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Bone marrow mesenchymal stem cells transport connexin43 via tunneling nanotubes to alleviate isopreterenol-induced myocardial hypertrophy

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Abstract

Background Paracrine signaling plays an important role in stem cell therapy. However, it alone cannot fully explain the therapeutic mechanisms of stem cell therapy in treating heart diseases. Recently, tunneling nanotubes (TNTs)—a novel type of long-distance intercellular connectional structure—have been identified between mesenchymal stem cells (MSCs) and cardiomyocytes (CMs). TNTs mediate the transmission of multiple signaling molecules, enabling cells to exert different biological functions. In the present study, we investigated the role of TNTs in MSC-based therapy for myocardial hypertrophy.

Methods MSCs And CMs were co Cultured for 24 h with or without isopreterenol (ISO) to induce myocardial hypertrophy. Confocal microscopy was used to quantify and analyze the number, morphology, composition, and cell source of TNTs between MSCs and CMs. the effects of ISO on CMs were assessed by comparing cell area (measured by confocal microscopy) and expression levels of hypertrophy Related genes (using qRT PCR) under co Culture and trans Well culture conditions. Flow cytometry was employed to assess the transfer of connexin43 (Cx43) from MSCs to CMs; lentivirus Mediated Cx43 overexpression and Cx43 siRNA were used to investigate the effects of Cx43 on ISO Induced myocardial hypertrophy.

Results ISO stimulation significantly increased the number, length, and thickness of TNTs between MSCs and CMs (Number: $P < 0.05$; length and thickness: $P < 0.01$). ISO also increased the proportion of TNTs containing microtubules and those derived from MSCs ($P < 0.05$). Co-culture conditions were more effective than trans-well culture in alleviating ISO-induced myocardial hypertrophy ($P < 0.05$). Furthermore, Cx43 was observed in TNTs, and ISO enhanced the transfer of Cx43-mCherry from MSCs to co-cultured CMs ($P < 0.05$). Overexpression of Cx43 in CMs alleviated myocardial hypertrophy, whereas knocking down of Cx43 in MSCs reduced their ability to alleviate myocardial hypertrophy ($P < 0.05$).

Conclusions Our results demonstrate that ISO promotes the formation of TNTs, particularly between MSCs and CMs, and induces changes in the morphology of TNTs (thickening and lengthening). Additionally, MSCs transmitted Cx43

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to CMs via TNTs, which contributes to the alleviation of ISO-induced myocardial hypertrophy. These results suggest that TNTs represent an important mechanism in MSC-mediated therapy for myocardial hypertrophy.

Keywords Mesenchymal stem cells, Cardiomyocytes, Tunneling nanotubes, Connexin43, Myocardial hypertrophy

Background

Pathological myocardial hypertrophy is a major risk factor for heart failure, sudden cardiac death, and various cardiovascular diseases such as arrhythmia and myocardial infarction [1–3]. Both basic and clinical studies show that stem cells have great potential for treating these heart diseases [4–8]. Identifying the mechanism underlying stem cell actions is crucial for promoting their therapeutic applications.

Tunneling nanotubes (TNTs) are long-distance cell junctions first reported in 2004 by Rustom et al. between rat pheochromocytoma PC12 cells [9]. Since then, TNTs have been found in various tissues and cell types [10–12]. They serve not only as connecting structures but also as novel intercellular junctions that allow cells to exchange small molecules and organelles, such as mitochondria, endosomes, and lysosomes, to perform different biological functions [13–16]. In 2014, a study found that TNTs can form between distressed cardiomyocytes and stem cells, demonstrating that damaged cardiomyocytes can promote the paracrine function of stem cells through TNTs [17]. In agreement, our previous study revealed that stem cells can transport fully functional mitochondria to damaged cardiomyocytes through TNTs, thereby reducing myocardial cell apoptosis [18]. It is becoming clear that TNTs may represent a new pathway for stem cell therapy in heart disease, alongside paracrine signaling and self-differentiation. Exploring the mechanism underlying TNT action could significantly advance the clinical applications of stem cells.

The intercellular hydrophilic channels between adjacent cells, formed by connexin 43 (Cx43), are the main conduits for calcium signal transmission between cardiomyocytes. They play an important role in maintaining normal heart function, including the coordination of heart contractions and relaxation [19, 20]. However, increased degradation and/or redistribution of Cx43 has been associated with several cardiac disorders such as myocardial ischemia, hypertrophy, arrhythmia, and heart failure [21–23]. Cx43 is also important in studies of TNTs as it is closely associated with the formation of intercellular TNTs [24, 25] and affects their biological functions by promoting mitochondrial transport [26].

In this study, we observed Cx43 in TNTs between mesenchymal stem cells (MSCs) and cardiomyocytes (CMs). We further demonstrated that MSCs can transmit Cx43 to CMs through TNTs, thereby alleviating isopreterenol (ISO)-induced cardiomyocyte hypertrophy.

Methods

Isolation and culture of cardiomyocytes (CMs)

Neonatal rat CMs were cultured as described previously [18]. According to the American Veterinary Association, the sensory nerves of newborn rats begin to develop 5–7 days after birth, and the nervous system of newborn rats is still immature at 2–3 days of age, with strong pain tolerance and no need for anesthesia. Therefore, in this study, 2–3-day old newborn rats were soaked in 75% alcohol for 5–10 s before conducting experiments. Briefly, the hearts of 2–3-day old Sprague Dawley (SD) Rats (provided by Beijing Huafukang BioScience Company, Beijing, China) were rapidly removed. Ventricles were finely cut and digested in 0.2% collagenase type II (Gibco, Waltham, Massachusetts, USA) at 37 °C for eight min, and the isolated cells were neutralized in cell culture medium. Cardiac tissues were digested until they disappeared. All suspensions were pelleted by centrifugation at 1000 rpm for five min and resuspended in Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F-12, Gibco, Waltham, Massachusetts, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin solution (100 U/mL). Cells were plated in 10-cm dishes and transferred to an incubator at 37 °C with an atmosphere containing 5% CO_2. After allowing two hours for fibroblast adherence, the CMs was seeded onto culture plates for subsequent treatments. Animal euthanasia was not used in this study, because removing the hearts of 2–3-day old newborn rats would cause circulatory and respiratory failure, leading to death. Euthanasia is not suitable for this situation. All animal experimental procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH, revised 1996) and with the approval of Anzhen Hospital (approval no. AZ2023LA014), which is affiliated with the Capital Medical University. This work was reported in accordance with the ARRIVE Guidelines 2.0.

Trans-well analysis and co-culturing of MSCs with CMs

Bone marrow-derived MSCs, derived from SD rats were purchased from Procell (Wuhan, China) and cultured in DMEM/F-12 containing 10% FBS at 37 °C

with an atmosphere containing 5% CO_2. To observe the role of TNTs in alleviating isopreterenol (ISO)-induced myocardial hypertrophy in MSCs, Trans-well culture and co-culture modes of MSCs and CMs were established in vitro, as previously described with some modifications [18]. Briefly, the Trans-well culture model consisted of indirectly culturing MSCs and CMs in a 2:1 ratio using a semipermeable membrane as a Trans-well insert (pore size, 0.4 μm; Corning, USA), which prevented the formation of TNTs between the two kinds of cells but allowed the diffusion of secreted factors. The co-culture model involved directly mixing and culturing MSCs and CMs in the same dish in a 2:1 ratio. All cells were maintained in DMEM/F-12.

Transmission electron microscopy

Co-cultured cells were grown in 35 mm dishes. After 24 h, cells were fixed by a fixative liquid (3% paraformaldehyde, 1.5% glutaraldehyde) for 3 h at room temperature (RT). Then the cells were washed three times with PBS and dehydrated in an ascending gradual series of ethanol (50%, 70%, 90%, 95%, and 100%) for 2 min each. After embedded in Epon812 resin, 70-nm-thick ultrathin sections were cut using a diamond knife, and collected on uncoated 200-mesh copper grids. The sections were stained with uranyl acetate, and observed by transmission electron microscopy (Japan, Hitachi HT 7800).

Immunoelectron microscopy

Cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde for 3 h. After washed with 0.1% PBS five times, cells were treated with 0.01% osmium acid. With 1% uranyl acetate staining and gradient alcohol dehydration, cells were embedded with Epon812 resin and sliced into sections with a thickness of 70–80 nm (Germany, Leica EM uc7).

The formvar-coated nickel grids with sections were blocked in 1%BSA contain 0.05% Tween20 for 5 min. Then the sections were incubated in the Cx43 antibody (Cell Signaling Technology, Boston, MA, USA) for 1 h at RT. After blocked again for 3 min, the sections were washed six times with 0.1% PBS. Incubated in the secondary antibody (goat anti-rabbit conjugated with 12 nm gold) (MINXHU, MS, Rat Sr Prot) for 45 min at RT, the sections were washed with 0.1% PBS and ddH_2O six and four times respectively. After stained with 3% uranyl acetate for 3 min, the sectioned were dried in the RT and examined in a transmission electron microscopy (Japan, Hitachi HT 7800).

Immunofluorescence staining analysis

MSCs and CMs were fixed in 4% paraformaldehyde for 20 min. After incubation, cells were penetrated with 0.2% Triton X-100, blocked with 5% BSA in PBS, and then stained with primary antibodies, α-tubulin (Abcam, Cambridge, UK), cardiac troponin T (Abcam) or Cx43 (Cell Signaling Technology) at 4 °C overnight, followed by Alexa Fluor 546/633 secondary antibodies. TRITC Phalloidin (Yeasen, China) and WGA Alexa Fluor® 488/555 conjugates (Invitrogen, Carlsbad, CA, USA) were incubated with the cells for 30 min at room temperature for F-actin or cell membrane staining. For living cell membrane staining, CellTracker™ Green CMFDA (Invitrogen) was incubated with the cells for 45 min under cell growth condition. Cells were imaged with a laser scanning confocal microscope equipped with multiple excitation lasers (405, 488, 561, and 633 nm).

Flow cytometry

Following labeling with CellTracker™ Green CMFDA for 45 min at 37℃ in serum-free DMEM/F-12, CMs were co-cultured with MSCs for 24 h. After stimulation with ISO (10 μM for 24 h), CMs were sorted from other co-cultured cells according to CellTracker™ Green CMFDA immunostaining. Sorted cells were used to detect the expression levels of myocardial hypertrophy markers.

Quantitative real-time PCR (qRT-PCR)

After 24 h of ISO (10 μM) treatment, total RNA was extracted from the sorted CMs using TRIZOL reagent, and reverse transcription reactions were performed using HiScript II Q RT SuperMix for qPCR (Vazyme, NanJing, China). QRT-PCR was performed on a PCR detection system using ChamQ Universal SYBR qPCR Master Mix (Vazyme). PCR conditions were 95 °C for 30 s, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s, 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. The expression levels of the target genes were normalized against GAPDH mRNA levels. Primer sequences are summarized in Table 1.

Table 1 Primers used for qRT-PCR

Primer	Sequence
SD-Rat-BNP-F	5′- CAGAAGCTGCTGGAGCTGATAAG –3′
SD-Rat-BNP-R	5′- TGTAGGGCCTTGGTCCTTTG –3′
SD-Rat-P-MHC-F	5′- GCGGACATTGCCGAGTCCCAG –3′
SD-Rat-P-MHC-R	5′- GCTCCAGGTCTCAGGGCTTCACA –3′
SD-Rat-GAPDH-F	5′- GCACCGTCAAGCTGAGAAC –3′
SD-Rat-GAPDH-R	5′-TGGTGAAGACGCCAGTGGA –3′

Lentiviral infection

To overexpress Cx43, Lentiviral-mCherry-Cx43 (LV-mCherry-Cx43) was constructed using the lentiviral vector pSLenti-CMV-mCherry (LV-mCherry). The Cx43 coding sequence was inserted between the EcoRI and XbaI genes of the vector, and mCherry was fused in frame to the carboxyl terminus of Cx43 with the addition of a ten-amino-acid polylinker (GGGSGGGGGS).

After infecting MSCs and CMs with LV-mCherry-Cx43 (100 MOI) for eight hours in serum-free medium containing 5 μg/mL polybrene, we replaced the serum-containing medium and continued to culture for 5–7 days. Control cells were infected with an empty vector (LV-mCherry, 80 MOI).

Transfection of siRNAs

We used rat small interfering RNA (siRNA) targeting Cx43 to silence its gene expression. A scramble was used as the non-targeting control. The sequences for the Cx43 siRNA and siRNA control were as follows: Cx43 siRNA#1-F: 5′-GCUUCUGGACAAGGUCCAAGCTT-3′, Cx43 siRNA#1-R: 5′-GCUUGGACCUUGUCCAGA AGCTT-3′; Cx43 siRNA#2-F: 5′-GGAAGGAAGAGA AGCUAAACATT-3′, Cx43 siRNA#2-R: 5′-UGUUUA GCUUCUCUUCCUUCCTT-3′; siRNA control-F: 5′-UUCUCCGAACGUGUCACGUTT-3′, siRNA control-R: 5′-ACGUGACACGUUCGGAGAATT-3′.

Transfection of MSCs with Cx43 siRNA#1 and #2 involved diluting the siRNA with DMEM/F-12 without serum to a final concentration of 100 nM, and incubating the cells with GP-transfect-Mate reagent (Genepharma, Shanghai, China) and the siRNA solution for four hours. The cells were further cultured in complete culture medium for three days before conducting subsequent experiments.

Statistical analyses

Group values are presented as means ± standard error of the mean (SEM). An unpaired two-tailed t-test or one-way analysis of variance (ANOVA) was applied to determine the significance of differences between two or more groups of parametric data, respectively. Statistical analyses were performed using GraphPad Prism software version 6.01 (GraphPad Software Inc., San Diego, CA, USA), and differences were considered significant at $P < 0.05$.

Results

Effect of ISO on the formation and morphology of TNTs between bone marrow (BM)-MSCs and neonatal rat CMs

Previously, we discovered that TNTs can form between CMs and MSCs [27]. In this study, we aimed to determine the biological functions of these TNTs in alleviating MSC-induced CM hypertrophy. First, we used a confocal microscope to observe and quantitatively analyze the effect of ISO, a compound that induces myocardial hypertrophy, on TNT formation between the two cell types. Our results revealed that TNTs were present, and their numbers significantly increased after 24 h of co-culture with ISO stimulation (Fig. 1A, B), indicating that ISO promoted the formation of intercellular TNTs.

Next, we quantified the length and thickness of 92 TNTs in the ISO-treated group and 118 TNTs in the without ISO (control) group. Both measurements significantly increased in the ISO group: length (38.39 ± 1.79 μm vs 26.83 ± 1.52 μm) and thickness (2.19 ± 0.07 μm vs 1.68 ± 0.07 μm) (Fig. 1C, D, E).

ISO stimulation significantly increases TNTs derived from MSCs

We further investigated the effect of ISO on the cellular origin of TNTs. Confocal microscopy was employed to observe TNTs derived from CellTracker™ Green/WGA555-labeled CMs or WGA 555-labeled MSCs (Fig. 2A). Almost 47% of the 94 TNTs were derived from MSCs, and this proportion significantly increased to about 60% of the 118 TNTs in the with ISO group (Fig. 2B). However, we did not observe any TNTs that originated from both types of cells simultaneously. These results suggest that ISO promoted the formation of TNTs from MSCs to CMs.

Effect of ISO on the cytoskeleton in TNTs

Consistent with previous reports, both F-actin and microtubules were observed in the TNTs between MSCs and CMs [18]. F-actin and microtubules are essential for the formation and function of TNTs [9, 18], with microtubules also influencing the morphology of these structures [18]. To further investigate the effects of ISO on the cytoskeleton in TNTs, we examined the presence of F-actin and microtubules. F-actin was observed in all TNTs regardless of ISO stimulation (Fig. 3A). Hollow tubular structures—Microtubules, were observed in TNTs by transmission electron microscopy (Fig. 3B). However, microtubules were present in only some TNTs, and ISO significantly increased the proportion of TNTs that contained microtubules (Fig. 3C, D).

Potential role of TNTs in MSC-mediated alleviation of ISO-induced myocardial hypertrophy

ISO can induce myocardial hypertrophy, and paracrine secretion is an important mechanism by which stem cell therapy addresses this pathological condition [4, 28]. As a novel form of intercellular "communication," TNTs mediate the transfer of multiple signaling molecules and functional proteins between cells

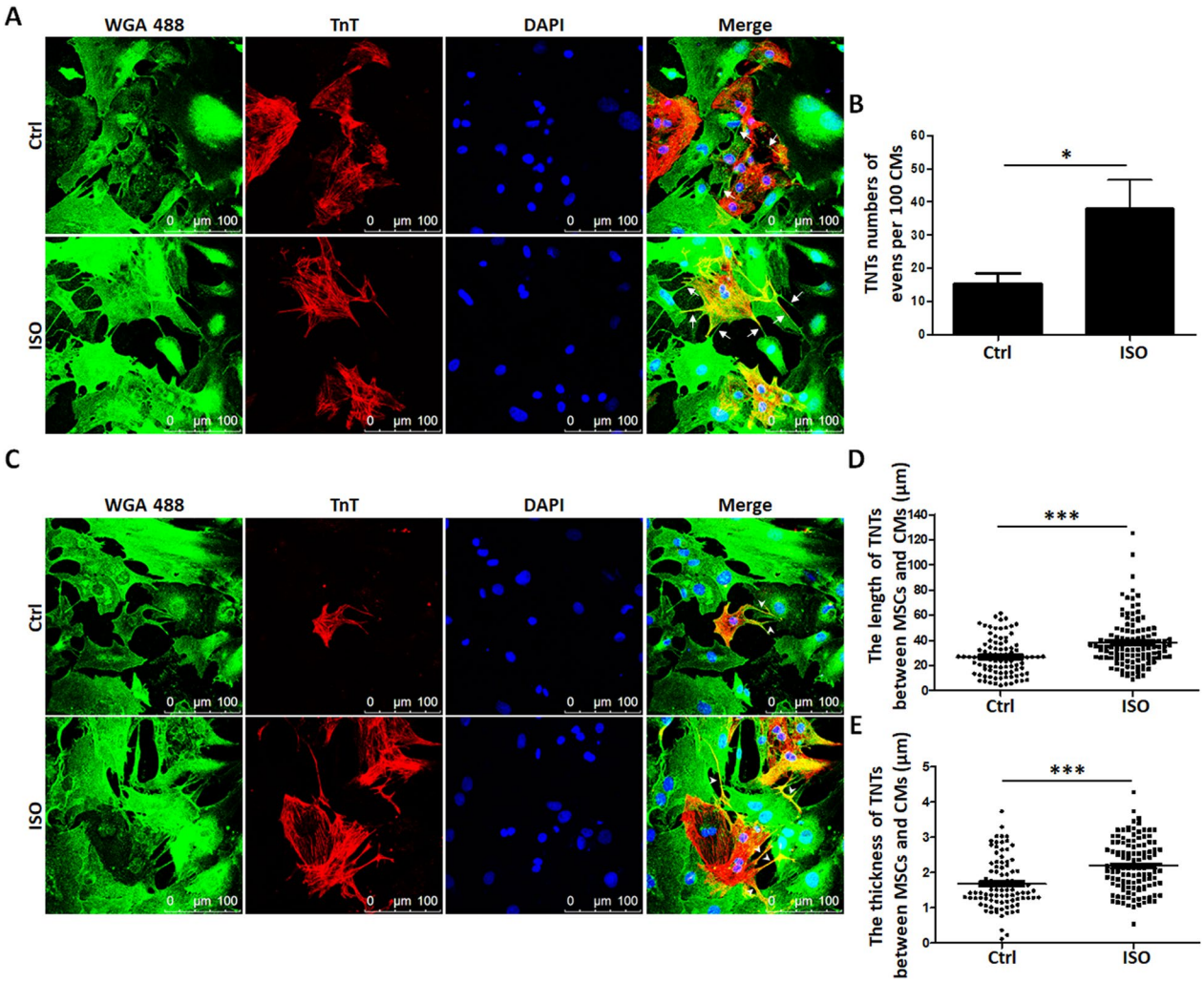

Fig. 1 Quantitative analysis of the effect of ISO on the formation and morphology of TNTs. Co-cultured MSCs and CMs were treated with ISO for 24 h, Cell membranes were labeled with WGA488 (green), and CMs were labeled by CM-specific marker protein Troponin T (TnT) (red). (**A**, **B**) TNTs (indicated by arrow heads) were observed under a 63 lens of a microscope, and TNT numbers per 100 CMs were evaluated by quantifying 10 randomly selected fields. The number of replicates: N = 4. (**C**, **D**, **E**) Quantifying the length and thickness of 95 and 112 TNTs (indicated by arrow tips) between 24 h–co-cultured cells with or without ISO treatment, respectively. N = 4, scale bar: 100 μm. Data are shown as the mean ± S.E.M. *P < 0.05 and ***P < 0.001 vs Ctrl

[29], potentially providing a new pathway for MSCs to alleviate ISO-induced myocardial hypertrophy. To verify this hypothesis, we quantified the cellular area of CMs when co-cultured with MSCs under ISO stimulation, using both Trans-well and direct co-culture systems. Our results showed that the area of ISO-treated CMs decreased when cells were trans-well cultured with MSCs, and the anti-hypertrophic effect further enhanced under co-culture conditions (Fig. 4A, B). We also analyzed the expression level of brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC), both indicators of myocardial hypertrophy, using quantitative RT-PCR (qRT-PCR). We obtained CellTracker™ Green CMFDA-labeled CMs, which co-cultured with MSCs, through flow cytometry sorting (Fig. 4C). The results showed that MSCs in trans-well culture inhibited the ISO-induced increase in BNP and β-MHC mRNA expression, with further reduction observed under co-culture conditions (Fig. 4D, E). These results indicate that, in addition to paracrine secretion, MSCs may alleviate ISO-induced myocardial hypertrophy through TNTs. The list of primers used in qRT-PCR is shown in Table 1.

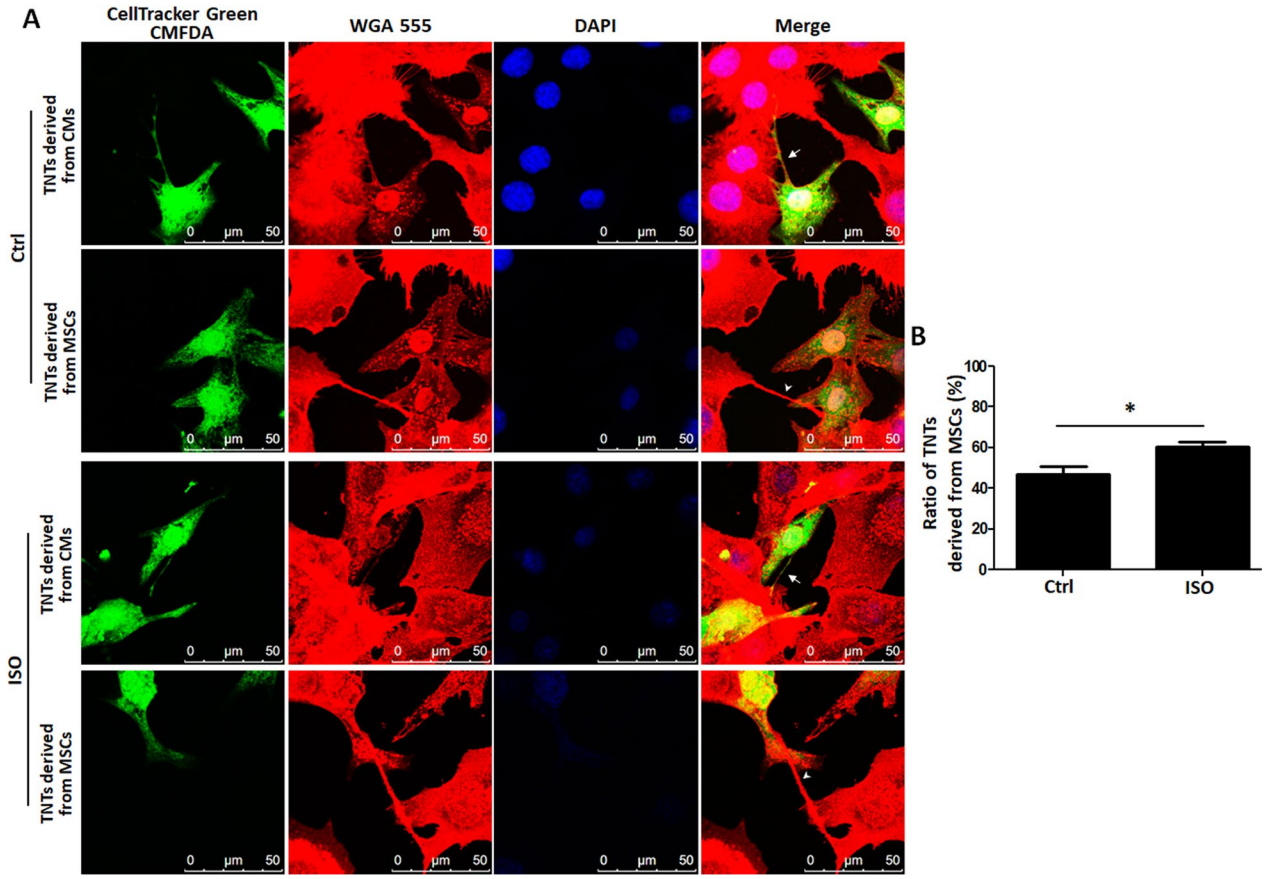

Fig. 2 Quantitative evaluation of the effect of ISO on the cellular source of TNTs. (**A**) CellTracker Green CMFDA-labeled CMs (green) and MSCs were co-cultured for 24 h, followed by WGA555 (red). TNTs derived from CMs or MSCs, which were indicated by arrow heads and arrow tips, respectively, were checked under a 40 × lens. (**B**) The ratio of different cellular originations of TNTs was quantified by randomly observing 94 and 118 TNTs with or without ISO. N = 4, scale bar: 50 μm. Data are shown as the mean ± S.E.M. *P < 0.05 vs Ctrl

ISO facilitates TNT-mediated Cx43 transfer from MSCs to CMs

How do TNTs promote MSC functions in inhibiting myocardial hypertrophy? Previous clinical and scientific studies have shown that Cx43 is closely associated with the occurrence and progression of myocardial hypertrophy [22, 27, 30]. In addition, Cx43 has been detected in various intercellular TNTs [25, 26, 31]. Based on these findings, we hypothesized that MSCs alleviate myocardial hypertrophy by transferring Cx43 via TNTs. To test this hypothesis, we investigated whether TNTs mediated the transfer of Cx43 from MSCs to CMs. Using immunofluorescence, we labeled Cx43 with red fluorescence and observed it under a confocal microscope. We detected Cx43 in TNTs using immunoelectron microscopy (Fig. 5A), and the proportion of TNTs containing Cx43 significantly increased following ISO stimulation (Fig. 5B, C). Additionally, we constructed a lentivirus (LV)-mCherry-Cx43 and expressed it in MSCs. By dynamically tracking the movement of Cx43, we observed the transfer of mCherry-Cx43 in TNTs (Supplementary movie 1). After co-culturing LV-mCherry-Cx43-expressing MSCs with CellTracker™ Green CMFDA-labeled CMs for 24 h, we detected mCherry-Cx43 in some CMs via flow cytometry (Fig. 6A). This proportion increased significantly with ISO stimulation (Fig. 6B). However, no mCherry-Cx43-positive CMs were detected in the Trans-well culture system. These results suggest that Cx43 is transferred from MSCs to CMs via TNTs, and ISO promoted this process. Similar to the characteristics of endogenous Cox43, we also observed aggregation of Cx43-mCherry at cell–cell junctions. Additionally, the presence of Cx43-mCherry in TNTs was verified (Supplementary Fig. 1).

TNTs inhibit ISO-induced myocardial hypertrophy by transmitting Cx43

To define the role of Cx43 in ISO-induced myocardial hypertrophy, we overexpressed LV-mCherry-Cx43

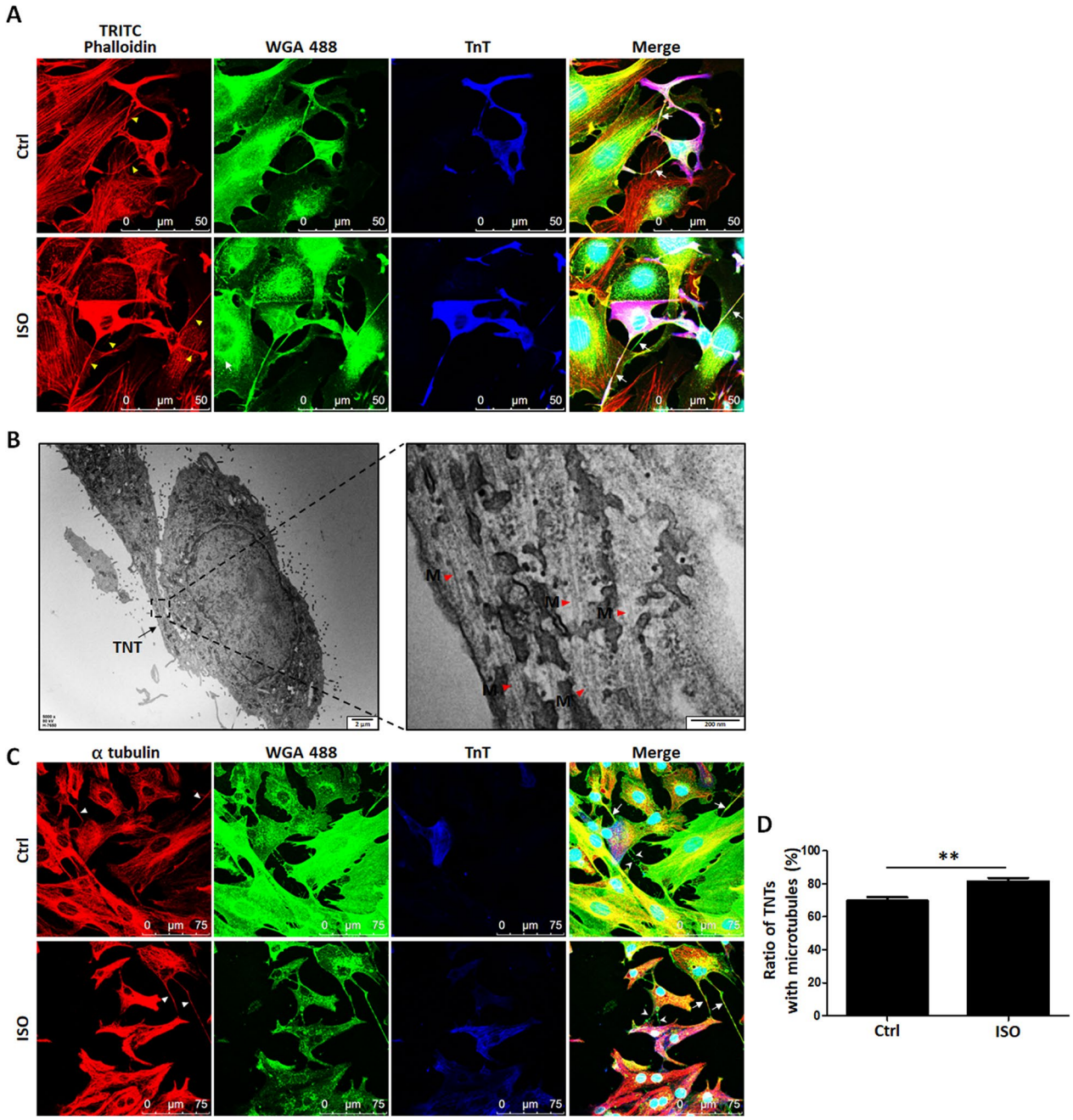

Fig. 3 The effect of ISO on the cytoskeleton in TNTs. 24 h-co-cultured cells were stimulated by ISO, followed labelled by WGA 488 (green) and TnTs (blue). (**A**) F-actin were labeled with TRITC Phalloidin (red), 175 TNTs were randomly observed under a 40 × lens. Arrows heads indicate TNTs containing F-actin, and yellow triangles indicate F-actin in TNTs. N = 4, Scale bar: 50 μm. (**B**) Representative transmission electron micrograph of microtubules in TNTs. Red triangles marked the microtubules in TNTs between the cells. Microtubule, M. scale bar: 2 μm and 200 nm. (**C**) Microtubules were stained by α-tubulin antibodies (red). TNTs with (+) or without (-) microtubules were indicated by arrows heads and arrow tips, respectively. White triangles indicate microtubules in TNTs. (**D**) Randomly observe 132 TNTs, and quantitative percentage of TNTs containing microtubules. N = 5, Scale bar: 75 μm. Data are shown as the mean ± S.E.M.** $0.001 < P < 0.05$

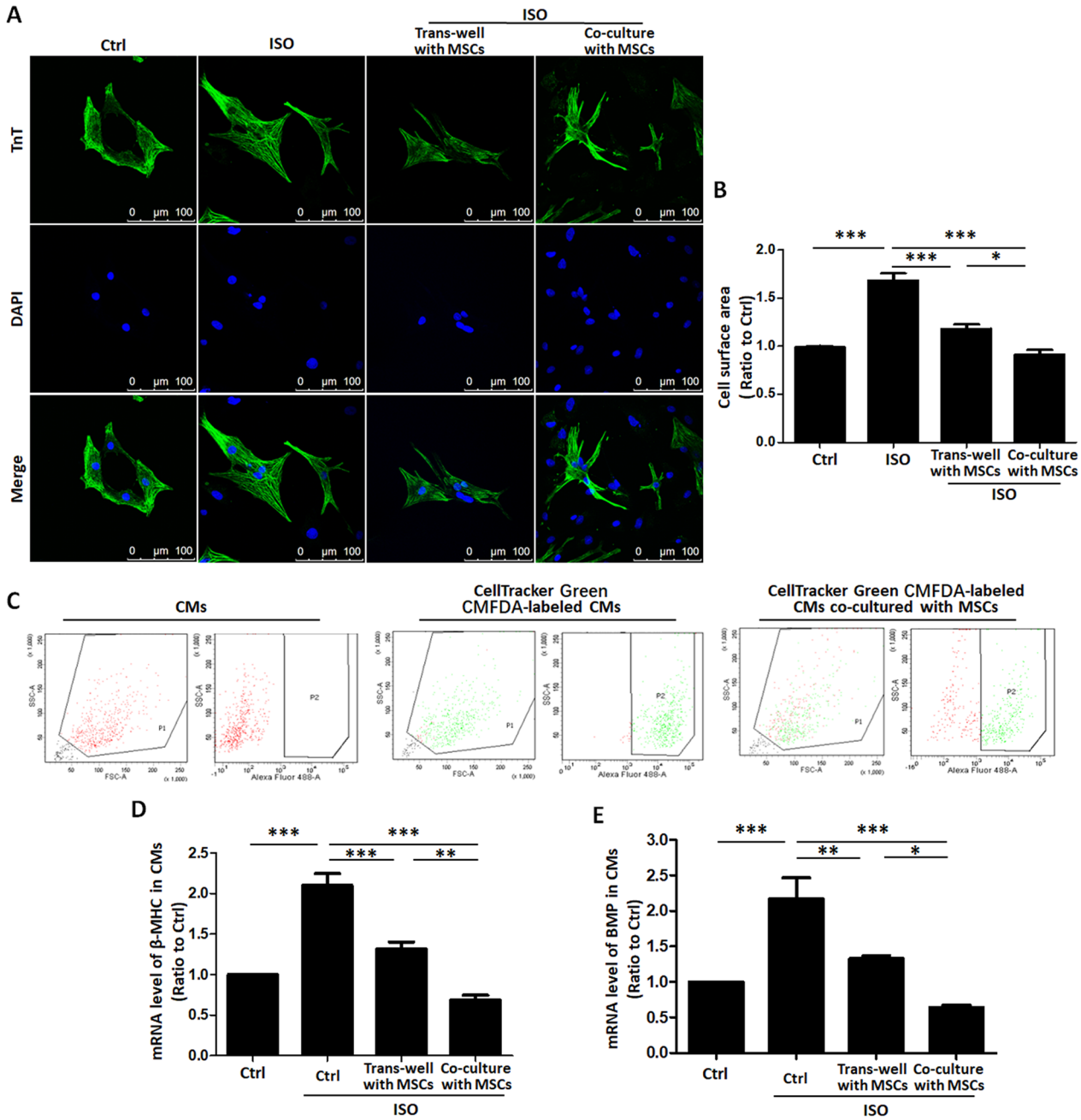

Fig. 4 Compare the suppressing effects of MSCs on ISO induced myocardial hypertrophy under different culture conditions. (**A**, **B**) The images represent immunostaining with TnT (Green), and the average cell surface area were quantified by detecting CMs in 10 randomly selected fields in four separate experiments. Scale bar: 100 μm. (**C**, **D**) Sorting CellTracker Green CMFDA-labeled CMs (Green) in co-culture system by flow cytometry, and the mRNA level of hypertrophic genes BNP, and β-MHC in CMs were analyzed by qPCR. N = 4, Data are shown as the mean ± S.E.M. * $P < 0.05$, ** $0.001 < P < 0.05$ and *** $P < 0.001$ vs Ctrl

in CMs and quantitatively analyzed the area of Cx43-positive CMs under ISO stimulation. The results showed that upregulating Cx43 expression significantly alleviated ISO-induced myocardial hypertrophy (Fig. 7A, B).

Building on previous results that TNTs may provide a novel pathway for MSCs to alleviate ISO-induced myocardial hypertrophy (Fig. 4), we next examined the effect of Cx43 depletion on TNT-mediated MSC activation. We transfected MSCs with a specific siRNA

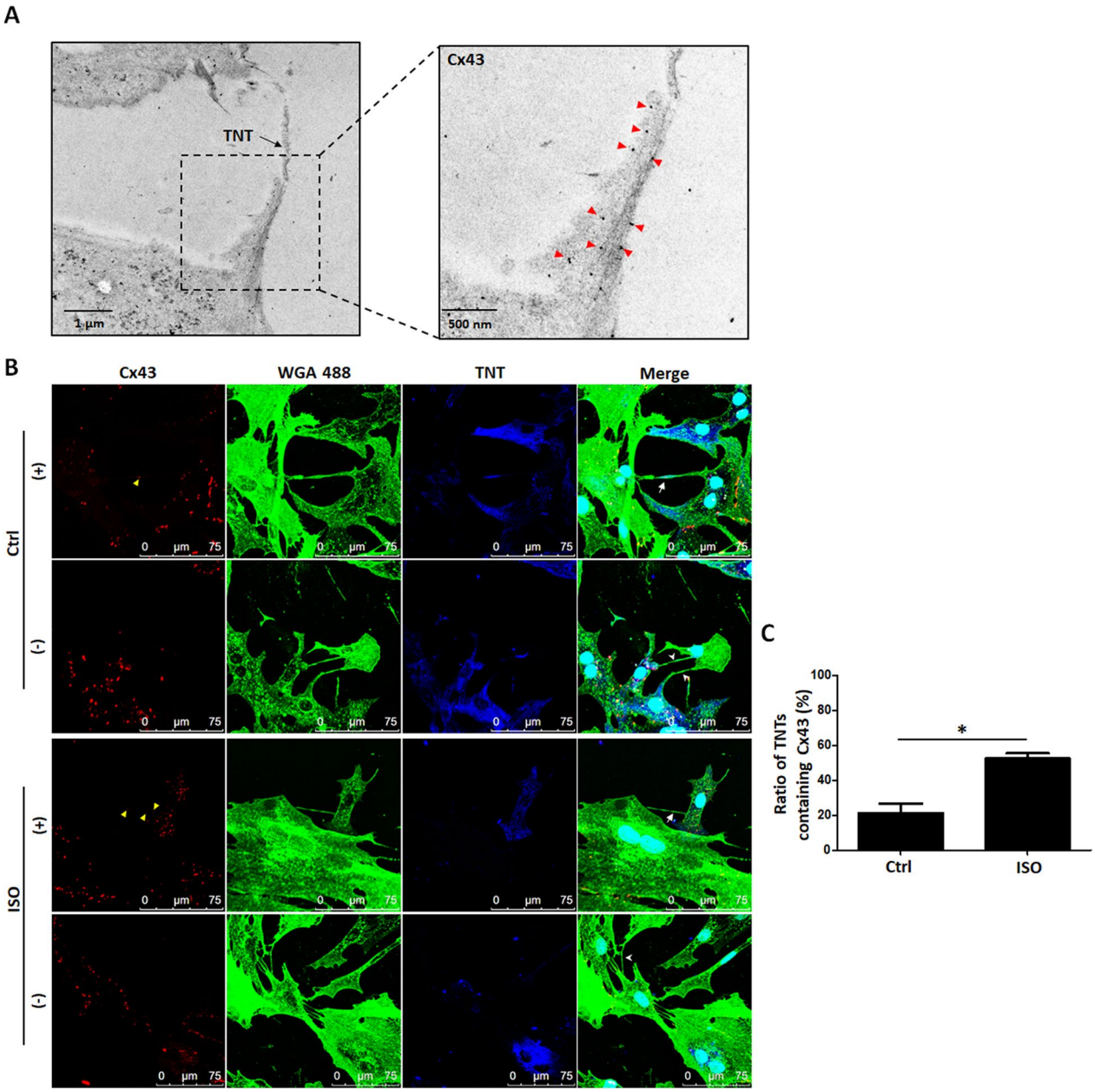

Fig. 5 Effect of ISO on the localization of Cx43 in TNTs. (A) Immunogold electron micrograph of Cx43 in TNTs. Red triangles marked Cx43 dots were enriched in TNTs between the cells. scale bar: 1 μm and 500 nm. (B) Co-cultured MSCs and CMs were triple labeled with WGA, Cx43, and TnT. Regardless of whether there was ISO, some TNTs contained Cx43 (indicated by arrow heads), and yellow triangles indicate Cx43 in TNTs. TNTs without Cx43 were indicated by arrow tips. N = 4, cale bar: 75 μm. (C) Randomly observe 125 TNTs and quantify the percentage of TNTs containing Cx43 with and without ISO stimulation. N = 4, Data are shown as the mean ± S.E.M. * $P < 0.05$ vs Ctrl

targeting Cx43 and co-cultured them with CMs for 24 h. Cx43 silencing strongly impaired the ability of MSCs to alleviate myocardial hypertrophy under co-culture conditions (Fig. 8B, C). The efficiency of siRNA-induced Cx43 silencing was detected by western blot analysis (Fig. 8A). Additionally, depletion of Cx43 did not affect the ability of ISO to promote TNT formation (Supplementary Figs. 2A, B). These data suggest that MSCs may alleviate ISO-induced myocardial hypertrophy by transmitting Cx43 to CMs via TNTs.

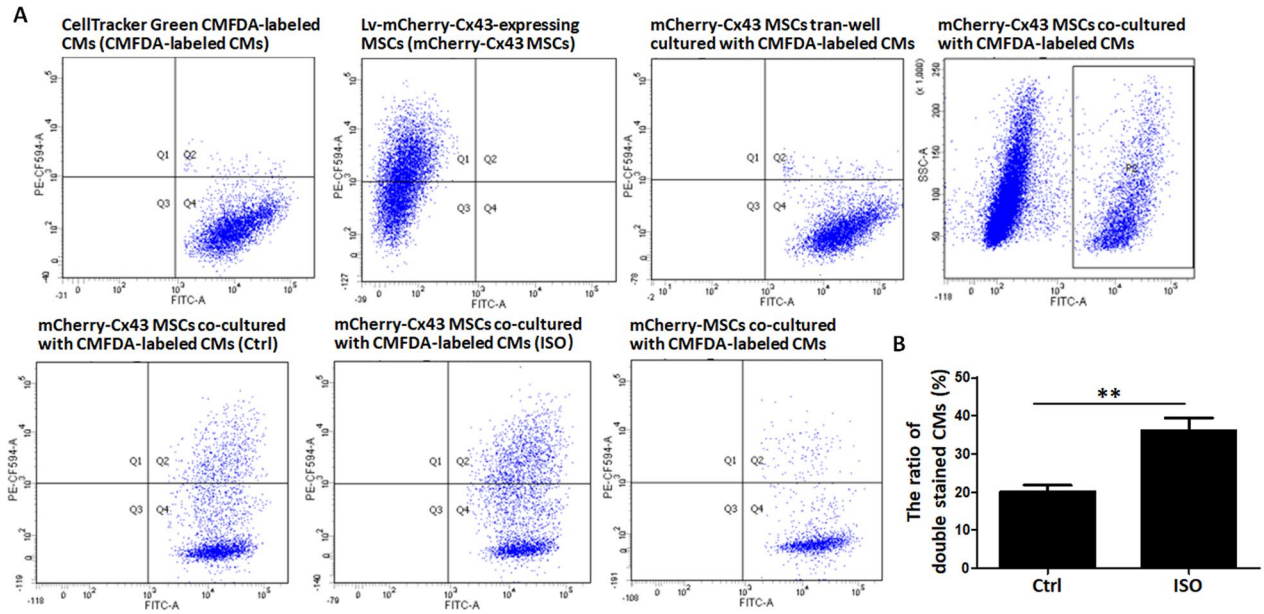

Fig. 6 Effect of ISO on the transfer of Cx43 from MSCs to CMs. (**A**) Transfecting Lentivirus (LV)-mCherry-Cx43 into MSCs (red) for 7 days, and then co-culturing with CMFDA-labeled CMs (green) for 24 h. CMs (green) containing LV-mCherry-Cx43 (red) were detected by flow cytometry regardless ISO treatment. N = 3. (**B**) Comparison of the percentage of CMFDA-labeled CMs (green) containing Lv-Cx43-mCherry(red) in all CMFDA-labeled CMs (green) under co-culture conditions with and without ISO stimulation. N = 3, Data are shown as the mean ± S.E.M. * P < 0.05 vs Ctrl

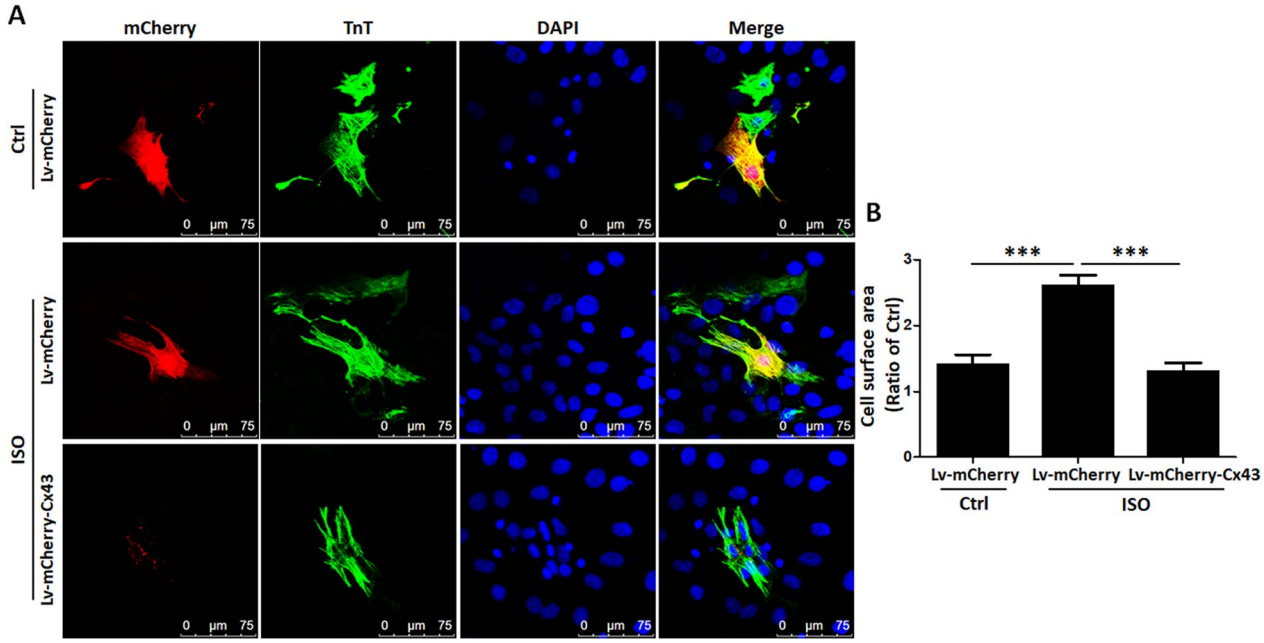

Fig. 7 Effect of overexpression of Cx43 on ISO induced myocardial hypertrophy. (**A**) Transfecting LV-mCherry or LV-mCherry-Cx43 (Red) into CMs for 5 days, the image shows transfected CMs stained with TnT (Green). N = 6 Scale bar: 75 μm. (**B**) Quantitative comparison of the area of transfected CMs. N = 6, Data are shown as the mean ± S.E.M. ***P < 0.001 vs Ctrl

Discussion

Five decades after the discovery of MSCs, these cells have become leading candidates for cell-based therapies [32]. Currently, MSCs are widely used to treat various heart diseases, including myocardial infarction, myocardial hypertrophy, and dilated cardiomyopathy [32–34].

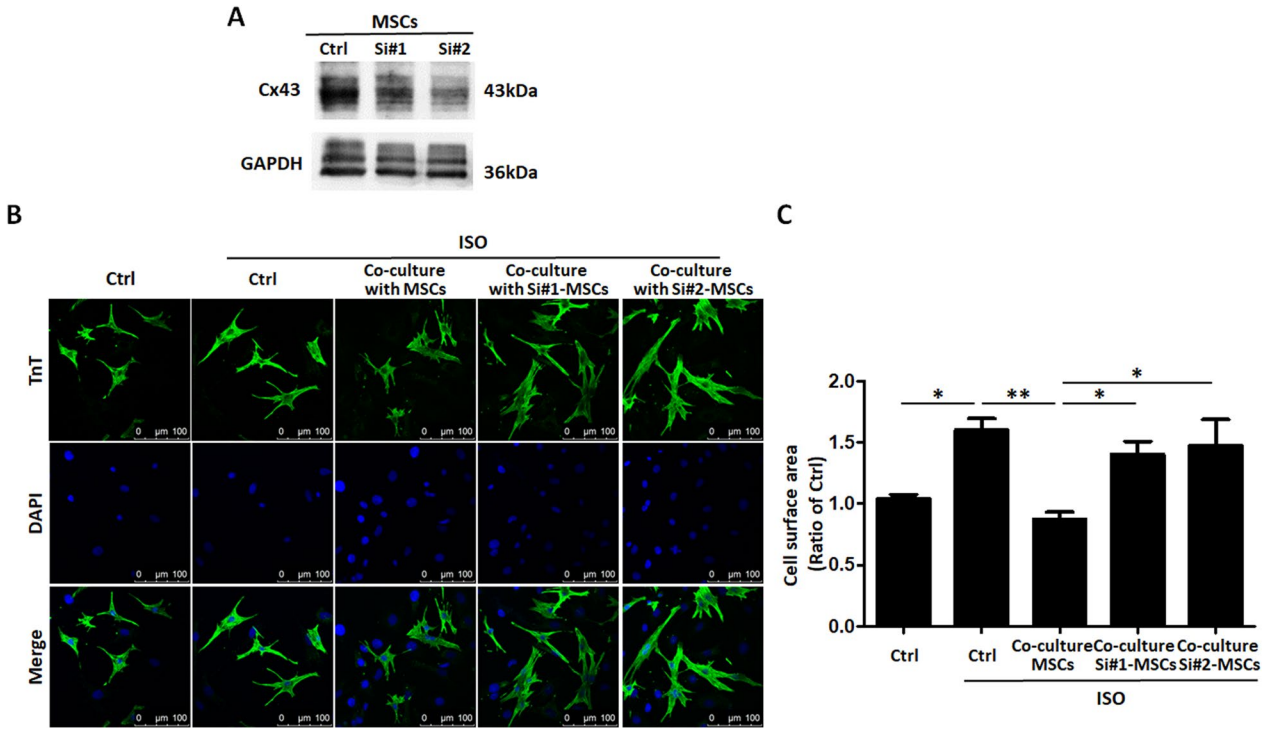

Fig. 8 Effect of knocking down Cx43 on MSCs to alleviate myocardial hypertrophy function. (**A**) Detecting the knock down efficiency of Cx43 SiRNA#1 and #2 (Si#1 and Si#2) in MSCs by western blot (WB). N = 3. (**B**) MSCs, pretreated with SiRNA control or Cx43 SiRNA, co-cultured with CMs for 24 h. CMs were labeled by TnT (Green). N = 4, Scale bar: 100 μm.(**C**) Quantifying the average surface area of CMs in 10 randomly selected fields. N = 4, Data are shown as the mean ± S.E.M. * $P < 0.05$ vs Ctrl, ** $0.001 < P < 0.05$

Significant progress has been made in understanding the mechanisms through which MSCs exert their therapeutic effects on cardiovascular diseases, but it mainly focuses on self-differentiation and paracrine signaling pathways [35]. Despite increasing interest and extensive research, many questions remain regarding the underlying biological mechanisms of MSCs.

TNTs are long-distance filamentous junctional structures that connect various types of cells, such as T cells, B cells, nerve cells (neurons), and cancer cells. These structures can mediate the transport of signaling molecules and organelles, thereby exerting certain biological effects [36–39]. Both our research group and Rodriguez et al. have reported that TNTs form between MSCs and damaged CMs[18, 31]. Furthermore, our research group revealed that MSCs can transport fully functional mitochondria along microtubules to damaged CMs through TNTs, thereby alleviating myocardial cell apoptosis [17, 18]. This observation indicates that TNTs may serve as a novel pathway by which MSCs contribute to the treatment of heart diseases.

Pathological myocardial hypertrophy, induced by stimuli such as hypertension and valvular disease, is a leading risk factor for heart failure, sudden cardiac death, and triggers various cardiovascular conditions such as arrhythmia and myocardial infarction [1, 2, 40]. The role of stem cells in treating myocardial hypertrophy has received extensive attention and considerable in-depth research has been devoted to understanding their mechanisms of action in this context [41, 42]. In this study, we investigated whether TNTs play an important role in alleviating myocardial hypertrophy in MSCs.

To clarify this, we investigated the effects of ISO, a β-receptor agonist that is widely used to induce myocardial hypertrophy [43, 44], on the quantity and morphology of intercellular TNTs. Our quantitative analysis showed that ISO stimulation significantly increased the number of TNTs formed between the cells (MSCs or CMs). Furthermore, a significant elongation and thickening of TNTs was also noticed. We also found that ISO significantly upregulated the ratio of TNTs originating from MSCs, suggesting that ISO enhances the ability of MSCs to influence CM function via TNTs. While the exact mechanism by which ISO regulates the formation and extension of TNTs remains unclear, recent studies have suggested that the F-actin cytoskeleton is essential for TNT formation in virtually all cell types [39, 45]. Several well-characterized proteins that modulate

cellular actin dynamics play key roles in TNT formation, such as the Arp2/3 complex, small GTPases, Cdc42, and Msec [46–50]. Moreover, ISO-induced activation of β−2 adrenergic receptors affects actin cytoskeleton organization [51], which may in turn affect TNT formation and its extension. We propose that future studies should explore the influence of ISO on actin dynamics and, subsequently, TNT formation.

Considering that ISO induces myocardial hypertrophy, whereas MSCs can treat it, we further explored whether TNTs are involved in MSC-mediated protection against myocardial hypertrophy. By analyzing the effects of ISO on CM area under different cultivation conditions, we found that direct co-culture with MSCs, as compared to transwell cultures, more effectively alleviated ISO-induced myocardial hypertrophy. This observation suggests that in addition to paracrine secretion, TNTs play an important role in the ability of MSCs to treat myocardial hypertrophy. This conclusion was further confirmed by qRT PCR analysis, which showed that MSC co-culture reduced the expression of BNP and β-MHC, two well-established markers of myocardial hypertrophy in CMs.

Cx43 has been implicated in the pathogenesis of myocardial hypertrophy. Studies have shown that the Cx43 expression is significantly reduced in ISO-induced hypertrophic CMs, and that maintaining or upregulating Cx43 levels can alleviate myocardial hypertrophy and improve cardiac function [22, 52]. Additionally, in patients with myocardial hypertrophy, Cx43 expression in the myocardium is significantly decreased [30]. These findings highlight the close association between Cx43 and the occurrence and development of myocardial hypertrophy. Cx43 is also present in TNTs, including those formed by pluripotent stem cell (iPSC)-derived MSCs, epithelial cells, and even cancer cells [25, 26, 53]. We observed the presence of Cx43 in some TNTs between MSCs and CMs and found that ISO significantly increased the proportion of TNTs containing Cx43. Using flow cytometry, we further demonstrated that MSCs could transmit Cx43 to CMs via TNTs, and this process was facilitated by ISO. These results suggest that ISO promotes Cx43 transmission from MSCs to CMs via TNTs. The cytoskeleton, motor proteins, and autophagy are known to be involved in cargo transport in TNTs [54, 55]. However, the precise mechanism by which ISO promotes Cx43 transmission remains unclear and warrants further investigation.

To further demonstrate the role of TNT-mediated Cx43 in MSC-mediated alleviation of myocardial hypertrophy, we depleted Cx43 in MSCs using siRNA. The results showed that depletion of Cx43 significantly impaired the ability of MSCs to reduce myocardial hypertrophy under co-culture conditions. Furthermore, we confirmed that overexpression of Cx43 in CMs inhibited ISO-induced myocardial hypertrophy. While these findings support the hypothesis that Cx43 plays a critical role in MSC-mediated protection against hypertrophy, several questions remain unanswered: how does TNT-mediated Cx43 transfer occur? How is Cx43 internalized by CMs? What specific mechanisms through which Cx43 alleviates myocardial hypertrophy remain to be clarified? Cx43 is an important protein that forms gap junctions between CMs, facilitating intracellular communication and calcium signaling. The localization and expression of Cx43 are critical for proper calcium signal transmission between CMS. Therefore, it is important to explore whether TNT-mediated Cx43 transfer affects calcium signaling in the heart, as this could provide important insights into how Cx43 contributes to the alleviation of myocardial hypertrophy. In addition, it has been reported that ISO-induced typical hypertrophic characteristics of cardiomyocytes were prevented after MSCs transplantation in vivo, and the structure of TNTs could also be found in heart tissue [15, 56]. These findings suggest that the vitro work of this study may be applied to the vivo situation and the efficacy of MSCs therapy may be able to enhance by promoting TNTs mediated Cx43 transport, which need to be further confirmed.

Conclusion

ISO stimulation caused significant changes in the quantity, morphology, composition, and origin of TNTs formed between MSCs and CMs. MSCs use TNTs to transport Cx43 to CMs, which plays a key role in alleviating ISO-induced myocardial hypertrophy. Our study suggests that, in addition to self-differentiation and paracrine secretion, TNTs provide a novel pathway for MSCs to treat pathological myocardial hypertrophy. Future investigations should focus on uncovering the molecular mechanisms underlying TNT formation, cargo transport, and the role of Cx43 in calcium signaling and myocardial remodeling, as well as validating the results of vitro experiments in the vivo level. These lines of investigations will be essential for optimizing the therapeutic potential of TNTs in regenerative cardiovascular medicine and enhancing the effectiveness of MSC-based therapies for myocardial hypertrophy and other cardiac disorders.

Abbreviations

TNTs	Tunneling nanotubes
MSCs	Mesenchymal stem cells
CMs	Cardiomyocytes
ISO	Isopreterenol
Cx43	Connexin43
LV-Cx43- mCherry	Lentiviral-Connexin 43-mCherry
LV- mCherry	PSLenti-CMV-mCherry

BNP	Brain natriuretic peptide
β-MHC	β-Myosin heavy chain
TnT	Troponin T

Supplementary Information

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Additional file 1

Additional file 2

Additional file 3

Additional file 4

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Author contributions
JZ, SL, LZ performed all experiments. ZX, YL, WL and CS participated in the data collection, data analysis. JZ and SC supervised the study and edited the manuscript. HJ and SC acquired the funding. All authors have read and approved the current version of the manuscript.

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Availability of data and materials
The authors confirm that all data generated or analyzed during this study are included in this published article and its supplementary file.

Declarations

Ethics approval and consent to participate
All animal experimental procedures were performed in adherence with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH, revised 1996) and with the approval of the Capital medical university affiliated Anzhen hospital (Approval No. AZ2023LA014, Data of Approval: February 11, 2023). And the title of the approved project is "The role of Tunneling Nanotubes between mesenchymal stem cells and cardiomyocytes in alleviating isoproterenol induced cardiac hypertrophy". This study did not involve human subjects and human cells.

Consent for publication
All authors and institutions have confirmed this manuscript for publication.

Competing interests
The authors declare that they have no competing interests.

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