure 1A**). The cardiac lesions induced by CVB3 were visible to naked eyes (**Figure 1B**). HE staining of heart showed scattered inflammation infiltration and myocardial lesion in the virus group (**Figure 1C**). Pathological score of the heart in virus group was significantly increased compared with the control group (**Figure 1D**).

CVB3 significantly increased the expression of CCN5/Wisp2 in mice hearts

RT-PCR and Immunohistochemistry were employed to detect the gene and protein expressions of CCN5/Wisp2 in mice heart. RT-PCR showed CCN5/Wisp2 mRNA expression increased markedly in the virus group (**Figure 2A**). Similarly, immunohistochemistry analysis revealed that CCN5/Wisp2 protein expression in mice heart was significantly higher in the virus group than those in the control group ($P<0.05$) (**Figure 2B**, **2C**). These results indicated that CVB3 can up-regulate CCN5 level *in vivo*.

CVB3 increased CCN5/Wisp2 expression in a time-dependent manner in cardiac microvascular endothelial cells

RT-PCR method was applied to detect CCN5/Wisp2 mRNA expression changes after CVB3 infection at different time points (24 h, 48 h

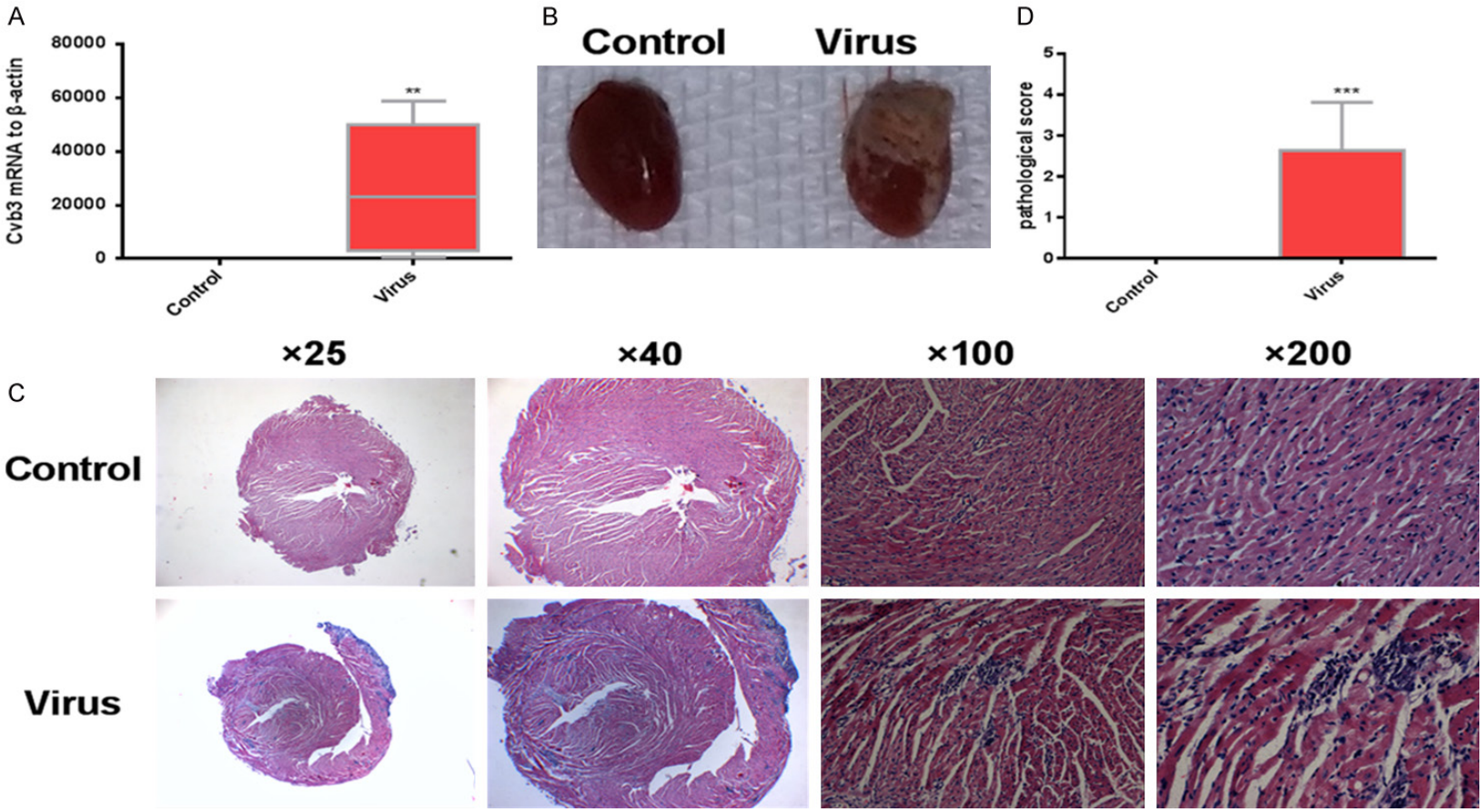

Figure 1. CVB3 infection induced inflammation and myocardial lesions. A. Data from real-time PCR indicated CVB3 infection of heart. (**P<0.01 vs. control). B. Cardiac lesions induced by CVB3 infection. C. Representative hematoxylin and eosin staining results. Red staining areas show the myocardium tissue; the blue staining areas show the inflammatory cells infiltration. D. Pathologic score comparison (***P<0.001 vs. control).

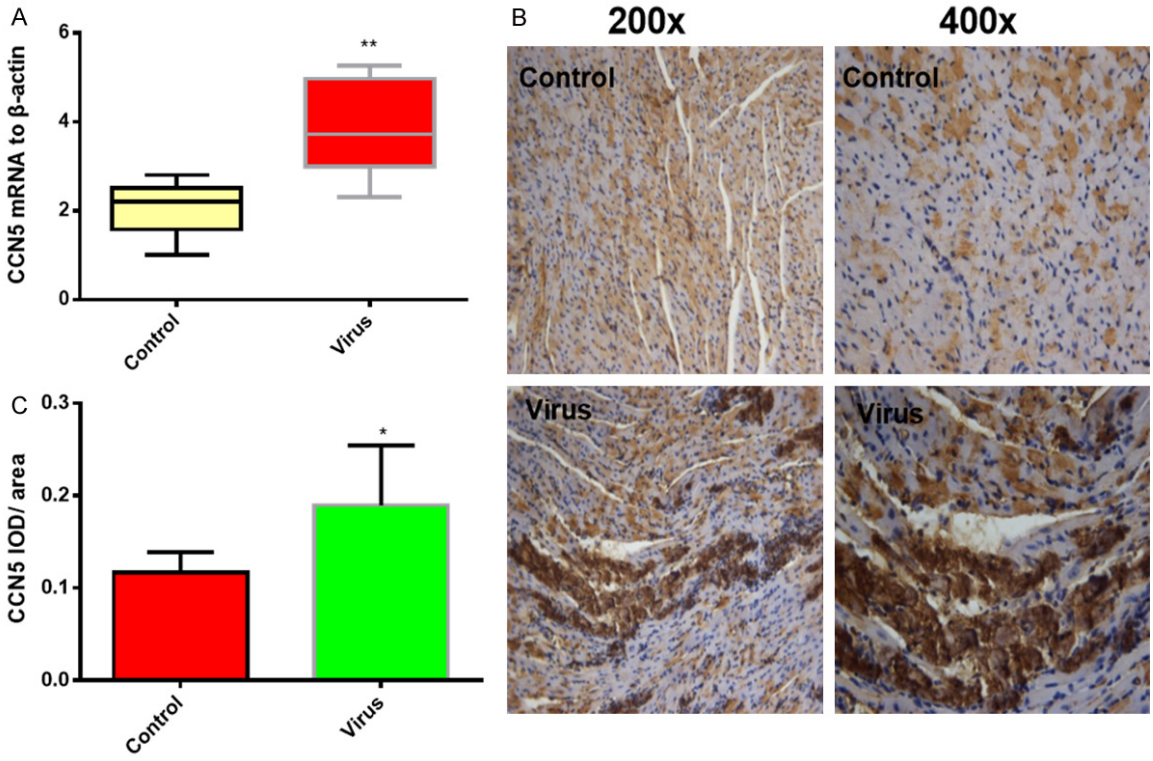

Figure 2. CVB3 increased the expression of CCN5/Wisp2 in mice hearts. A. Data from real-time PCR indicated up-regulation of CCN5/Wisp2 mRNA in mice hearts infected with CVB3 (**P<0.01 vs. control). B. Representative immunohistochemistry staining indicated up-regulation of CCN5/Wisp2 in mice hearts infected with CVB3. C. An immunohistochemical semi-quantitative analysis of CCN5/Wisp2 expression was conducted by analyzing average optical density (integrated optical density/area) of positive reactions. Immunohistochemistry analysis revealed that CCN5/Wisp2 protein expression in mice hearts infected with CVB3 was significantly up-regulated (*P<0.05 vs. control). IOD = integralopticaldensity.

and 72 h) in cardiomyocytes and cardiac microvascular endothelial cells. We found that CCN5/Wisp2 mRNA was expressed in the cardiomyocytes and cardiac microvascular endothelial cells. In the cardiomyocytes, CCN5/Wisp2 mRNA expression increased in the virus group, although it was not significant (**Figure 3A**). After infection with CVB3, CCN5/Wisp2 mRNA expression in cardiac microvascular endothelial cells increased in a time-dependent manner (**Figure 3B**). At 72 h, the CCN5/Wisp2 mRNA expression in the virus group was significantly higher than those in the control group. Immunofluorescent staining images also revealed CCN5 was up-regulated when cardiac microvascular endothelial cells were exposed to CVB3 (**Figure 3C**). Next, we examined the relationship between CCN5 mRNA and CVB3 mRNA (**Figure 3D**). Spearman analysis showed that there was a positive correlation between levels of CCN5/Wisp2 mRNA and CVB3 mRNA ($r = 0.88$, $P<0.05$).

Discussion

In the current study, we reported that CVB3 infection increased the expression of CCN5/Wisp2 in vivo and in vitro. We found that CCN5/Wisp2 mRNA was expressed in cardiomyocytes and cardiac microvascular endothelial cells. We also showed CVB3 increased the expression of CCN5/Wisp2 in a time-dependent manner in cardiac microvascular endothelial cells. The findings are beneficial to elucidating the molecular mechanisms of viral myocarditis.

CCN5/Wisp2 is a member of the CCN family proteins. It was originally reported in the late 1990s by several research teams [5]. CCN family comprises six members. The molecular structure of six members reaches high consistency which lies in that all six members contain three domains with sequence homology to IGFBP, vWC, and TSP. However, unlike the other members of the CCN family, CCN5/Wisp2 lacks the

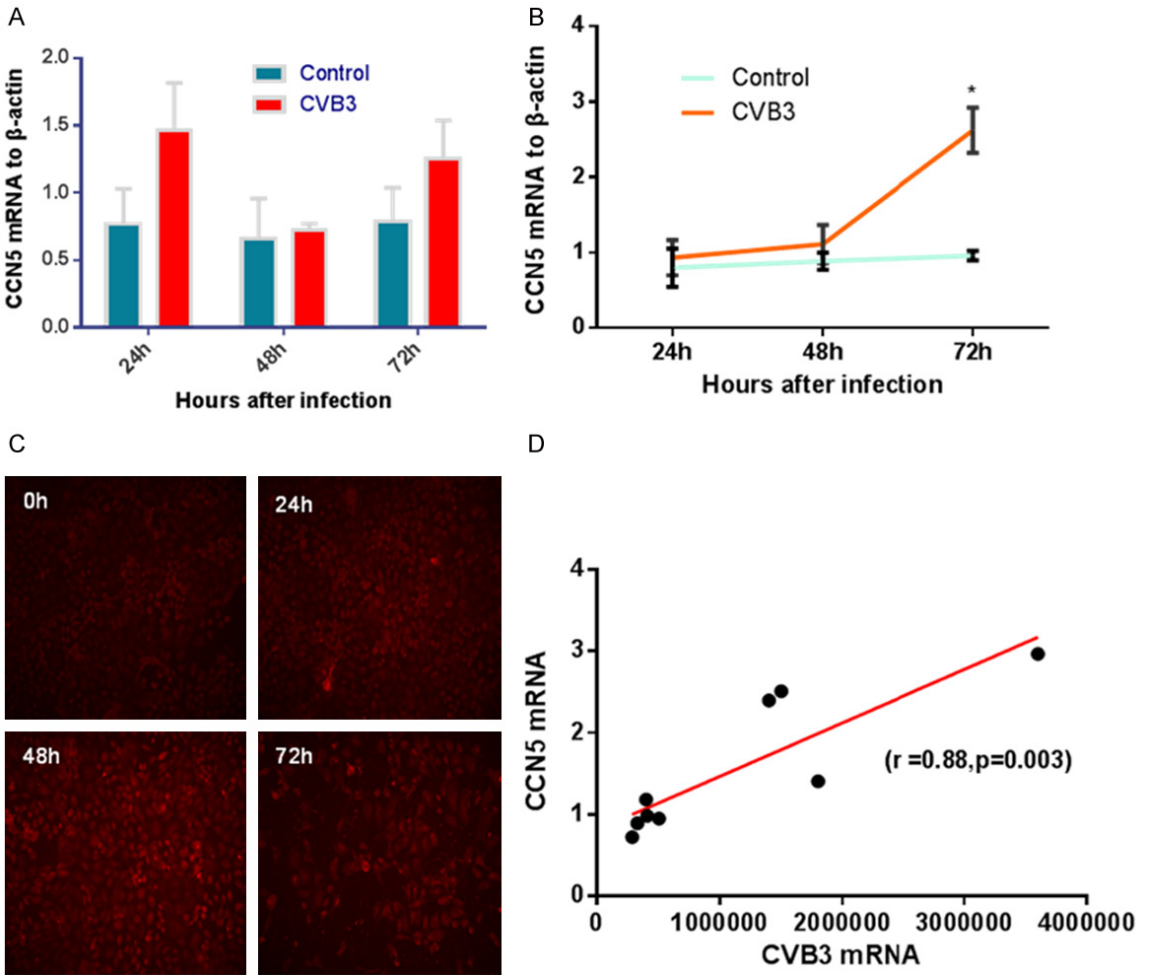

Figure 3. CVB3 increased CCN5/Wisp2 expression in vitro. A. Data from real-time PCR indicated CVB3 increased CCN5/Wisp2 mRNA in cardiomyocytes. B. CVB3 significantly increased CCN5/Wisp2 mRNA expression in a time-dependent manner in cardiac microvascular endothelial cells. C. Representative immunofluorescence of CNN5/Wisp2 in cardiac microvascular endothelial cells infected with CVB3. D. Graph showed CCN5 mRNA had linearly correlation with CVB3 mRNA in cardiac microvascular endothelial cells (Spearman r = 0.88, P = 0.003).

CT domain. This has motivated many researchers to hypothesize that CCN5/Wisp2 might act as a dominant negative regulator against other CCN family members. In fact, several studies reported CCN5/Wisp2 could antagonize the effect of CCN2 [9, 15]. One study [9] found the opposing effects of CCN2 and CCN5/Wisp2 on the development of cardiac hypertrophy and fibrosis. In cardiomyocytes, overexpression of CCN2 induced hypertrophic growth which could be inhibited by the overexpression of CCN5/Wisp2. In animals, CCN2 transgenic mice showed significantly increased fibrosis and an accelerated deterioration of cardiac function in response to pressure overload. On the contrary, hypertrophy and fibrosis were both significantly inhibited in CCN5/Wisp2 transgenic mice. Similarly, another study [15] reported CCN2 overexpression led to promoted proliferation and elevated collagen and α-smooth muscle actin expression, which were inhibited by CCN5/Wisp2 overexpression. Moreover, the CT domain played an essential part in fibroblast proliferation and differentiation.

The role of CCN2 in a CVB3-induced myocarditis animal model has been explored [16-18]. CCN2 is a crucial molecule in the development of fibrosis associated with viral myocarditis [16]. Downregulation of cardiac CCN2 expression may offset cardiac fibrosis and the subsequent heart muscle dysfunction in ongoing enteroviral myocarditis [16]. TGF-β is a specific

inducer of CCN2 expression in both cardiac fibroblasts and cardiomyocytes [19]. CCN5/Wisp2 represses expression of key components of the transforming growth factor signaling pathway [20]. In the present study, we found cardiac CCN5/WISP2 expression was up-regulated at the gene and protein levels both in mice and cells. We speculate the increase of CCN5/Wisp2 may be a compensatory mechanism against the effects of CCN2. Basing on our preliminary results, the role of CCN5/Wisp2 in viral myocarditis and its relationship with CCN2 are worth further studying.

In summary, we showed that CCN5/Wisp2 expression was elevated at both gene and protein level in CVB3-induced myocarditis. We also found that CCN5/Wisp2 was elevated in cardiomyocytes and cardiac microvascular endothelial cells when they were infected with CVB3. Although the molecular mechanism requires further study, these results indicated that CCN5/Wisp-2 may be involved in the pathogenesis of CVB3-induced acute viral myocarditis. The exact role of CCN5/Wisp2 in viral myocarditis deserves further investigation.

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Disclosure of conflict of interest

None.

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