博　　士　　論　　文

題目：人工多能性幹細胞（iPSCs）由来心筋細胞の生体内における分化
（In vivo differentiation of induced pluri-potent stem cell-derived cardiomyocytes）

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目次

博士論文の内容要旨

〔題　　名〕人工多能性幹細胞（iPSCs）由来心筋細胞
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（In vivo differentiation of induced pluripotent
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〔背　　景〕心不全は致死的な病態であり、薬物や人工臓器による治療では限界があり、移植治療もドナー不足から多数例に施行することは難しい。新しい治療法として、再生医療が注目を集め、骨髄細胞、筋芽細胞を用いた細胞治療が臨床応用され、一定の効果は得られたが不十分で、最大の問題点として心筋細胞への分化が認められないことが報告された。近年、iPS（induced pluripotent stem）細胞の研究の進歩とともに、in vitro で心筋細胞への分化も可能となったが、生体に移植された心筋細胞の構造・機能の詳細は明らかにされていない。今まで iPS 細胞由来心筋細胞の心筋転写因子、構造蛋白、収縮蛋白などの発現や局在は報告されたが、微細構造や特に生体に移植した際、ホスト心筋細胞外基質への接着に必要な接着分子α-dystroglycan、α-sarcoglycan、laminin-α2 などの発現、および iPS 細胞由来心筋細胞成熟化の原因がほとんど報告されなかった。

〔目　　的〕iPS 細胞由来心筋細胞が心筋細胞特異的な収縮蛋白、構造蛋白などをその細胞内に局在性を持って発現しており、ミトコンドリアを初めとする細胞内小器官が心筋と同様の構造、機能を有していて、生体に移植した際にホスト心筋細胞外基質に接着しうる接着分子を発現していることを仮説とし、これらのことを分子病理学的に検討した。

〔方　　法〕6 週齢の F344/NJcl ヌードラットの心臓に、分化 16 日目の iPS 細胞由来心筋細胞シートを移植し、2 週間後に移植した細胞シートを取り出して、免疫蛍光染色法及び電子顕微鏡法などによって解析を行った。iPS 細胞から心筋細胞への分化は無血清で Insulin-Transferrin-Selenium-X の添加によって行った。

〔 結　　果 〕iPS 細胞から作成された心筋細胞（iPSC-CM）は胚様体（EB）形成 12 日目頃から、心筋の最大の特徴である自律的収縮が観察され、16 日目に 7 割の EB に拍動が認められた。心筋への分化過程において心筋特異的転写因子 GATA4、Nkx2.5 が早期から強く発現し、構造蛋白 MLC2a、収縮蛋白 cTnT の発現は徐々に増加した。

免疫染色にて iPSC-CM には胎児心筋細胞と同様に骨格蛋白 MHC と胎児型心筋マーカーβMHC が強く発現し、成体型心筋マーカーαMHC はほとんど発現が見られなかった。電子顕微鏡では、心筋に特徴的とされる横紋構造、Z 盤、多数のミトコンドリアなども観察されたが、胎児心筋細胞と比較して、ミトコンドリアのクリスタは疎であり、量的にも少ない傾向が見られた。

iPSC-CM は細胞外基質への接着に必要な接着分子 N-cadherin、α7-integrin、dystrophin、α-dystroglycan、α-sarcoglycan、laminin-α2 が強く発現し、それを反映してラット心への移植に際して良好な生着を示した。しかも、生着した iPSC-CM には in vitro で認められなかったαMHC の高発現が認められ、胎児型心筋細胞から成熟心筋細胞への転換が起こったと考えられた。また、規則的な細胞内局在の見られなかったギャップ結合タンパク connexin43 も in vivo では心筋細胞の介在板に発現していた。これらの形態学的変化は in vitro における増殖因子の刺激だけでは見られなかったので、心筋細胞が生体環境で stretch されることにより誘導された可能性が推測された。そこで、in vitro で iPSC-CM に対して、種々の増殖因子や機械的な進展刺激を与えた所、IGF1 添加と機械的伸展刺激によって成熟心筋細胞への分化が誘導される結果が得られた。

〔 考　　察 〕iPSC-CM は胎児型心筋の phenotype を有しており、dystrophin-dystroglycan complex 系の接着分子を介して生体心臓に生着する可能性が考えられた。in vivo に移植すると iPSC-CM は胎児型から成熟型心筋に分化し、connexin43 の発現パターンも変化していた。これらの in vivo における分化は生体環境における収縮伸展による物理的刺激がトリガーになっている可能性が考えられた。以上より、iPSC-CM は生体心臓へ移植すると成熟型心筋に分化ことから、将来、再生医療に応用できる可能性が示唆された。

【背景】

Despite medical advances, end-stage heart failure is still a life-threatening disorder (1,2). New treatments for ischemic heart disease, including thrombolytic agents and percutaneous angioplasty, have not been proven to replace scar tissue with functional contractile cardiomyocytes, and it is still difficult to improve the quality of life or prognosis for patients with end-stage heart failure (3-5). Cell therapy has been introduced as a promising new treatment. The initial clinical applications, involving myoblast or bone marrow cell transplants, modestly improved cardiac performance through (mainly) paracrine effects rather than through the differentiation of transplanted cells into functional cardiomyocytes (6-8). To contribute directly to cardiac function, somatic tissue-derived stem or progenitor cells, such as c-kit+, Sca-1+, and mesenchymal stem cells, have been used to treat heart failure. However, these cells were also found to improve cardiac function through paracrine effects; cardiomyocyte-specific markers have not been detected in most implanted cell populations (9,10).

Research into "true" cardiomyocytes, which have specific molecular phenotypes and are contractile both in vitro and in vivo, has generated a great deal of

enthusiasm. Experimentally developed embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are expected to provide a basis for new cell therapies (11-13). Cardiomyocytes differentiated from these stem cells not only contract in vitro, but also express specific proteins appropriately. These cardiomyocytes are considered to have good in vivo transplantation potential for both survival and function (14,15). However, no studies have demonstrated that these cells have the necessary molecular patterns and microstructures, such as the cardiomyocyte-specific structural proteins or mitochondria found in differentiated cardiomyocytes, or that they have the ability to attach to the damaged heart after heterogeneous implantation.

The physiological stimulus of mechanical stretch is thought to be required for cardiac development and growth, and for the regular arrangement of cardiomyocytes that creates effective heart contractions (16-19). Cells in the cardiovascular system are constantly subjected to mechanical forces from the pulse of blood flow and shear stress in the beating heart. Mechanical stretch modulates growth, apoptosis, electric remodeling, autocrine and paracrine effects, and alterations in gene expression in cardiac myocytes (20). IGF-1 and

other growth factors are also important for cardiac development, maturation, and survival (21,22).

In this study, we hypothesized that cardiomyocytes derived from iPS cells (iPSC-CMs) might contain sufficient cardiac-specific microstructures to restore the damaged heart after in vivo implantation, as well as adhesive systems able to attach to and function in extracellular matrices after heterogeneous implantation into the heart. We studied phenotypic changes in transplanted iPSC-CMs in vivo, as well as the effect of growth factor stimulation or mechanical stretch on these cells in vitro.

【材料と方法】

Cell culture

Cardiomyocytes derived from the germline-competent mouse iPSC line 20D-17 and 256H18 (23,24) (generously contributed by Professor S Yamanaka, Kyoto University, Japan). The 20D-17 cell line was carrying a Nanog promoter-driven green fluorescent protein (GFP), internal ribosome entry site (IRES), and puromycin-resistance gene (Nanog-iPS cells), were maintained as previously described. The 256H18 cell line was established by introducing only Oct3/4, Sox2, and Klf4 (without c-Myc), expressed red fluorescent protein (DsRed) constitutively. The iPSCs were cultured and maintained on feeder layers of mitomycin C-treated mouse embryonic fibroblasts (MEFs, Chemicon, Billerica, Massachusetts) in Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque, Kyoto, Japan) containing 15% fetal bovine serum (FBS, Biofill, Victoria, Australia), 100 μM non-essential amino acids (NEAA, Invitrogen, Tokyo, Japan), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 50 U/mL penicillin, 50 mg/mL streptomycin (Invitrogen), and 1000 U/mL recombinant leukemia inhibitory factor (LIF, Chemicon). The medium was replenished daily. MEFs were grown in in a 37°C incubator in 5% CO_2, in a medium containing

high-glucose DMEM (Nacalai Tesque) supplemented with 10% FBS, 1 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. The medium was replenished daily. As the MEFs reached confluence, they were exposed to 50 mg/mL mitomycin C (Wako, Osaka, Japan) for 2 hr and 15 min. They were then washed in phosphate- buffered saline (PBS), trypsinized (0.05% Trypsin-EDTA, Invitrogen), plated at 75,000 cells/cm^2 on gelatin-coated tissue culture dishes as a feeder layer, and incubated overnight before plating the iPS cells.

Differentiation of iPS cells to beating cardiomyocytes

The iPSCs were trypsinized to prepare a single-cell suspension, and counted. For myocardial differentiation, 500 iPS cells were resuspended in 30-μl aliquots of differentiation medium containing DM and a growth medium lacking leukemia inhibitory factor (LIF). Cells were cultured in 96-well HydroCell plates (CellSeed, Tokyo, Japan) for 2 days. On day 2, an additional 30 μl of DM containing 4 μM 6-bromoindirubin-3′-oxime (a glycogen synthase kinase-3β inhibitor) (BIO, Calbiochem, Darmstadt, Germany) was added to each well (25,26). On day 5, EBs (embryoid bodies) were plated individually on gelatin-coated dishes; on day 8, these were differentiated in serum-free Modified Eagle Medium (MEM,

Invitrogen) with insulin-transferrin-selenium-X (Invitrogen). From day 10 onward, contracting areas could be observed in the iPSC EBs.

iPSC-derived cardiomyocytes sheet formation

On day 5 (Figure 1A), EBs were plated on 12-wells temperature-responsive culture dishes (provided by Prof. Okano, Tokyo Women's Medical University) at 37°C with the EB numbers adjusted to 20 per well, thereafter on day 8 EBs were differentiated in the serum free Modified Eagle Medium (MEM, Invitrogen) with insulin-transferin-selenium-X (Invitrogen). From day 10, contracting areas could be observed in EBs of iPS cells. On day 19 the dishes were removed to refrigerator set at 20°C, a few minutes after iPSC-derived cardiomyocyte sheets detached spontaneously from the dish surfaces (27).

Neonatal mouse cardiac myocyte isolation

Hearts from 1-day-old female mice (C57BL/6JJcl) (Crea, Osaka, Japan) were excised and placed immediately into cold Hanks Balanced Salt Solution (HBSS). The blood vessels and atria were removed, and the remaining right and left ventricular tissues were minced, washed with HBSS, and subjected to

sequential digestion with 1% Collagenase type II (Invitrogen)/HBSS solution at 37°C for 10 min, with 4 repetitions. The fragments were then collected, filtered through a cell strainer, centrifuged at 3,000 rpm for 10 min, placed in 60 mm-diameter dishes, and cultured in DMEM/Ham's F-12 (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ/ml streptomycin.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) from mouse iPSC-derived EBs on days 6, 8, 12, and 19; cDNA was synthesized using the Super Script III First-strand Synthesis System (Invitrogen). PCR was performed with KOD-plus (Toyobo, Osaka, Japan). PCR conditions included denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 55°C for 1 min for 35 cycles, with a final extension at 72°C for 7 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primer sequences are listed in Table 1.

Table 1

Primers for RT-PCR

Genes	Direction	Sequences
GATA4	forward	CTC CAT GTC CCA GAC ATT CAG TAC T
	reverse	GAT TAT GTC CCC ATG ACT GTC AGC
Nkx2.5	forward	CAG TGG AGC TGG ACA AAG CC
	reverse	TAG CGA CGG TTC TGG AAC CA
cTroponin T	forward	GCG GAA GAG TGG GAA GAG ACA
	reverse	CCA CAG CTC CTT GGC CTT CT
MLC-2a	forward	CCC ATC AAC TTC ACC GTC TTC CT
	reverse	AGA GAA CTT GTC TGC CTG GGT CA
GAPDH	forward	AGT ATG ACT CCA CTC ACG GCA A
	reverse	TCT CGC TCC TGG AAG ATG GT

Western blotting

EBs at 6, 8, 12, and 19 days were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 10% glycerol, 0.1% SDS, 1.0% deoxycholic acid, 50 mM NaF, 10 mM $Na_3P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin. Sample proteins were separated on SDS-polyacrylamide gels (8% -12%) and transferred to nitrocellulose membranes. The membranes were blocked with milk for 1 hr, incubated overnight with primary antibody (Table 2-a), washed, incubated with secondary antibody (anti-rabbit goat IgG) (Dako, Tokyo, Japan) for one hr, and visualized using chemiluminescent substrate. Light emission was detected by autoradiography and quantified using an image-analysis system (Bio-Rad, Tokyo, Japan).

Table 2-a

Antibodies used for western blot studies

Antibody specificity	Source	Clonality	Dilution used
Anti-GATA4	Santa Cruz	Polyclonal	1:1000
Anti-sarcomeric α-actinin	Abcam	Polyclonal	1:2000
Anti-MHC	Abcam	Monoclonal	1:1000
Anti-connexin43	Sigma	Monoclonal	1:2000
Anti-α-sarcoglycan	Santa Cruz	Polyclonal	1:1000
Anti-N-cadherin	Abcam	Polyclonal	1:1000
Anti- GAPDH	Santa Cruz	Monoclonal	1:2000

Immunofluorescent staining

Neonatal or iPSC-derived cardiomyocytes were fixed with 4% paraformaldehyde at room temperature for 20 min, washed 3 times with PBS, permeabilized with 0.3% Triton X-100 (PBST, Nacalai Tesque) for 20 min, and blocked with 3% bovine serum albumin (BSA) in PBST for 1 hr. The samples were incubated overnight at 4°C with a primary antibody (Table 2-b). The secondary antibody, Alexa Fluor 488 anti-rabbit goat IgG (1:1000; Invitrogen) or Alexa Fluor 488 rabbit anti-rat IgG (1:1,000; Invitrogen), was applied to the sections for 1 hr at room temperature. To visualize the F-actin cytoskeleton, cells were stained using Alexa Fluor 594 phalloidin (1:40; Invitrogen). Nuclei were stained with DAPI (Invitrogen, 1:1000 dilution) for 5 min at room temperature, then triple-washed with PBS. One drop of anti-fade reagent (Invitrogen) was placed

on each slide, and coverslips were applied the cell surface down. Images of the stained cell samples were acquired with an FV1000D (Olympus, Tokyo, Japan).

Table 2-b
Antibodies used for immunostaining studies

Antibody specificity	Source	Clonality	Dilution used
Anti-MHC	Abcam	Monoclonal	1:100
Anti-αMHC	Abcam	Monoclonal	1:100
Anti-βMHC	Sigma	Monoclonal	1:100
Anti-MLC-2a	Synaptic Systems	Monoclonal	1:100
Anti-MLC-2v	Synaptic Systems	Monoclonal	1:100
Anti-N-cadherin	Abcam	Polyclonal	1:100
Anti-α7-integrin	Santa Cruz	Polyclonal	1:50
Anti-dystrophin	Santa Cruz	Polyclonal	1:50
Anti-α-dystroglycan	Millipore	Monoclonal	1:100
Anti-α-sarcoglycan	Leica	Monoclonal	1:100
Anti-laminin-α2	Thermo	Monoclonal	1:100
Anti-connexin43	Sigma	Monoclonal	1:100
Anti-SDHA	Cell Signaling	Polyclonal	1:50

Electron microscopy

Neonatal or iPSC-derived cardiomyocytes were fixed with 2% glutaraldehyde in 0.1 mM phosphate buffer (pH 7.4) at 4°C for 60 minutes, washed and immersed overnight in PBS at 4°C, and fixed in 1% buffered osmium tetroxide. Specimens were dehydrated through graded ethanol and embedded in epoxy resin. Ultrathin sections (85 nm) were double-stained with uranyl acetate and lead citrate, and were observed under electron microscopy (H-7600; Hitachi, Tokyo, Japan).

iPS-derived cardiomyocyte transplantation in vivo

Six-week-old female F344/NJcl-rnu/rnu rats were obtained from Japan Crea (Osaka, Japan) and maintained under specific pathogen-free conditions in the animal facilities at the Osaka University Graduate School of Medicine. A sheet of iPS-derived cardiomyocytes was transplanted to the surface of each rat's heart (n=6). Two weeks later, the hearts were excised, quickly frozen in liquid nitrogen-cooled isopentane, sectioned in 6-μm-thick slices in a cryostat, and analyzed by immunofluorescent staining as described above. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Osaka University Graduate School of Medicine before beginning this study.

Stretch or growth-factor induced differentiation of iPS cells to cardiomyocytes

On day 8, when iPSC-derived EBs began to differentiate in serum-free medium with insulin-transferin-selenium-X, we tested the effect of growth factor or mechanical stretch stimulation on cardiomyocyte differentiation. We added recombinant IGF1 (20 ng/ml) (Abcam, Tokyo, Japan), hepatocyte growth factor

(HGF, 10 ng/ml) (R&D Systems, Minneapolis, Minnesota), or growth hormone (GH, 20 ng/ml) (BioVision, Mountain View, California) to iPSC-derived EBs from day 8 to day 15 to enhance maturity in vitro.

Mechanical stretch was provided by an FX-4000 (Flexcell, Denver, Colorado) strain unit consisting of a vacuum unit linked to a computer-controlled valve (28,29). From day 8 to day 15, iPSC-CMs cultured on a flexible membrane base were subjected to cyclic stretch, with a sinusoidal negative pressure peaking at 15 kPa at a frequency of 1 Hz (60 cycles per minute) for varying amounts of time. On day 15, iPSC-CMs that had been stimulated by growth factors or mechanical stretch were analyzed for αMHC by immunofluorescent staining.

Statistical Analysis

All data were obtained from at least three independent experiments. All values are expressed as the mean ± standard deviation (SD). Statistical comparison of the data was performed using unpaired t-tests. A p-value of <0.05 was considered statistically significant.

【結果】

Figure 1

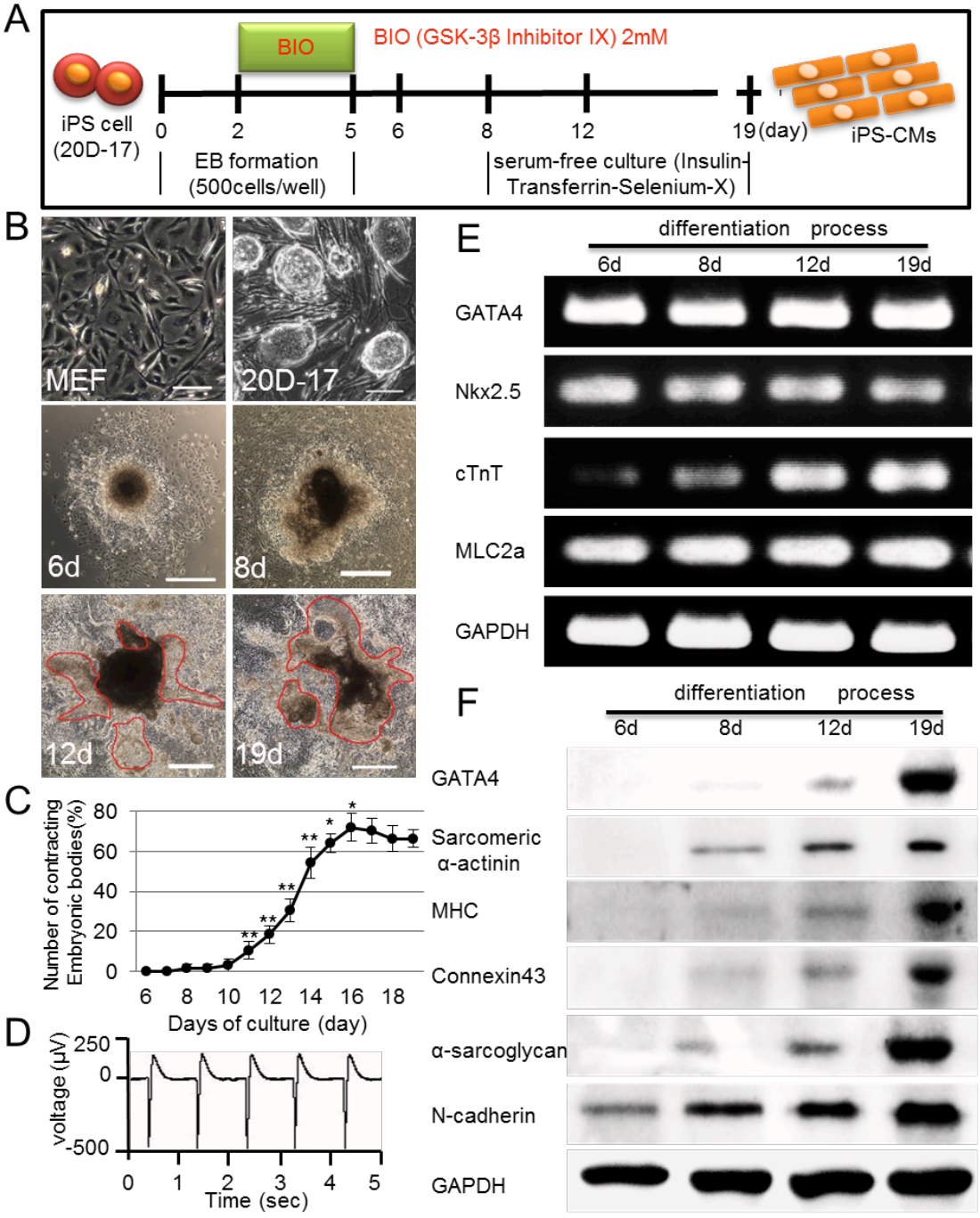
A
BIO
BIO (GSK-3β Inhibitor IX) 2mM
iPS cell
(20D-17)
0
2
5
6
8
12
19 (day)
iPS-CMs
EB formation
(500cells/well)
serum-free culture (Insulin-
Transferrin-Selenium-X)
B
MEF
20D-17
6d
8d
12d
19d
C
Number of contracting
Embryonic bodies(%)
Days of culture (day)
D
voltage (μV)
Time (sec)
E
differentiation process
6d
8d
12d
19d
GATA4
Nkx2.5
cTnT
MLC2a
GAPDH
F
differentiation process
6d
8d
12d
19d
GATA4
Sarcomeric
α-actinin
MHC
Connexin43
α-sarcoglycan
N-cadherin
GAPDH

Figure 1. Differentiation of iPSCs into cardiomyocytes.

(A) Protocol for in vitro differentiation of iPSCs into cardiomyocytes. (B) Serial morphological changes during iPSC differentiation: iPSCs (20D-17) on a feeder layer (MEFs) were observed as undifferentiated embryoid bodies (EBs) until day 5, when the EBs were individually placed on gelatin-coated dishes. On days 6 and 8, no beating areas were found. Under serum-free medium with insulin-transferrin-selenium-X starting on day 8, beating areas with differentiated cells (inside red line) were detected on days 12 and 19. Scale bars = 100 μm. (C) Percentage of spontaneously beating EBs with the differentiation process. (D) Field-potential recordings of re-plated beating iPSC-derived cardiomyocyte (iPSC-CM) colonies, measured with the MED 64 system. (E) RT-PCR analysis of cardiac marker gene expression (GATA4, Nkx2.5, Cardiac Troponin T, and MLC2a) during the differentiation of iPSCs into cardiomyocytes (days 6-19). GAPDH was used as a loading control. (F) Western blot analysis of the cardiac-specific proteins GATA4, sarcomeric α-actinin, MHC, connexin43, α-sarcoglycan, and N-cadherin from days 6 to 19. GAPDH was used as a loading control. * $p<0.05$, ** $p< 0.01$.

Myocardial differentiation of mouse iPS cells in vitro

In vitro myocardial differentiation of iPS cells was induced using an original protocol established in our laboratory (Figure 1A). Serial phenotypic changes in

iPS cells during the differentiation process were analyzed for spontaneous contraction and for the expression of cardiac-specific molecules such as GATA4, Nkx2.5, cardiac troponin (cTn) T, MLC2a, sarcomeric α-actinin, MHC, connexin43, α-sarcoglycan, or N-cadherin. The electrophysiology of terminally differentiated iPS cells in vitro was analyzed using a MED 64 system (Panasonic SU-MED640).

From day 10 onwards, some iPSC-derived EBs spontaneously began to contract in a regular, synchronous beating cycle under the culture conditions (Figure 1B). The number of contracting EBs gradually increased, reaching a plateau on day 16 (Figure 1C). Field-potential recordings of the re-plated beating colonies, measured by the MED 64 system, showed that the EBs generated regular beats at consistent voltages (Figure 1D). RT-PCR showed similar, high expression levels of GATA4, Nkx2.5, and MLC2a on days 6, 8, 12, and 19, whereas the cTnT expression increased over time (Figure 1E). In contrast, western blotting revealed that the GATA4, sarcomeric α-actinin, MHC, connexin43, α-sarcoglycan, and N-cadherin protein levels gradually increased during the differentiation process (Figure 1F).

Figure 2

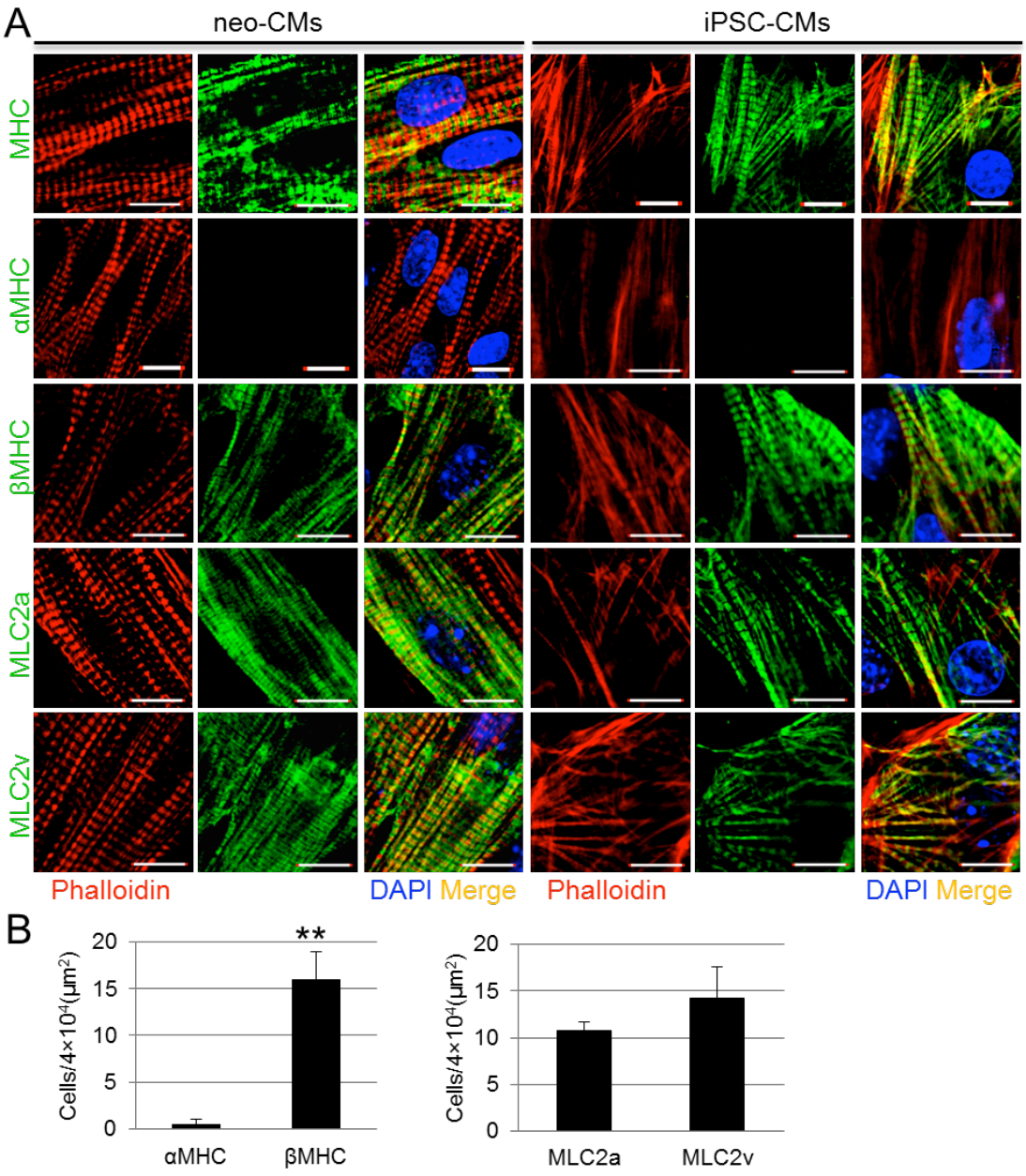

Figure 2. Cytoskeletal proteins in iPSC-CMs

(A) Immunofluorescent analysis of cytoskeletal proteins in neonatal cardiomyocytes (neo-CMs) and iPSC-derived CMs (iPSC-CMs). Cells were immunostained with the cytoskeletal proteins MHC, α-MHC (an adult cardiomyocyte marker), β-MHC (a neonatal

cardiomyocyte marker), MLC2a, and MLC2v in the sarcomeres (green). Actin was stained with phalloidin. Nuclei were stained with DAPI (blue). Scale bars=10μm. (B) The number of iPSC-CMs expressing α-MHC, β-MHC, MLC2a, or MLC2v. * $p<0.05$, ** $p< 0.01$.

Immunohistochemical analyses of iPSC-derived cardiomyocytes

Immunohistochemical analysis of cytoskeletal showed that the expression and distribution of the cardiac-specific protein MHC, the major contractile protein, was similar in iPSC-CMs and neo-CMs (Figure 2A). The MHC α-isoform, which is the adult cardiomyocyte marker, was rarely expressed by either iPSC-CMs or neo-CMs, but the β-isoform, which is the neonatal cardiomyocyte marker, was strongly expressed by both (Figures 2A, 2B). Neo-CMS and iPSC-CMs did not differ in their expression of myosin light chain (MLC)2a and MLC2v, which are atrial and the ventricular markers, respectively (Figures 2A, 2B).

Figure 3

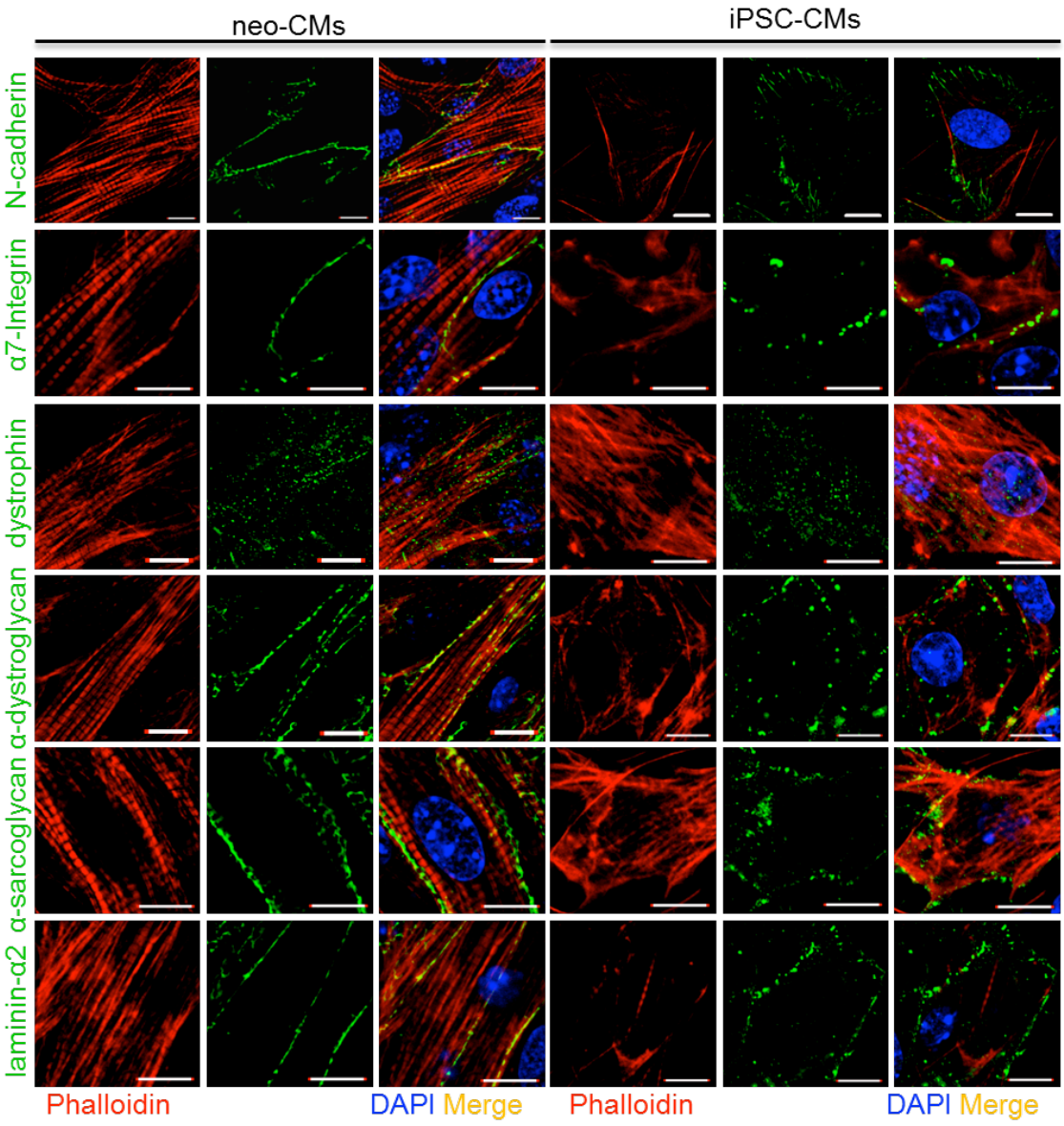

Figure 3. Detection of adhesion molecules in iPSC-CMs.

Immunofluorescent analysis of adhesion molecules in neo-CMs and iPSC--CMs. Cells were immunostained for the adhesion molecules N-cadherin, α7-integrin, dystrophin, α-dystroglycan, α-sarcoglycan, and laminin-α2 on the cell membrane (green). Actin was stained with phalloidin. Nuclei were stained with DAPI (blue). Scale bars=10μm.

Immunohistochemical analyses of iPSC-derived cardiomyocytes

Immunohistochemical analysis of adhesion molecules showed that the expression and distribution of the cardiac-specific protein N-cadherin, α7-integrin, α-dystroglycan, α-sarcoglycan, and laminin-α2 were expressed at lower levels on the iPSC-CM cell membrane than on the neo-CM membrane (Figure 3). The dystrophin distribution was similar in iPSC-CMs and neo-CMs (Figure 3).

Figure 4

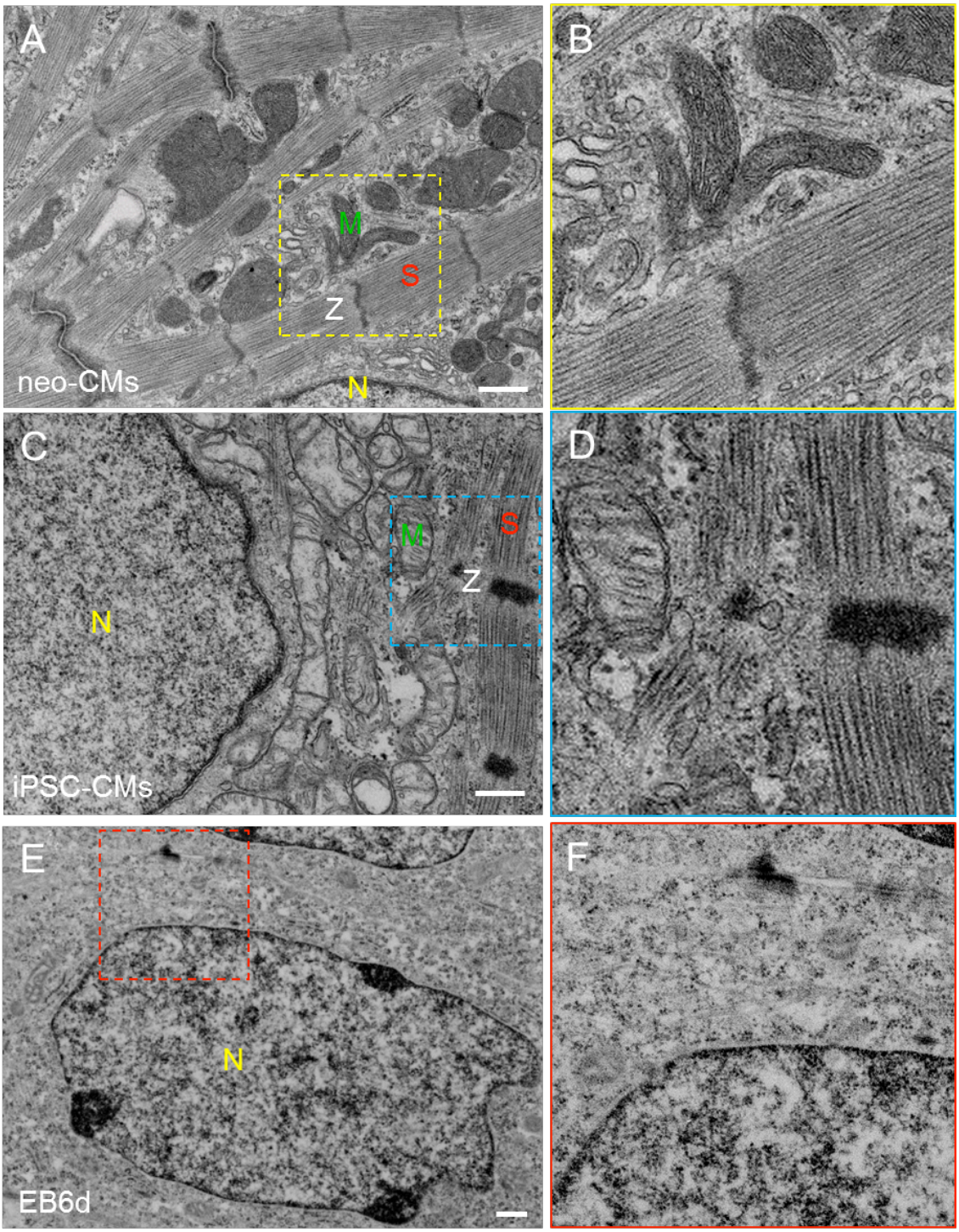

Figure 4. Ultrastructural analysis of iPSC-CMs.

Ultrastructure of neo-CMs (A, B) and iPSC-CMs (beating colonies); B shows a magnified view of A. (C, D) EBs on day 6. (E, F). M: mitochondria, S: sarcomere, Z: Z-bands, N: nucleus. Scale bar = 1 μm.

Ultrastructural analysis using transmission electron microscopy in vitro

We used transmission electron microscopy to compare the neo-CM ultrastructure with that of iPSC-CMs on day 19 and EBs on day 6. On day 19, the iPSC-CM ultrastructure resembled that of the neo-CM, with abundant myofibrillar bundles with transverse Z-bands and a developed mitochondrial structure. However, there were fewer mitochondria, with lower-density cristae, in the iPSC-CM than the neo-CM sarcomeric structure. There was no observable sarcomeric structure in the day-6 EBs (Figure 4).

Figure 5

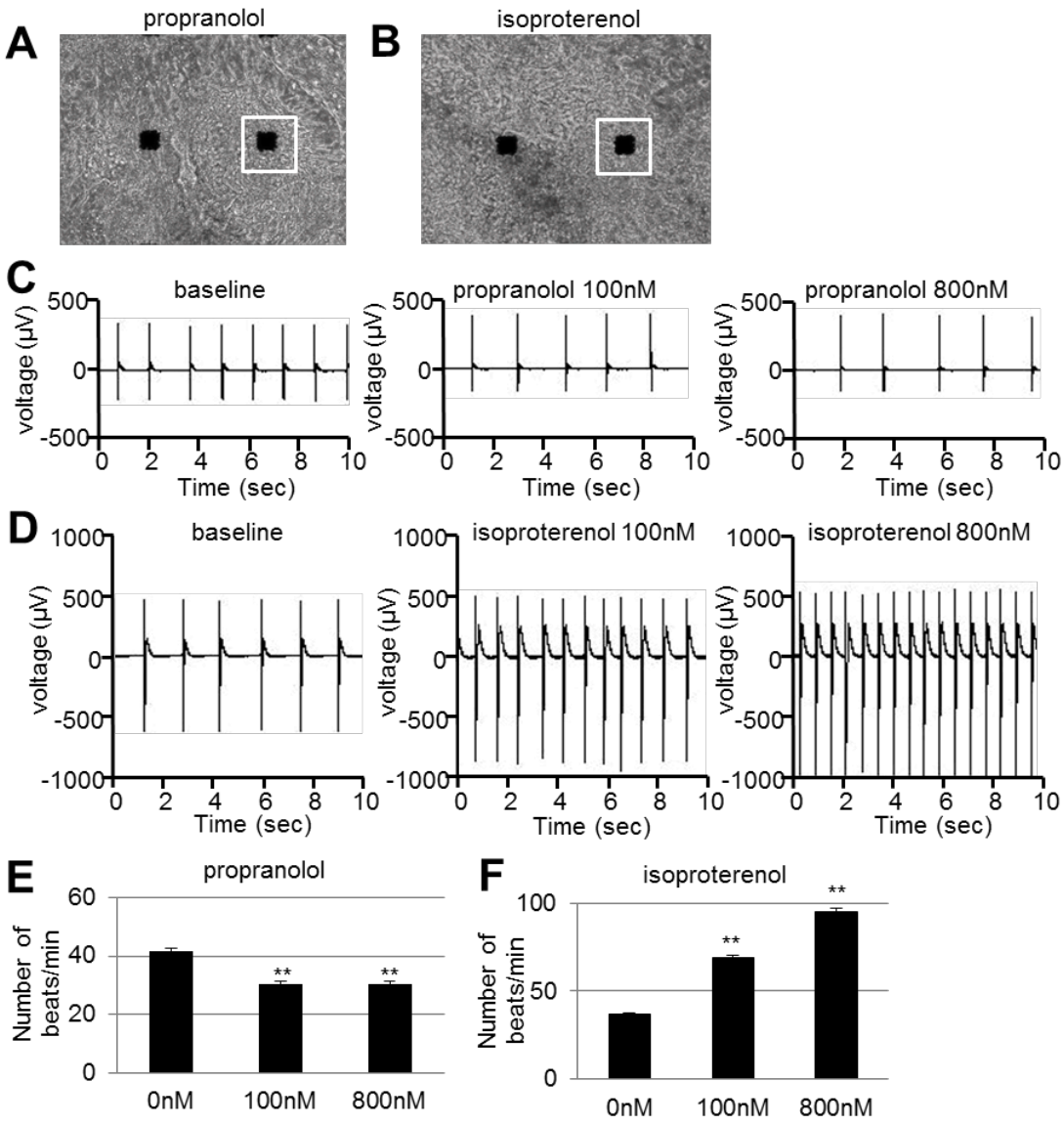

Figure5. The effects of cardioactive drugs on the beating rates of contractile colonies derived from iPS cells.

(A) Effect of propranolol on FP frequency. (B) Effect of isoproterenol on FP frequency. (C, E) The graph shows a summary of the changes in the beating frequency caused by

propranolol. (D, F) The graph shows a summary of the changes in the beating frequency caused by isoproterenol. *P<0.05, **P<0.01.

Electrophysiological studies in-vitro

Action potential of the iPSC-CMs was assessed in-vitro by using the MED 64 system (Panasonic SU-MED640). The contracting iPSC-CMs at day 19 were dispersed into the small clumps on the microelectrodes and then cultured for 3 days till full-confluence was achieved (Figure 5A, 5B). Spontaneous and induced beats of the iPSC-CMs were recorded. iPSC-CMs had spontaneous regular beats in a culture condition. Addition of propranolol decreased beat rate in a dose-dependent manner, though it did not reduce the voltage (Figure 5C, 5E) In contrast, addition of isoproterenol increased both beat rate and voltage in a dose-dependent manner (Figure 5D, 5F).

Figure 6

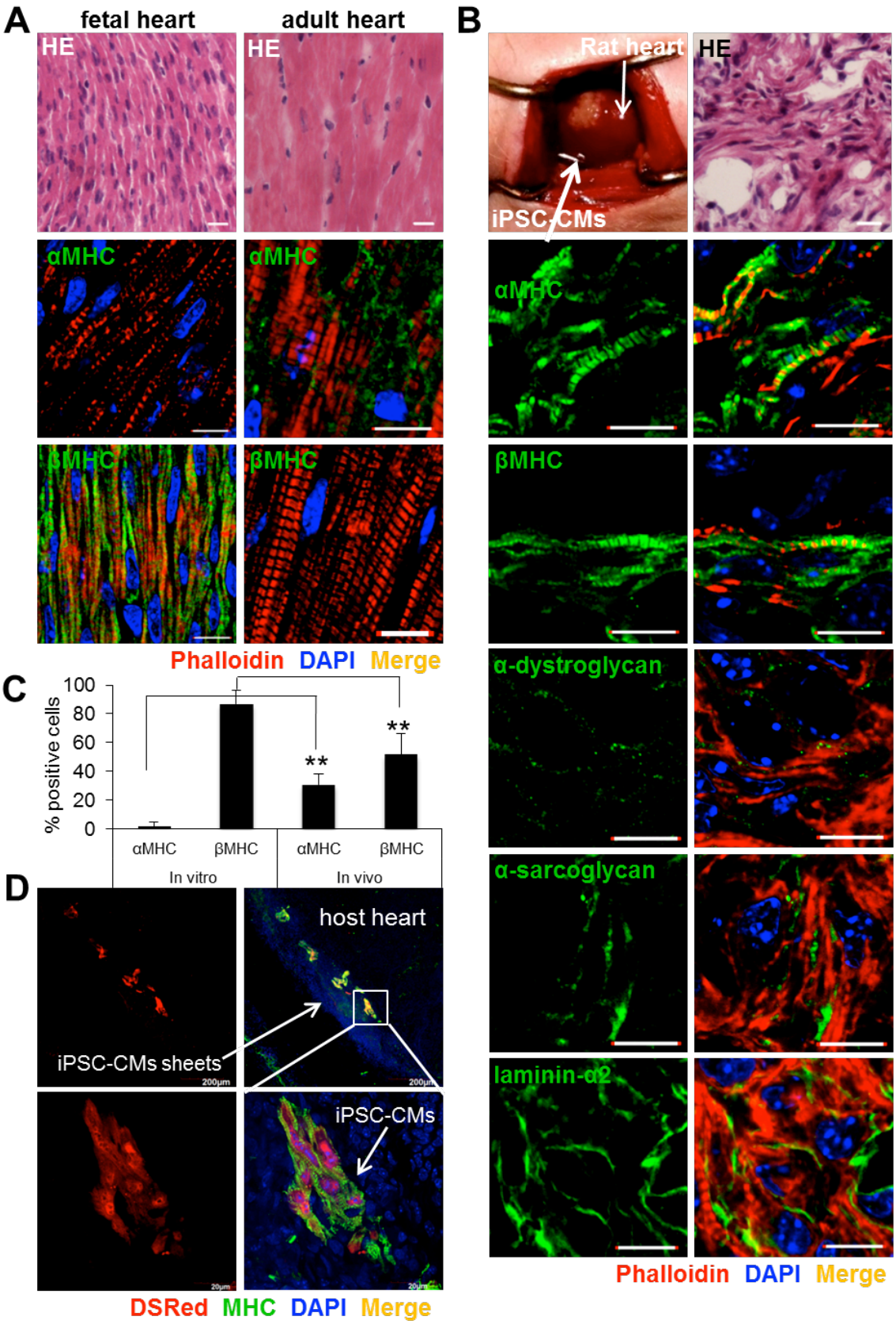
A
fetal heart
adult heart
HE
HE
αMHC
αMHC
βMHC
βMHC
Phalloidin DAPI Merge
B
Rat heart
iPSC-CMs
HE
αMHC
βMHC
α-dystroglycan
α-sarcoglycan
laminin-α2
Phalloidin DAPI Merge
C
% positive cells
100
80
60
40
20
0
**
**
αMHC
βMHC
αMHC
βMHC
In vitro
In vivo
D
host heart
iPSC-CMs sheets
iPSC-CMs
DSRed MHC DAPI Merge

Figure 6. Detection of cytoskeletal and adhesion molecules in transplanted iPSC-CMs.

(A) Expression of α-MHC and β-MHC in fetal and adult cardiomyocytes. (B) Immunohistochemical analysis of the cytoskeletal and adhesion molecules α-MHC, β-MHC, α-dystroglycan, α-sarcoglycan, and laminin-α2 (green) in transplanted iPSC-CMs. Actin was stained with phalloidin. Nuclei were stained with DAPI (blue). Scale bars=10μm. (C) The number of α-MHC-positive cells in cultured iPS-CMs (in vitro) or transplanted iPS-CMs (in vivo). (D) DsRed was expressing in iPS-derived cardiomyocytes. iPSC-CMs were immunostained with the cytoskeletal proteins MHC (green). Nuclei were stained with DAPI (blue). * $p<0.05$, ** $p< 0.01$.

Phenotypic changes in iPSC-derived cardiomyocytes transplanted in vivo

Cell sheets of iPSC-CMs were transplanted onto an adult nude rat heart. After 2 weeks, we used immunohistochemistry to analyze the expression of cytoskeletal and adhesion molecules in iPSC-CMs compared to that in fetal or adult murine hearts.

The transplanted iPSC-CMs expressed and distributed αMHC and βMHC at similar levels diffusely, while αMHC or βMHC was more predominant in the adult and fetal heart, respectively (Figure 6A). Furthermore, αMHC was much more

strongly expressed in cardiomyocytes transplanted in vivo than in cardiomyocytes cultured in vitro (Figures 6B, 6C). The transplanted iPSC-CMs expressed adhesion molecules such as α-dystroglycan, α-sarcoglycan, and laminin-α2 (Figure 6B). DsRed was expressing in iPS-derived cardiomyocytes. iPSC-CMs were immunostained with the cytoskeletal proteins MHC(Figure 6D).

Figure 7

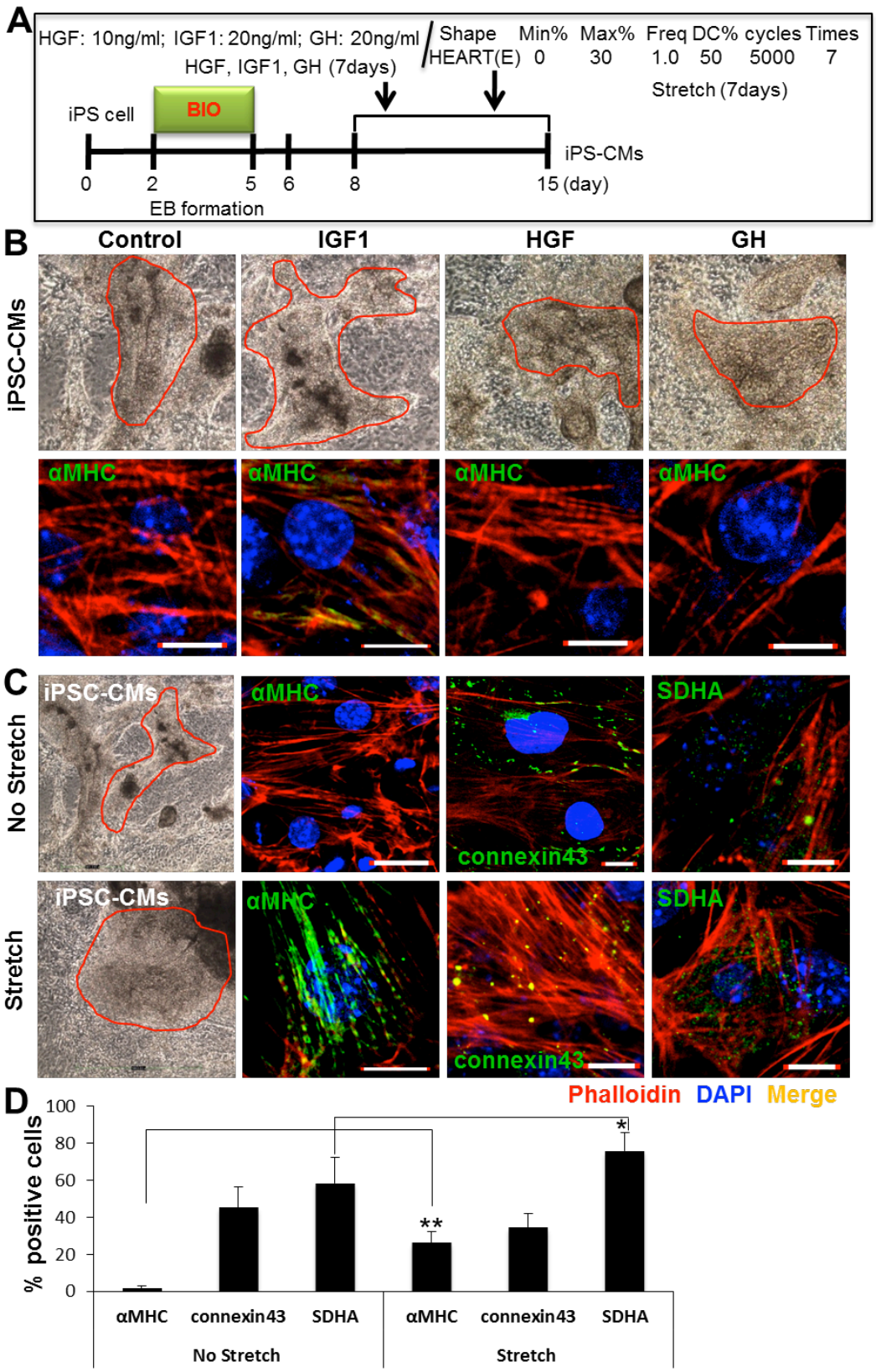
A
HGF: 10ng/ml; IGF1: 20ng/ml; GH: 20ng/ml
HGF, IGF1, GH (7days)
Shape Min% Max% Freq DC% cycles Times
HEART(E) 0 30 1.0 50 5000 7
Stretch (7days)
iPS cell
BIO
iPS-CMs
0 2 5 6 8 15 (day)
EB formation
B
Control
IGF1
HGF
GH
iPSC-CMs
αMHC
C
No Stretch
Stretch
iPSC-CMs
αMHC
connexin43
SDHA
Phalloidin DAPI Merge
D
% positive cells
100
80
60
40
20
0
αMHC connexin43 SDHA αMHC connexin43 SDHA
No Stretch
Stretch
*
**

Figure 7. Effects of growth factor or mechanical stretch on iPSC-CM differentiation.

(A) Protocol for cardiomyocyte differentiation of iPS cells by growth factor or mechanical stretch in vitro. (B) Effects of IGF-1, HGF or GH on the beating areas of EBs (inside red line) and αMHC expression. (C) Effects of mechanical stretch on the beating areas of EBs (inside red line), αMHC, connexin43 and SDHA expression. (D) αMHC and SDHA were up-regulated in stretch iPSC-CM. Connexin43 was down-regulated in stretch iPSC-CM. Actin stained with phalloidin (red). Nuclei stained with DAPI (blue). Scale bars=10μm. * $p<0.05$, ** $p< 0.01$.

Effects of growth factor or mechanical stretch on iPSC differentiation

We tested the effects of growth factor stimulation and mechanical stretch on iPSC differentiation (Figure 7A). Immunofluorescent studies showed that when EBs were stimulated by recombinant IGF-1 for 7 days, the number of contracting EBs increased, and αMHC expression was enhanced (Figure 7B). HGF or GH, however, had no effect on the number of contracting EBs or on αMHC expression. Mechanical stretch increased the number of beating EBs and elevated the αMHC and SDHA levels in iPSC-CMs (Figure 7C). αMHC and SDHA were up-regulated in stretch iPSC-CM. Connexin43 was down-regulated in stretch iPSC-CM (Figure 7D).

【考察】

Although it has been shown that iPSCs can differentiate into cardiomyocytes in vitro, the structure and function of iPSC-CMs have not been fully studied (30). Therefore, we conducted a comprehensive study of iPSC-CM ultrastructure and molecular expression. We found that iPSCs of murine origin differentiated in vitro into cardiomyocytes with a spontaneous, regular, and synchronous beating cycle. In addition, the differentiated iPSCs exhibited cardiomyocyte-specific markers, such as GATA4, Nkx2.5, cTnT, MLC2a, sarcomeric α-actinin, MHC, connexin43, α-sarcoglycan, and N-cadherin (31). The expression pattern of cardiac-specific markers and our ultrastructural findings showed that the morphology and characteristics of iPSC-CMs are similar to neo-CMs.

Adhesion molecules such as α-sarcoglycan or α-dystroglycan on the cell membrane are important for cardiomyocyte cell-cell and cell-matrix communications, which are the cardiomyocyte's functional connections in the myocardium (32). Although the expression of adhesion molecules in iPSC-CMs is poorly understood, this study clearly showed that iPSC-CMs express these molecules at levels similar to those expressed by neo-CMs. This may indicate

that transplanted iPSC-CMs can be efficiently integrated into the myocardium and form functional connections with native cardiomyocytes and extracellular matrices. In addition, iPSC-CMs expressed laminin-α2, suggesting that an efficient transplantation protocol might induce the formation of adherens junctions with the basement membrane, possibly enhancing the integration of transplanted iPSC-CMs into the heart.

When iPSC-CMs were transplanted into nude rat hearts, they acquired the ability to express α-MHC, an adult cardiomyocyte-specific molecule. Interestingly, stimulation with mechanical stretch or IGF1 enhanced the expression of this marker in iPSC-CMs in vitro.

Mechanical stretch modulates cell alignment and determines cell polarity (16) by altering mechanoreceptor-related gene expression (33), causing individual cardiomyocytes to integrate into the functional myocardium. Mechanical stretch has also been shown to induce cardiomyocytes to secrete or synthesize bioactive molecules such as angiotensin II (AngII), IGF-1, tumor necrosis factor α (TNFα), or vascular endothelial growth factor (VEGF), which exert both

autocrine and paracrine effects on adjacent cells (34). Thus, mechanical stretch regulates the mechanical and biological functions of cardiomyocytes and the myocardium in vivo. These findings suggest that the cardiac environment may be crucial for iPSC-CMs to fully mature.

Our ultrastructural analysis showed that the sarcomeric structure and the mitochondrial quantity, function, and structure were less developed in the iPSC-CMs than in neo-CMs. However, transplanting iPSC-CMs into the heart induced a transition from the MHC β to the α isoform, a transition seen during cardiac development and maturation (35). While this study did not investigate changes in mitochondrial quantity or structure in iPSC-CMs after transplantation into the heart, the maturing of MHC after transplantation could imply a functional improvement of the mitochondria.

Transplantation of iPSC-CMs is a promising method for regenerating or replacing lost or damaged myocardial tissues in situ, although the optimal transplantation method is still under debate (36). The cell-sheet method used in this study has been shown to preserve the functionality, and thus the quantity, of

cells transplanted into the heart, with minimal damage (32). This study showed that further differentiation was induced in situ in iPSC-CMs transplanted by the cell-sheet method, although the contribution of these cells to regional or global cardiac function was not studied. These findings warrant further functional, electrophysiological, and biological studies to optimize the transplantation protocol of iPSC-CMs for the greatest therapeutic effect against cardiac failure.

In conclusion, iPS cells of murine origin were not only efficiently differentiated in vitro into cardiomyocytes with a phenotype and function like that of neo-CMs, but could also be differentiated to express adult cardiomyocyte-specific molecules. This differentiation process might be promoted in vitro by IGF-1 stimulation or mechanical stretch. These results suggest iPSC-CMs as a useful source for cell therapy for the future treatment of heart failure.

【結語】

iPSC-CM は dystrophin-dystroglycan complex 系の接着分子を介してホスト心筋組織に生着する可能性が考えられた。生体に移植すると iPSC-CM は胎児型から成熟型心筋に分化し、connexin43 の発現パターンも変化していた。これらの in vivo における分化は生体環境における収縮伸展による物理的刺激がトリガーになっている可能性が考えられた。以上より、iPSC-CM は生体心臓へ移植すると成熟型心筋に分化ことから、将来、再生医療に応用できる可能性が示唆された。

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【引用文献】

1. Towbin JA, Bowles NE. The failing heart. *Nature* 2002; 415: 227－233.

2. Hosseinkhani M, Hasegawa K, Ono K, Kawamura T, Takaya T, Morimoto T, et al. Trichostatin A induces myocardial differentiation of monkey ES cells. *Biochem Biophys Res Commun* 2007; 356: 386－391.

3. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 2005; 115: 572－583.

4. Dinsmore J, Ratliff J, Deacon T, Pakzaban P, Jacoby D, Galpern W, et al. Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. *Cell Transplant* 1996; 5: 131－143.

5. Segers VF, Lee RT. Stem-cell therapy for cardiac disease. *Nature* 2008; 451: 937－942.

6. Fukuda K. Progress in myocardial regeneration and cell transplantation. *Circ J* 2005; 69: 1431－1446.

7. Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. *Circ Res* 2006; 98: 1414－1421.

8. Choi SH, Jung SY, Kwon SM, Baek SH. Perspectives on stem cell therapy for cardiac regeneration. *Circ J* 2012; 76: 1307－12.

9. Perino MG, Yamanaka S, Li J, Wobus AM, Boheler KR. Cardiomyogenic stem and progenitor cell plasticity and the dissection of cardiopoiesis. *J Mol Cell Cardiol* 2008; 45: 475－494.

10. Yuasa S, Fukuda K. Cardiac regenerative medicine. *Circ J* 2008; 72: 49-55.

11. Chen H, Hattori F, Murata M, Li W, Yuasa S, Onizuka T, et al. Common marmoset embryonic stem cell can differentiate into cardiomyocytes. *Biochem Biophys Res Commun* 2008; 369: 801－806.

12. Iwamuro M, Komaki T, Kubota Y, Seita M, Kawamoto H, Yuasa T, et al. Comparative analysis of endoderm formation efficiency between mouse ES cells and iPS cells. *Cell Transplant* 2010; 19: 831－839.

13. Mandai M, Ikeda H, Jin ZB, Iseki K, Ishigami C, Takahashi M. Use of lectins to enrich mouse ES-derived retinal progenitor cells for the purpose of transplantation therapy. *Cell Transplant* 2010; 19: 9－19.

14. Li X, Yu X, Lin Q, Deng C, Shan Z, Yang M, et al. Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. *J Mol Cell Cardiol* 2007; 42: 295－303.

15. Reinecke H, Minami E, Zhu WZ, Laflamme MA. Cardiogenic differentiation and transdifferentiation of progenitor cells. *Circ Res* 2008; 103: 1058－1071.

16. Matsuda T, Takahashi K, Nariai T, Ito T, Takatani T, Fujio Y, et al. N-cadherin-mediated cell adhesion determines the plasticity for cell alignment in response to mechanical stretch in cultured cardiomyocytes. *Biochem Biophys Res Commun* 2005; 326: 228－232.

17. Sadoshima J, Jahn L, Takahashi T, Kulik TJ, Izumo S. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J Biol Chem* 1992; 267: 10551－10560.

18. Komuro I, Kaida T, Shibazaki Y, Kurabayashi M, Katoh Y, Hoh E, et al. Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* 1990; 65: 3595－3598.

19. Ruwhof C, van der Laarse A. Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. *Cardiovasc Res* 2000; 7: 23－37.

20. Shyu KG. Cellular and molecular effects of mechanical stretch on vascular cells and cardiac myocytes. *Clin Sci Lond* 2009; 16: 377－389.

21. Shyu KG, Ko WH, Yang WS, Wang BW, Kuan P. Insulin-like growth factor-1 mediates stretch-induced upregulation of myostatin expression in neonatal rat cardiomyocytes. *Cardiovasc Res* 2005; 68: 405－414.

22. Eschenhagen T, Didié M, Heubach J, Ravens U, Zimmermann WH. Cardiac tissue engineering. *Transpl Immunol* 2002; 9: 315－321.

23. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007; 48: 313－317.

24. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 2008; 26: 101－106.

25. Naito AT, Shiojima I, Akazawa H, Hidaka K, Morisaki T, Kikuchi A, et al. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci USA* 2006; 03: 19812－19817.

26. Zhang CM, Gao L, Zheng YJ, Yang HT. Berbamine protects the heart from ischemia/reperfusion injury by maintaining cytosolic Ca(2+) homeostasis and preventing calpain activation. *Circ J*2012; 76: 1993－2002.

27. Miyagawa S, Saito A, Sakaguchi T, Yoshikawa Y, Yamauchi T, Imanishi Y, et al. Impaired myocardium regeneration with skeletal cell sheets-a preclinical trial for tissue-engineered regeneration therapy. *Transplantation* 2010; 90: 364－372.

28. Shyu KG, Chen CC, Wang BW, Kuan PL. Angiotensin II receptor antagonist blocks the expression of connexin43 induced by cyclical mechanical stretch in cultured neonatal rat cardiac myocytes. *J Mol Cell Cardiol* 2001; 3: 691－698.

29. Chang H, Wang BW, Kuan P, Shyu KG. Cyclical mechanical stretch enhances angiopoietin-2 and Tie2 receptor expression in cultured human umbilical vein endothelial cells. *Clin Sci* 2003; 04: 421－428.

30. Rajala K, Pekkanen-Mattila M, Aalto-Setälä K. Cardiac differentiation of pluripotent stem cells. *Stem Cells Int* 2011; 2011: 4061－4073.

31. Gao XR, Tan YZ, Wang HJ. Overexpression of Csx/Nkx2.5 and GATA-4 enhances the efficacy of mesenchymal stem cell transplantation after myocardial infarction. *Circ J*2011; 75: 2683－2691.

32. Ozawa E, Mizuno Y, Hagiwara Y, Sasaoka T, Yoshida M. Molecular and cell biology of the sarcoglycan complex. *Muscle Nerve* 2005; 2: 563－576.

33. Aikawa R, Nagai T, Kudoh S, Zou Y, Tanaka M, Tamura M, et al. Integrins play a critical role in mechanical stress-induced p38 MAPK activation. *Hypertension* 2002; 9: 233－238.

34. Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* 1993; 75: 977－984.

35. Nishi H, Ono K, Horie T, Nagao K, Kinoshita M, Kuwabara Y, et al. MicroRNA-27a regulates beta cardiac myosin heavy chain gene expression by targeting thyroid hormone receptor beta1 in neonatal rat ventricular myocytes. *Mol Cell Biol* 2011; 31: 744－755.

36. Gonzales C, Pedrazzini T. Progenitor cell therapy for heart disease. *Exp Cell Res* 2009; 315: 3077－3085.