


Trop2 Guarantees Cardioprotective Effects of Cortical Bone-Derived Stem Cells on Myocardial Ischemia/Reperfusion Injury

Cell Transplantation
2018, Vol. 27(8) 1256–1268
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DOI: 10.1177/0963689718786882
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Abstract

Stem cell transplantation represents a promising therapeutic approach for myocardial ischemia/reperfusion (I/R) injury, where cortical bone-derived stem cells (CBSCs) stand out and hold superior cardioprotective effects on myocardial infarction than other types of stem cells. However, the molecular mechanism underlying CBSCs function on myocardial I/R injury is poorly understood. In a previous study, we reported that Trop2 (trophoblast cell-surface antigen 2) is expressed exclusively on the CBSCs membrane, and is involved in regulation of proliferation and differentiation of CBSCs. In this study, we found that the Trop2 is essential for the ameliorative effects of CBSCs on myocardial I/R-induced heart damage via promoting angiogenesis and inhibiting cardiomyocytes apoptosis in a paracrine manner. Trop2 is required for the colonization of CBSCs in recipient hearts. When Trop2 was knocked out, CBSCs largely lost their functions in lowering myocardial infarction size, improving heart function, enhancing capillary density, and suppressing myocardial cell death. Mechanistically, activating the AKT/GSK3 β / β -Catenin signaling axis contributes to the essential role of Trop2 in CBSCs-rendered cardioprotective effects on myocardial I/R injury. In conclusion, maintaining the expression and/or activation of Trop2 in CBSCs might be a promising strategy for treating myocardial infarction, I/R injury, and other related heart diseases.

Keywords

myocardial ischemia/reperfusion injury, CBSCs, Trop2, apoptosis, angiogenesis

Introduction

Ischemia of the heart induced by the interruption of blood flow, commonly in myocardial infarction (MI), is one of the leading cardiovascular pathologies that carries an alarmingly increased prevalence and mortality^{1,2}. Early reperfusion by thrombolytic agents or primary percutaneous coronary intervention (PCI) remain the most common treatment methods for myocardial ischemia^{3,4}. However, the restoration of blood flow evokes multiple deleterious responses and tissue lesions, such as inflammation, oxidative stress, and, ultimately, cell death, leading to functionally and structurally cardiac remodeling or even, in severe cases, that of heart failure^{5–8}. Despite advancements in understanding the pathogenesis of myocardial ischemia-reperfusion (I/R) and the development of pharmacological and interventional strategies, the outcomes of I/R-triggered heart injury treatment are far from being satisfactory^{9,10}. Thus, new therapeutic approaches are urgently required to improve the prognosis of post-infarction myocardial remodeling and the subsequent development of heart failure.

The emergence of stem cell therapy has provided an encouraging solutions for myocardial I/R injury^{11,12}. Since the first experimental application of stem cells in diseased hearts, numerous studies have shown the therapeutic potential of transplantation of autologous cardiac stem cells, bone

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Submitted: December 16, 2017. Revised: May 6, 2018. Accepted: June 11, 2018.

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marrow-derived stem cells, induced pluripotent stem cell and endogenous progenitor cells in improving cardiac function after injury^{13–17}. Although the safety and efficacy of these stem/progenitor cells in clinical trials have been demonstrated, overall functional improvements are limited. Thus, exploring the optimal stem cell type for cardiac repair is a key step towards improving clinical outcomes.

Recently, a new technology-derived isolation of cortical bone-derived stem cells (CBSCs) showed that, and in comparison with the widely used cardiac stem cells, CBSCs exhibited a more effective capacity to improve cardiac remodeling after ischemic injury^{18,19}. However, the underlying mechanisms of CBSCs-exerted a beneficial function in cardiac repair is largely unknown.

Trophoblast cell-surface antigen 2 (Trop2, also known as TACSTD2), is a type I transmembrane glycoprotein of the tumor-associated calcium signal transducer (TACSTD) family that was originally identified in human placental trophoblastic tissue²⁰. Despite the low, or even undetectable, levels of expression in adult healthy tissues, Trop2 is upregulated in tumors²¹. The over-expression of Trop2 is correlated with poor prognosis in patients with solid tumors, which might be attributed by the promotive function of Trop2 on cancer cell proliferation, migration, and metastasis by modulating PI3K/AKT and ERK pathways^{22–24}. Notably, Trop2 is expressed in stem cell-like prostatic basal cells and hepatic oval cells. In addition, only cells with high expression of Trop2 hold the capacity to self-renew and differentiate to mature cells^{25–27}. Recent studies from our group further found that Trop2 is expressed in CBSCs^{28,29}, while myocardial ischemia can activate the Trop2-positive cardiac c-kit⁺ cells²⁸. Furthermore, Trop2 facilitates the survival and proliferation of CBSCs²⁹. We therefore hypothesize that Trop2 might be a key factor in determining the beneficial function of CBSCs in cardiac repair, which, however, has not been clarified yet.

Herein, we demonstrated that the Trop2-positive CBSCs are more effective in repairing heart injury induced by ischemia/reperfusion than CBSCs with Trop2 deficiency, which are based primarily on the negative regulation of Trop2 on CBSCs apoptosis and its positive regulation on VEGF and FGF2 section.

Materials and Methods

The C57BL6/J mice were purchased from HUAUFU-KANG Bioscience Co. Inc. (Beijing, China). The Trop2-knockout (KO) mice were generated as previously described by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China) via targeting gene interruption through homologous recombination^{29,30}. All mice were maintained in standard SPF facilities in Tongji Medical College of Huazhong University of Science.

Primary CBSC Isolation, Culture and Treatment

Primary CBSCs were isolated from male mice at the age of 2–3 weeks according to the method of Zhu and colleagues³¹, which was also adopted by Duran and colleagues¹⁹, who first coined the term CBSCs to describe compact bone-derived mesenchymal stem cells. In our previous study²⁹, we followed the same method and isolated the cells for comparative study: the characterization and identification of the phenotype and the induced differentiation between wild type (WT)-CBSCs and trop2-KO CBSCs. Briefly, the mice were sacrificed by cervical dislocation, and then the femur, humerus, and tibia were isolated. After removing the epiphyses, bone marrow was flushed three times with minimal essential medium (α -MEM; Hyclone Laboratories, Logan, UT, USA). The remaining cortical bone was crushed and digested using type II Collagen (Gibco BRL, Grand Island, NY, USA) at the concentration of 1 mg/ml for 90 min. The bone chunks were washed three times with α -MEM, and then plated in CBSC culture medium that included α -MEM, 10% MSC-qualified FBS (Gibco BRL), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich), and 100 μ g/ml streptomycin (Sigma-Aldrich). To examine the regulatory effects of Trop2 on CBSC viability and apoptosis, CBSCs were exposed to the presence of H₂O₂ at the dose of 0.2 mM for indicated periods. Cells were then collected by 0.025% trypsin/0.02% EDTA (Sigma) digestion for subsequent studies.

Primary Mouse Cardiomyocytes Isolation and Treatment

Primary cardiomyocytes were isolated by enzymatic digestion from 1- to 3-day-old neonatal mice with a C57BL6/J background³². After the mice were killed by cervical dislocation, their whole hearts were excised, minced, and digested with a collagenase/dispase mixture (Roche, Indianapolis, IN). Tissue fragments were then centrifuged, and supernatants containing suspended cells were preplated for 2 h to remove fibroblasts and endothelial cells. The purified cardiomyocytes were cultured on fibronectin-coated dishes in DMEM with 10% FBS at a temperature of 37°C in the presence of 5% CO₂.

For the co-culture experiments that served as the control, the isolated CBSCs were co-cultured with primary cardiomyocytes for 24 h, followed by hypoxia with 1% O₂ stimulation for 2 h. The cells were then collected for subsequent examinations.

Mouse Myocardial I/R Model and Intramyocardial CBSCs Transplantation

Myocardial I/R injury was established in female mice at the age of 10–12 weeks by ligating the left coronary artery; subsequent reperfusion was performed according to previously

reported methods^{33,34}. In brief, the mice were anesthetized and exposed to a left thoracotomy at the fourth intercostal space. The pericardium of the mouse was then opened, and the proximal left anterior descending coronary artery was ligated using a silk suture to occlude blood flow. After 30 min of ischemia, the silk suture was removed to enable reperfusion.

To evaluate the cardioprotective function of CBSCs in myocardial I/R injury, the isolated (wild type) WT-CBSCs and Trop2-KO CBSCs were injected intramyocardially into the border zone of hearts with 5 μ l medium including 10⁵ cells at 24 h after reperfusion. Note that the mice were sacrificed at each indicated time point followed by the collection of their hearts for the following study.

Heart Function Assay by Echocardiography

The heart functions of the mice were detected using echocardiography with a MyLab 30CV ultrasound system (Biosound Esaote Inc. Indianapolis, IN, USA) at baseline, 1 week, and 6 weeks after reperfusion respectively, according to the manufacture's instruction. Left ventricular (LV) fractional shortening, LV end systolic diameter (LVESD), LV end systolic volume, posterior wall thickness, and infarct wall thickness were recorded.

Histological Examination and Immunofluorescence Staining

The paraffin-embedded hearts were sectioned serially at 5- μ m thickness for histological and immunofluorescence staining. Evans blue and triphenyl-tetrazolium chloride (TTC) staining was performed on heart sections of mice after myocardial I/R surgery to visualize infarct volumes influenced by CBSCs treatment as described in a previous study³⁵. Periodic acid-Schiff (PAS) staining (Sigma-Aldrich, St. Louis, MO, USA) was performed to evaluate myocardial I/R-induced fibrosis of mice in each group according to a standard protocol. The histological images were then observed and captured under a light microscope (Olympus, Tokyo, Japan).

Microvascular generation in infarcted hearts were examined by anti-CD31 staining using the primary antibody of mouse monoclonal to CD31 (ab24590; Abcam) at 4°C overnight followed by incubating with goat anti-mouse-IgG (H+L) (Jackson Laboratory, Bar Harbor, ME, USA) as the secondary antibody at 37°C for 1 h. The cell apoptosis in heart sections and in vitro were visualized by TUNEL staining, which was performed using an ApopTag Plus In Situ Apoptosis Fluorescein Detection Kit (S7111; Millipore, Burlington, MA, USA) according to the manufacturer's instructions. DAPI (4',6-diamidino-2-phenylindole) was used to label the nuclei. These images of immunofluorescence staining were obtained under a fluorescence microscope (Olympus DX51).

Real-Time RT-PCR

The relative mRNA expression of RBMY (RNA binding motif protein, Y-linked) in the CBSCs-injected heart tissues was examined via real-time RT-PCR after total RNA was isolated from heart samples using an RNeasy RNA isolation kit (Qiagen, Hilden, Germany). The appropriate quantity and concentration of isolated RNA was detected using a Nanodrop 2000 (Thermo Fisher Scientific, Madison, WI, USA). Next, 2 g of total RNA was exposed to reverse transcription using oligo (dT) and Superscript II RNase reverse transcriptase. The emerging cDNA was used as a template for the amplification of target genes by PCR using La-Taq and GC buffer I (Takara Bio, Shiga, Japan). The PCR amplification products were then analyzed and quantified by LightCycler 480 SYBR Green 1 Master Mix. Primers used for RBMY and β -actin were as follows: RBMY-fwd: 5'-GACAA-GAAGTGCTTCCACCA-3', RBMY-rev: 5'-CAGCC-CATCCTTAGGTGAAT-3'; β -actin-fwd: 5'-AGACTTCG AGCAGGAGATGG-3', β -actin-rev: 5'-CAGGCAGCTCATAGCTCTTCT-3'. The relative mRNA expression of RBMY was normalized to that of β -actin.

Western Blot Analysis

The protein expression of indicated factors in heart and cell samples were examined by western blot as described in a previous studies³⁶. In brief, proteins were isolated via the PhosphoSafe Extraction Reagent (Merck KGaA, Darmstadt, Germany) and quantified using a Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific). The obtained proteins were loaded and separated on SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blockage with 5% skim milk, PVDF membranes were incubated with primary antibody at 4°C overnight followed by corresponding secondary antibody at 37°C for 1 h. The primary antibodies used in this study included VEGF (GTX 20119; GeneTex, Inc, Irvine, CA, USA), FGF, BAX, BCL2, and MMP9, while Peroxidase AffiniPure goat anti-rabbit-IgG (H+L) (Jackson Laboratory, Bar Harbor, ME, USA) and goat anti-mouse-IgG (H+L) were used as secondary antibodies at the dilution of 1:10000. The images were recorded and analyzed using a UVP AutoChem Image System (UVP, Upland, CA, USA).

Flow Cytometry

After 12 h of H₂O₂ (0.2 mM) exposure, CBSCs were harvested and incubated using the antibodies of FITC-ANNEXIN V/PI (eBioscience, San Diego, CA, USA) for 30 min at room temperature. Fluorescein isothiocyanate (FITC)-conjugated IgG was used as a negative control. Stained cells were then resuspended in phosphate buffered saline (PBS) for flow cytometry. Detection channel FL1 or FL2 was used for the signal collection. Data were then

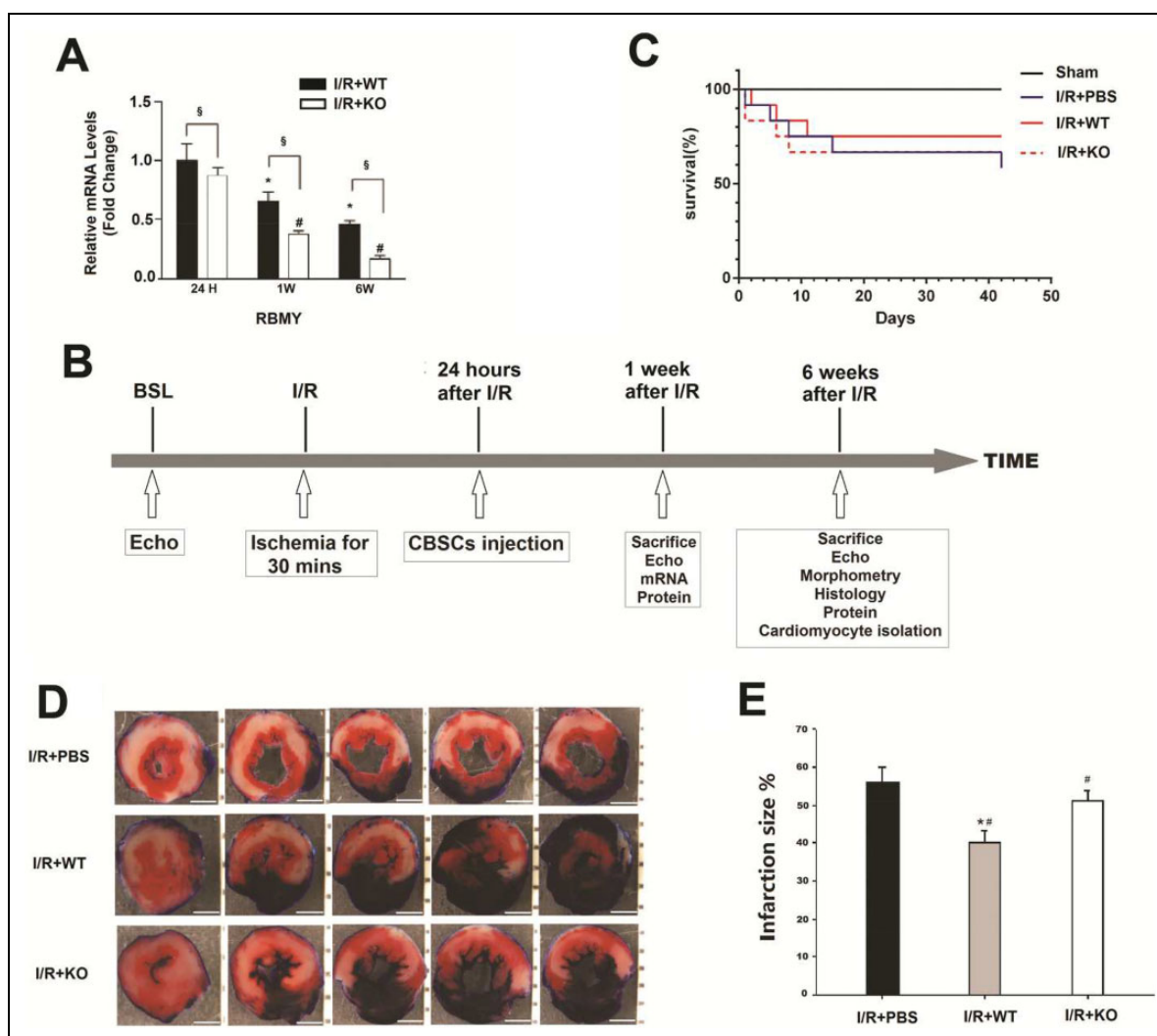


Fig. 1. Trop2 deficiency reduces the protective effect of CBSCs on myocardial I/R injury. (A) Relative mRNA expression of RBMY in the heart tissues of mice receiving WT CBSCs or Trop2-KO CBSCs. The expression levels of RBMY were examined at 24 h, 1 week, and 6 weeks after CBSCs injection. $n = 6$ mice in each group at each detected time points. * $P < 0.05$ compared with I/R+WT 24 h group, # $P < 0.05$ compared with I/R+Trop2-KO CBSCs 24 h group, § $P < 0.05$ indicate statistical significance between WT CBSCs and Trop2-KO CBSCs at the same time point. (B) The schematic figure showing experimental protocols to evaluate the function of WT and Trop2-KO CBSCs on the myocardial I/R injury. (C) The 6-week survival rate of mice subjected to myocardial I/R surgery and injected with WT (Trop2^{+/+}) CBSCs, Trop2-KO CBSCs, or PBS at 24 h after reperfusion. Mice without blood blocking were served as sham controls. $n = 12$ mice in each group. (D and E) Representative images of Evans blue and TTC staining (C) and the ratio of infarction size (D) in the heart sections of mice subjected to myocardial ischemia (30 min) followed by 6 weeks of reperfusion. Mice were injected with WT CBSCs, Trop2-KO CBSCs, or PBS at 24 h after reperfusion. * $P < 0.05$ compared with I/R+PBS group, # $P < 0.05$ compared with I/R+WT CBSCs group. $n = 12$ mice in each group.

analyzed by the CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Enzyme-Linked Immunosorbent Assays

The concentrations of IGF-1, VEGF and SDF-1 in the media of cultured CBSCs and cardiomyocytes were examined using mouse DuoSet enzyme-linked immunosorbent assays (ELISA) Kits, purchased from R&D Systems (Minneapolis, MN, USA). Data for each factor was obtained at least three times

from independent experiments. Unconditioned medium was utilized as blank background, and the value was subtracted.

Statistical Analysis

All statistical analyses used in this study were performed using SPSS (Version 21.0; IBM, Armonk, NY, USA), and the data are expressed as the mean \pm standard deviation (SD). The statistical differences between each two groups were analyzed by two-tailed Student's *t*-test, while one-way

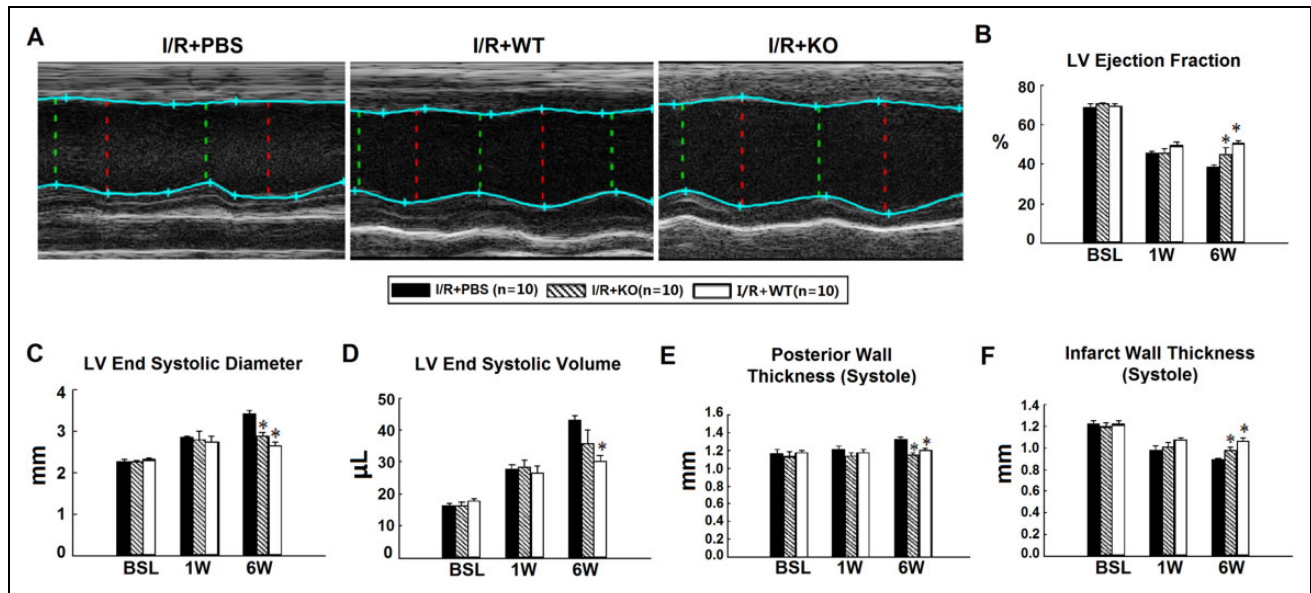


Fig. 2. Losing Trop2 diminished the protection of CBSCs against left ventricle systolic function. (A) The representative echocardiography showing LV function of WT CBSCs, Trop2-KO CBSCs, or PBS-injected mice at 6 weeks after myocardial I/R surgery. $n = 6$ mice in each group. (B–F) The LV EF (%; B), LVESD (C), LV end systolic volume (D), posterior wall thickness (systole) (E) and infarct wall thickness (systole) (F) of mice treated with WT CBSCs, Trop2-KO CBSCs, or PBS at 24 h after reperfusion. The heart functions were examined by echocardiographic analysis after 1 week and 6 weeks of reperfusion or at baseline. $n = 6$ mice in each group at each time point. * $P < 0.05$ compared with I/R+PBS group, # $P < 0.05$ compared with I/R+WT CBSCs group.

analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test was carried out for statistical analysis among more than two groups. A P value less than 0.05 was considered as significant.

Results

Trop2 Deficiency Reduces the Protective Effect of CBSCs on Myocardial I/R Injury

To evaluate the potential regulation of Trop2 in CBSCs-mediated protection against MI, we first applied male to female donor–recipient transplant pairs by injecting intramyocardially equal contents of CBSCs isolated from WT or Trop2-KO male mice into the heart of female mice. The expression of RBMY, a sex-determining factor specifically expressed in males³⁷, was applied to evaluate the colonization of CBSCs in the organ recipient. As shown in Fig. 1A, both WT- and Trop2-KO CBSCs were successfully transplanted to the heart of female mice at 24 h, 1 week and 6 weeks after injection. However, the relative mRNA expression of RBMY in the heart injected with Trop2-KO CBSCs was significantly lower than that of the WT- CBSCs-injected group (Fig. 1A), indicating that Trop2 contributes greatly to the essential colonization capacity of CBSCs.

In the established myocardial I/R models with ischemic period of 30 min, WT and Trop2-KO CBSCs were injected into the border zone of I/R-challenged hearts at 24 h after reperfusion. Mice injected with PBS were served as the

controls. The schematic image of experimental protocol is shown in Fig. 1B. It was found that the myocardial I/R procedure led a 6-week survival in 77% of the mice receiving PBS injection. The survival rate was increased to 81% in WT CBSCs-treated mice, whereas no significant improvement in survival was observed in mice injected with Trop2-KO CBSCs when compared with the PBS controls (Fig. 1C). Accordingly, Evans blue and TTC staining exhibited an effective amelioration for myocardial necrosis in WT CBSCs-injected mice when compared with the PBS controls (Fig. 1D, E). However, the protective effect of CBSCs on MI was almost completely abolished by Trop2 deletion (Fig. 1D, E). These data suggest that Trop2 has an indispensable role of Trop2 in CBSCs-mediated protection against I/R-induced death and MI.

Losing Trop2 Diminished the Protection of CBSCs Against Left Ventricle Systolic Function

As key indexes of heart injury, the left ventricle (LV) along with its internal diameter and contractility were examined by echocardiography at 1 and 6 weeks after myocardial I/R operation, respectively. The WT CBSCs therapy significantly improved cardiac function and attenuated adverse cardiac remodeling features at 6 weeks after reperfusion compared with PBS-treated controls (Fig. 2A). The echocardiograph analysis showed that both animals receiving WT or Trop2 deficiency CBSCs, exhibited improved LV ejection fraction (EF; Fig. 2B), decreased LVESD (Fig. 2C), and

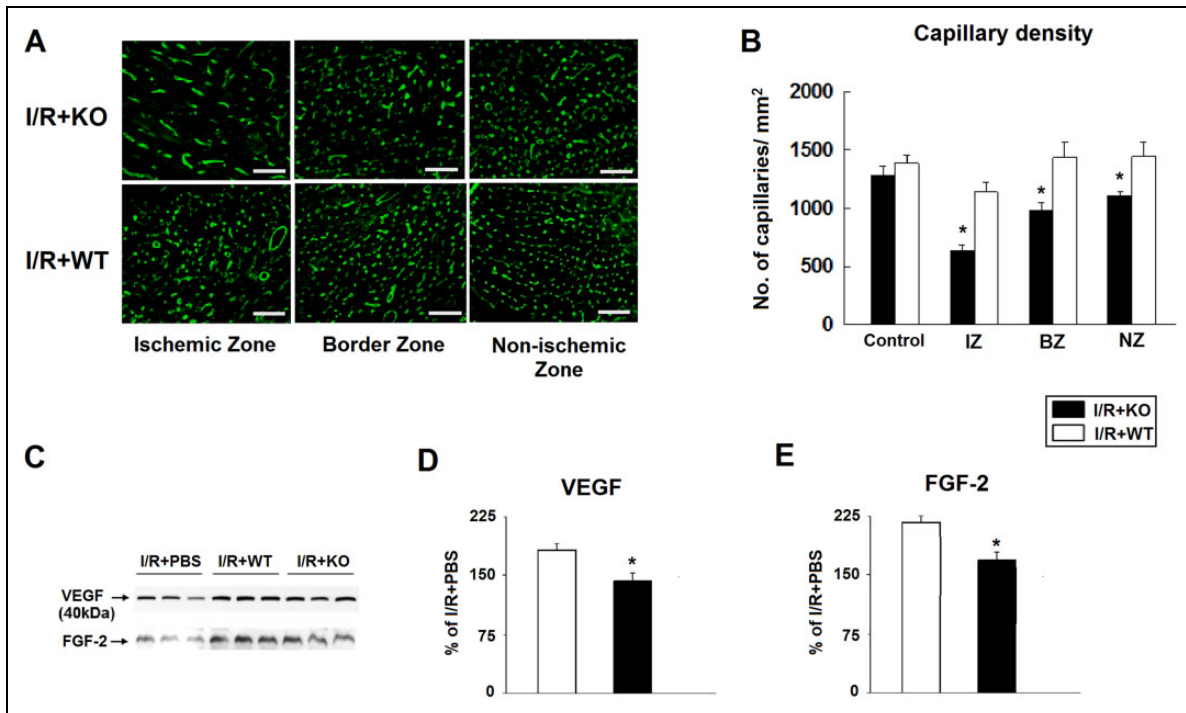


Fig. 3. Trop2 promotes VEGF and FGF-2 production to accelerate angiogenesis. (A) Representative images of anti-CD-31 staining on the infarct zone (IZ), border zone (BZ) and normal zone (NZ) of heart sections from mice in the WT CBSC and Trop2-KO CBSC groups at 6 weeks after myocardial I/R surgery. (B) The quantification of capillary density in the IZ, BZ, NZ of myocardial I/R-challenged heart sections or sham control from mice treated with WT CBSCs or Trop2-KO CBSCs. (C–E) The representative Western blot bands (C) and quantification results (D and E) of VEGF and FGF-2 in the heart tissues from mice treated with WT CBSCs and Trop2-KO CBSCs at 6 weeks after reperfusion. The protein expression of VEGF and FGF-2 were examined at 6 weeks after myocardial I/R surgery. * $P < 0.05$ compared with the I/R-KO group. $n = 6$ mice in each group.

inhibited the enlargement of LV end systolic volume (Fig. 2D) after a 6-week reperfusion compared with PBS-injection controls. In line with the improved cardiac function, CBSCs treatment inhibited the increased posterior wall thickness (Fig. 2E) and infarct wall thickness (Fig. 2F) induced by I/R insult. No significant effect can be observed as early as 1 week after I/R surgery (Fig. 2B–F). Notably, compared with the WT cells, Trop2 deficiency significantly reduced this protective function of CBSCs (Fig. 2), which indicates that Trop2 is necessary for the therapeutic effect of CBSCs on LV systolic dysfunction after myocardial I/R insult.

Trop2 Promotes VEGF and FGF-2 Production to Accelerate Angiogenesis

Previous studies have shown that stem cells can promote neo-vascularization effects after myocardial infarction³⁸. To investigate the effects of Trop2 on neo-vascularization, we examined the capillary density in the hearts of mice injected with WT or Trop2-KO CBSCs at 6 weeks after I/R surgery. Fig. 3A shows that the CD31-positive cells were greatly lower in all the infarct zone, border zone and normal zone of hearts from the Trop2-KO group than in the WT CBSCs group (Fig. 3A, B). Since VEGF and FGF-2 are vital

markers of angiogenesis, we followed up by testing their protein expression in the hearts of mice in each group. Western blot analysis revealed a dramatic increase in the protein expression of both VEGF and FGF-2 in two CBSCs-treated groups compared with PBS group, whereas the increase of VEGF and FGF-2 in Trop2-KO CBSCs-treated mice was much lower than that of WT CBSC group (Fig. 3C–E). Overall, Trop2 is required for the effect of CBSCs treatment in order to promote angiogenesis and the selection of VEGF and FGF-2.

Trop2 Ablation Blunts the Anti-Apoptosis Function of CBSCs During Myocardial I/R Injury

In addition to the regulatory effect on promoting angiogenesis, CBSCs holds the protective function of repairing heart injury dependent on their potent capacity to inhibit the apoptosis of cardiomyocytes. As shown in Fig. 4A and B, Trop2 deletion significantly attenuated the anti-apoptotic effect of CBSCs on cardiomyocytes in the heart sections after I/R insult. Consistently, the protein expression of pro-apoptotic factor Bax was increased in the heart tissue of Trop2-KO CBSCs-treated mice when compared with the WT CBSC group, whereas the expression level of anti-apoptotic Bcl2

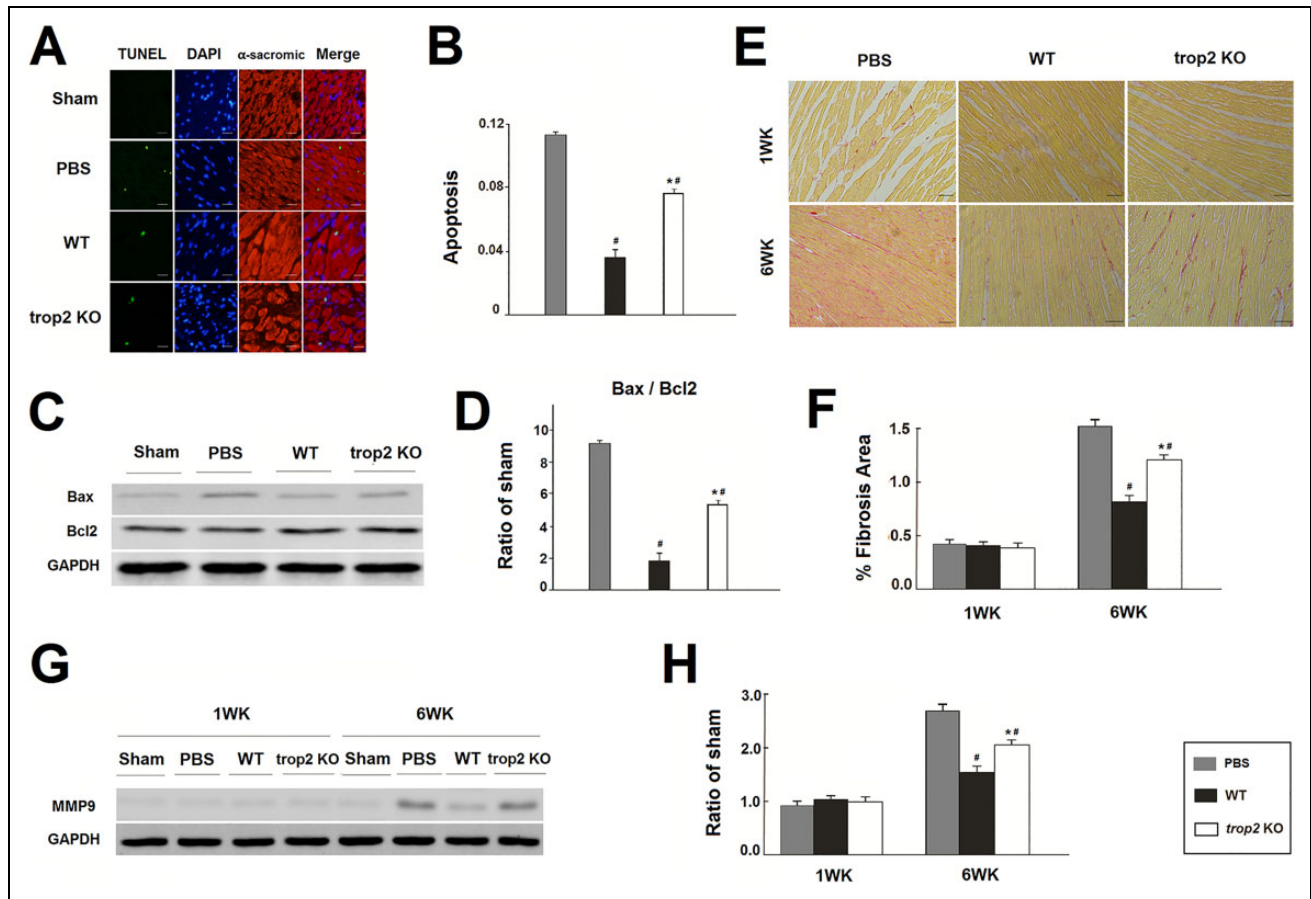


Fig. 4. Trop2 ablation blunts the anti-apoptosis function of CBSCs during myocardial I/R injury. (A and B) Representative TUNEL images (A) and quantification of TUNEL-positive cells (green and highlighted by white arrows) ratio in total cardiomyocytes (B) of heart sections from WT and KO CBSCs injection mice after 6 weeks of sham or myocardial I/R surgery. DAPI staining (blue) is used as a marker for the cell nucleus. Sections were co-stained with anti-monoclonal α -actinin (red) to identify cardiac myocytes. $n = 6$ mice in each group. Scale bar: 50 μ m. * $P < 0.05$ compared with the WT CBSC group; # $P < 0.05$ compared with the PBS group. (C and D) Representative Western blot (C) showing the protein expression of pro-apoptotic Bax and anti-apoptotic Bcl2 and the quantification of Bax to Bcl2 ratio (D) in the heart from WT CBSCs or Trop2-KO CBSCs-injected mice after 6 weeks after myocardial I/R surgery. * $P < 0.05$ compared with the WT CBSC group; # $P < 0.05$ compared with the PBS group. $n = 3$ mice in each group. (E and F) The representative picrosirius red staining images (E) and the ratio of fibrotic area (F) in the heart sections from WT CBSCs or Trop2-KO CBSCs-treated mice at 1 week and 6 weeks after reperfusion. * $P < 0.05$ compared with the WT CBSC/6w group; # $P < 0.05$ compared with the PBS/6w group. $n = 6$ mice in each group. (G and H) Representative Western blot (G) and quantification (H) showing the protein expression of pro-fibrotic factor matrix metalloproteinase 9 (MMP9) in mice with Trop2-KO CBSCs or WT CBSCs injection at 1 week and 6 weeks after myocardial I/R surgery. $n = 3$ mice in each group. * $P < 0.05$ compared with the WT CBSC/6w group; # $P < 0.05$ compared with the PBS/6w group.

showed an opposing trend (Fig. 4C). As a result, the ratio of Bax to Bcl2 was much higher in the hearts from mice injected with Trop2-KO CBSCs than that of the WT group (Fig. 4D).

In terms of cardiac interstitial fibrosis, picrosirius red staining (PRS) revealed that mice in the Trop2-KO group exhibited more severe fibrosis in their heart than the WT CBSCs-treated mice at 6 weeks after I/R surgery (Fig. 4E, F), accompanied by significantly higher expression of fibrosis-related factor matrix metalloproteinase 9 (MMP9) compared with WT group (Fig. 4G, H). No significant differences were observed in fibrotic content among WT CBSCs-injected mice, Trop2-KO CBSCs-injected mice and PBS-injected mice at 1 week after I/R insult. These data

implied that Trop2 is required for the protective effect of CBSCs on apoptosis and fibrosis in the heart after myocardial I/R surgery.

Trop2 Inhibits CBSCs Apoptosis and Promotes Cardiomyocytes Survival in a Paracrine Manner

Stem cell apoptosis is a major obstacle to its benefits in myocardial I/R injury. Thus, to explore cellular events underlying the effect of Trop2 on the functional role of CBSCs in heart injury, we examined the response of Trop2-KO and WT CBSCs to H_2O_2 challenge—a common inducer mimicking *in vivo* oxidative stress triggering cell death. It was found that, in comparison to the WT controls,

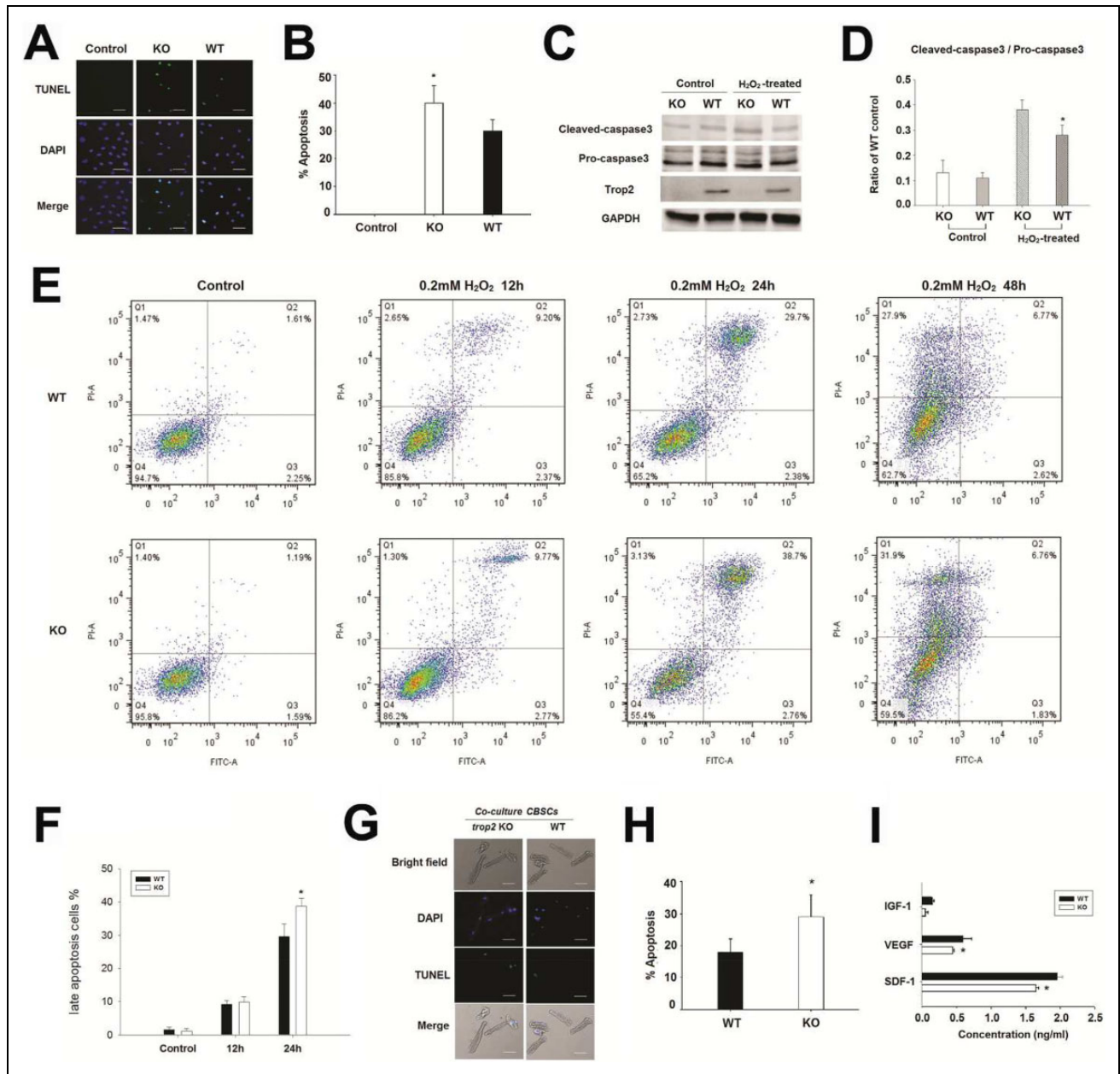


Fig. 5. Trop2 inhibits CBSCs apoptosis and promotes cardiomyocytes survival in a paracrine manner. (A and B) The representative TUNEL images (A) and quantified apoptosis (B) of WT CBSCs and Trop2-KO CBSCs in response to H₂O₂ exposure at the dose of 200 μ M for 24 h. Cells treated with PBS served as controls. TUNEL-positive cells were visualized by green, and nuclei were stained by DAPI (blue). Scale bar: 50 μ m. **P* < 0.05 compared with the Trop2-KO CBSC group. (C and D) Representative western blot showing the protein expression of cleaved-caspase 3 and pro-caspase 3 (C) and the quantified ratio of cleaved-caspase 3 to pro-caspase 3 of WT CBSCs and Trop2-KO CBSCs after 12 h of H₂O₂ administration. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the loading control. **P* < 0.05 compared with the Trop2-KO CBSC group. (E and F) Flow cytometry (E) and quantification (F) showing the ratio of ANNEXIN V/PI-labeled positive CBSCs with Trop2 KO or not after H₂O₂ challenge for 12, 24, and 48 h. **P* < 0.05 compared with the Trop2-KO CBSC/24 h group. (G and H) Primary cardiomyocytes isolated from C57BL/6 J mice and co-cultured with WT CBSCs or Trop2-KO CBSCs for 24 h followed by hypoxia for 2 h. Representative TUNEL staining image (G) and quantification of apoptotic ratio (H) of cells. Scale bar: 50 μ m. **P* < 0.05 compared with the WT CBSC group. (I) The concentrations of pro-angiogenesis IGF-1, VEGF and SDF-1 in the medium of culture system in (G). **P* < 0.05 compared with the WT CBSC group. *n* = 3 independent experiments.

Trop2 deficiency significantly exacerbated the ratio of TUNEL-positive cells (Fig. 5A, B). In line with the increased TUNEL cells, CBSCs with Trop2 deficiency

exhibited higher expression of pro-apoptotic protein cleaved-caspase3 and the ratio of cleaved-caspase3 to pro-caspase3 after H₂O₂ stimulation for 12 h (Fig. 5C, D).

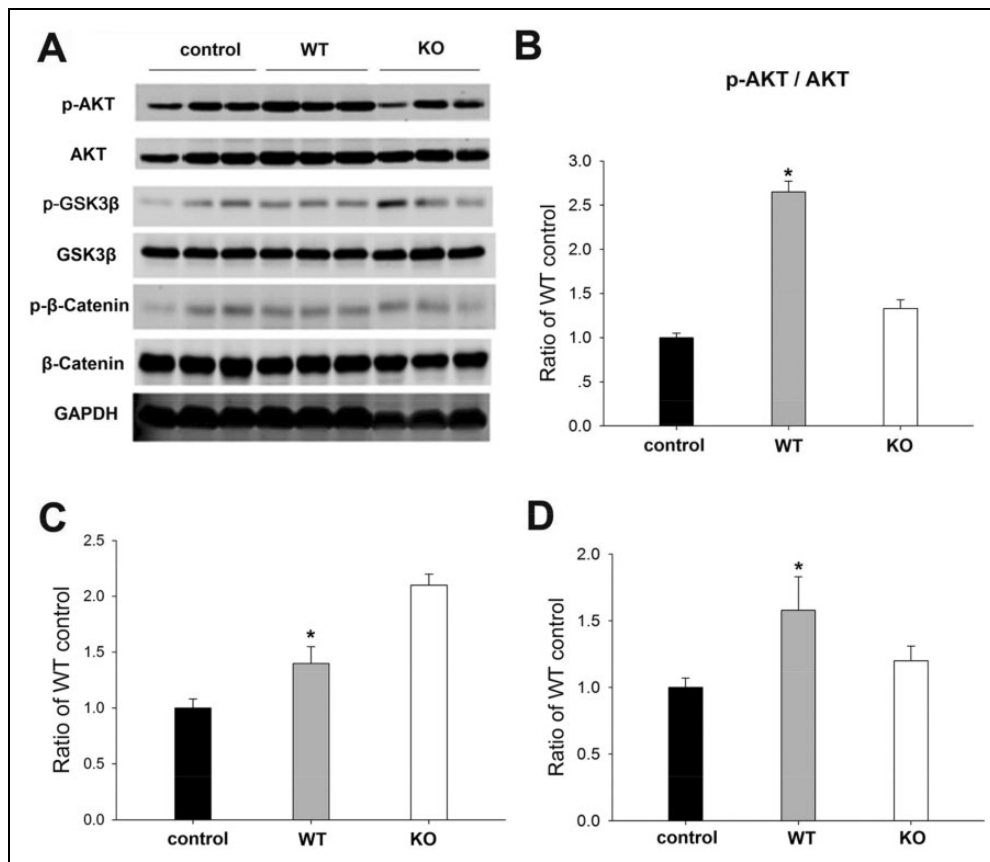


Fig. 6. Trop2 inhibits CBSCs apoptosis by activating AKT/GSK3 β / β -Catenin pathway. (A) Representative western blot showing the phosphorylated and total expression of AKT, GSK3 β and β -Catenin in WT CBSCs and Trop2 KO CBSCs after H₂O₂ treatment for 12 h. PBS served as a control. GAPDH is the loading control. (B–D) The quantification of p-AKT to total AKT ratio (B), p-GSK3 β to total GSK3 β (C), and p- β -Catenin to total β -Catenin (D). **P* < 0.05 compared with the Trop2-KO CBSC group. *n* = 3 independent experiments.

Furthermore, flow cytometry assay indicated the percentage of ANNEXIN V and PI-double labeled cells were significantly and gradually increased in Trop2-KO CBSCs in response to H₂O₂ treatment, especially at 24 h after H₂O₂ treatment (29.7% in WT group vs. 38.7% in Trop2-KO group in 24 h) (Fig. 5E, F). These data indicated that Trop2 is a pivotal controller for CBSCs survival and thus guarantees the protective effect of CBSCs on myocardial I/R-induced heart damage.

The direct influence of CBSCs Trop2 on cardiomyocytes was further determined, in which isolated mice primary cardiomyocytes were co-cultured with CBSCs with Trop2 deficiency or not followed by hypoxia treatment for 2 h. TUNEL staining showed that the apoptotic ratio of cardiomyocytes was significantly higher in the group that co-cultured with Trop2 deficiency CBSCs than that in the WT-CBSC co-culture group (Fig. 5G, H). Furthermore, the concentrations of IGF-1, VEGF and SDF-1 in the medium of co-culture system was decreased by Trop2 deletion in CBSCs (Fig. 5I). Collectively, Trop2 in CBSCs is required for CBSC-rendered protective effects on cardiomyocyte survival *via* promoting the secretion of growth factors.

Trop2 Inhibits CBSCs Apoptosis by Activating AKT/GSK3 β / β -Catenin Pathway

For CBSC survival and self-renewal, AKT signaling has been reported as a key transduction pathway³⁹. More importantly, Trop2 holds a potent capacity to activate AKT signaling in tumor cell proliferation⁴⁰. Thus, to explore the regulatory mechanisms underlying the anti-apoptotic effect of Trop2 on CBSCs, we examined the activation of AKT signaling by detecting the phosphorylated and total levels of AKT, GSK3 β and β -Catenin. As shown in Fig. 6, the expression levels of phosphorylated AKT, GSK3 β and β -Catenin consistently increased in WT-CBSCs after H₂O₂ exposure compared with un-treated control group. However, the activation of AKT/GSK3 β / β -Catenin signaling was significantly reduced when Trop2 was absent (Fig. 6). These findings clearly showed that the anti-apoptotic function of CBSCs was largely mediated by Trop2-mediated activation of AKT/GSK3 β / β -Catenin signaling.

Discussion

Stem cell treatment is a promising strategy for post-infarction injury of the heart, especially for MI or myocardial

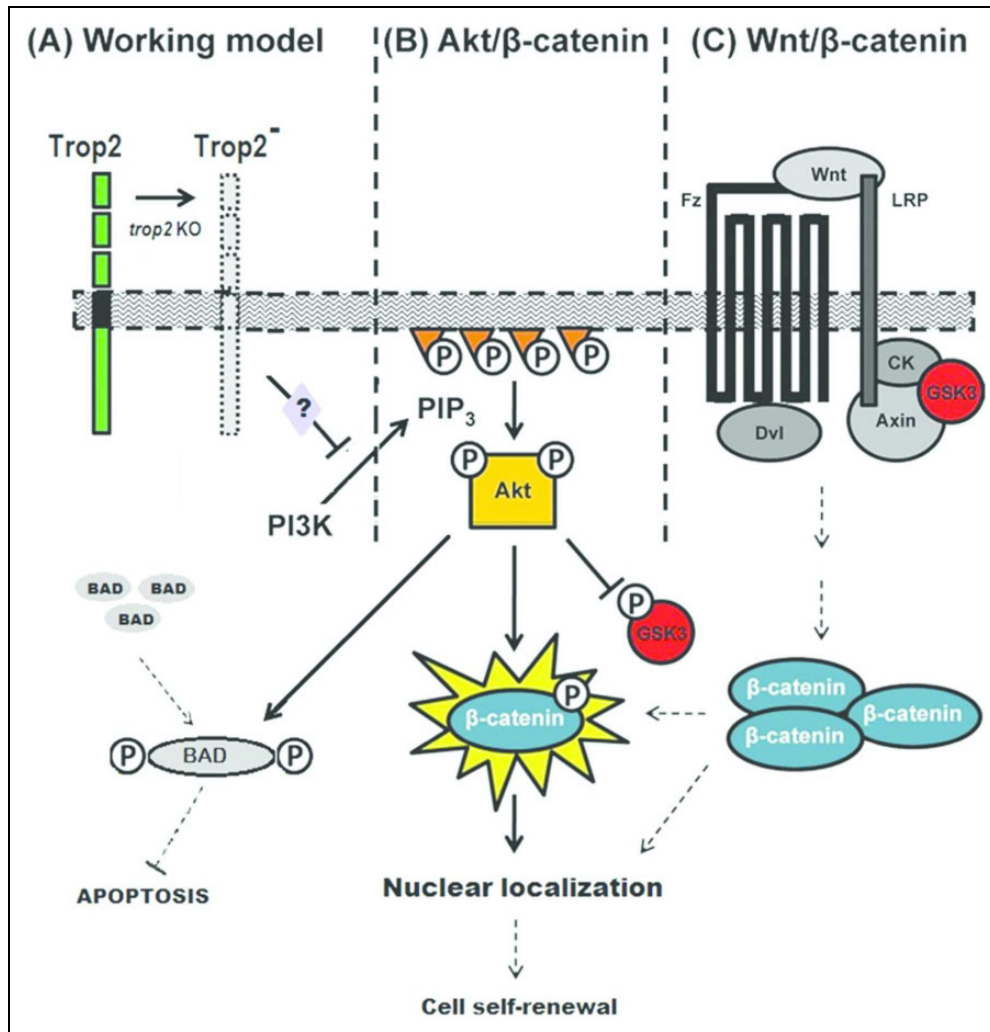


Fig. 7. Schematic figures showing the underlying mechanism of the essential role of Trop2 in CBSCs-mediated cardioprotective effect during myocardial I/R injury via an AKT/GSK3 β / β -Catenin-dependent manner.

I/R insult^{11,12}. Recent studies have emphasized that CBSCs possess greater protective effects on self-renewal than other types of stem cell, more superior even than that of c-kit-positive cardiac stem cells¹⁹. However, the detailed mechanisms of CBSCs physiological function remain largely unknown. In this study, we found that Trop2 is a key factor playing an essential role in CBSC-rendered protective effects on myocardial I/R injury by diminishing LV systolic dysfunction, promoting angiogenesis, and inhibiting cardiomyocyte apoptosis. The underlying cellular mechanisms of Trop2 functions have been demonstrated as maintaining CBSCs renewal and secreting pro-growth factors to promote cardiomyocytes survival in a paracrine manner, while activating AKT/GSK3 β / β -Catenin signaling largely contributes to the Trop2-induced potentially protective capacity of CBSCs on cardiomyocyte damage and heart injury after myocardial I/R surgery. Thus, this study provides new understandings on the mechanisms of CBSCs function on MI and I/R injury.

More importantly, our findings demonstrate that upregulation Trop2 expression is a promising approach for cardiac stem cell therapeutic strategies for I/R-induced heart damage in clinic.

Healing following myocardial I/R is a complex process that is fine-tuned by neovascularization, cell proliferation, and cardiomyocytes death^{41,42}. Angiogenesis is an important ischemia-driven cellular event that represents a generation of new capillaries from existing microvasculature to mediate cell survival, and thus is intimately associated with prognosis following MI^{43,44}. It has been shown that CBSCs possess a stronger capacity to secrete proangiogenic factors and to promote neovascularization more than any other cardiac stem cells^{19,45}. In line with previous studies, we observed a significant increase of proangiogenic factors VEGF and FGF-2 production in CBSCs-treated mice when compared with the PBS-administrated controls. These findings suggest that the pro-angiogenesis ability of CBSCs might contribute

greatly to their cardioprotective function. However, the underlying mechanism of CBSCs-regulated angiogenesis remains elusive. In addition, we found that Trop2 in CBSCs dramatically promoted angiogenesis in infarcted heart tissues. Consistently, maintaining Trop2 in CBSCs kept its potent function to inhibit cardiomyocyte apoptosis and thus ameliorate heart damage. Paracrine-induced neovascularization and cell survival promote the endogenous repair mechanism for the functional regulation of Trop2 on myocardial I/R treatment. Thus, our findings provide the first evidence supporting the fundamental role of Trop2 in pro-angiogenesis and inhibiting cell death in myocardial I/R development.

The AKT signaling is a key regulator of hypoxia and ischemia-mediated genes, *e.g.* VEGF, and activation and phosphorylation of AKT signaling are benefit for improving cardiac function post-MI or myocardial I/R injury through promoting angiogenesis and blunting cardiomyocyte apoptosis^{46,47}. AKT undergoes phosphorylation and subsequently phosphorylates GSK3 β leading to its inactivation. The phosphorylation of AKT/GSK3 can further promote β -Catenin phosphorylation and translocation into the nuclear where β -Catenin binds to transcriptional coactivators and induces the expression of genes related to angiogenesis and proliferation, including cyclin D1, DKK, VEGF, and YAP^{48,49}. In the absence of Trop2, the activation of AKT signaling in CBSCs was largely abolished, and, consequently, the renewal capacity of CBSCs as well as its promoting effect on cardiomyocyte survival and angiogenesis was greatly compromised (Fig. 7). These data indicate that Trop2 guarantees the cardioprotective effects of CBSCs, dependent on its activation in AKT signaling.

In closing, this study provides the first evidence that Trop2 is required for CBSCs-rendered cardioprotective function on myocardial I/R injury by promoting angiogenesis and inhibiting cardiomyocyte apoptosis through the AKT/GSK3 β / β -Catenin cascade. Targeting Trop2 and maintaining its expression represent promising strategies for improving the therapeutic capacity of stem cells on myocardial I/R and other heart-related diseases.

Author Contributions

Tianyu Li and Yunshu Su contributed equally to this work. Jianye Yang conceived and designed all the experiments. Tianyu Li and Yunshu Su performed the experiments. Xiongli Yu and Durgahee S.A. Mounir analyzed the data and made the interpretation. Jianye Yang, Xiang Wei, and Jackson Ferdinand Masau drafted and revised the article. All authors read and approved the final version.

Ethics Approval

All animal procedures used in this study were approved by the Ethics Committee of Animal Use for Teaching and Research, Tongji Medical College of Huazhong University of Science (Wuhan, China).

Statement of Human and Animal Rights

The animals received humane treatment according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences and the National Institutes of Health.

Statement of Informed Consent

Statement of Informed Consent is not applicable for this article.

Availability of Data and Materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grants from National Natural Science Foundation of China (No. 81400226)

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