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CRISPR/Cas9-mediated knock-in of a fluorescent reporter into the target locus of interest in human pluripotent stem cells ☆

A-Hyeon Kim [a,b,1], Ha Myoung Lee [a,b,1], Hong-Sik Kim [a], Seong Woo Jeong [a], Jong Kwan Jun [b], Jiho Jang [a,b,c,*]

[a] Department of Biomedical Sciences, College of Medical Convergence, Catholic Kwandong University, Gangneung-si, Gangwon-do, Republic of Korea
[b] Institute of Reproductive Medicine and Population, Medical Research Center, Dept. of Obstetrics & Gynecology, Seoul National University College of Medicine, Seoul 03080, Korea
[c] Institute for Aging and Metabolic Diseases, College of Medicine, The Catholic University of Korea, Seoul 06591, South Korea

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ABSTRACT

The method presented herein is associated with the Lab Resource article titled "Generation of αMHC-EGFP knock-in in human pluripotent stem cell line, SNUe003-A-3, using CRISPR/CAS9-based gene targeting" [1]. The cardiac muscle-specific protein, α-myosin heavy chain (αMHC), is encoded by the human *MYH6* gene, which is expressed in both the atria and ventricles during embryonic development and is predominantly expressed in the atria after birth [2]. Herein, the methods used to achieve CRISPR/SpCas9-mediated introduction of an EGFP reporter into *αMHC*, the target locus in human pluripotent stem cells (hPSCs) for cardiac lineage tracing and clinical cell sorting are described. The CRISPR-Cas9 system enables efficient replacement of the stop codon in the last exon of *αMHC* with a 2A non-joining peptide (T2A)-EGFP cassette. First, hPSCs are transfected with the donor construct and Cas9/sgRNA plasmids via electroporation and selected with neomycin for approximately 3 weeks. Thereafter, the established cell line exhibits typical characteristics of human embryonic stem cells (hESCs). When these cells differentiate into cardiomyocytes, the expression of EGFP is confirmed using confocal microscopy, flow cytometry analysis, and immunostaining.

- The line enables monitoring of cell maturation events during human cardiac development.
- The line is a valuable platform for cardiotoxicity tests and drug screening.
- This method has already been employed in two original studies, as previously reported for reporter cell line generation using CRISPR/Cas9.

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* Corresponding author.
E-mail address: jhjang@cku.ac.kr (J. Jang).

[1] These authors contributed equally to this work.

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Resource availability:	http://www.cosmogenetech.com/cosmo/account/account_dna.jsp, https://www.thermofisher.com/order/catalog/product/MPK5000

Method details

Background

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), have a broad potential to differentiate into all somatic cell types. This robust capability makes hPSCs invaluable for powerful cellular disease modeling, cell replacement therapy, and drug discovery. However, these approaches have frequently been challenging due to the heterogeneity of differentiated cells [1–3].

Stable hPSC reporter lines can be used to improve differentiation efficiency or optimized for in vivo tracking of transplanted cells. Conventionally, generating reporter hPSC lines using classical genome editing technology through homologous recombination has been known to be inefficient and arduous in hPSCs [4]. Currently, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9) system, as genomic scissors, has become widely used as a new tool to edit the genome at desired genome loci of hPSCs [5]. This system consists of a single guide RNA (sgRNA) with a sequence of about 20 bp that binds to the target DNA to be corrected, and the Cas9 protein that cuts a specific base sequence. After the Cas9 enzyme is activated by binding to sgRNA, the Cas9/sgRNA complex binds the target DNA sequence followed by the PAM (protospacer adjacent motif, NGG) sequence and cleaves both strands, leaving them in a double-strand break (DSB). These DSBs are repaired by the intrinsic repair mechanisms: non-homologous end joining (NHEJ) or homologous direct repair (HDR). The NHEJ typically results in insertion/deletion (indel) of a short base sequence at the cut site. While HDR pathway precisely leads to foreign gene substitution or gene correction when exogenous (donor) template DNA with a homologous DNA sequence is added to the cut genome region. By manipulating the donor cassette on both sides of the homology arm, it was possible to create hPSC reporter lines containing fluorescent proteins in various genes using CRISPR/Cas9 via HDR.

CRISPR/Cas9-based genome engineering technologies with high accuracy and efficiency have been widely applied for clinical and basic research [6]. hPSC reporter lines are necessary for studying differentiation, lineage tracing, and cell-based therapies to further expand the potentiality of therapeutic applications. However, reporter genes such as EGFP knock-in hPSCs remain challenging and inefficient despite enhancement by CRISPR/Cas9. Here, we provide a stepwise protocol and a detailed guide for the generation of hPSC reporter lines using the CRISPR/Cas9-mediated homology recombination. We explain in detail the design of the donor vector, the delivery of plasmids into hPSCs via electroporation, and the selection of clones with antibiotics, and the validation of the selected clones. We also describe the process of producing the αMHC-EGFP Knock-in hPSC line as an example.

• Before you begin

Prepare donor, sgRNA, and CRISPR/Cas9 plasmid to generate the αMHC-EGFP Knock-in hPSC line. Of note, the donor plasmid was designed to insert the *EGFP* gene and puromycin resistance genes into the C-terminal region of the *αMHC* gene using Snapgen software (Fig. 1).

STEP 1: Transformation

Materials

- DH5a chemically competent *E. coli* (Enzynomics, CP010)
- Ampicillin, Sodium Salt (Merck Millipore, Cat. 171254)

  Note: Dilute to 50 mg/ml (1000x) in UPW

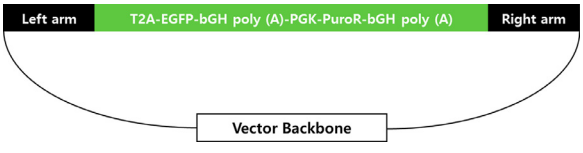

Fig. 1. Donor Plasmid Design. A donor plasmid was designed using Snapgene software. The donor plasmid contains the existing vector backbone sequence, genome sequence homology left arm, cassette to be inserted (T2A-EGFP-bGH poly(A)-PGK-Puro-bGH poly(A)), and genome sequence homology right arm.

- Difco™ LB Broth, Miller (Luria Bertani) (BD, Cat. 244620)
- Bacto™ Agar (BD, Cat. 214010)

  Note 1: The LB broth consists of trypton, NaCl, and Yeast, and is dissolved in UPW according to the appropriate ratio. To prepare the agar plate, add agar to the medium.
  Note 2: During the preparation of LB broth and the agar plate, 50 μg/ml ampicillin should be added to the medium.

- Compact shaking incubator (JSR, JSSI-100T)
- CHEMICAL FUME HOOD (DONGJAE)
- General incubator 96 L (JSR, JSGI-100T)

Methods

1. DNA concentration: Add 0.5 μL of DNA to 20 μL of competent cells. After 5 min on ice, heat shock for 30 sec at 42 ˚C, and place on ice for 2 min. Add LB broth (+ ampicillin 50 μg/mL) or SOC 100 μL with the alcohol lamp on.
2. Incubate for 1 h in a shaking incubator at 225 rpm and 37 ˚C. After shaking, turn on the alcohol lamp and apply a smear. Incubate overnight (16 h) in an incubator at 37 ˚C.

   Note 1: Add DNA to competent cells to achieve 0.6 μg/mL - 1 μg/mL
   Note 2: At the start of shaking incubation, dry the agar plate (+ ampicillin 50 μg/mL) in the hood for 30 min - 1 h.

STEP 2: 250 mL culture

Materials

- Ampicillin, Sodium Salt (Merck Millipore, Cat. 171254)

  Note: Dilute to 50 mg/ml (1,000x) in UPW.

- Difco™ LB Broth, Miller (Luria Bertani) (BD, Cat. 244620)
- Vertical Autoclave 85 L (JSR, JSAT-85)
- Compact shaking incubator (JSR, JSSI-100T)

Methods

1. In a 1 L Erlenmeyer flask, add 6.25 g of Difco™ LB Broth followed by 250 mL of UPW. Autoclave the mixture and then cool in a refrigerator.
2. When the media is cool enough, add 250 μL of ampicillin (final 50 μg/mL) and incubate overnight (16 h) in a shaking incubator at 225 rpm and 37 ˚C (2.5 mL of 10 mL culture microbial media in 250 mL).

   Note: If the medium is not used on the same day, add ampicillin, seal well with foil, and store at 4 ˚C.

STEP 3: Midi prep

Materials

- NucleoBond Xtra Midi (MACHEREY-NAGEL, Cat. 740410.50)
- Ethyl alcohol, Pure (Sigma, E7023)

  Note: Dilute to 70 % EtOH in UPW.

- Isopropanol (Merck Millipore, Cat. 109634)
- High-speed centrifuge (himac, CR22N)

  Note: For use at 4 ˚C, pre-cool at 3,000 rpm for 30 min at 4 ˚C.

Methods

1. Pour 250 mL of the microbial culture into a High-Speed Refrigerated Centrifuge dedicated container. Weigh on a scale with UPW as a balance and then perform centrifugation with a High-Speed Centrifuge at 5,000 rpm for 10 min at RT. Discard the supernatant and add 10 – 30 mL of UPW to resuspend the pellet. Transfer to a 50 mL tube and perform centrifugation with a High-Speed Centrifuge at 3,000 rpm for 10 min at RT.
2. Remove the supernatant and dissolve the pellet with 8 mL of RES. After adding 8 mL of LYS, invert and incubate for 5 min at RT. Dispense 12 mL of EQU onto the filter for 5 min at RT. Add 8 mL of NEU to the mixture and invert slightly strongly (Check the white sediment). Hang on the filter.
3. (1^{st} wash) When the dripping solution is completely removed, wet 5 mL of EQU around the edges.
4. (2^{nd} wash) After removing the filter, add 8 mL of wash buffer along the wall of the column.

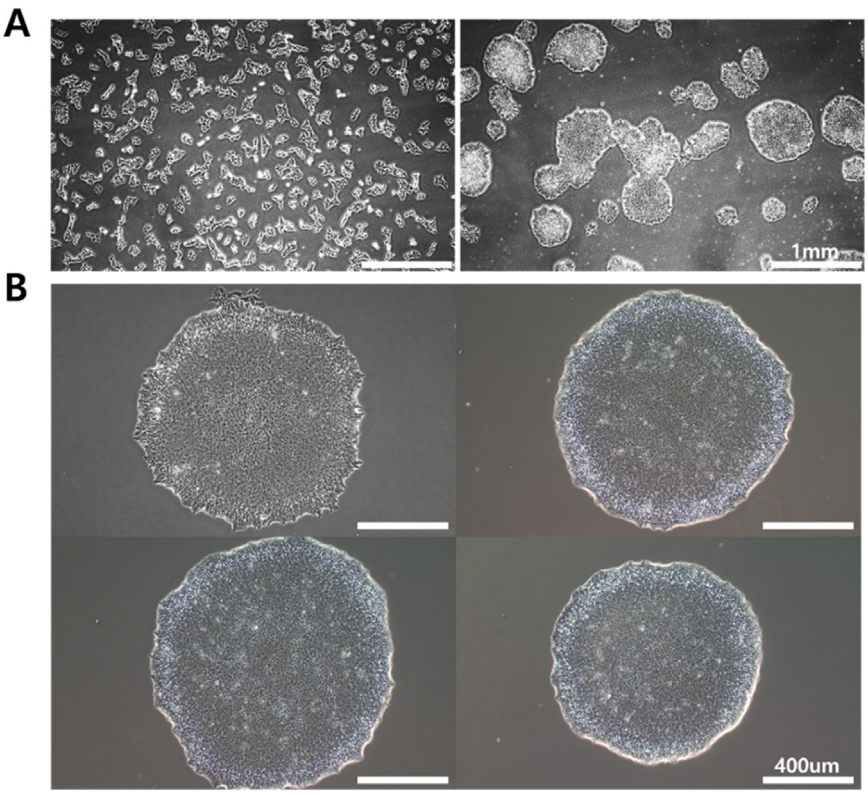

Fig. 2. hESC morphology before and after puromycin selection. (A) (left) hESC without puromycin selection after 1 day of transfection, (right) hESC that survived 7 days after puromycin selection and formed a colony; Scale bar: 1 mm. (B) Clone picked and maintained in 35 mm dishes after puromycin selection for 2 – 3 weeks; Scale bar: 400 μm.

5. Support the 50 mL tube on the column and add 5 mL of ELU to the column. Add 3.5 mL of isopropanol to a 50 mL tube and mix well via vortex. Weigh on a scale, with UPW as a balance, and then perform centrifugation with a High-Speed Centrifuge at 12,000 rpm for 30 min at 4 °C.
6. Mark the position of the pellet and discard the supernatant. Add 2 mL of 70 % EtOH and vortex to ensure 70 % EtOH directly disrupts the pellet. Weigh on a scale, with 70 % EtOH as a balance. Perform centrifugation with a High-Speed Centrifuge at 12,000 rpm for 5 min at RT.
7. Discard the supernatant and dry for 10 – 15 min at RT. Add 200 μL of UPW and vortex to ensure UPW directly disrupts the pellet. Spin down at 1000 rpm for 30 sec - 1 min. Collect solution, transfer to an autoclaved EP tube, and store at -20 °C.

• Generation of the αMHC-EGFP Knock-in reporter hPSC line

The αMHC-EGFP Knock-in reporter hPSC line is established using the CRISPR/Cas9-mediated KI technology to monitor mature human cardiomyocyte. To construct this line, prepare sgRNA plasmid, donor plasmid, and CRISPR/Cas9 plasmid, and perform electroporation, puromycin selection (Fig. 2 A), and single-cell clone generation using hESCs (Fig. 2 B).

STEP 1: hESC preparation

Materials

- Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Gibco, A14700)
- DPBS (Hyclone, SH30028.02)

  Note 1: Before VTN is used, thaw for 20 – 24 h at 4 °C, create 200 μL aliquots, and store at -20 °C.
  Note 2: To perform VTN coating, thaw VTN for 2 – 3 h at 4 °C and dilute 1:100 with DPBS. Dispense 1 mL of the VTN solution into a 6-well plate or 35 mm dishes and incubate for 30 min - 1 h at RT before use (Coat at 1 mL/well for a 6-well plate, 0.5 mL/well for a 12-well plate, and 0.25 mL/well for a 24-well plate). Cover with foil and store at 4 °C if not used immediately.

- Essential 8 Basal Med. DMEM/F12 (Gibco, A15169–01)
- Essential 8 Supplement (50X) (Gibco, A15171–01)

- Penicillin/Streptomycin (Gibco, Cat. 15140–122)

  Note: Divide Essential 8 Basal Medium + Essential 8 Supplement (50X) + 1 % Penicillin/Streptomycin into 50 mL portions in 50 mL conical tubes and store at 4 ˚C. Penicillin/Streptomycin is not added to the E8 medium for transformation. The medium should be left for 30 min - 1 h at RT before use.

- Accutase (Merck Millipore, SCR005)

  Note: Thaw accutase for 20 – 24 h at 4 ˚C, then divide into 10 mL aliquots and store at -20 ˚C.

- Trypan Blue Stain (0.4 %) (Gibco, Cat. 15250–061)
- Y-27632 (hydrochloride) (Cayman Chemical, Cat. 988000)

  Note: Dilute to 100 mM in DMSO then 10 mM in DPBS.

- Neon^{TM} Transfection System 100 μL Kit (Thermo Fisher Scientific, MPK10096)

Methods

1. 1 μg sgRNA plasmid +1 μg CRISPR/Cas9 Plasmid, either 2 μg or 3 μg of donor plasmid should be used. Prepare the sample in an autoclaved EP tube.
2. Remove the coating matrix from the VTN-coated 6-well plate, add 2 mL of E8 medium (with Essential 8 supplement, without Penicillin/Streptomycin) + Y-27632 10 μM and incubate at 37 ˚C, 5 % CO_2.
3. hESCs are maintained in Essential 8 Medium. Perform the passages in 35 mm culture dishes at a ratio of 1:20 every 4 – 5 days using 1X accutase. When the confluence of hESC reaches 70 - 80 %, suction the medium.
4. Wash the cells once with DPBS, add 0.5 mL accutase, and incubate for 3 – 5 min at 37 ˚C. Add 1.5 mL of DPBS, collect the cells in a 15 mL conical tube by pipetting, and count the cells using a hemocytometer.
5. Count the cells using trypan blue and prepare the appropriate number of cells; $1x10^5$ cells are required per sample. Centrifuge the cells collected in a 15 mL conical tube at 850 rpm for 3 min. Remove the supernatant and add R buffer. After resuspension, add 120 μL to the prepared plasmid.

   Note 1: For the number of cells, prepare one plate (N+1) more than the required amount (N).
   Note 2: For the R buffer, 110 μL is required per plate. N+1 should be prepared.

STEP 2: Neon^{TM} transfection system preparation

Materials

- Neon^{TM} Transfection System 100 μL Kit (Thermo Fisher Scientific, MPK10096)
- Neon^{TM} Transfection System (Thermo Fisher Scientific, MPK5000)

Methods

1. Load the Neon^{TM} Tube into the Neon^{TM} Pipette Station and add 4 mL of E2 buffer. Use the Neon^{TM} Pipette and Neon^{TM} Tip, mounted on the Neon^{TM} Pipette Station, to perform electroporation (Voltage: 850 V, Width: 30 ms, Pulse: 1 or 2).
2. After electroporation, transfer to a pre-incubated VTN-coated 6-well plate. Incubate the plate in a 37 ˚C, 5 % CO_2 incubator for 48 h. After 48 h of electroporation, replace the medium in each well with E8 medium (with Essential 8 supplement, Penicillin/Streptomycin) every day.

   Note 1: Avoid creating bubbles in the Neon^{TM} Tip.
   Note 2: Gently shake the plate forward, backward, left, and right to spread the cells evenly. Avoid spreading in circles as cells will aggregate in the center or at the edges of the well.

STEP 3: Puromycin Selection and Collection of the Surviving Clones

Materials

- Puromycin, Solution (10 mg/mL) (InvivoGen, ant-pr-1)

Methods

1. Continue cell culture for 1 – 2 days until the cell density reaches 50 – 60 %, and then replace medium with 2 mL of E8 medium (with Essential 8 supplement, Penicillin/Streptomycin) + puromycin.

   Note 1: Proceed with selection by gradually increasing the puromycin concentration (minimum 0.05 μg/mL, maximum 0.2 μg/mL). Some differentiated colonies will form that should be removed.
   Note 2: Puromycin selection is maintained for 7 – 14 days.
   Note 3: Increased apoptosis occurs 7 days after the initiation of puromycin selection and surviving colonies appear.

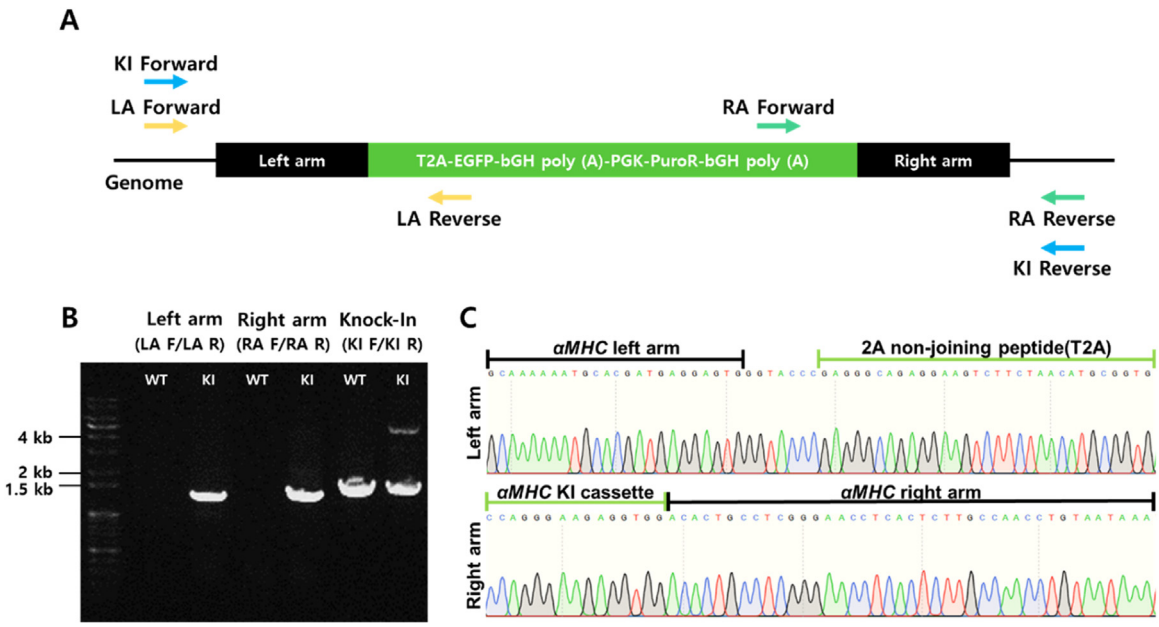

Fig. 3. Confirmation of the targeted allele of the clone. (A) KI Forward and KI Reverse confirm the entire size from the outer area of the cassette. Left arm junction and right arm junction were verified using each LA primer and RA primer. (B) Genomic PCR bands for the untargeted and targeted loci confirmed that the clone was heterozygous. (C) The PCR product of the targeted allele of the clone confirmed through DNA sequencing.

STEP 4: Single-cell clone generation

Materials

- Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Gibco, A14700)
- DPBS (Hyclone, SH30028.02)
- Essential 8 Basal Med. DMEM / F12 (Gibco, A15169–01)
- Essential 8 Supplement (50X) Gibco, A15171–01)
- Penicillin/Streptomycin (Gibco, Cat. 15140–122)
- Accutase (Merck Millipore, SCR005)
- Y-27632 (hydrochloride) (Cayman Chemical, Cat. 988000)
- Puromycin, Solution (10 mg/mL) (InvivoGen, ant-pr-1)

Methods

1. Passage single colonies using a mechanical method. Create dissecting pipettes with a Pasteur pipette, Use the prepared dissecting pipette to create clumps of colonies and transfer to a VTN-coated 35 mm dishes.
2. Place the dish in a 37 ˚C, 5 % CO_2 incubator. Treat with puromycin from 2 days after passage. After puromycin selection is complete, add 0.5 mL of 1X accutase to each well and incubate for 3 – 5 min at 37 ˚C.
3. Add 1.5 mL of DPBS and collect the cells in a 15 mL conical tube by pipetting. Centrifuge the cells collected in the 15 mL conical tube at 850 rpm for 3 min. Remove the supernatant and transfer the cells to a VTN-coated 35 mm dishes.
4. Place the dish in a 37 ˚C, 5 % CO_2 incubator. After 2 days, change the medium. When the cell density reaches ~80 %, perform PCR, sequencing, maintenance, and storage of each clone.

• Validation of the αMHC-EGFP reporter hES cell line

The primer for screening the αMHC-EGFP clones are shown in Fig. 3 A. The genomic DNA was checked with the primers produced to confirm effective insertion of the desired gene into the surviving clone (Fig. 3 B). Upon differentiation into cardiomyocytes, EGFP expression was confirmed using a fluorescence microscope (Fig. 4 A), Flow cytometry (Fig. 4 B), and immunostaining (Fig. 4 C).

STEP 1: PCR and sequencing

Materials

- Phusion High-Fidelity DNA Polymerase (Thermo scientific molecular biology, F-530 L)

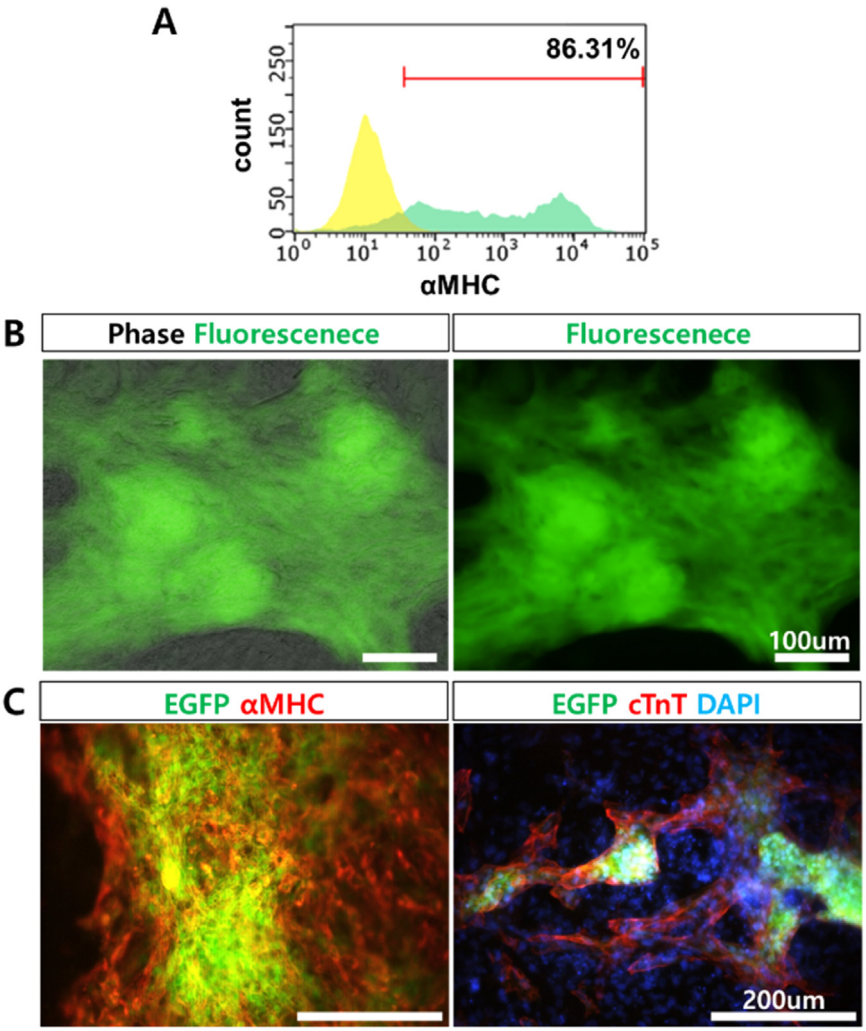

Fig. 4. Confirmation of EGFP expression after differentiation into cardiomyocytes. (A) Percentage of the EGFP population at day 15 of cardiac differentiation based on flow cytometry, (B) Verification of EGFP expression in cardiomyocytes; Scale bar: 100 μm. (C) Immunostaining results verifying that the EGFP co-expressed *α*MHC and CTNT; Scale bar: 200 μm.

Methods

1. Extract gDNA (QIAGEN, DNeasy blood & tissue kit) from the selected clones. Dilute the extracted gDNA to a concentration of 100 ng/μL using UPW. Perform PCR using phusion polymerase. Prepare the reaction mix (20 μl total) using 2 μl 10X HF buffer, 0.2 μl Phusion polymerase, 0.4 μl dNTP (10 mM), 2 μl forward primer (5 μM), 2 μl reverse primer (5 μM), 200 ng extracted DNA, and UPW.
2. Use the following cycling conditions for PCR: pre-denaturation, 98 °C for 1 min; cycling protocol (30 cycles): denaturation, 98 °C for 20 sec; extension 72 °C for 2 min; and final extension, 72 °C for 3 min.
3. Check the band via 1 % agarose gel electrophoresis. For clones with a confirmed band, perform sequencing using the primers used for PCR.

   Note: Following addition of the secondary antibody, perform subsequent steps in the dark.

STEP 2: Cardiomyocyte differentiation

Materials

- Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Gibco, A14700)
- DPBS (Hyclone, SH30028.02)
- Essential 8 Basal Med. DMEM / F12 (Gibco, A15169–01)

- Essential 8 Supplement (50X) (Gibco, A15171–01)
- Penicillin/Streptomycin (Gibco, Cat. 15140–122)
- Accutase (Merck Millipore, SCR005)
- Y-27,632 (hydrochloride) (Cayman Chemical, Cat. 988000)
- RPMI-1640 (Sigma, R8758)
- B-27 Supplement, Serum Free (50X) (Gibco, Cat. 17504–044)

  Note: Differentiation medium for cardiomyocyte comprises RPMI-1640 + B-27 Supplement, Serum Free (50X) + 1 % Penicillin/Streptomycin

- CHIR99021 (StemMACS, Cat. 10005583)

  Note: Dilute to 10 mM in DMSO.

- IWR-1-endo (STEMCELL, Cat. 72562)

  Note: Dilute to 5 mM in DMSO

- Fluorescence microscopy (OLYMPUS, IX73)

Methods

(single) Use 2 mL of E8 medium (with Essential 8 supplement, Penicillin/Streptomycin) and replace medium daily. Subculture at 1:40 using accutase when the density is 80 – 90 %.

(colony) On day 3 after the subculture of single cells, mechanically transfer approximately 60 colonies using a glass Pasteur pipette.

1. From the second day after the mechanical transfer, use 2 mL of E8 medium (with Essential 8 supplement, Penicillin/Streptomycin) and replace medium daily for 6 – 7 days. Remove the existing E8 media (with Essential 8 supplement, Penicillin/Streptomycin) and wash with 1 mL of DPBS.
2. Add 2 mL of RPMI-1640 (with B-27 supplement, Penicillin/Streptomycin) and CHIR99021 6 μM, and incubate for 2 days. Remove the old media and wash with 1 mL of DPBS. Add 2 mL of RPMI-1640 (with B-27 supplement, Penicillin/Streptomycin) followed by IWR-1 5 μM, and incubate for 2 days.
3. Remove the old media and wash with 1 mL of DPBS. Add 2 mL of RPMI-1640 (with B-27 supplement, Penicillin/Streptomycin) and incubate for approximately 10 days, replacing media every 2 days.
4. Confirm EGFP expression using a fluorescence microscope and FACS on day 14 after the start of differentiation.

STEP 3: Flow cytometry

Materials

- FETAL BOVINE SERUM CHARACTERIZED (Hyclone, SH30919.03)
- Sodium azide 0.1 M solution (Sigma, 08591–1ML-F)
- DPBS (Hyclone, SH30028.02)

  Note: Prepare FACS buffer that 2 % FBS, 0.1 % sodium azide in DPBS.

- TrypLE Express (Gibco, Cat. 12605–028)
- Guava® easyCyte™ Flow Cytometers (Luminex, Guava® easyCyte™ 5)

Methods

1. Remove the media of cultured αMHC KI ES and αMHC KI cardiomyocytes via suction. Add 1 mL of DPBS and remove via suction (repeat twice).
2. Add 500 μL of TrypLE and incubate for 3 min at 37 ˚C. Add 1.5 mL of DPBS and collect the cells in a 15 mL conical tube using a pipette (total: 2 mL). Perform centrifugation at 1,000 rpm for 5 min. Remove the supernatant.
3. Add 500 μL of FACS buffer, resuspend, and transfer to an EP tube.
4. Analyze using the Guava® easyCyte™ Flow Cytometer.

   Note 1: As pellet are easy to loosen, remove using pipette.
   Note 2: Adjust the FACS buffer volume according to the cell density.
   Note 3: Samples are loaded after resuspending with a pipette immediately before measurement. Avoid creating air bubbles.

STEP 4: Immunostaining

Materials

- 20X PBS Buffer (Biosesang, PR2007–100–00)

Note: Dilute to 1:20 in UPW and sterilize via autoclave (1X PBS).

- 4 % Paraformaldehyde (Biosesang, PC2031–100–00)
- Bovine Serum Albumin Fraction V IgG Free (Gibco, Cat. 30063–572)

Note: Dilute to 2 % in 1X PBS

- TWEEN 20 (Sigma, P7949)

Note: Dilute to 0.1 % in 1X PBS

- Triton X-100 (Merck Millipore, Cat. 1.12298.1001)

Note: Dilute to 0.1 % in 1X PBS

- αMHC (MYH6) (R&D Systems, MAB8979)
- Cardiac Troponin T Antibody (13–11) (Invitrogen, MA5–12,960)
- Alexa Fluor 594 donkey anti-mouse IgG (H+L) (Thermo Fisher Scientific, A21203)

Note: When used, dilute the primary antibody to 1:200 and the secondary antibody to 1:1000 in 2 % Bovine Serum Albumin.

- VECTASHIELD PLUS antifade Mounting Medium with DAPI (Vector Laboratories, H-2000)
- Confocal (OLYMPUS, FV-3000)

Methods

1. Dispense 500 μL of culture medium per well into a 4-well plate and seed at 1 – 1.5x10^4 cells/well for incubation (37 ˚C, 5 % CO_2).
2. After 32 – 36 h, remove culture medium and wash with 1X PBS 500 μL (repeat thrice). Add 4 % paraformaldehyde 500 μL and incubate for 10 min at 37 ˚C. Remove the paraformaldehyde solution, add 1X PBS 500 μL, wait 3 min, and remove (repeat thrice).
3. Add 0.1 % Triton X-100 400 μL and incubate for 15 min at RT. Remove the Triton X-100 solution, add 1X PBS 500 μL, wait 3 min, and remove (repeat thrice).
4. Add 300 μL of 2 % BSA and incubate for 60 min at RT. Dilute the primary antibody to the desired concentration in 200 μL of 2 % BSA and incubate cells overnight with the diluted antibody at 4 ˚C. Remove the primary antibody solution, add 1X PBS 500 μL, wait 3 min, and remove (repeat thrice).
5. Dilute the fluorescent labeled secondary antibody to the desired concentration in 2 % BSA 500 μL and incubate cells with the diluted antibody for 1 – 2 h at RT. Add 0.1 % Tween-20 500 μL, wait 3 min, and remove (repeat thrice).
6. Dry the cover slide and add the mounting medium (with nuclear stain (DAPI)). Seal the cover slide and check the target antigen using a fluorescence microscope.

• Troubleshooting

Generation of the αMHC-EGFP Knock-in reporter hPSC line		
STEP	Problem	Solution
STEP 2: Neon™ transfection system preparation	Cells do not attach and death occurs after electroporation.	Confirm the electroporation program is optimized for your cells and is suitable for the culture conditions. Ensure sufficient VTN coating for at least an hour before tranfection.
STEP 3: Puromycin selection and collection of the surviving clones	Cells do not die after puromycin selection.	In our case, rapid cell death appeared 4 – 5 days after puromycin treatment. If the cells do not die after that period, gradually increase the concentration of antibiotics.
Validation of the αMHC-EGFP reporter hPSC line		
STEP 1: PCR and sequencing	No band appears, or a non-specific band appears in PCR verification	Optimize the PCR program by adjusting the annealing temperature and time. If performance does not improve, try designing the primer under different conditions.
	Sequencing failure.	Amplify more PCR products and use gel extraction or PCR purification to derive a sample with improved purity.
STEP 2: Cardiomyocyte differentiation	Fluorescence is not observed when the validated clone is differentiated.	Ensure that the termination codon of the target gene is lost on the used donor. If there is no problem with termination codon, confirm that all frames are correct. The target gene may have been properly inserted in the desired location for other reasons such as being off-target. In this case, select another clone among the picked clones for which knock-in has been verified.
STEP 4: Immunostaining	A lack of a clean fluorescent signal	Please purchase another antibody and try again. Check the origin of the primary antibody and ensure that the secondary antibody is compatible. Increase the blocking time by up to 2 h. If the signal does not improve, increase the washing time to 5 min and try again. If the target protein is clean, but the DAPI is not, check to ensure cells are mycoplasma negative.

Ethics statement

Approval was granted by the Institutional Review Board (IRB) of Seoul National University, MRC, IRMP (Approval no. 219,932–201,904–04–01–01)

CRediT author statement

A-Hyeon Kim: Conceptualization, Validation, Data curation, Writing-Original draft; Ha Myoung Lee: Conceptualization, Validation, Data curation; Hong-Sik Kim: Validation, Data curation; Seong Woo Jeong: Validation, Data curation; Jong Kwan Jun: Writing – Reviewing and editing; Jiho Jang: Writing – Reviewing and editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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