Silencing of Tenascin-C inhibits hypoxia reoxygenation injury of cardiomyocyte through TLR4 $\text{NF-}\kappa\text{B}$ pathway

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Abstract

Background: Myocardial infarction (MI) is one of the leading causes of morbidity and mortality worldwide, and its main pathophysiological mechanism is myocardial ischemia/reperfusion (I/R) injury. TNC is an extracellular matrix glycoprotein, and high TNC expression was associated with MI and ventricular remodeling. The present study aimed to investigate the effect and the underlying mechanisms of TNC on myocardial I/R injury.

Methods: Cardiomyocyte H9c2 cells was used to establish an in vitro hypoxia/reoxygenation (H/R) model, which were under hypoxia for 4 h and reoxygenation for 12 h. TNC was silenced by small interfering RNA (siRNA) in H9c2 cells.

Results: TNC mRNA and protein expressions were increased by H/R. TNC knockdown by siRNA improved the cell viability in H/R-stimulated H9c2 cells. TNC knockdown reversed the H/R-induced increase in the apoptosis, as evidenced by reduced TUNEL+ cells and caspase-3 activity, and inhibited oxidative stress and inflammatory cytokine production. TNC silencing inhibits TLR4 mRNA and protein expressions and NF-kappa B signals, as evidenced by reduced nuclear NF- κ B p65 and increased cytoplasmic I- κ B α in H/R-stimulated H9c2 cells. All these cardioprotection against H/R by siTNC was reversed by TLR4 overexpression.

Conclusions: TNC silencing prevents H/R-induced injury by inhibiting apoptosis and oxidative stress by inhibition of TLR4-NF- κ B pathway.

Background

Myocardial ischemia/reperfusion (I/R) injury is a complex pathological mechanism of myocardium tissue that often induced by cardiac surgery or coronary artery disease. [1, 2]. Myocardial I/R injury is composed of two phase, the early myocardial ischemia caused by blockade of the coronary artery, and the late myocardial injury caused by restoration of coronary blood flow [3]. Multiple biological mechanisms involve myocardial I/R injury, including apoptosis, oxidative stress and inflammatory response in cardiomyocytes, which further aggravate myocardium tissue infarction [4, 5]. Myocardial I/R injury is an important factor that contributes to high disability and mortality. However, the effective treatments for the disease are limited. Therefore, it is important to explore new molecules and uncover the precise mechanisms underlying myocardial I/R injury, so as to provide basis for the development of potential agents for therapeutic and preventive strategies.

Toll-like receptor 4 (TLR4) is a primary receptor of the innate immune system and can induce inflammatory response in myocardial ischemia through activating signaling pathways, like nuclear factor kappa B (NF- κ B) [6]. Activation of NF- κ B promotes production of proinflammatory cytokines, such as interleukin 1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [7]. It is reported that TLR4 plays a pivotal role in the initiation and progression of atherosclerosis, and contributes myocardial I/R injury of

cardiomyocytes [8, 9]. Therefore, blockade of excessive TLR4 expression is an potential therapeutic strategy for protection of myocardial I/R injury.

Tenascin-C (TNC) is an extracellular matrix (ECM) protein and is abundantly expressed during embryogenesis and is normally undetectable in most healthy adult tissues. TNC is rapidly induced in adult tissues in response to pathological stress, such as inflammation, wound healing, and various cancers [10–12]. TNC is rarely detected in heart of healthy adults, but is highly expressed in the myocardium during the acute post-infarction stage [13]. TNC accelerates ventricular remodeling after MI, and high expression of TNC is associated with poor long-term outcome of MI patients [14]. However, it has not been fully elucidated about the detailed molecular mechanisms of TNC in myocardial ischemia/reperfusion injury.

To further understand the role and mechanisms of TNC in myocardial I/R injury, H9c2 cardiac cell line was applied in this study to establish in vitro hypoxia/reoxygenation (H/R) model. We hypothesized that TNC silencing protects cardiomyocytes against H/R injury by inhibiting TLR4-NF- κ B signaling.

Methods

Cell culture and treatment

The H9c2 cardiac myoblast cell line was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Thermo Fisher Scientific, Inc., Waltham, MA, USA), in a 5% CO₂ incubator at 37°C. After cells grow to 70–80% confluence, H9c2 cells were exposed to hypoxia (1% O₂, 94% N₂ and 5% CO₂) for 4 h, followed by reoxygenation for 4, 8, 12 or 16 h. The control group was incubated in normoxic conditions (5% atmosphere and 5% CO₂) at 37°C.

Cell transfection

To knockdown TNC, H9c2 cells were transfected with TNC siRNAs and negative control (NC) siRNAs (GenePharma, Shanghai, China). The cDNA of TLR4 was inserted into the pcDNA3.1 expression vector to construct TLR4 overexpressing plasmids (pcDNA-TLR4). H9c2 cells were seeded into 6-well plates (5×10^5 cells/well) and cultured with complete medium. The next day, subconfluent (50–60%) cells were replaced with DMEM with 0.1% PBS, and were transfected with siRNA-TNC (siTNC) or vectors overexpressing TLR4 using Lipofectamine Reagent (Life Technologies, NY, USA). After 48 h, the transfection efficacy was confirmed by RT-qPCR or western blot.

Cell viability assay

For cell viability assay, H9c2 cells were seeded in a 96-well plate (5×10^3 cells/well). After 18 h of various treatments, cells were incubated with CCK-8 solution (10 μ L) for each well, and then kept at 37°C for 2 h. The absorbance at 450 nm was measured using a microplate reader.

lactate dehydrogenase (LDH) release assay

The damaged cardiomyocytes release LDH into extracellular fluid, and LDH content was measured to evaluate cell injury. Culture medium was collected after 18 h of various treatments, and the concentration of LDH was determined by spectrophotometry using an LDH assay kit (Jiancheng, Nanjing, China).

Measurement of caspase-3 activity

Caspase-3 activity was determined by colorimetric assay using a commercialized assay kit (Biovision, Inc.). H9c2 cells were harvested and lysed, followed by centrifugation at 10000 g for 10 min at 4 °C. The supernatant (30 µL) was incubated with synthetic peptide substrate Ac-DEVD-pNA (10 µL, 0.2 mM, Sigma, St. Louis, MO, USA) at 37 °C for 2 h. Caspase-3 activity was evaluated by measuring optical absorbance at 405 nm in a spectrophotometer.

TUNEL Assay

H9c2 cells were fixed with 4% paraformaldehyde in PBS at room temperature for 1 h, and after washing with PBS solution, cells were blocked by 3% H₂O₂ in 100% methanol for 10 min at room temperature. Samples were then incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) on ice for 2 min. Cells were added with TUNEL reagent at 37 °C in the dark for 1 h. Cells were then washed with PBS, and incubated with DAPI solution for 30 min at room temperature in the dark. Finally, the cells were observed under fluorescence microscope (detection range: 515–565 nm). The number of TUNEL + cell were counted and normalized to that of total cells (DAPI + cells).

Determination of malondialdehyde (MDA) and superoxide dismutase (SOD)

After 18 h of various treatments, cells were harvested and lysed by ultrasonic pyrolysis. After centrifuged at 3000 × g for 10 min at 4 °C, the supernatant was collected and incubated with detection working fluid for MDA (Cat. No. A003-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) or superoxide dismutase (SOD; Cat. No. A001-1) at 37 °C for 15 min. Then the reaction mixture was used to measure absorbance values at 532 nm (MDA) or 520 nm (SOD) by a microplate reader. All experiments were repeated three times independently.

Measurement of pro-inflammatory cytokines

After 18 h of various treatments, supernatant was collected and ELISA assay was performed to measure the IL-6 level by a ELISA test kit (Cat. No. R6000B, R&D Systems, Minneapolis, MN, USA). The concentrations of IL-6 in each group was determined based on the standard curve, and experiments were repeated three times independently.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed to obtain cDNA from RNA template, using Superscript II reverse transcriptase (Toyobo Life Science, Osaka, Japan). Real-time PCR was carried out by SYBR Premix Ex Taq (Takara Bio, Japan) using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). PCR primers included: Tenascin-C sense (5'-GCT CTC CTA TGG CAT CAA GG-3') and Tenascin-C antisense (5'-TCA TGT GTG AGG TCG ATG GT-3'); IL-6 sense (5'-AGT TGC CTT CTT GGG ACT GA-3') and IL-6 antisense (5'-ACT GGT CTG TTG TGG GTG GT-3'); TLR4 sense (5'-AGC CAT TGC TGC CAA CAT CA-3') and TLR4 antisense (5'-GCC AGA GCT ACT CAG AAA C-3'); GAPDH sense (5'-CCT CTA TGC CAA CAC AGT GC-3') and GAPDH antisense (5'-GTA CTC CTG CTT GCT GAT CC-3'). The conditions for real-time PCR were as follows: denaturation at 94 °C for 5 min, then amplified by 40 cycles at 94°C for 15 s and 58°C for 30 s. The 2- $\Delta\Delta C_t$ method was used to determine the relative mRNA levels.

Total (TNC, TLR4), cytoplasmic (I- κ B α) and nuclear (NF- κ B p65) proteins were extracted from H9c2 using RIPA lysis buffer, cytoplasmic and Nuclear Extraction Reagents (Pierce Biotechnology, Inc., Rockford, IL, USA). and quantified by BCA protein assay kit (Beyotime Biotechnology, China). Protein samples (50 μ g) were separated on 10% SDS-PAGE gel and transferred onto PVDF membranes. Membranes were then incubated with primary antibodies against TNC (1:200, ab233198, Abcam, UK), TLR4 (1:1000, Cell Signaling, USA), NF- κ B p65 (1:500, Santa Cruz Biotechnology, USA), and I- κ B α (1:500, Santa Cruz Biotechnology, USA) overnight at 4 °C. The blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody. The proteins were detected using chemiluminescent detection system (Thermo Scientific, Waltham, MA, USA), and analyzed using ImageJ software.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation, and were analyzed by SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used to analyze the differences among multiple groups, followed by Bonferroni-test. $P < 0.05$ was considered as statistically significant.

Results

H/R treatment induced TNC expression in cardiomyocytes

To investigate the potential relevance of TNC in H/R injury, we first investigated whether H/R treatment affected TNC expression in cardiomyocytes. Rat cardiomyocytes H9c2 were subjected to hypoxia for 4 h, followed by reoxygenation for 4, 8, 12, or 16 h under normoxic conditions. The results showed that after reoxygenation cell viability was markedly reduced compared to cells with hypoxia alone (Fig. 1A). Moreover, H/R treatment significantly increased mRNA and protein expressions of TNC in H9c2 cells (Fig. 1B, 1C). This results suggests that TNC may involve pathological process of H/R injury.

Knockdown of TNC attenuated H/R-induced injury in cardiomyocytes

siRNA was performed to specifically targeting TNC expression, so as to investigate the biological function of TNC in H/R injury of cardiomyocytes. The results showed that TNC mRNA and protein expressions were significantly decreased in cells transfected with TNC siRNAs (Fig. 1D, 1E). Moreover, knockdown of TNC significantly increased the cell viability and reduced LDH release in supernatant of H9c2 cells with H/R treatment (Fig. 1F, 1G). These results suggest that TNC plays a protective effect on H/R injury of cardiomyocytes.

Knockdown of TNC ameliorated H/R-induced cardiomyocytes apoptosis

To evaluate the effect of TNC on H/R-induced apoptosis, TUNEL staining was performed detect apoptosis. Representative photomicrographs of H9c2 cardiomyocytes are shown (magnification, $\times 200$). Apoptotic nuclei were stained with TUNEL (green), total nuclei were stained with DAPI (blue) (Fig. 2A). Quantification of TUNEL staining showed that H/R exposure led to a significant increase in the TUNEL-positive cells, however, TNC knockdown reduced TUNEL-positive cells in comparison to H/R group ($P < 0.05$) (Fig. 2B). Consistently, H/R also increased the caspase-3 activity, which indicates the induction of apoptosis. This effect was obviously suppressed by knockdown of TNC (Fig. 2C). These findings indicated the anti-apoptotic actions of siTNC on H/R-induced cardiomyocytes.

Knockdown of TNC inhibited oxidative stress in H/R-induced cardiomyocytes

To test whether the oxidative stress is modulated by TNC, we assessed the lipid oxidation product MDA level and ROS-scavenging enzyme SOD activity in H9c2 cells. H/R stimulation significantly increased MDA content and decreased SOD activity in H9c2 cells compared with the control cells, while these changes were markedly attenuated by knockdown of TNC (Fig. 2D, 2E). In addition, RT-qPCR and ELISA showed that IL-6 mRNA level and content in culture media were significantly increased in the H/R group compared to the control, and knockdown of TNC attenuated the increase in this inflammatory factor (Fig. 2F, 2G). Collectively, these findings indicate that knockdown of TNC has substantial suppression on oxidative stress and inflammatory responses in cardiomyocytes induced by H/R.

Knockdown of TNC on TLR4 expression and NF- κ B translocation in cardiomyocytes undergoing H/R

RT-qPCR showed that H/R stimulation significantly increased the expression of TLR4 mRNA ($P < 0.05$). However, compared with the H/R group, cells with siTNC showed reduced TLR4 mRNA expression ($P < 0.05$) (Fig. 3A). Western blot also showed that the TLR4 protein level was significantly lower in siTNC group compared with the H/R group ($P < 0.05$) (Fig. 3B, 3C). Moreover, H/R stimulation induced nuclear translocation of NF- κ B p65 protein in H9c2 cells, and this effect was markedly reversed by siTNC ($P < 0.05$) (Fig. 3D). H/R also reduced cytoplasmic I- κ B α protein levels ($P < 0.05$), which was reversed by siTNC (Figure. 3E). These results indicate that knockdown of TNC reversed H/R induced NF- κ B nuclear translocation and degradation of cytoplasmic I- κ B α , thus inhibiting NF- κ B signaling pathway.

Overexpression of TLR4 reverses the protective Effects of siTNC

We then investigated the role of TLR4 in siTNC-mediated cardioprotection by overexpression of TLR4 using pcDNA3.1 vector. The efficiency of gene transfection was evaluated by western blot (Fig. 4A). siTNC increased cell viability and decreased caspase-3 activity in H9c2 cells exposed to H/R. However, these effects were reversed by TLR4 overexpression (Fig. 4B, 4C). Similarly, transfection with pcDNA-TLR4 markedly reversed the siTNC-mediated decrease in MDA and increase in SOD in H/R-exposed cells (Fig. 4D, 4E). Furthermore, TLR4 overexpression also attenuated the reduction in supernatant IL-6 by siTNC in H/R-exposed cells (Fig. 4F). These results indicate that TLR4 signaling pathway mediates siTNC-induced cardioprotection against H/R injury.

Discussion

In the present study, we explored the effect of TNC in H9c2 cells subjected with H/R, in vitro model of myocardial I/R injury. The principal findings were that (1) TNC silencing by siRNA markedly attenuated the H/R-induced decrease in cell viability and increase in culture medium LDH content in H9c2 cells; (2) TNC silencing decreased TUNEL + cells, caspase-3 activity and attenuated the level of oxidative stress and proinflammatory cytokine (IL-6) induced by H/R in H9c2 cells; (3) TNC silencing suppressed TLR4 expression and blocked NF- κ B translocation from the cytoplasm to the nucleus. Our data showed that TNC silencing play a protective role by inhibiting cell death and inflammation in H/R cardiomyocytes, which may be related to TLR4/NF- κ B pathway.

In ischemic heart disease, myocardial blood reperfusion is important therapeutic method, but this method often aggravates myocardial injury. Therefore, it is urgent to explore the more detailed mechanisms underlying myocardial IR injury, so as to find new therapeutic strategies to protect cardiomyocytes during treatment. In this study, TNC was found to increase in a time-dependent manner in H9c2 induced with H/R, and TNC inhibition increased cell viability and decreased LDH release and apoptotic cells. These results are consistent with a previous report that TNC mRNA expression was increased by oxygen and glucose deprivation, and TNC content was elevated in plasma and myocardial tissue in patients 7 days post MI [15]. Moreover, the aggravating effect of TNC on ischemic injury is also reported in other tissues. For instance, ischemia enhanced TNC expression in the optic nerve [16]. TNC deficient mice were more resistant to liver I/R injury [17].

In myocardial H/R injury is a complex pathological process, hypoxia leads to cardiomyocyte apoptosis through destroying microenvironment homeostasis [18]. Further, cardiomyocyte apoptosis promotes the major pathological processes of myocardial H/R injury, such as myocardial injury, cardiac dysfunction and eventually heart failure [19]. Cardiomyocytes under myocardial IR injury also show excessive production of reactive oxygen species (ROS), thus inducing oxidative stress. IR injury can induce cardiomyocyte apoptosis by triggering oxidative stress [20]. Therefore, inhibition of oxidative stress has become a major preventing and therapeutic strategy for myocardial IR injury [21]. TNC expression was upregulated by oxidative damage in lung artery endothelial cells and lung epithelial cells [22, 23], which is consistent with our results. Moreover, our data showed that TNC might aggravate the oxidative damage of cardiomyocytes under myocardial IR injury, as evidenced by decreased MDA and increased SOD in

cells with siTNC. Excessive oxidative stress is often associated with inflammation in cardiovascular disease, including myocardial I/R injury, with myeloperoxidase (MPO) as link of these two mechanisms [24]. Interestingly, TNC markedly upregulated MPO activity in neutrophils, and potentiated the neutrophil-mediated injury in steatotic livers [17]. However, whether TNC also regulate MPO in myocardial IR injury and remain further in vivo investigation.

The present study showed that TNC increased TLR4 expression and promoted NF- κ B translocation from cytoplasm to nucleus of cardiomyocytes with H/R injury, which is dependent on I κ B α . In response to MI, TLR4 is activated and induce cardiomyocytes express proinflammatory cytokines and initiate a local inflammatory response [6]. TLR4 induced the NF- κ B dependent expression of proinflammatory cytokines, such as TNF- α , IL-1 β and IL-6 [25]. Recent report showed that inhibition of TLR4/NF- κ B signaling pathway protected cardiomyocytes from H/R-induced injury [26]. TNC accelerated left ventricular remodeling after MI by modulating M1/M2-macrophage polarization [27]. TNC could activate inflammasomes in epicardium-derived cells (EPDCs) via TLR4, thus promoting various proinflammatory factors production from EPDCs [28]. These also support our experiment results that siTNC-mediated protection on myocardial IR injury was reversed by TLR4 overexpression. Therefore, our study propose a TNC-TLR4-NF- κ B signaling pathway in myocardial IR injury. This pathway has been recently reported in promotion of inflammation in white adipose tissue [29]. TLR4 also mediated the increased mRNA expression and protein secretion of IL-6 in cultured human cardiac myofibroblasts [30]. Given the fact that TNC-TLR4 pathway links between inflammation and ECM remodeling of visceral adipose tissue [31], whether it also contribute to the cardiac hypertrophy after MI remains further investigate [32].

Conclusion

silencing TNC by siRNA protects cardiomyocytes from H/R injury through inhibiting apoptosis, oxidative stress and inflammatory response. Moreover, TNC-TLR4-NF- κ B signaling mediate this process. Future in vivo studies is needed to confirm the protective role and mechanisms of TNC silencing for myocardial I/R injury.

Abbreviations

ECM, extracellular matrix; H/R: hypoxia/reoxygenation; IL-1 β , interleukin 1 β ; I/R: ischemia/reperfusion; LDH, lactate dehydrogenase; MDA, malondialdehyde; MPO, myeloperoxidase; MI: myocardial infarction; NF- κ B, nuclear factor- κ B; ROS, reactive oxygen species; siRNA, small interfering RNA; SOD, superoxide dismutase; TNC, Tenascin-C; TLR4, Toll-like receptor 4; TNF- α , tumor necrosis factor- α

Declarations

Availability of data and materials

The data in support of the results are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This is an in vitro study based on cell line, and no formal ethics approval was required in this particular in vitro study.

Consent for publication

Not applicable.

Competing interests

The authors declared no conflict of interest with other people or organizations.

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Author's contribution

Zhiru Ge designed and supervised the study, and wrote the manuscript; Chenjun Zhang performed experiments and analyzed data; Jie Lin and Hong Pan analyzed the data. Zijun Lin performed the statistical analysis; Wei Zhang revised the manuscript. All authors have read and approved the manuscript.

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References

1. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med*. 2007; 357:1121-1135.
2. Binder A, Ali A, Chawla R, Aziz HA, Abbate A, Jovin IS. Myocardial protection from ischemia-reperfusion injury post coronary revascularization. *Expert Rev Cardiovasc Ther*. 2015;13(9):1045-57.
3. Donato M, Evelson P, Gelpi RJ. Protecting the heart from ischemia/reperfusion an update on remote ischemic preconditioning and postconditioning. *Curr Opin Cardiol*. 2017;32(6):784-790.
4. Cadenas S. ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection. *Free Radic Biol Med*. 2018;117:76-89.
5. Ong SB, Hernández-Reséndiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek XY, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic Pharmacol Ther. 2018;186:73-87.

6. Lee SM, Hutchinson M, Saint DA. The role of Toll-like receptor 4 (TLR4) in cardiac ischaemic-reperfusion injury, cardioprotection and preconditioning. *Clin Exp Pharmacol Physiol.* 2016;43(9):864-71.
7. Yang Y, Lv J, Jiang S, Ma Z, Wang D, Hu W, et al. The emerging role of Toll-like receptor 4 in myocardial inflammation. *Cell Death* 2016 ;7:e2234.
8. Jia SJ, Niu PP, Cong JZ, Zhang BK, Zhao M. TLR4 signaling: a potential therapeutic target in ischemic coronary artery disease. *Int Immunopharmacol.* 2014 ;23(1):54-9.
9. Lin L, Knowlton AA. Innate immunity and cardiomyocytes in ischemic heart *Life Sci.* 2014;100(1):1-8.
10. Chiquet-Ehrismann R, Chiquet M. Tenascins: regulation and putative functions during pathological stress. *J Pathol* 2003;200:488–99.
11. Yuan W, Zhang W, Yang X, Zhou L, Hanghua Z, Xu K. Clinical significance and prognosis of serum tenascin-C in patients with sepsis. *BMC Anesthesiol.* 2018 ;18(1):170.
12. Midwood KS, Orend G. The role of tenascin-C in tissue injury and tumorigenesis. *J Cell Commun Signal* 2009;3:287–310.
13. Imanaka-Yoshida K. Tenascin-C in cardiovascular tissue remodeling: from development to inflammation and repair. *Circ J* 2012;76:2513–2520.
14. Sato A, Aonuma K, Imanaka-Yoshida K, Yoshida T, Isobe M, Kawase D, et al.. Serum tenascin-C might be a novel predictor of left ventricular remodeling and prognosis after acute myocardial infarction. *J Am Coll Cardiol* 2006;47:2319–2325.
15. Gonçalves IF, Acar E, Costantino S, Szabo PL, Hamza O, Tretter EV, et al. Epigenetic modulation of tenascin C in the heart: implications on myocardial ischemia, hypertrophy and metabolism. 2019;37(9):1861-1870.
16. Reinhard J, Renner M, Wiemann S, Shakoor DA, Stute G, Dick HB, et al. Ischemic injury leads to extracellular matrix alterations in retina and optic nerve. *Sci Rep.* 2017;7:43470.
17. Kato H, Duarte S, Miller MG, Busuttill RW, Coito AJ. Overproduction of Tenascin-C Driven by Lipid Accumulation in the Liver Aggravates Hepatic Ischemia/Reperfusion Injury in Steatotic Mice. *Liver Transpl.* 2019 ;25(2):288-
18. Zhang B, Zhou M, Li C, Zhou J, Li H, Zhu D, et al. MicroRNA-92a inhibition attenuates hypoxia/reoxygenation-induced myocardiocyte apoptosis by targeting Smad7. *PLoS One.* 2014;9(6):e100298.
19. Baines CP. How and when do myocytes die during ischemia and reperfusion: the late phase. *J Cardiovasc Pharmacol Ther.* 2011;16(3-4):239-43.
20. Kurian GA, Rajagopal R, Vedantham S, Rajesh M. The Role of Oxidative Stress in Myocardial Ischemia and Reperfusion Injury and Remodeling: Revisited. *Oxid Med Cell Longev.* 2016;2016:1656450.
21. Tao L, Huang K, Wang J, Xue Y, Zhou Y, He F, et al. Retinol palmitate protects against myocardial ischemia/reperfusion injury via reducing oxidative stress and inhibiting apoptosis. *Am J Transl*

- Res. 2019;11(3):1510-1520. eCollection
22. Aziz SM, Toborek M, Hennig B, Mattson MP, Guo H, Lipke DW. Oxidative stress mediates monocrotaline-induced alterations in tenascin expression in pulmonary artery endothelial cells. *Int J Biochem Cell Biol.* 1997 ;29(5):775-87.
 23. Fitch PM, Howie SE, Wallace WA. Oxidative damage and TGF- β differentially induce lung epithelial cell sonic hedgehog and tenascin-C expression: implications for the regulation of lung remodelling in idiopathic interstitial lung *Int J Exp Pathol.* 2011;92(1):8-17.
 24. Ndrepepa G. Myeloperoxidase - A bridge linking inflammation and oxidative stress with cardiovascular disease. *Clin Chim Acta.* 2019;493:36-51.
 25. Frantz S1, Ertl G, Bauersachs J. Mechanisms of disease: Toll-like receptors in cardiovascular disease. *Nat Clin Pract Cardiovasc Med.* 2007 A;4(8):444-54.
 26. Wang Y, Wang Y, Wang X, Hu P. Tilianin-loaded Reactive Oxygen Species-Scavenging Nano-Micelles Protect H9c2 Cardiomyocyte Against Hypoxia/Reoxygenation-Induced Injury. *J Cardiovasc Pharmacol.* 2018 ;72(1):32-39.
 27. Kimura T, Tajiri K, Sato A, Sakai S, Wang Z, Yoshida T, et al. Tenascin-C accelerates adverse ventricular remodelling after myocardial infarction by modulating macrophage *Cardiovasc Res.* 2019;115(3):614-624.
 28. Hesse J, Leberling S, Boden E, Friebe D, Schmidt T, Ding Z, et al. CD73-derived adenosine and tenascin-C control cytokine production by epicardium-derived cells formed after myocardial infarction. *FASEB J.* 2017 ;31(7):3040-3053.
 29. Cao W, Huang H, Xia T, Liu C, Muhammad S, Sun C. Homeobox a5 Promotes White Adipose Tissue Browning Through Inhibition of the Tenascin C/Toll-Like Receptor 4/Nuclear Factor Kappa B Inflammatory Signaling in Mice. *Front* 2018;9:647.
 30. Maqbool A, Spary EJ, Manfield IW, Ruhmann M, Zuliani-Alvarez L, Gamboa-Esteves FO, et al, Turner NA. Tenascin C upregulates interleukin-6 expression in human cardiac myofibroblasts via toll-like receptor 4. *World J* 2016;8(5):340-50.
 31. Catalán V, Gómez-Ambrosi J, Rodríguez A, Ramírez B, Rotellar F, Valentí V, et al. Increased tenascin C and Toll-like receptor 4 levels in visceral adipose tissue as a link between inflammation and extracellular matrix remodeling in obesity. *J Clin Endocrinol Metab.* 2012;97(10):E1880-9.
 32. Podesser BK, Kreibich M, Dzilic E, Santer D, Förster L, Trojanek S, et al, Trescher K, Kiss A. Tenascin-C promotes chronic pressure overload-induced cardiac dysfunction, hypertrophy and myocardial fibrosis. *J Hypertens.* 2018 ;36(4):847-856.

Figures

Figure 1

TNC expression in cardiomyocytes with H/R injury. Human cardiomyocytes H9c2 were subjected to hypoxia for 4 h, and then were under normoxic conditions for 4, 8, 12, or 16 h. The control cells were kept under normoxic conditions. (A) Cell viability was assessed using an CCK-8 assay. (B) Relative mRNA expression of TNC was measured by RT-qPCR. (C) Protein expression of TNC was measured by western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. hypoxia group. Cardiomyocytes were transfected with TNC siRNA or NC siRNA before H/R treatment. Relative mRNA (D) and protein (E) expression of TNC was determined by RT-qPCR and western blot. ** $P < 0.01$, *** $P < 0.001$ vs. control group; ## $P < 0.01$, ### $P < 0.001$ vs. blank group. (F) Cell viability was detected by CCK-8 assay. (G)

Supernatant LDH level was detected using an LDH assay. ***P<0.001 vs. control group; ###P<0.001 vs. H/R group.

Figure 2

Effects of TNC knockdown on H/R-induced apoptosis, oxidative stress and inflammation of cardiomyocytes. H9c2 cells were transfected with TNC-specific siRNA (siTNC) or negative control (siNC) and then subjected to H/R for 16 h. (A) Representative photomicrographs of H9c2 cells with TUNEL

staining are shown. Magnification, $\times 200$. (B) Quantification of TUNEL+ cells. (C) Cell apoptosis was determined by measurement of caspase-3 activity. The markers of oxidative stress including MDA (D) and SOD (E) in H9c2 cells were determined by colorimetric method. (F) IL-6 mRNA expression was evaluated by RT-qPCR. (G) Supernatant IL-6 protein level was evaluated by ELISA. *** $P < 0.001$ vs. control group; ### $P < 0.001$ vs. H/R group.

Figure 3

TNC silencing inhibits TLR4 and NF-kappa B signals. H9c2 cardiomyocytes were transfected with TNC-specific siRNA (siTNC) or negative control (siNC) and then subjected to H/R for 16 h. (A) TLR4 mRNA expression was examined by RT-qPCR. (B) Representative western blot analyses of TLR4, nuclear NF-κB p65 and cytoplasmic I-κBα expression. Bands were analyzed and quantified by densitometry and the TLR4/β-actin (C), NF-κB p65/β-actin (D), and I-κBα/β-actin (E) Ratios were evaluated. ** $P < 0.01$, *** $P < 0.001$ vs. control group; ## $P < 0.01$, ### $P < 0.001$ vs. H/R group.

Figure 4

Overexpression of TLR4 reduces TNC knockdown-mediated protection against H/R injury. (A) TLR4 protein expression was measured in H9c2 cells transfected with TLR4 DNA/pcDNA3.1 or control DNA/pcDNA3.1 by western blot. H9c2 cardiomyocytes were cotransfected with TNC-specific siRNA (siTNC) and TLR4 DNA (pcDNA-TLR4) or control DNA (pcDNA), and then subjected to H/R for 16 h. *** $P < 0.001$ vs. control group. (B) Viability of H9C2 cells were measured by CCK-8. (C) Cell apoptosis was determined by measurement of caspase-3 activity. The markers of oxidative stress MDA (C) and SOD (D) were measured. Inflammation was evaluated by measuring supernatant IL-6 protein. ** $P < 0.01$, *** $P < 0.001$ vs. H/R group; ## $P < 0.01$, ### $P < 0.001$ vs. siTNC group.