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Corresponding Author:	Wuqiang Zhu University of Alabama at Birmingham College of Arts and Sciences Birmingham, AL UNITED STATES
Corresponding Author's Institution:	University of Alabama at Birmingham College of Arts and Sciences
Corresponding Author E-Mail:	wzhu@uab.edu
Order of Authors:	Wuqiang Zhu
	Meng Zhao
	Yawen Tang
	Patrick J. Ernst
	Asher Kahn-Krell
	Chengming Fan
	Danielle Pretorius
	Hanxi Zhu
	Xi Lou
	Lufang Zhou
	Jianyi Zhang
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TITLE:

Enhancing the Engraftment of Human Induced Pluripotent Stem Cell-derived Cardiomyocytes via a Transient Inhibition of Rho Kinase Activity

AUTHORS AND AFFILIATIONS:

Meng Zhao[1], Yawen Tang[1], Patrick J. Ernst[1,2], Asher Kahn-Krell[1], Chengming Fan[1,3], Danielle Pretorius[1], Hanxi Zhu[1], Xi Lou[1], Lufang Zhou[2], Jianyi Zhang[1], Wuqiang Zhu[1]

[1]Department of Biomedical Engineering, School of Medicine, School of Engineering, University of Alabama at Birmingham, Birmingham, AL, USA

[2]Division of Cardiovascular Diseases, School of Medicine, School of Engineering, University of Alabama at Birmingham, Birmingham, AL, USA

[3]Department of Cardiac Surgery, Xiangya Medical University, Changsha City, Hunan Province, China

Corresponding authors:

Wuqiang Zhu (wzhu@uab.edu)

Jianyi Zhang (jayzhang@uab.edu)

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SUMMARY:

In this protocol, we demonstrate and elaborate on how to use human induced pluripotent stem cells for cardiomyocyte differentiation and purification, and further, on how to improve its transplantation efficiency with Rho-associated protein kinase inhibitor pretreatment in a mouse myocardial infarction model.

ABSTRACT:

A crucial factor in improving cellular therapy effectiveness for myocardial regeneration is to safely and efficiently increase the cell engraftment rate. Y-27632 is a highly potent inhibitor of Rho-associated, coiled-coil-containing protein kinase (RhoA/ROCK) and is used to prevent dissociation-induced cell apoptosis (anoikis). We demonstrate that Y-27632 pretreatment for human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs^{+RI}) prior to implantation results in a cell engraftment rate improvement in a mouse model of acute myocardial infarction (MI). Here, we describe a complete procedure of hiPSC-CMs differentiation, purification, and cell pretreatment with Y-27632, as well as the resulting cell contraction, calcium transient measurements, and transplantation into mouse MI models. The proposed method provides a simple, safe, effective, and low-cost method which significantly increases the cell engraftment rate. This method cannot only be used in conjunction with other methods to further enhance the cell transplantation efficiency but also provides a favorable basis for the study of the mechanisms of other cardiac diseases.

INTRODUCTION:

Stem cell-based therapies have shown considerable potential as a treatment for cardiac damage caused by MI[1]. The use of differentiated hiPSCs provides an inexhaustible source of hiPSC-CMs[2] and opens the door for the rapid development of breakthrough treatments. However, many limitations to therapeutic translation remain, including the challenge of the severely low engraftment rate of implanted cells.

Dissociating cells with trypsin initiates anoikis[3], which is only accelerated once these cells are injected into harsh environments like the ischemic myocardium, where the hypoxic environment accelerates the course toward cell death. Of the remaining cells, a large proportion is washed out from the implantation site into the bloodstream and spread throughout the periphery. One of the key apoptotic pathways is the RhoA/ROCK pathway[4]. Based on previous research, the RhoA/ROCK pathway regulates the actin cytoskeletal organization[5,6], which is responsible for cell dysfunction[7,8]. The ROCK inhibitor Y-27632 is widely used during somatic and stem cell dissociation and passaging, to increase cell adhesion and reduce cell apoptosis[9–11]. In this study, Y-27632 is used to treat hiPSC-CMs prior to transplantation in an attempt to increase the cell engraftment rate.

Several methods aimed at improving the cell engraftment rate, such as heat shock and basement membrane matrix coating[12], have been established. Aside from these methods, genetic technology can also promote cardiomyocyte proliferation[13] or reverse nonmyocardial cells into cardiomyocytes[14]. From the bioengineering perspective, cardiomyocytes are seeded onto a biomaterial scaffold to improve the transplantation efficiency[15]. Unfortunately, the majority of these methods are complicated and costly. On the contrary, the method proposed here is simple, cost-efficient, and effective, and it can be used as a basal treatment before transplantation, as well as in conjugation with other technologies.

PROTOCOL:

All animal procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham and were based on the National Institutes of Health Laboratory Animal Care and Use Guidelines (NIH Publication No 85-23).

1. Preparation of culture media and culture plates

1.1. Medium preparation

1.1.1. For hiPSC medium, mix 400 mL of human pluripotent stem cell (hPSC) basal medium (**Table of Materials 1**) and 100 mL of hPSC 5x supplement; store the mixture at 4 °C.

1.1.2. For RPMI 1640/B27 minus insulin (RB-) medium, mix 500 mL of RPMI 1640 and 10 mL of B27 supplement minus insulin.

1.1.3. For RPMI 1640/B27 (RB+) medium, mix 500 mL of RPMI 1640, 10 mL of B27 supplement, and 5 mL of penicillin-streptomycin (pen-strep) antibiotic.

1.1.4. For purification medium[16], mix 500 mL of no-glucose RPMI 1640, 10 mL of B27 supplement, 5 mL of pen-strep antibiotic, and 100 µL of sodium DL-lactate solution (at a final concentration of 4 mM).

1.1.5. For neutralizing solution, mix 200 mL of RPMI 1640, 50 mL of fetal bovine serum (FBS), 2.5 mL of pen-strep antibiotic, and 50 µL of Y-27632 (at a final concentration of 10 µM).

1.1.6. For freezing medium, mix 9 mL of FBS, 1 mL of dimethyl sulfoxide (DMSO), and 10 µL of Y-27632 (at a final concentration of 10 µM).

1.1.7. For extracellular matrix solution (EMS), thaw concentrated extracellular matrix (**Table of Materials 1**) at 4 °C until it has reached an evenly consistent liquid state. Precool pipette tips and tubes prior to making matrix aliquots of 350 µL each on ice, and store the aliquots at -20 °C. Before coating the plates, dilute one thawed aliquot into 24 mL of ice-cold DMEM/F12 medium (EMS); keep the EMS in 4 °C for up to 2 weeks.

NOTE: Perform all coating steps on ice to prevent basement membrane matrix solidification.

1.1.8. For Tyrode's solution, take 90% of the final required volume of tissue culture grade water, add the appropriate amount of the following reagents, and stir until dissolved. Then, use 1 N NaOH to adjust the pH to 7.4. Add extra water to a final concentration of 140 mmol/L sodium chloride, 1 mmol/L magnesium chloride, 10 mmol/L HEPES, 5 mmol/L potassium chloride, 10 mmol/L glucose, and 1.8 mmol/L calcium chloride. Sterilize by filtration, using a 0.22 µm membrane.

1.2. Cell culture plate coating

1.2.1. For hiPSC culture plates, apply 1 mL of EMS to each well of a 6-well plate and incubate the plate for at least 1 h at 37 °C. Aspirate DMEM/F12 solution before seeding cells.

1.2.2. For gelatin coating, dissolve 0.2 g of gelatin powder in tissue culture grade water to make a 0.2% (w/v) solution. Sterilize by autoclaving at 121 °C, 15 psi for 30 min. Coat each well of a 6-well plate with 2 mL of the solution and incubate for 1 h at 37 °C. Before passaging the cells, aspirate the solution and allow the plate to dry for at least 1 h at room temperature inside the tissue culture hood.

2. hiPSC maintenance and cardiomyocyte differentiation

2.1. Culture the hiPSCs in hiPSC medium (see step 1.1.1) with a daily medium change until the cells reach 80%–90% confluence per well.

NOTE: For maintenance purposes, hiPSCs are generally ready for passage every 4 days.

2.2. To passage hiPSCs, aspirate the medium and wash the well 1x with 1x phosphate-buffered saline (PBS). Add 0.5 mL of stem cell detachment solution (**Table of Materials 1**) to each well and incubate at 37 °C for 5–7 min.

NOTE: The incubation time depends on the density of the cells and the rate of dissociation, which can be observed under a microscope.

2.3. Neutralize the stem cell detachment solution with 1 mL of hiPSC medium supplemented with 5 µM Y-27632. Collect the mixture in a 15 mL centrifuge tube. Centrifuge the tube for 5 min at 200 x *g*, aspirate the supernatant without disturbing the cell pellet, and then, resuspend the cells in 1 mL of hiPSC medium containing 5 µM Y-27632.

2.4. Seed the hiPSCs onto a new EMS-coated 6-well plate at a ratio between 1:6 to 1:18. Change the medium to hiPSC medium without Y27632 after 24 h.

2.5. To freeze the cells, resuspend the dissociated hiPSCs in freezing medium at a concentration of 0.5–1 million/500 µL, place the suspension in cryovials, and store them in a -80 °C freezer. Transfer the frozen vials to liquid nitrogen after 24 h.

2.6. To differentiate, replace the hiPSC medium with 2 mL of RB- medium supplemented with 10 µM GSK-3β inhibitor CHIR99021 (CHIR) at 80%–90% cell-confluence. Replace the medium with 3 mL of RB- medium after 24 h and incubate for an additional 48 h (day 3).

2.7. On day 3, change the medium to 3 mL of RB- medium with 10 µM Wnt inhibitor IWR1 for 48 h (day 5), and then replace the medium with 3 mL of RB- medium and maintain for 48 h (day 7).

2.8. On day 7, replace the medium with 3 mL of RB medium and change the medium every 3 days going forward. Spontaneous beating of hiPSC-CMs should be observed between days 7–10.

3. hiPSC-CMs purification and small molecule pre-treatment

NOTE: Highly purified, recombinant cell-dissociation enzymes (**Table of Materials 1**) were used to dissociate hiPSC-CMs.

3.1. On day 21 after hiPSC-CM differentiation, aspirate the medium and wash the cells 1x with sterile PBS. Incubate the cells with 0.5 mL of cell-dissociation enzymes per well for 5–7 min at 37 °C. Repeatedly pipette the cell suspension using a 1 mL pipette in order to thoroughly dissociate the cells.

3.2. After the cells are dissociated, add 1 mL of neutralizing solution to each well, collect the cell mixture into a 15 mL centrifuge tube and centrifuge at 200 x *g* for 3 min. Discard the supernatant and resuspend the cells in neutralizing solution.

3.3. Replant the cells onto gelatin-coated 6-well plates at a density of 2 million cells per well.

3.4. After 24–48 h, replace the culture medium with purification medium for 3–5 days.

NOTE: Purification is complete when more than 90% of the cells in each microscope view field are beating. To prevent further damage to the cardiomyocytes, do not extend the purification duration beyond 5 days. If the first round does not yield the necessary purity, replace the purification medium with RB+ medium for 1 day, and then complete a second round of purification.

3.5. Prior to transplantation, culture the cells in the treatment group for 12 h in RB+ medium supplemented with 10 µM Y-27632. Similarly, perform verapamil treatment on the cells in the RB+ medium with 1 µM verapamil for 12 h (hiPSC-CMs^{+VER}).

3.6. After treatment, wash the hiPSC-CMs 1x with PBS and incubate the cells with 0.5 mL of cell-dissociation enzymes per well for a maximum of 2 min. Repeatedly pipette the cell suspension, using a 1 mL pipette, to thoroughly dissociate the cells. Neutralize the cells with neutralizing solution, collect the cell mixture in a 15 mL centrifuge tube, and centrifuge at 200 x *g* for 3 min. Resuspend the cells in PBS at a concentration of 0.1 million cells/5 µL in preparation for injection.

4. Myocardial infarction and cell transplantation

NOTE: All surgical instruments are presterilized with autoclave and are maintained in aseptic condition during multiple surgeries via a hot bead sterilizer (**Table of Materials 2**).

4.1. Anesthetize NOD/scid mice (**Table of Materials 2**) with inhaled isoflurane (1.5%–2%). Monitor the anesthesia levels by the mice's responses to a toe pinch. Place vet optical ointment onto the eyes to prevent them from drying during surgery.

4.2. Supply 0.1 mg/kg buprenorphine subcutaneously for pain management prior to surgery.

4.3. Position and secure the mouse in a supine position on a heated operating table, remove the hair from the ventral neck region and the left thorax using a depilatory cream, expose the skin, and disinfect it with 70% alcohol.

4.4. Cut a 0.5 cm incision at the center of the neck using surgical scissors, separate the subcutaneous fat with sterile forceps, and expose the trachea. Introduce orally the intubation cannula into the trachea, connect the cannula to a small animal ventilator, and adjust the ventilation settings (set the tidal volume at 100–150 µL and the respiration rate at 100x–150x per minute).

4.5. After the mouse stabilizes, cut a 0.5 cm incision in the middle of the left chest skin. Use forceps to bluntly separate the muscle layer and make a small incision in the fifth intercostal space to expose the chest cavity. Place the retractor in the incision to open the thoracic cavity

and locate the left descending coronary artery (LAD). Under a dissecting surgical microscope, ligate the LAD with an 8-0 nonabsorbable suture.

4.6. Immediately following MI induction, inject 5 µL of hiPSC-CMs^{-RI}, hiPSC-CMs^{+RI}, hiPSC-CMs^{-VER}, hiPSC-CMs^{+VER}, or an equal volume of PBS into the mouse's myocardium at each site (3 x 10^5 cells/animal, 1 x 10^5 cells/site), one in the infarct area and two in the areas around the infarct.

NOTE: Since 5 µL is very small volume, it is not easy to accurately control the volume by directly sucking it with a syringe. The lid of a sterile Petri dish can be turned over, and 5 µL of the cells can be first deposited on the lid with a pipette. Due to the surface tension, it will not spread but will condense into a small liquid mass. This can be easily absorbed and injected into the mouse's myocardium.

4.7. Eliminate residual air in the thoracic cavity by filling it up with warm isotonic saline solution. Stitch the ribs, muscles, and skin in sequence with a 6-0 absorbable suture. Turn off the isoflurane anesthesia injection. Keep the surgery animals on the heating pad and closely observe them while they regain full consciousness. Then, place the animals in a clean cage. Do not return the animals to the company of other animals until they are fully recovered.

4.8. Following surgery, inject the mice intraperitoneally with buprenorphine (0.1 mg/kg) every 12 h for 3 consecutive days and inject them with ibuprofen (5 mg/kg) every 12 h for one day. Perform subsequent analysis studies at specified time points.

5. Calcium transient and contractility recording

5.1. Autoclave 15 mm-diameter coverslips (**Table of Materials 2**) and tweezers in a small glass beaker at 121 °C, 15 psi for 15 min. Precool the coverslips and tweezers at 4 °C.

5.2. Using the tweezers, place the coverslip into a 12-well plate and pipette 300 µL of extracellular matrix onto each coverslip.

NOTE: Do not let the matrix exceed the edge of the coverslip to prevent reducing the matrix coating amount. Incubate the 12-well plate in a 37 °C cell culture incubator for 1 h.

5.3. Aspirate the medium in the gelatin-coated 6-well plate with purified hiPSC-CMs, wash it 1x with PBS, and then digest it with 0.5 mL of cell-dissociation enzymes for 1.5 min. Add 1 mL of neutralizing solution, pipette repeatedly for no more than 5x–10x with a 1 mL pipette, and centrifuge the mixture at 200 x *g* for 3 min.

5.4. Aspirate the coated extracellular matrix from the coverslips. Count the cell number and resuspend the cells in the neutralizing solution to a concentration of 40,000/300 µL, and then add 300 µL of the cell suspension onto each coverslip. Culture the plate in a 37 °C incubator.

5.5. After 24 h, gently aspirate the neutralized solution, add 500 µL of RB+ medium to each well of the plate, and continue to culture for 2 days.

5.6. Add Y-27632 (10 µM), Rho kinase activator (RA, 100 nM), verapamil (1 µM), or an equal volume of PBS into the culture medium of hiPSC-CMs, 12 h before calcium transient and contractility detection.

NOTE: In addition to the detection of the cells' calcium transient and contractility after 12 h of chemical treatment, the detection of these parameters was also tested at 12, 24, 48, and 72 h after chemical withdrawal.

5.7. Take out the plate, discard the medium, and wash the plate 1x with PBS. Change the medium to a phenol-red-free DMEM containing 0.02% (w/v) of surfactant polyol (**Table of Materials 1**), 5 µM calcium indicator (**Table of Materials 1**), and the corresponding Y-27632 (10 µM), RA (100 nM), verapamil (1 µM), or an equal volume of PBS, and incubate the plate for 30 min at 37 °C.

5.8. Discard the supernatant, wash the cells 3x with dye-free DMEM medium, and let the medium rest for 30 min to de-esterify the mapping dye.

5.9. Place the cell-seeded coverslip in an open bath chamber and insert the chamber into a microincubation system equilibrated to 37 °C with an automatic temperature controller (**Table of Materials 2**), perfuse it with Tyrode's solution, and continuously add Rho kinase inhibitor (RI), RA, or PBS to the perfusion solution.

5.10. Use an inverted fluorescence microscope (**Table of Materials 2**) and a laser scanning head (**Table of Materials 2**) to record spontaneous calcium transient by employing an x-t line scan, using a 488 nm argon laser for dye excitation and a 515 ± 10 nm barrier filter to collect emission light. Record the spontaneous contraction of hiPSC-CMs using the transmitted light function of the microscope in conjunction with a 1,200 s high-speed camera (**Table of Materials 2**).

NOTE: Calcium transient recordings were processed and analyzed using MATLAB R2016A software (**Table of Materials 4**). Cell contraction change assays were performed using ImageJ software (**Table of Materials 4**).

REPRESENTATIVE RESULTS:

The hiPSC-CMs used in this study were derived from human origin with luciferase reporter gene; therefore, the survival rate of the transplanted cells in vivo was detected by bioluminescence imaging (BLI)[17] (**Figure 1A,B**). For histological heart sections, human-specific cardiac troponin T (hcTnT) and human nuclear antigen (HNA) double-positive cells were classified as engrafted hiPSC-CMs (**Figure 1C**). Both results indicated that Y-27632 pretreatment significantly improved the cell engraftment rate. The luciferase activity of the hiPSC-CM^{+RI} group was increased roughly sixfold on days 3, 7, and 28 after the transplantation, compared to that of the hiPSC-CM^{-RI} group (**Figure 1B**). Moreover, the hcTnT/HNA expression increased close to sevenfold in the hiPSC-CM^{+RI} group, relative to that in the hiPSC-CM^{-RI} group (**Figure 1C**).

The results also indicated that Y-27632 pretreatment regulated cytoskeletal changes in transplanted cells. On days 7 and 28 of transplantation, hiPSC-CMs^{+RI} exhibited a larger and more defined rod-shaped cytoskeletal structure compared to hiPSC-CMs^{-RI} (**Figure 1D**).

Moreover, Y-27632 pretreatment has the potential to reduce transplanted hiPSC-CM apoptosis in vivo. TUNEL staining showed that the number of TUNEL-positive cells was significantly decreased in the hiPSC-CM^{+RI} group relative to that in the hiPSC-CM^{-RI} group on day 2 after transplantation (**Figure 1E**).

ROCK inhibition promoted the adhesion of transplanted cells and had the potential to further increase the retention of implanted cells at administration sites. Western blot and cardiac tissue immunostaining suggested that Y-27632 pretreatment reversibly promoted the increased expression of integrin β1 and N-cadherin and decreased the expression of phospho-myosin light chain 2 (p-MLC2) (**Figure 2A,B**).

The mouse cell line HL-1 was selected as the control CMs for in vitro cell attachment experiments. The results indicated that Y-27632 pretreatment significantly increased hiPSC-CM adherence relative to HL-1. To further confirm these findings, hiPSC-CMs^{+RI} were incubated with N-cadherin or integrin β1 neutralizing antibody for 1 h, resulting in a vanishing of the improved adhesion seen previously (**Figure 2C**).

Compared with the hiPSC-CM^{-RI} group, the contractile force in the hiPSC-CM^{+RI} group was reduced by 32%, and in the hiPSC-CM^{+RA} group (hiPSC-CMs pretreated with ROCK activator, **Table of Materials 1**), it was increased by 42% (**Figure 3A**). Meanwhile, compared with the hiPSC-CM^{-RI} group, peak calcium transient fluorescence (Peak $\Delta F/F_0$) for the hiPSC-CM^{+RI} group was reduced by 41%, and the calcium transient duration (CaTD50) was reduced by 11% (**Figure 3B,C**). In contrast, Peak $\Delta F/F_0$ for the hiPSC-CM^{+RA} group was increased by 48%, and CaTD50 was increased by 13% (**Figure 3B,C**).

In addition, a pretreatment of the cardiomyocytes with Y-27632 for 12 h prior to the transplantation significantly reduced the expression of cTnI and cTnT (**Figure 3D**), both of which are troponin (Tn) subunits that regulate cardiomyocyte contraction.

Similar to Y-27632, a verapamil pretreatment (1 μM, 12 h) significantly increased the engraftment rate of hiPSC-CMs in induced MI mice. The hypothesis was confirmed through the observation of an increased luciferase signal in the bioluminescence assay (**Figure 4A**) and increased numbers of hcTnT/HNA double-positive cells on days 7 and 28 after cell transplantation (**Figure 4B**).

FIGURE LEGENDS:

Figure 1: Y-27632 pretreatment increased the engraftment rate of hiPSC-CMs in MI mice hearts. (**A**) Standard curve of BLI measurements. (**B**) Luciferin signal in NOD/scid mice treated with PBS, hiPSC-CMs^{-RI}, or hiPSC-CMs^{+RI} on days 3, 7, and 28 after surgery. n = 6–9 mice per group. *P <

0.05 vs. PBS; $^{\dagger}P < 0.05$ vs. hiPSC-CMs^{-RI}. (**C**) Immunostaining of heart sections for hcTnT and HNA. For 10X images, the scale bars = 100 µm; for 40x images, the scale bars = 20 µm. *n* = 5 mice per group. *$P < 0.05$ vs. hiPSC-CMs^{-RI}. (**D**) Representative images of heart sections stained with phalloidin and hcTnT. The scale bar = 20 µm. *n* = 5 mice per group. *$P < 0.05$ vs. hiPSC-CMs^{-RI}. (**E**) Representative images of heart sections for TUNEL staining. The scale bar = 20 µm. *n* = 4–6 mice per group. *$P < 0.05$ vs. hiPSC-CMs^{-RI}. This figure has been modified from Zhao et al.[18].

Figure 2: Y-27632 enhanced the adhesion of hiPSC-CMs by maintaining the expression of adhesion proteins. (**A** and **B**) Western blot analysis of the expression of integrin ß1, N-cadherin, and phosphorylation of MLC2 (p-MLC2) in hiPSC-CMs treated with RI and in nontreated hiPSC-CMs. *n* = 5 replicates per group. (**C**) hcTnT immunofluorescence staining analysis of -RI and +RI hiPSC-CMs with and without a pretreatment of anti-N-cadherin (N-Cad) and anti-integrin ß1 (Integ) antibodies. The scale bars = 100 µm. *$P < 0.05$ vs. hiPSC-CMs^{-RI}; $^{\dagger}P < 0.05$ vs. hiPSC-CMs^{+RI}. This figure has been modified from Zhao et al.[18].

Figure 3: Pretreatment with Y-27632 reduced the contractility of hiPSC-CMs and down-regulated the expression of cardiac troponin subunits. (**A**) Quantification of the percentage of shortening of hiPSC-CMs exposed to RI and RA treatment. *$P < 0.05$ vs. hiPSC-CMs^{-RI}; $^{\dagger}P < 0.05$ vs. hiPSC-CMs^{+RI}. (**B** and **C**) Representative images and quantification of calcium transient measurements of hiPSC-CMs treated with RI and RA and of a nontreated group. *$P < 0.05$ vs. hiPSC-CMs^{-RI}; $^{\dagger}P < 0.05$ vs. hiPSC-CMs^{+RI}. (**D**) Western blot analysis of the expression of cardiac troponin subunits (cTnC, cTnT, and cTnI) in hiPSC-CMs treated with RI and RA and in a nontreated group. This figure has been modified from Zhao et al.[18].

Figure 4: Verapamil pretreatment improved the engraftment rate of hiPSC-CMs in MI mice. (**A**) Luciferin signal in NOD/scid mice treated with PBS, hiPSC-CMs^{-VER}, or hiPSC-CMs^{+VER} on days 3, 7, and 28 after surgery. *n* = 8 mice per group. *$P < 0.05$ vs. PBS; $^{\dagger}P < 0.05$ vs. hiPSC-CMs^{-VER}. (**B**) Immunostaining of heart sections for hcTnT and HNA. For 10x images, the scale bars = 100 µm; for 40x images, the scale bars = 20 µm. *n* = 5 mice per group. *$P < 0.05$ vs. hiPSC-CMs^{-VER}. This figure has been modified from Zhao et al.[18].

DISCUSSION:

The key steps of this study include obtaining pure hiPSC-CMs, improving the activity of hiPSC-CMs through Y-27632 pretreatment, and finally, transplanting a precise amount of hiPSC-CMs into a mouse MI model.

The key issues addressed here were that, first, we optimized the glucose-free purification methods[19] and established a novel efficient purification system. The system procedure included applying cell-dissociation enzymes, replanting cells in gelatin-coated plates, culturing the cells in the neutralization medium for 24 h after replating, and minimizing the digestion time of the cells before transplantation, all of which were performed to achieve the highest activity of the cells while obtaining pure cardiomyocytes.

Second, we elaborated on the pretreatment of hiPSC-CMs with Y-27632 at 12 h before cell injection. The anoikis is usually induced by the modification of cells' adhesion proteins, such as integrins[4,20] and N-cadherin[21], that lead to the activation of the apoptotic pathway. We demonstrated that Y-27632 pretreatment could promote the adhesion of hiPSC-CMs to the transplantation site through maintaining the expression of integrin β1 and N-cadherin, suppressing the expression of p-MLC2, which is related to changes in the cytoskeletal architecture[22,23]. Seven days after hiPSC-CM transplantation, hiPSC-CMs^{+RI} resulted in a greater cell engraftment area, finer defined rod shapes, and a more complete cytoskeletal organization compared to hiPSC-CMs^{-RI} (**Figure 1B–D**).

Third, we established a novel intracellular injection system in mouse MI models, with 5 µL per injection point to accurately inject the required number of cells, while avoiding a decrease in the mouse survival rate due to excessive injection volume.

Fourth, we elaborated on how to determine the changes in cell contraction and calcium transient in hiPSC-CMs after pretreatment with RI or RA. We found that Y-27632 can reversibly reduce the cell contractility and calcium transient. The hypothesis here is that due to the lower energy requirements of the transplanted cells, the engraftment rate increased. Similar effects can be achieved using the calcium channel inhibitor verapamil. The proposed mechanism resulting in the contractility inhibition is that Y-27632 reduces the expression of troponin subunits cTnT and cTnI in hiPSC-CMs.

The main limitation is that Y-27632 only played a transient role during the transplantation. How to inhibit Rho kinase for a longer period, thus further improving the transplantation efficiency of hiPSC-CMs, is a problem to be solved in the future. As for more applications of this method, ROCK inhibitor can not only improve the activity of cardiomyocytes during transplantation but also enhance the activity of other cells; therefore, it can also be used during the pretreatment in the transplantation processes of other cells. Moreover, the approach presented here lays a good experimental foundation for more research on heart disease.

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DISCLOSURE:

The authors have nothing to disclose.

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Figure

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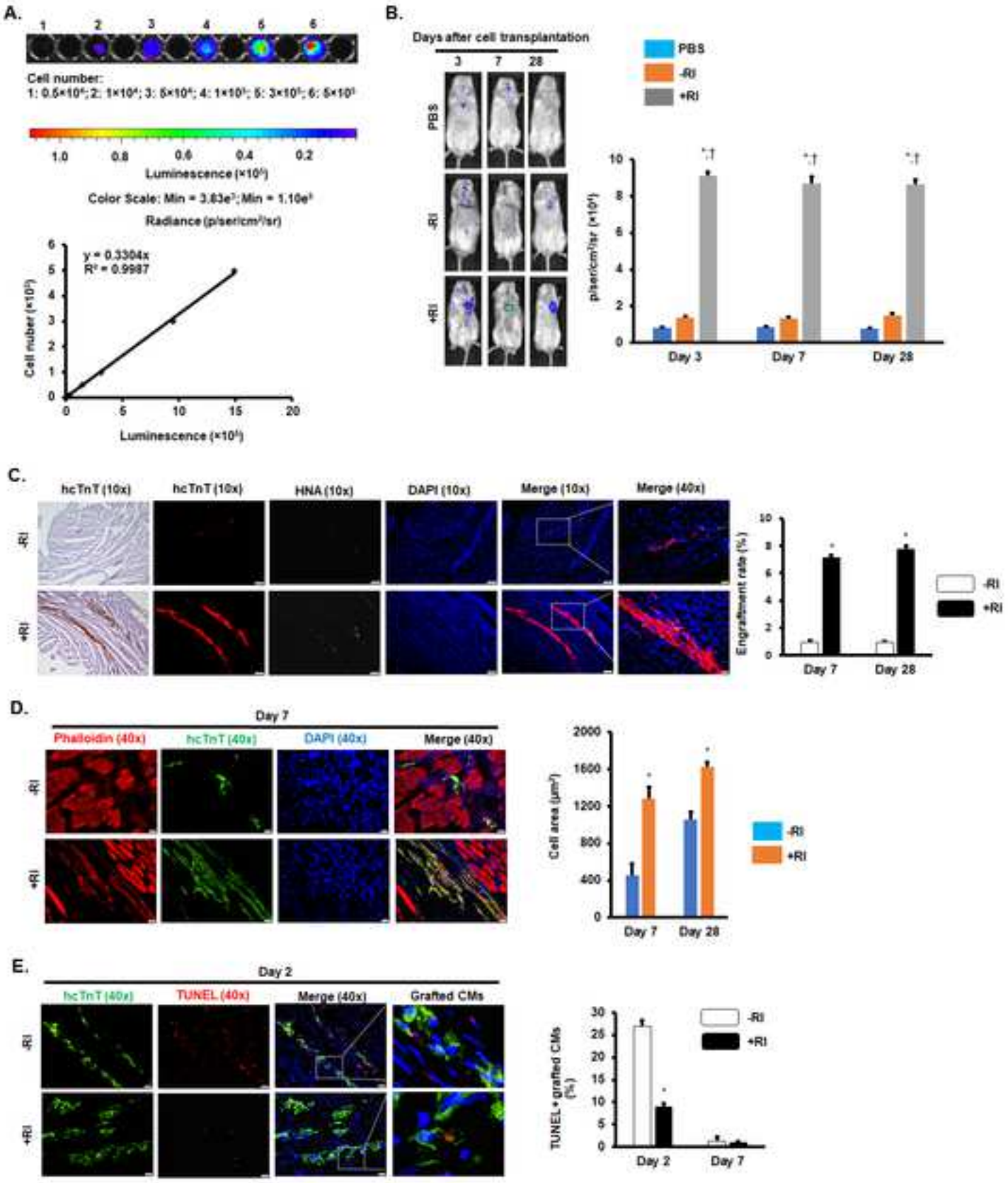


Figure 2

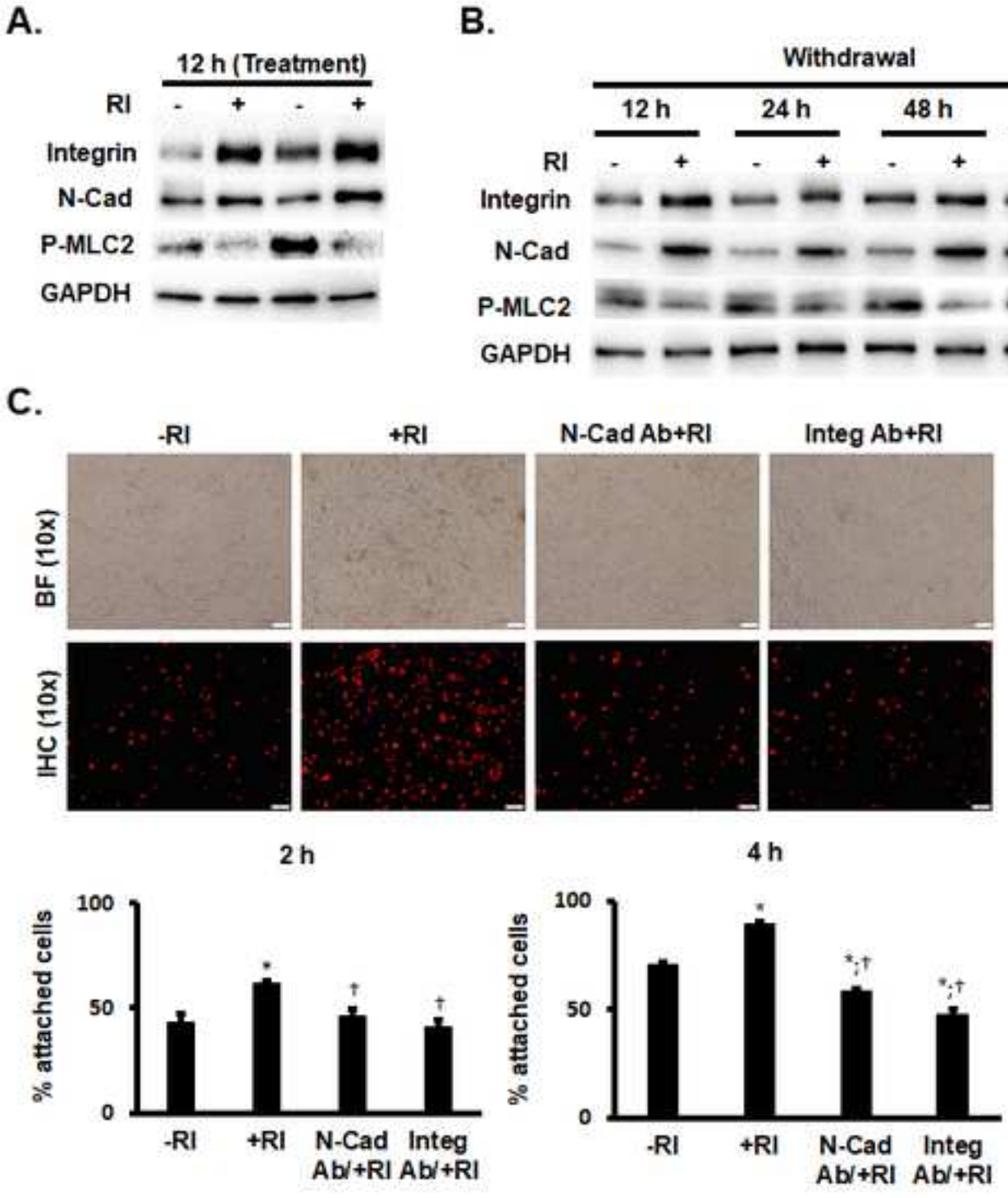
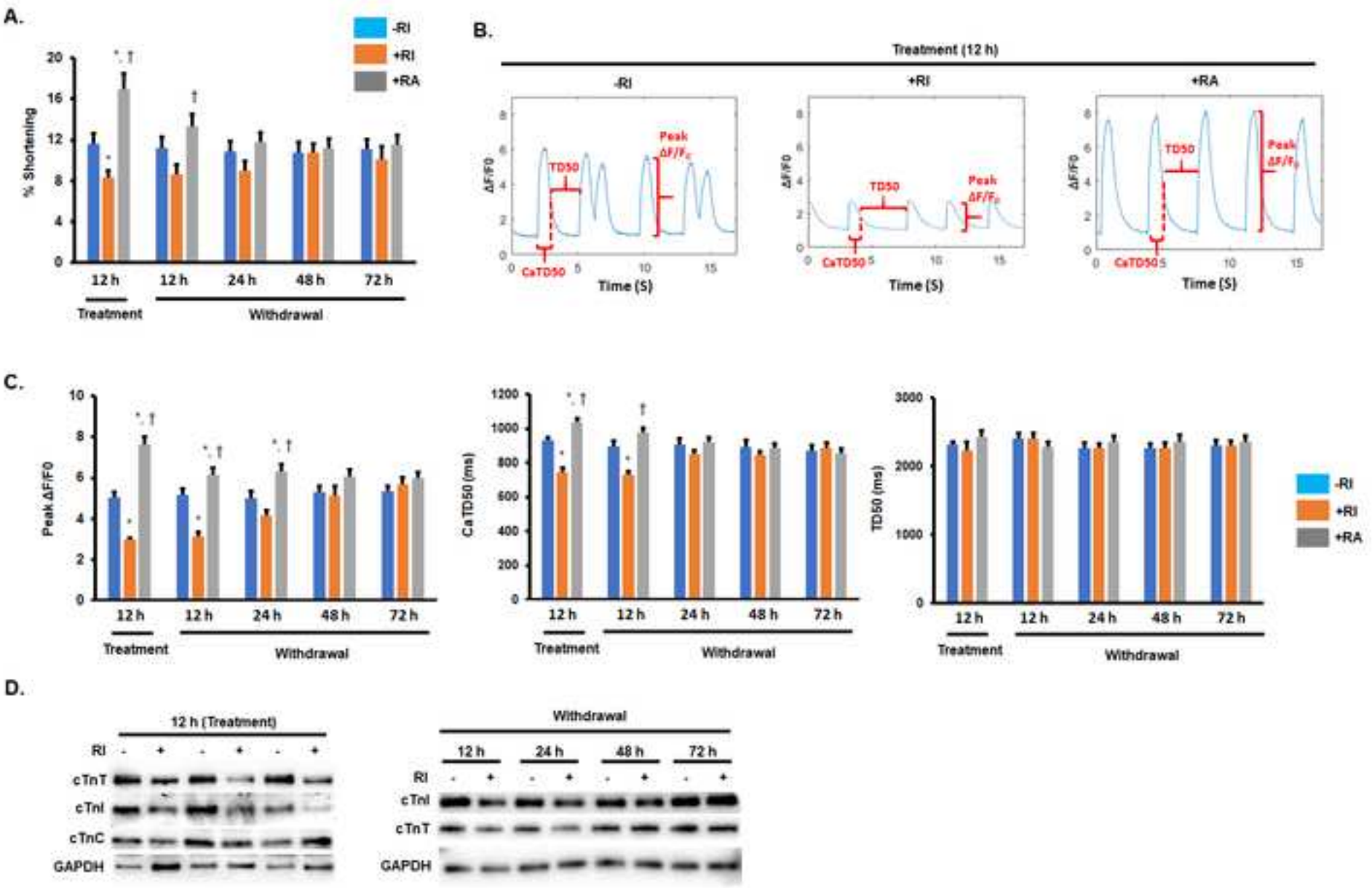


Figure 3



Figure

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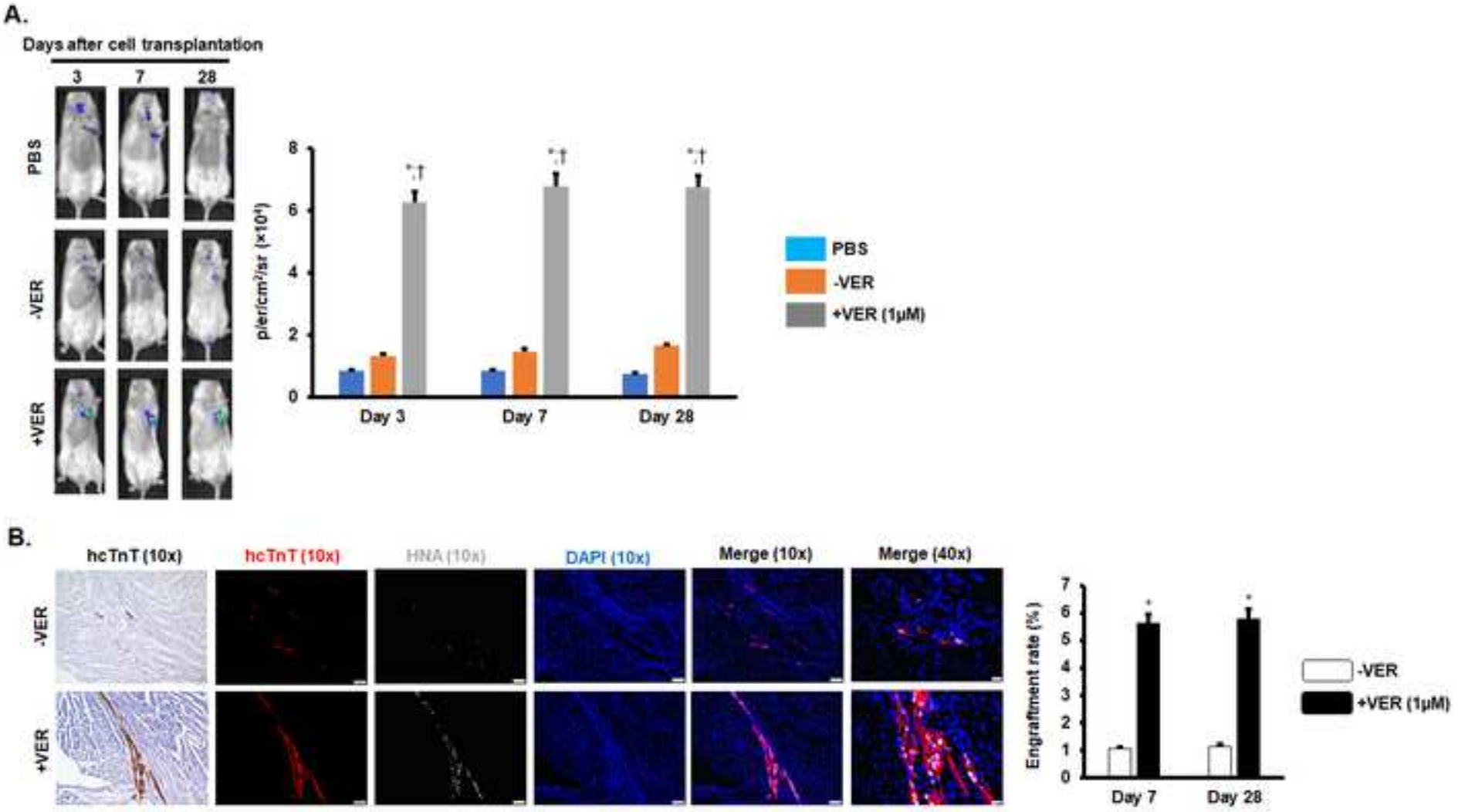

Table 1

Reagent
Accutase (stem cell detachment solution)
B27 minus insulin
B27 Supplement
CHIR99021
DMEM (1x), high glucose, HEPES, no phenol red
Fetal bovine serum
Fluo-4 AM (calcium indicator)
Glucose-free RPMI 1640
IWR1
Matrigel (extracellular matrix)
mTeSR (human pluripotent stem cells medium)
Pen-strep antibiotic
Pluronic F-127 (surfactant polyol)
Rho activator II
RPMI1640
Sodium DL-lactate
TrypLE (cell-dissociation enzymes)
Verapamil
Y-27632

Table 2

Equipment and Supplies
IVIS Lumina III Bioluminescence Instruments
15 mm Coverslips
Centrifuge
Confocal Microscope
Cryostat
Dual Automatic Temperature Controller
Electrophoresis Power Supply
Fluoresence Microscope
High Speed Camera
Laser Scan Head
Low Profile Open Bath Chamber (mounts into above mi
Microincubation System
Minivent Mouse Ventilator
NOD/SCID mice
Precast Protein Gels

PVDF Transfer Packs
Trans-Blot System
Hot bead sterilizer

Table 3

Antibody
Anti-human Nucleolin (Alexa Fluor 647)
Cardiac Troponin T
Cardiac Troponin C
Cardiac Troponin I
Cy5-donkey anti-mouse
Cy3-donkey anti-rabbit
Fitc-donkey anti-mouse
GAPDH
Human Cardiac Troponin T
Integrin β1
Ki67
N-cadherin
Phospho-Myosin Light Chain 2

Table 4

Software
Matlab
Image J

Manufacturer	Catalog Number
STEMCELL Technologies	#07920
Fisher Scientific	A1895601
Fisher Scientific	17-504-044
Stem Cell Technologies	72054
Thermofisher	20163029
Atlanta Biologicals	S11150
Invitrogen/Thermofisher	F14201
Fisher Scientific	11879020
Stem Cell Technologies	72562
Fisher Scientific	CB-40230C
STEMCELL Technologies	85850
Fisher Scientific	15-140-122
Sigma-Aldrich	P2443
Cytoskeleton	CN03
Fisher Scientific	11875119
Sigma-Aldrich	L4263
Fisher Scientific	12-605-010
Sigma-Aldrich	V4629
STEMCELL Technologies	72304

Manufacturer	Catalog Number or Type Number
PerkinElmer	CLS136334
Warner	CS-15R15
Eppendorf	5415R
Olympus	IX81
Thermo Scientific	NX50
Warner Instruments	TC-344B
BIO-RAD	1645050
Olympus	IX83
pco	1200 s
Olympus	FV-1000
Warner Instruments	RC-42LP
Warner Instruments	DH-40iL
Harvard Apparatus	845
Jackson Laboratory	001303
BIO-RAD	4561033

BIO-RAD	1704156
BIO-RAD	Trans-Blot Turbo
Fine Science Tools	18000-45

Manufacturer	**Catalog Number**
Abcam	ab198580
R&D Systems	MAB1874
Abcam	ab137130
Abcam	ab47003
Jackson ImmunoResearch Laboratory	715-175-150
Jackson ImmunoResearch Laborat	711-165-152
Jackson ImmunoResearch Laborat	715-095-150
Abcam	ab22555
Abcam	ab91605
Abcam	ab24693
EMD Millipore	ab9260
Abcam	ab18203
Cell Signaling Technology	3671s

Manufacturer	**Version**
MathWorks	R2016A
NIH	1.52g

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Author(s):	Meng Zhao, Yawen Tang, Patrick J. Ernst, Asher Kahn-Krell, Chengming Fan, Danielle Pretorius, Hanxi Zhu, Xi Lou, Lufang Zhou, Jianyi Zhang, Wuqiang Zhu

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CORRESPONDING AUTHOR

Name:	Wuqiang Zhu
Department:	Biomedical Engineering
Institution:	University of Alabama at Birmingham
Title:	A Method to Enhances Engraftment of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes via Transient Inhibition of Rho Kinase Activity
Signature:	Wuqiang Zhu
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Response to editors

Manuscript #JoVE59452

"A Method to Enhance Engraftment of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes via Transient Inhibition of Rho Kinase Activity"

By Meng Zhao, et al

We appreciate the careful and constructive review given to our manuscript. We have revised the manuscript to address the comments from editor and reviewers. All changes were marked in red. A point by point response is as follows.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly proofread the manuscript to revise all the spelling or grammar issues.

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Response: Each Figure has been uploaded to our Editorial Manager individually as a .tif file.

4. Figure 1C-E and Figure 4: Please use lowercase x for the magnification (i.e., 10x, 40x). Please define the error bars in the figure legend.

Response: Lowercase x for the magnification has been used and the error bars in the figure legend has been defined.

5. Figure 2 and Figure 3: Please include a space between all numerical values and their corresponding units (12 h, 24 h, 48 h, etc.). Please change "Time(s)" to "Time (s)" (i.e., include a space).

Response: We checked the entire manuscript to ensure that a space between numbers and units was included. "Time(s)" has been changed to "Time (s)".

6. Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment.

Response: We have generated 4 tables to include all relevant supplies, reagents, equipment and software in separate columns in an .xls file. The items were sorted in alphabetical order according to the name.

7. Please revise the title to reflect the content of the method.

Response: The title has been changed to *"A Method to Enhance Engraftment of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes via Transient Inhibition of Rho Kinase Activity"*.

8. Please provide an email address for each author.

Response: We provided a table to include an email address for each author.

9. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response: A Summary section has been added before Abstract to describe the protocol applications.

10. Please rephrase the Abstract to more clearly state the goal of the protocol.

Response: We have rephrased the Abstract to more clearly state the goal of the protocol.

11. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Information that can help readers to determine if the method is appropriate for their application.

Response: We have expanded our introduction to include the advantages over alternative techniques to help readers to determine if this method is appropriate for their application.

12. Please define all abbreviations before use.

Response: we have checked the entire manuscript to define all abbreviations.

13. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.

Response: All units has been changed to SI abbreviations. micro symbol µ was used instead of u, and liters symbol was used as L.

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Response: All commercial language from our manuscript were removed and generic terms were used instead. All commercial products were referenced in the Table of Materials and Reagents.

16. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response: An ethics statement indicating that the protocol follows the animal care guidelines has been included.

17. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

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18. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We checked the entire manuscript to exclude all personal pronouns.

19. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: The protocol has been revised to use only action items which described in the imperative tense. Some texts that cannot be written in the imperative tense have been added as a "Note." The discussion about the protocol has been moved to the Discussion.

20. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Response: We have added more details to our protocol to help viewers to replicate the protocol. We have tried to answer every question about how the step is performed.

21. Line 71: Please specify what supplement is used.

Response: We have specified the supplement used (mTeSR supplement).

22. Line 103: What volume of mTeSR medium is used to neutralize and resuspend the cells?

Response: Information about the volume of medium was added to 2.3.

23. Line 140: Please describe how to dissociate using TrypLE by referring to the previous steps.

Response: Information about how to dissociate using TrypLE was added to 3.7.

24. Lines 167-168: What volumes of such solutions are injected?

Response: The volume of injected solution was added to 4.6.

25. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have highlighted all the Protocol that identifies the essential steps for the video.

26. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We highlighted complete sentences which ensure that the highlighted step includes at least one action that is written in imperative tense.

27. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: We included all relevant details that are required to perform the step in the highlighting.

28. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: We revised the discussion to focus on the methods and the protocol and made sure the discussion covered the above questions.

29. References: Please do not abbreviate journal titles.

Response: We changed all journal titles to full journal titles.

30. The author license agreement you uploaded is for UK authors. Please sign the attached agreement and replace the form in your Editorial manager account.

Response: We have signed the attached agreement and replaced the form in our Editorial manager account.

Reviewer #1:

Manuscript Summary:
The manuscript describes that the 12-hr pretreatment of human iPSC-derived cardiomyocytes with small molecule inhibitor of Rho kinase (ROCK) inhibitor, Y27632, or inhibitor of L-type calcium channel improved retention of cardiomyctes after injected into mouse with myocardium infarction. The data suggest that ROCK inhibition improve the adhesion of cardiomyocytes to substrate, which might be a potential mechanism of ROCK inhibitor induced improve retention of the injected cells.

Major Concerns:
The mouse data of improved retention rate is impressively high. However, the description of protocol lacks details, which is very important for the JoVE readers. While it might be prepared at the video production stage, we strongly encourage to include schematic to summarizing the experiments and time course of procedures. Author should include original protocols from previous citations. The protocol to measure contractility or calcium transient is missing entirely.

Response: We have revised the protocol to include more details and schematics to summarizing the experiments and time course of procedures. The protocol to measure contractility or calcium transient was also included.

Minor:

1. *Add reference for Purification medium. Tohyama et al. 2016 Cell Metabolism

Response: The reference was added.

2. *The hiPSC-CMs+RA is defined only in Abstract. It should be defined again in the body as well as caption for readers' continence.

Response: The hiPSC-CMs+RA was defined again in the body as well as caption.

3. *Figure 2A what is the MCL-2 phospho-antibody is against (non-muscle or cardiac myosin light chain?)

Response: The cells we used are hiPSC-CMs. MCL-2 phospho-antibody is against cardiac myosin light chain.

4. *Antibody against non-phosphoryated MCL-2 should be used to normalize to analyze the ROCK activity phosphorylating the MCL-2.

Response: We should do that, but the effect of Y-27632 on phospho-MLC2 has been proved in lots of studies.

5. *Figure 2C, Bright Field images are difficult to see and what was used to stain IHC image (IHC should be spelled out somewhere).

Response: The cells in bright field are HL1 cells, they are covered with the entire field of view. We used human cTnT to stain IHC image, as HL1 cell is of mouse source, only attached hiPSC-SMs can express human cTnT.

6. *How force, fraction of shortening or Calcium transients were measured

Response: The protocol about the cell contraction and calcium transient detection has been added.

7. *Figure 3: TD50 appears to be longer in the +RT condition in Figure 3B but does not change significantly in Figure 3C.

Response: The histogram shows the statistical results of large amounts of data.

8. *Figure 3D, in the 12h treatment gel, cTnC looks like it is also affected by +RI treatment except for the last lane of the gel. GAPDH loading looks much larger for the second lane of the gel.

Response: By quantification from at least 5 times of experiment, we found that cTnC was not affected by RI treatment. GAPDH is uneven but can be used for quantification.

9. *Figure 3B, why is there arrhythmia (i.e. double peaks) in -RI condition?

Response: That is not arrhythmia but noise signal.

10. *Why does the PBS control condition look like it decreases cell engraftment? Is this significant?

Response: Without Y-27632 treatment, implanted cells are susceptible to apoptosis due to ischemia, hypoxia, inflammation, and tissue damage. Of the remaining fraction of cells, a large proportion is washed out from the implantation site into the blood stream. It is very significant. The data was shown in figure 1.

11. *What is the dose range of Y-27632 treatment effectiveness for improving cell engraftment? Authors show 10 μM concentration, were other doses tested?

Response: For hiPSC-CMs transplantation, concentrations of 0.1 μM, 1 μM , 10 μM , 50 μM and 100 μM were tested. We found that 10 μM is better than 0.1 μM and 1 μM, but 50 μM and 100 μM showed no difference relative to 10 μM.

12. *Please include a list of antibodies (manufactures etc).

Response: Antibody list was included in table 3 of materials.

13. *Not all the reagents are listed; please include.

Response: We added all the reagents, equipment, supplies, antibody and software in the tables of materials.

Reviewer #2:

Manuscript Summary:
In this manuscript, Zhu et al introduced a protocol to improve hiPSC-CM survival and retention in mouse ischemic heart via Y-27632 preconditioning, it is an important study, which have high impact on cell therapy field. The data support the conclusion. The protocol is detailed prescribed for audience to follow.

Major Concerns:
None.

Minor Concerns:
The authors should give full name of RI in its first appearance in abstract.

Response: Full name of RI was given in the first appearance in abstract.

Reviewer #3:

1. Line 112: Why did you use Wnt inhibitor?

Response: At the 72 h of cardiomyocytes differentiation, Wnt inhibitor will induce cardiac mesoderm production.

2. Line 169: What you chose 3 injection?

Response: We injected iPSC-CMs into infarct zone and both border zones to compensate for the loss of cardiomyocytes after infarction. We tried to inject at more points, however, the mouse heart is too small for more injection points.

3. Line 157: The anesthesia protocol is not complete.

Response: We added more details into the anesthesia protocol.

4. Line 193: The tunel staining must be performed in various time. The methodology part is not perfect and must be completed.

Response: The TUNEL staining was performed at day 2 and day 7 after myocardial infarction. The method has been further improved.

Response to editors

Manuscript #JoVE59452

"A Method to Enhance Engraftment of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes via Transient Inhibition of Rho Kinase Activity"

By Meng Zhao, et al

We appreciate the careful and constructive review given to our manuscript. We have revised the manuscript to address the comments from editor and reviewers. All changes were marked in red. A point by point response is as follows.

Editorial comments:

The manuscript has been modified and the updated manuscript, ***59452_R1.docx****, is attached and located in your Editorial Manager account.* ***Please use the updated version to make your revisions.***

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly proofread the manuscript to check for spelling or grammar issues.

2. The manuscript has been rearranged and the Figure Legend has been moved before Discussion. Please adjust the numbering of all references and ensure that all references are numbered in the order of the appearance in the manuscript.

Response: Thank you! We have adjusted the numbering of all references and ensured that all references are numbered in the order of the appearance in the manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include mTeSR, Matrigel, Pluronic, Olympus, Fluoview, etc.

Response: We have removed all commercial language from our manuscript and used generic terms instead. All commercial products can be sufficiently referenced in the Table of supplement.

4. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: We have removed all the trademark (™) and registered (®) symbols.

5. Please use standard SI unit symbols and prefixes such as µm, µL, mL, L, g, m, etc.

Response: We checked the entire manuscript to ensure that we used standard SI unit symbols and prefixes.

6. Please use h, min, s for time units.

Response: We checked the entire manuscript to ensure that we used h, min, s for time units.

7. Please use a single space between numerical values and their units.

Response: We have correct this in the revised manuscript.

8. Step 2.1: Please specify the culture condition.

Response: The culture condition has been specified in Step 2.1.

9. 3.2: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Response: All centrifuge speeds in the entire manuscript have been converted to centrifugal force (x g).

10. Discuss maintenance of sterile conditions during survival surgery.

Response: The maintenance of sterile conditions during survival surgery has been discussed in surgery section (section 4, line 188-189).

11. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Response: We added this information to Section 4.7 (line 226-228).

12. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Response: We added this information to Section 4.7 (line 226-228).

13. Please remove all headers from Representative Results.

Response: All headers have been removed from Representative Results.

14. Please do not abbreviate journal titles for all references.

Response: The abbreviations were generated by Endnote automatically. We will manually correct this before production of this manuscript.

15. Figure 3C: Please define the scale bar.

Response: We defined the scar bar in figure legend.

E-Mail of all co-authors

Author	**Email**
Meng Zhao	mengzhao@uab.edu
Yawen Tang	yawen@uab.edu
Patrick J. Ernst	pernst@uab.edu
Asher Kahn-Krell	akrell@uab.edu
Chengming Fan	markfan@uab.edu
Danielle Pretorius	dpret@uab.edu
Hanxi Zhu	
Xi Lou	xlou@uab.edu
Lufang Zhou	lufangzhou@uabmc.edu
Jianyi Zhang	jayzhang@uab.edu
Wuqiang Zhu	wzhu@uab.edu