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Generation of Electrophysiologically Functional Cardiomyocytes from Mouse Induced Pluripotent Stem Cells

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Abstract

Induced pluripotent stem (iPS) cells can efficiently differentiate into the three germ layers similar to those formed by differentiated embryonic stem (ES) cells. This provides a new source of cells in which to establish preclinical allogeneic transplantation models. Our iPS cells were generated from mouse embryonic fibroblasts (MEFs) transfected with the Yamanaka factors, the four transcription factors (Oct4, Sox2, Klf4 and c-Myc), without antibiotic selection or MEF feeders. After formation of embryoid bodies (EB), iPS cells spontaneously differentiated into Flk1-positive cardiac progenitors and cardiomyocytes expressing cardiac-specific markers such as alpha sarcomeric actinin (α-actinin), cardiac alpha myosin heavy chain (α-MHC), cardiac troponin T (cTnT), and connexin 43 (CX43), as well as cardiac transcription factors Nk2 homebox 5 (Nkx2.5) and gata binding protein 4 (gata4). The electrophysiological activity of iPS cell-derived cardiomyocytes (iPS-CMs) was detected in beating cell clusters with optical mapping and RH237 a voltage-sensitive dye, and in single contracting cells with patch-clamp technology. Incompletely differentiated iPS cells formed teratomas when transplanted into a severe combined immunodeficiency (SCID) mouse model of myocardial infarction. Our results show that somatic cells can be reprogrammed into pluripotent stem cells, which in turn spontaneously differentiate into electrophysiologically functional mature cardiomyocytes expressing cardiac-specific makers, and that these cells can potentially be used to repair myocardial infarction (MI) in the future.

1. Introduction

Heart failure caused by the dysfunction or damage of cardiomyocytes is a leading cause of death and disability [1]. Dysfunctional or damaged cardiomyocytes cannot be replaced

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because human postnatal cardiomyocytes become terminally-differentiated after birth and lose their ability to proliferate [2]. Other studies have shown that the adult heart has limited capacity to regenerate cardiomyocytes through cardiac stem cell (CSC) differentiation or through proliferation of cardiomyocytes *in vivo*, although CSCs can easily be induced to differentiate into cardiomyocytes *in vitro* [3–5]. Furthermore, there is no evidence that stem cells derived from other sources, such as the bone marrow, are able to give rise to contractile cardiac cells after transplantation into the heart [6]. The therapeutic effect of stem cells is, thought to be, mediated by paracrine mechanisms, such as the release of cytokines and growth factors, which inhibit apoptosis and fibrosis formation, enhance contractility, and activate endogenous regenerative mechanisms [7,8]. Embryonic stem (ES) cells, a type of pluripotent stem cell derived from early developing embryos, can potentially differentiate into all cell types derived from the three germ layers, including cardiac lineages [9–11]. However, several ethical and practical issues limit the use of human ES cells and thus these cells are not readily accessible as an autologous cell source [12]. Current stem cell therapeutic approaches are still limited by the availability of reliable cell sources.

The recent generation of iPS cells has brought new hope to the field of regenerative medicine. Similar to ES cells, iPS cells are able to differentiate into all cell types of the three primary germ layers, which offers a unique approach for regenerative cell therapy [13–15]. Especially, human iPS cell-derived cardiomyocytes (iPS-CMs) show nodal-, atrial-, and ventricular-like phenotypes of gene expression and electrophysiological properties [16–18] and are responsive to β-adrenergic stimulation [18,19]. However, transplantation of undifferentiated iPS cells into the heart can result in teratomas because of the embryonic characteristics of the cells or possible activation of oncogenes [20, 21]. Mature iPS-CMs or iPS cells derived from viral-free and non-integrating methods bypass the risk of teratoma formation; engrafts remain within the infarcted heart and result in improved cardiac function after ischemic damage [22–25]. However, the generation of sufficient functional iPS-CMs with high purity and maturity [26–28] remains challenging.

Here we describe the generation of iPS cells from mouse embryonic MEFs using four transcription factors (Oct4, Sox2, Klf4 and C-myc), without feeder cells nor antibiotic selection. The cardiac markers, including α-actinin, MHC, cTnT, CX43, Nkx2.5, and Gata4, were tested. The electrophysiological action potential was verified by patch-clamp in single contracting cells, and recorded by optical mapping with RH237, a voltage-sensitive dye, in iPS-CM clusters. These results show that mature functional cardiomyocytes with cardiac-specific markers and cardiomyocyte potential can be derived from somatic cells via cellular reprogramming methodologies.

2. Materials and Methods

All surgical operations related to mice in the experiments were performed under anesthesia, and mice were sacrificed by euthanasia. These experiments were approved by Institutional Animal Care And Use Committee (IACUC) of Baylor College of Medicine.

2.1 Generation of iPS cells

The original Yamanaka pMXs-based retroviral vectors encoding Oct4, Sox2, Klf4, and C-myc (cat# 13366, 13367,13370 and 13375, Addgene) [13] were introduced individually into each 10 cm plate with Plat-E cells with Fugene 6 transfection reagent (cat# 11814443001, Roche) according to the manufacturer's recommendations. After 24 hours of transfection, virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45 μm cellulose acetate filter and supplemented with 4 μg/ml polybrene.

One day before infection, 8×10^5 MEF cells were seeded into 10 cm 0.1% gelatin-coated plates. MEF cells were then incubated with virus/polybrene-containing supernatants overnight. MEFs were infected twice. After 24 hours of infection, the media were replaced with 10 ml fresh ES cell media. The iPS cells were generated without antibiotics selection [29]. Three weeks later, alkaline phosphatase staining (cat# SK-5100, Vector Laboratories) was carried out to evaluate reprogramming efficiency, and colonies were selected according to a morphology characteristic of ES colonies [29]. To establish stable iPS cell lines, a single iPS colony was picked into one well of a 24-well plate. The iPS colonies used in our experiments, were lines in which the viral ectopic genes Oct4, Sox2, Klf4 and c-Myc were shown to be silenced [30].

2.2 Differentiation of iPS cells into cardiomyocytes

Both iPS and ES cells were maintained on tissue culture dishes coated with 0.1% gelatin. Subsequently, cell colonies were dissociated into single cell suspensions with 0.25% trypsin-EDTA (cat# 25200-056, Invitrogen). To induce embryoid body (EB) formation, cells were plated in Petri dishes containing differentiation medium composed of Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen), 15% fetal bovine serum, 0.2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 0.1 mmol/L β-mercaptoethanol. The cells aggregated spontaneously to from EBs. At day 5 of differentiation, 10 EBs per well were seeded into a 0.1% gelatin-coated 6-well culture dish or 3 EBs per well on a gelatin-coated 12-well culture dish (Nunc) for immunofluorescence analyses. Differentiation medium was replaced every second to third day. Starting on day 6 of differentiation (1 day after plating), each EB outgrowth was examined daily for beating areas.

2.3 Immunofluorescence staining

The iPS cells and the cells at day 22–28 of iPS EB differentiation were fixed with 4% paraformaldehyde, and stained following a standard immunostaining procedure. Briefly, cells were fixed in 4% formaldehyde solution in phosphate-buffered saline (PBS), permeated with 5% bovine serum albumin (BSA) plus 0.1 Triton X100, and blocked with 5% BSA plus 0.01% tween 20. The primary and secondary antibodies were diluted in blocking solution. The primary antibodies against mouse Nanog (cat# ab80892, Abcam), Sox2 (cat# ab5603, Millipore), Oct4 (cat# sc-5279, Santa Cruz), Connex43 (cat# C6219, Sigma);α-sarcomeric actinin (cat# A7732, Sigma); MHC (ab15, Abcam); cTnT (ab8295, Abcam); Laminin (cat# ab11575, Abcam); SM22 alpha (cat# ab14106, Abcam); alpha smooth muscle Actin (cat# ab5694,Abcam); Calponin (cat# ab46794, Abcam); GATA4 (cat# AF2606, R&D system); and NKx2.5 (cat# AF2444, R&D system) were used at a 1:200 dilution. Secondary antibodies, Texas Red AffiniPure Donkey Anti-Mouse IgG (cat#715-075-150, Jackson

ImmunoResearch), Texas Red AffiniPure Donkey Anti-Goat IgG (H+L) (cat# 705-075-003, Jackson ImmunoResearch), FITC AffiniPure Donkey Anti Rabbit IgG (H+L) (cat# 711-095-152, Jackson ImmunoResearch), and Texas Red AffiniPure Donkey Anti- Rabbit IgG (H+L) (Cat#711-075-152, Jackson ImmunoResearch) were used at a 1:200 dilution.

2.4 Fluorescence-activated cell sorting (FACS)

Cells at day 4 or day 8 of EB differentiation were dispersed with 2 mM EDTA in DMEM, stained with anti-Flk 1 antibody (1:200 dilution) (cat# 555307, BD Pharmingen) as the primary antibody; and then stained with goat anti-rat IgG-PE (sc-3740, Santa Cruz Biotechnology) diluted 1:200 as secondary antibody. Flk1-positive cells were analyzed with a Flow Cytometer BD LSRFortessa (BD Biosciences).

2.5 Preparation of the acute myocardial infarction (AMI) mouse model and transplantation of EB cells

The 12 acute myocardial infarction (AMI) model was created by surgically ligating the left anterior descending artery of 8-week-old SCID C57/BL6 mice anesthetized with isoflurane (2%). The AMI model was created within 2 minutes after ligation. The surface of the infarcted region looked white, instead of red, and a surface electrocardiograph detected dynamic changes in the T and the Q waves. We injected 1×10^6 iPS cells at day 5 of EB differentiation into six model infarcted region right after the ligation. The rest six AMI models were taken as control. The mice were euthanized and hearts were surgically excised at days 7 and 28 after AMI, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained. Cell survival, proliferation, differentiation, and presence of teratomas were evaluated. The IACUC of Baylor College of Medicine approved the experimental protocol.

2.6 Detection of action potentials of iPS-CMs by optical mapping

The electrophysiological activity of clusters of iPS-derived beating cells was recorded by an optical mapping system using RH237 a voltage-sensitive dye as previously described [31]. Briefly, beating clusters (22–28 days after differentiation) were cultured in 35 mm dishes overnight, and then surface perfused at 37°C with modified Tyrode's solution containing 126 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L HEPES, (pH 7.4 adjusted with 1 mol/L NaOH). After 5 minutes, the clusters were incubated with the voltage-sensitive dye RH237 (1 μmol/L) for 5 minutes. The optical signal was excited with custom-made LED and recorded with a high frequency CCD camera (710 frames/sec). The data was processed with an algorithm on the Matlab platform and phase maps were constructed with a time-delay embedding method [32].

2.7 Recording of whole-cell action potentials

Beating clusters were dissociated with 0.25% trypsin-EDTA and single cell suspensions were plated onto 0.1% gelatin-coated glass coverslips and continuously cultured for 1–2 days. Spontaneously beating individual iPS-CMs were selected for patch-clamp experiments. For action potential recording, we used the perforated patch technique with a pipette solution containing amphotericin B (240 mg/L, Sigma, MO) with a current-clamp mode of Axon multiclamp 700A amplifier and patch clamp 10.0 software (Molecular

Devices, CA). The pipette solution used to record action potentials and K^+ current contained 120 mmol/L K-Aspartate, 10 mmol/L Na_2ATP, 2 mmol/L $MgCl_2$, 10 mmol/L EGTA, and 10 mmol/L HEPES (pH 7.35 adjusted with 1mol/L KOH). Cells were perfused with Tyrode's solution during these experiments.

2.8 RNA isolation and analysis

Total RNAs were isolated from cell samples using TRizol reagent (catalog# 15596018, Invitrogen). RNA samples were analyzed with reverse transcription (RT)-PCR or quantitative real-time polymerase chain reaction (qRT-PCR) with sybr green Q-PCR reagent (catalog # 208056, Qiagen) and gene specific PCR primers, as listed in supplemental table 1.

2.9 Statistical analysis

All data were obtained from triplicate experiments and presented as mean ± standard deviation (SD). Student's T-test was performed to determine the differences among grouped data. * indicates no statistical significance with $P \geq 0.05$; ** indicates statistical significance with $P < 0.05$.

3. Results

3.1 Differentiation of iPS cells into spontaneously contracting cells and mesoderm progenitor cells in vitro

Mouse iPS cells were generated by viral introduction of the four Yamanaka factors—Oct4, Sox2, c-Myc and Klf4—into mouse embryonic fibroblasts and then subsequently selecting morphologically 'good' iPS colonies, generated without antibiotic selection or MEF feeders [30]. The selected iPS colonies were positive for endogenous Oct4, Sox2, and Nanog expression using fluorescently labeled antibodies and stained for alkaline phosphatase (ALP) activity [30]. Exogenous viral expression of Oct4, Sox2, c-Myc, and Klf4 was silenced in the iPS clone used in this experiment [13]. In addition the iPS cells were confirmed to have the ability to form EBs, and chimeras, and to be germ line competent [13, 30]. In order to induce the iPS cells to differentiate into functional cardiomyocytes, the cells were dissociated into a single cell suspension by trypsinization, after which they spontaneously aggregated to form EBs in Petri dishes to which they do not adhere. After 5 days, the iPS EBs were transferred to 0.1% gelatin-coated tissue culture plates to which they adhered. Five to seven days later, beating cells were observed in the cultures (Supplementary video 1); different colonies were beating at different frequencies, which were distinguished by different motions. (Figs. 1A and 1B).

Flk1 is a receptor for vascular endothelial growth factor A (VEGF-A), that is expressed in early lateral mesodermal progenitor cells at the primitive streak stage. Flk1-positive progenitor cells are multipotent; they can differentiate into endothelial cells, smooth muscle cells, and cardiomyocytes [33,34], which indicates that they could potentially be used to establish more useful therapeutic approaches for human cardiovascular diseases such as ischemia or MI. In order to validate the ability of iPS cells to differentiate into cardiac progenitor cells, we analyzed Flk1 expression during the differentiation process cells using FACS. The results show that 11.6% of iPS EB cells differentiated into Flk1-positive cells at

day 4 of differentiation (Fig. 1C). By day 8, 17.3% of iPS EB cells had differentiated into Flk1-positive cells (Fig. 1D). While, 2.7% of ES cells at days 4 (Fig. 1E) and 5.4% at day 8 (Fig. 1F) were Flk1-positive. These data demonstrate that iPS cells can differentiate into mesodermal progenitor-Flk-1 positive cells, and thus had the potential to differentiate into cardiomyocytes, endothelial, and vascular smooth muscle lineages [34].

3.2 iPS cell-derived differentiated cells express cardiac-specific markers

The expression of cardiac-specific markers is a key feature of cardiomyocytes derived from iPS cells. At days 22–28 of differentiation some iPS-derived cells expressed the cardiac transcription factors Nkx2.5 (Figs. 2A–2C) and gata4 (Figs. 2D–2F) in the nucleus confirmed by immunostaining. This suggests that iPS cells differentiated spontaneously toward cardiac lineages.

To evaluate the expression of myofilament proteins and the sarcomeric organization in iPS-CMs, we performed immunostaining of differentiated EB cells at days 22–28 with antibodies for cardiac-specific markers. The MHC (Figs. 3A–3C), α-actinin (Figs. 3D–3F), and cTnT (Figs. 3G–3I) markers were expressed in the cytoplasm and sarcomere of cells, indicating that those sarcomere proteins were well organized. CX43, an important component of gap junctions in ventricular cells, was expressed in the cell membranes between the cells in a cluster (Figs. 4A–4C), and was expressed on sites where the membranes made contact with other cells (Figs. 4D–4F). CX43 was also detected in the cytoplasm of single cells (Figs. 4G–4I).

Other markers related to cardiac cells were also tested. Calponin, a calcium binding protein that is indispensable for muscle contraction, was also expressed in iPS-derived cells (Figs. 5A–5C). Moreover, the smooth muscle cell markers SM22 (Figs. 5D–5F) and SMA (Figs. 5G–5I) were expressed in differentiated iPS cells too. Laminin was also found in differentiated iPS cells (Figs. 5J–5L). These results show that iPS-CMs express mature cardiac protein markers.

3.3 iPS-CMs possess electrophysiological activity

The important feature of cardiacmyocytes is their electrophysiological activity. The action potentials of iPS-CMs were determined after the iPS cells were induced to spontaneously differentiate into contracting cells. We recorded their electrophysiological activity with an optical mapping system for action potentials using the voltage-sensitive dye RH237 (Fig. 6A and Supplementary Video 2). We found that cells were electrophysiologically active beating at a rate of 360 bpm. The electrical waves propagated throughout the beating area. This indicates that iPS cells can differentiate into electrophysiologically functional cardiomyocytes.

To further test the action potentials of single beating cells, beating clusters were trypsinized to obtain a single cell suspension and subsequently re-plated on 0.1% gelatin coated-cover slides, and then tested by Patch-clamping of single beating cells. The resulting maps from the Patch-clamp studies showed that the pattern of action potentials was similar to that of cardiomyocytes (Fig. 6B).

3.4 Transplantation of incompletely differentiated iPS cells results in teratomas in an AMI mouse model

To validate the safety of transplanting incompletely differentiated iPS cells, we created a mouse AMI model by surgically ligating the left anterior descending coronary arteries of SCID C57/BL6 mice. We dispersed iPS EB cells at day 5 of differentiation into a single cell suspension and 1×10^6 cells in PBS were transplanted into a broad zone of damaged tissue within the hearts of female AMI SCID mice (n=3) after AMI models were made. The hearts were collected at days 7 or 28 after transplant. Hematoxylin and eosin (HE) staining results showed that the injected cells were present around the site of injection after 7 days of transplantation. The proliferating cells showed teratoma characteristics (Figs. 7A and 7B). Twenty-eight days later, a number of proliferating cells were found in the site where the original cardiomyocytes had died and formed a teratoma (Figs. 7C and 7D). Thus, incompletely differentiated iPS cells transplanted into damaged areas of the heart can survive and grow, but they present the risk of becoming a tumor.

4. DISCUSSION

ES cells have been reported to improve the function of damaged hearts by differentiating into cardiac lineages and enhancing neovascularization via the release of specific factors. [35]. However, the results were more diverse among different iPS cell lines than ES cells, despite the fact that iPS cells closely resemble ES cells in morphology and developmental potential [13,30]. To further establish stable iPS cell lines, we selected iPS cells whose exogenously transfected Oct3/4, Sox2, Klf4, and c-Myc were silenced; these cells were confirmed to have pluripotent properties *in vitro* and *in vivo*, including germ line transmission capabilities [30,36]. Our iPS cells generated from fibroblast cells shared similar undifferentiated characteristics with ES cells [13,37]. Moreover, our iPS cells were able to spontaneously differentiate into mature contractile cardiomyocytes expressing specific cardiac markers, such as α-actinin, MHC, and cTnT, and having a well-organized distribution and indistinguishable sarcomeric organization. This suggests that iPS cells are capable of differentiating into mature cardiomyocytes. Although the iPS cells have the capacity to spontaneously differentiate into CMs, the efficiency is low. Thus, induction factors, such as cardiac specific transcription factors, should be included in the process to improve the efficiency of cardiac differentiation.

Gap junctions play an important role in intercellular impulse propagation within the myocardium. Cardiac gap junctions are composed of proteins called connexins (Cxs). Cxs form low resistance channels that enable electrical coupling of adjacent myocytes and intercellular electrical communication. These Cxs are distributed in specific cells, exhibit diverse properties, and are regulated by different gating mechanisms. There are as many as three major isoforms of Cxs in the heart. Studies have shown that a homomeric composition of gap junctions, e.g., Cx40/Cx40, and Cx43/Cx43, is required for efficient communication between cells in exogenous expression systems. CX43 is the only Cx known to be expressed in the adult working myocardium of the ventricle [38]. Alterations in the localization, expression level, and nature of Cx43 cause abnormal electrical conduction, leading to arrhythmias [39]. Transplantation of non-Cx43-expressing cells would promote arrhythmia

in the area of the myocardial infarction [40,41]. Therefore, engraftment of Cx43-expressing myocytes can potentially reduce life-threatening post-infarct arrhythmias through the augmentation of intercellular coupling in a cardiac cell-based therapy [42]. In the present study, we have shown that Cx43 is only expressed on the membranes of adjacent iPSC-CMs, which need communication or material exchange. However, further functional studies are warranted to determine functional gap junction formation.

Electrophysiological activity is an important feature and a basic function of cardiomyocytes. It ensures that cardiomyocytes contract synchronously in response to stimulation from the sinoatrial node. We used an optical mapping system to test the electrophysiological activity of beating clusters and patch-clamping to test this activity in single beating single cells. The results showed that both spontaneously beating clusters of iPS-CMs and single of iPS-CMs cells fire action potentials. The rate of beating of iPS-CMs was similar to that of mouse cardiomyocytes. This indicates that iPS-CMs possess not only the markers of mature cardiomyocytes but also electrophysiological function, and would be prone to couple or synchronize with host cells, and potentially avoid arrhythmia after transplantation into the myocardium.

We also determined the safety of transplanting incompletely differentiated iPS cells. It has been demonstrated that iPS-CMs are safe after transplantation into the heart, but their proliferation is limited [20]. Therefore, the selection of adequate time points during differentiation of iPS cells is critical. Flk1-positive cells were selected as cardiac progenitors as they possess the capability to differentiate into cardiac lineages [34]. In the present study, EBs of iPS cells at days 4 to 8 of differentiation showed a significant increase in Flk1-positive cells. However, unpurified Flk1-positive cells formed teratomas after injection into the hearts of our AMI mouse models; therefore it is unsafe to inject incompletely differentiated iPS cells into the heart. Expression of the Yamanaka factors was silenced in the iPS cells and was not re-activated during formation of iPSC-CMs (Supplemental Figure 1), so engrafts derived from more mature cells seemed to be safe [43]. However, this raised another important issue of whether iPS cell-derived cardiac myocytes would couple with host cells successfully. Engrafts will promote arrhythmia if injected cells cannot couple or synchronize with host cells [44].

5. Conclusion

The present study shows that iPS-CMs express cardiac specific markers, express membrane connexin-43, and display cardiac electrophysiological activity. These results indicate that iPS cells might be a promising cell source for the generation of patient-specific cardiomyocytes [45,46], even for patients with severe heart disease [47,48], because the cells can be made available in unlimited amounts and are capable of cardiomyocyte generation. However, further studies are warranted to determine the right subgroup of iPS-CMs at a right stage of differentiation to clarify safety issues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

In this study we show that iPS derived cardiomyocytes (iPS-CMs) express cardiac specific markers, e.g. MHC and cTnT, and importantly express membrane connexin-43, a key gap junction protein that is responsible for synchronous communication between cardiac cells. Further investigations demonstrated that these iPS-CMs display cardiac electrophysiological activity verified by patch-clamp in single contracting cells, and optical mapping with RH237, a voltage-sensitive dye, in iPS-CM clusters. These results indicate that iPS cells might be a promising cell source for the generation of patient-specific cardiomyocytes even for patients with severe heart disease, because the cells can be made available in unlimited amounts and are capable of cardiomyocyte generation.

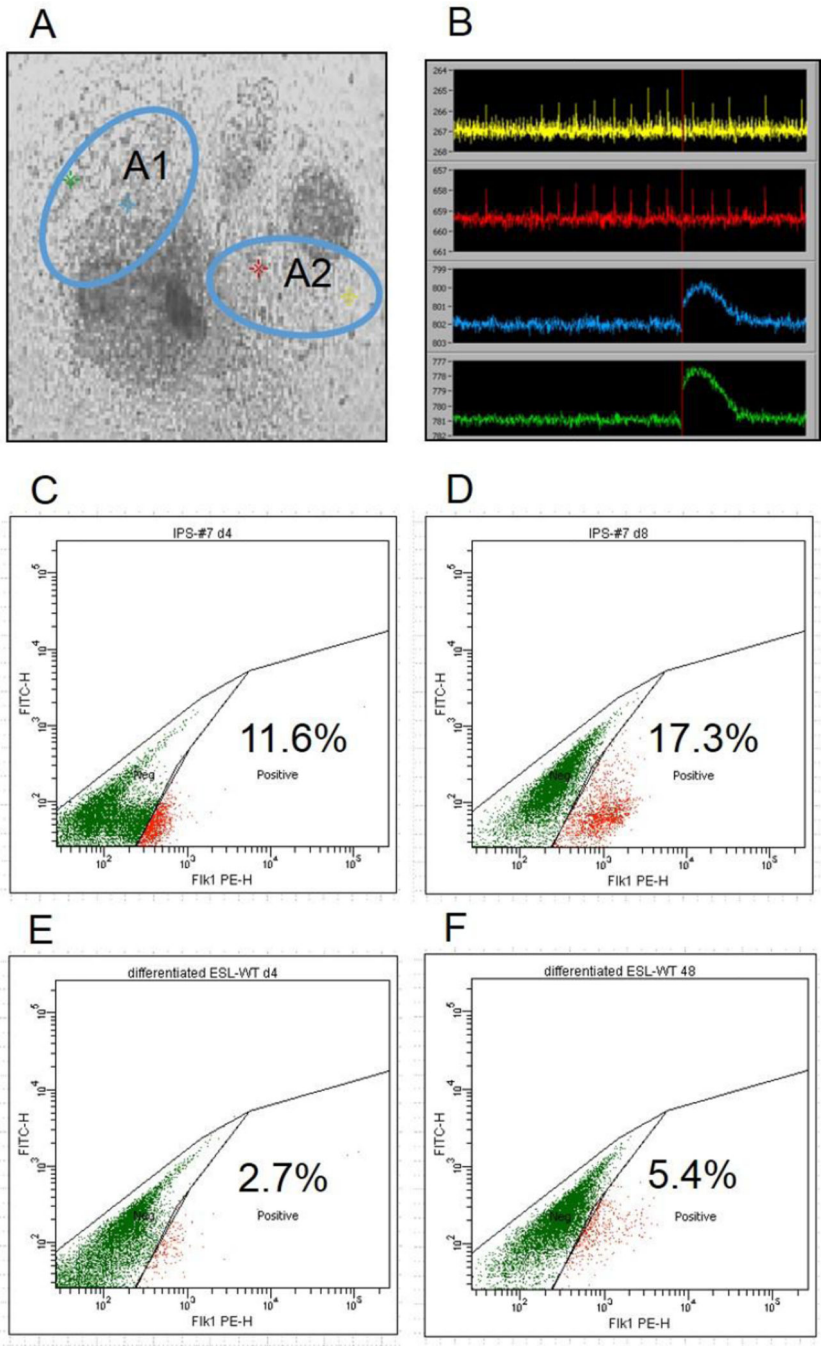

Figure 1. iPS cells differentiate into Flk1-positive beating cells with diverse rates

(A) Two beating clusters (A1 and A2) in the iPS-derived EB cultures. (B) The colors trace the beating frequency of A1 cluster (green and blue) and A2 cluster (red and yellow) corresponding to the clusters in A. (C) FACS analysis of Flk1-positive cells on day 4 of iPS EB differentiation. (D) FACS analysis of Flk1-positive cells on day 8 of iPS EB differentiation. (E) FACS analysis of Flk1-positive cells on day 4 of ES EB differentiation. (F) FACS analysis of Flk1-positive cellson day 8 of ES EB differentiation.

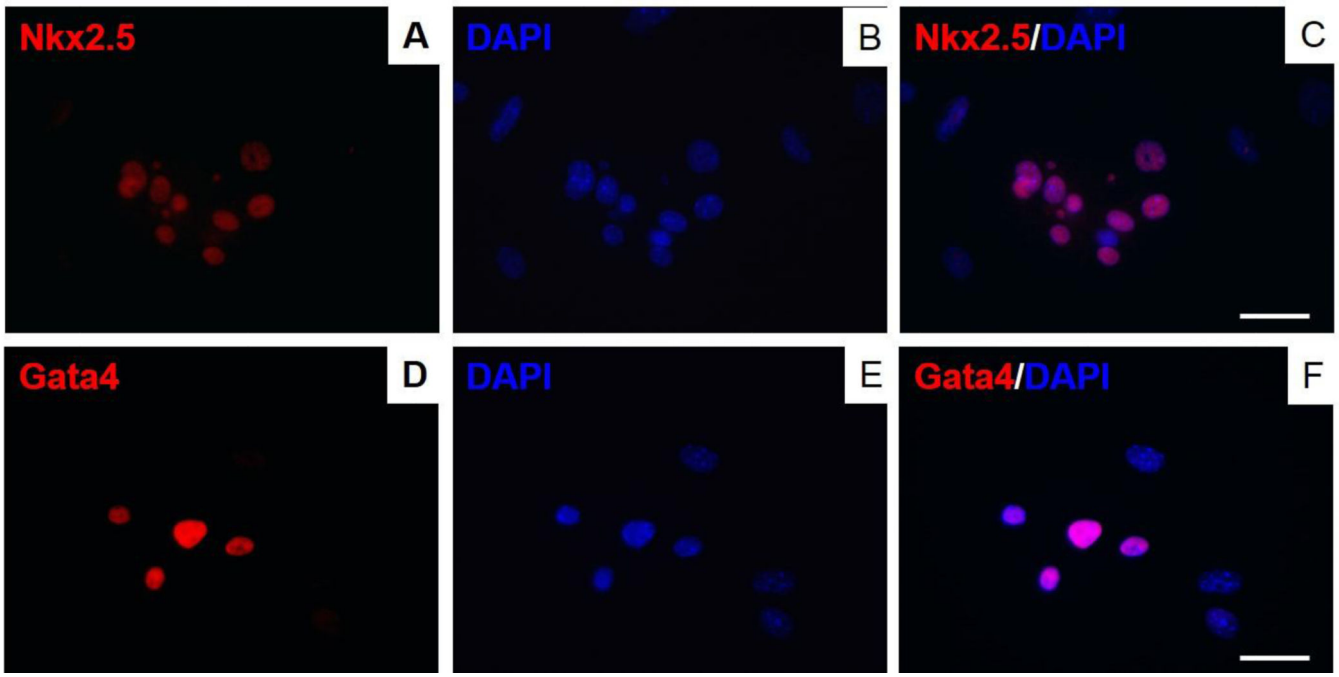

Figure 2. Expression of transcription factors related to cardiac development at days 22–28 of iPS EB differentiation

Immunostaining of: (A) Nkx2.5 (red); (B) DAPI (blue) and (C) merge of Nkx2.5 (red) and DAPI (blue). Immunostaining of: (D) Gata4 (red); (E) DAPI (blue) and merge of gata4 (red); and (F) DAPI (blue). Bar scale = 20 μm.

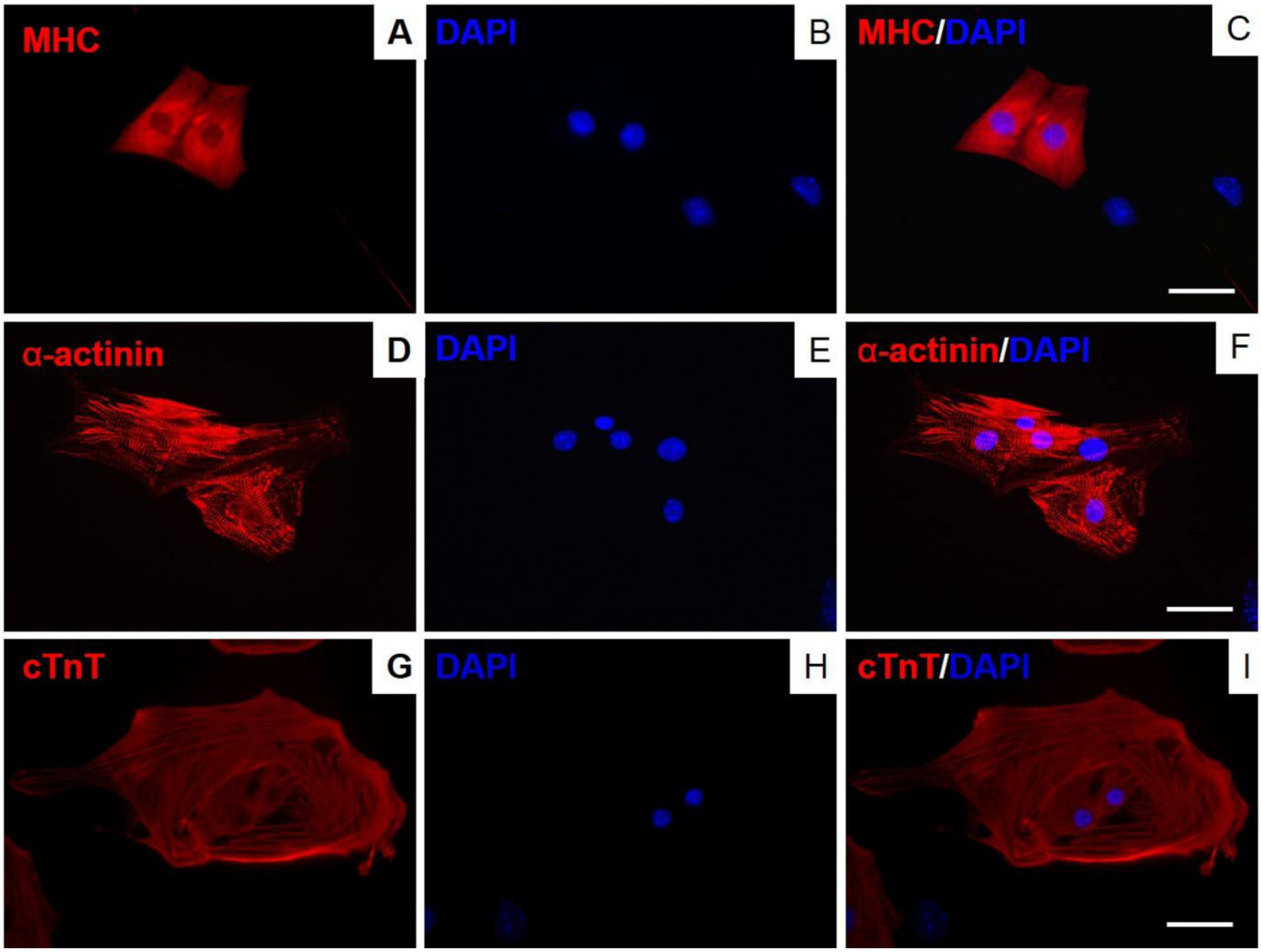

Figure 3. Expression of cardiac-specific markers at days 22–28 of iPS EB differentiation

Immunostaining of: (A) MHC (red); (B) DAPI (blue) and (C) merge of MHC (red) and DAPI (blue). Immunostaining of: (D) α-actinin (red); (E) DAPI (blue) and (F) merge of α-actinin (red) and DAPI (blue. Immunostaining of: (G) cTnT (red); (H) DAPI (blue) and merge of cTnT (red); (I) and DAPI (blue). Bar scale = 30 μm.

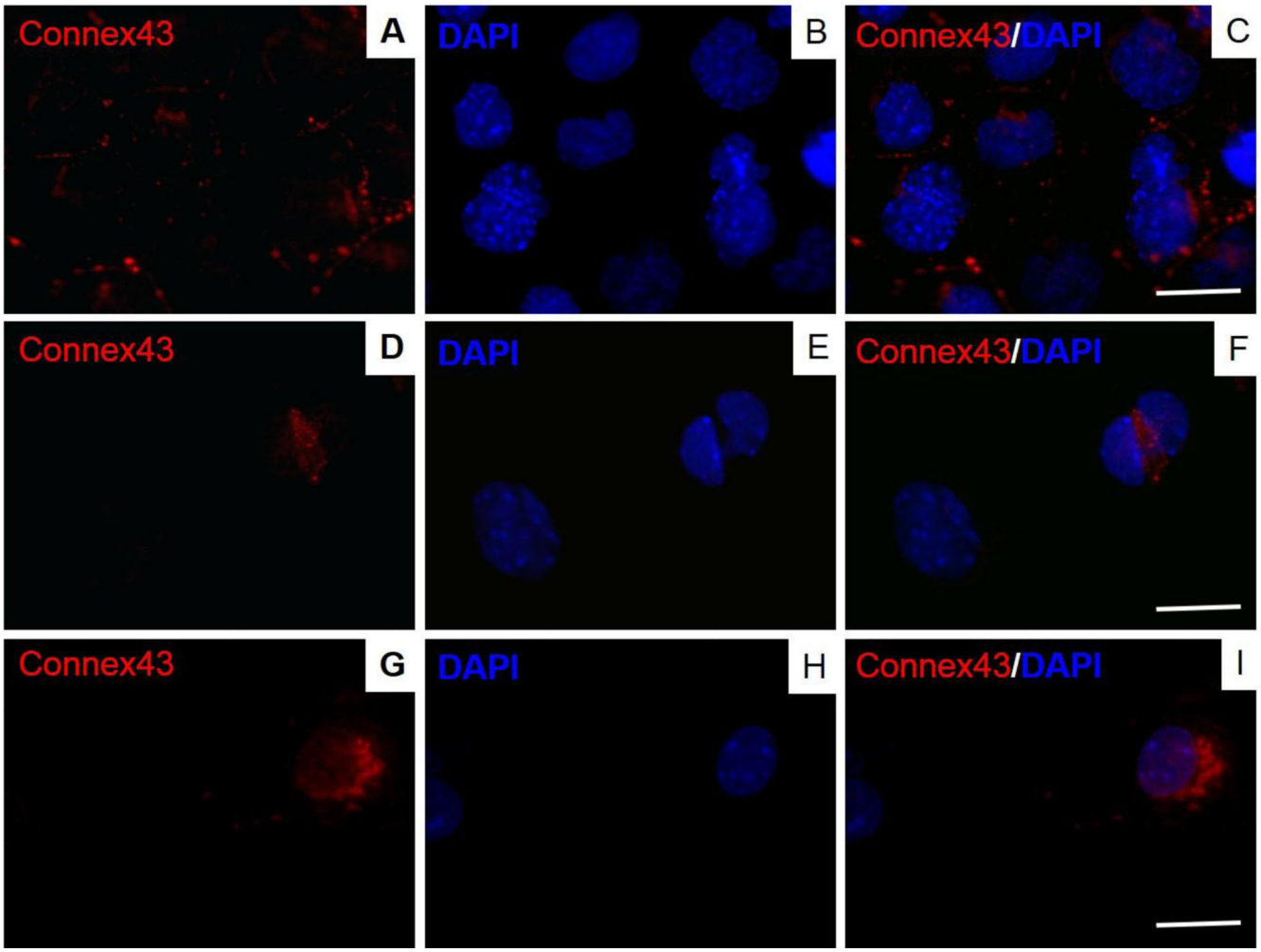

Figure 4. Distribution of gap junction protein CX 43 at days 22–28 of iPS EB differentiation

Cluster Immunostaining of: (A) CX43 (red); (B) DAPI (blue) and (C) merge of CX43 (red) and DAPI (blue). Two-cell Immunostaining of: (D) CX43 (red); (E) DAPI (blue) and (F) merge of CX43 (red) and DAPI (blue).Single cell immunostaining of: (G) CX43 (red); (H) DAPI (blue) and (I) merge of CX43 (red) and DAPI (blue). Bar scale = 10 μm.

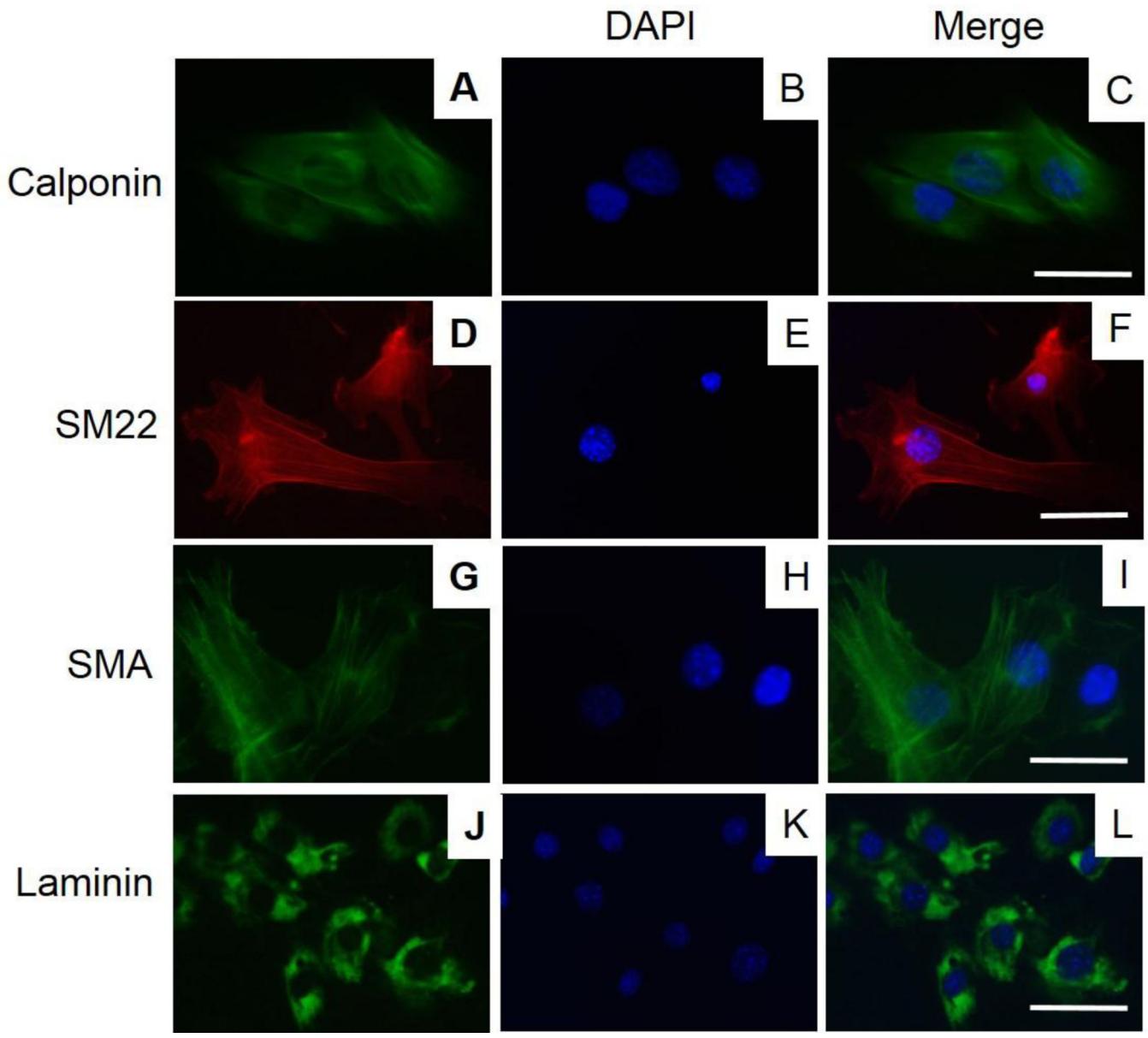

Figure 5. Expression of other genes during days 22–28 of iPS EB differentiation

Immunostaining of: (A) Calponin (green); (B) DAPI (blue) and (C) merge of Calponin (green) and DAPI (blue). Immunostaining of: (D) SM22 (red); (E) DAPI (blue) and (F) merge of SM22 (red) and DAPI (blue). Immunostaining of: (G) SMA (green); (H) DAPI (blue) and (I) merge of SMA (green) and DAPI (blue). Immunostaining of: (J) Laminin (green); (K) DAPI (blue) and (L) merge of Laminnin (green) and DAPI (blue). Bar scale = 10 μm.

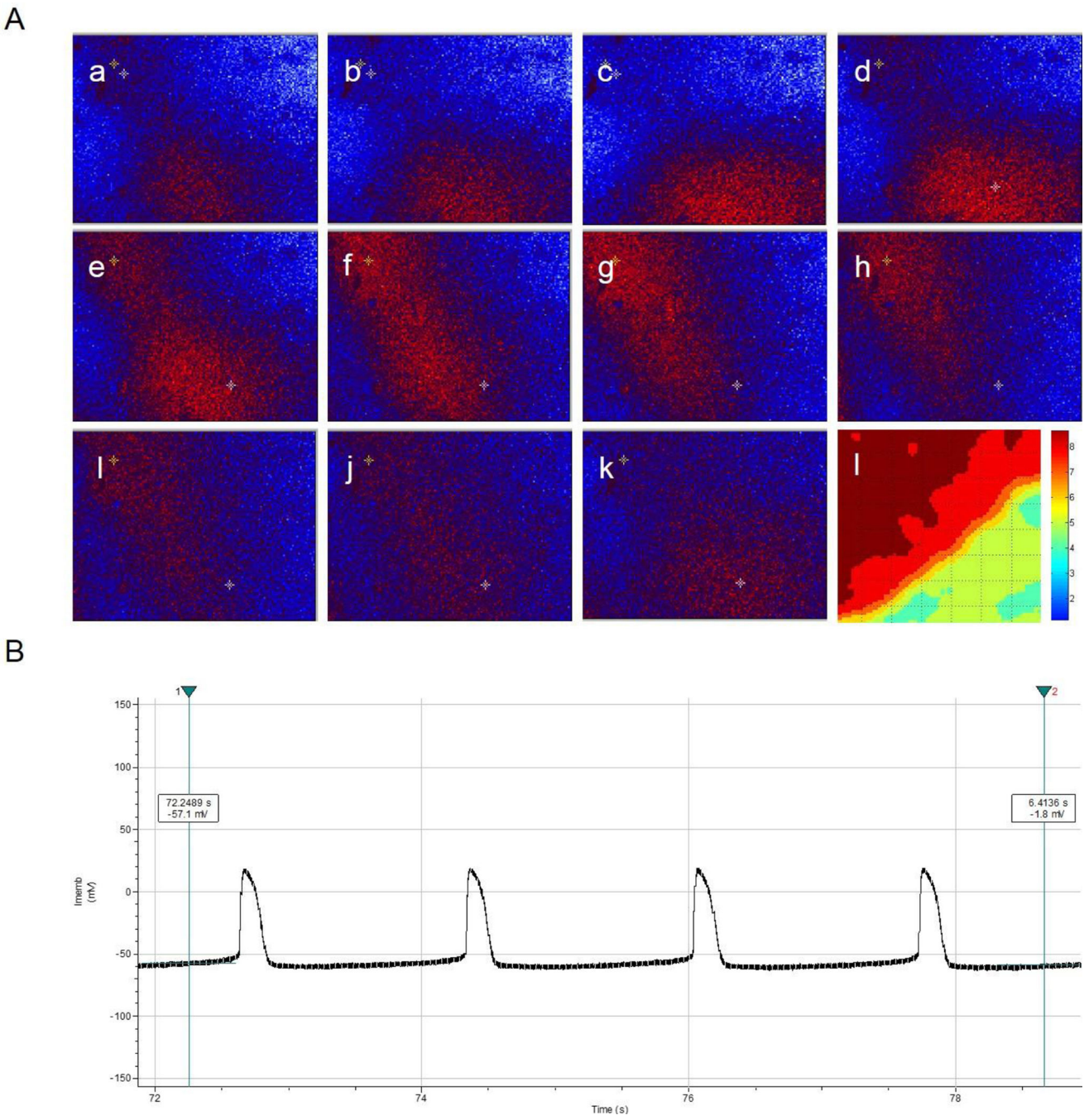

Figure 6. Evaluation of eletrophysiological activity

(A) Recorded by optical mapping using the voltage-sensitive dye RH237. Intervals between pictures = 28 mS. Beat Rate = 360 bpm. A (a–k), Propagation within the beating cluster. l, phase map. (B) Electrophysiological potential of single beating cells recorded using patch-clamping.

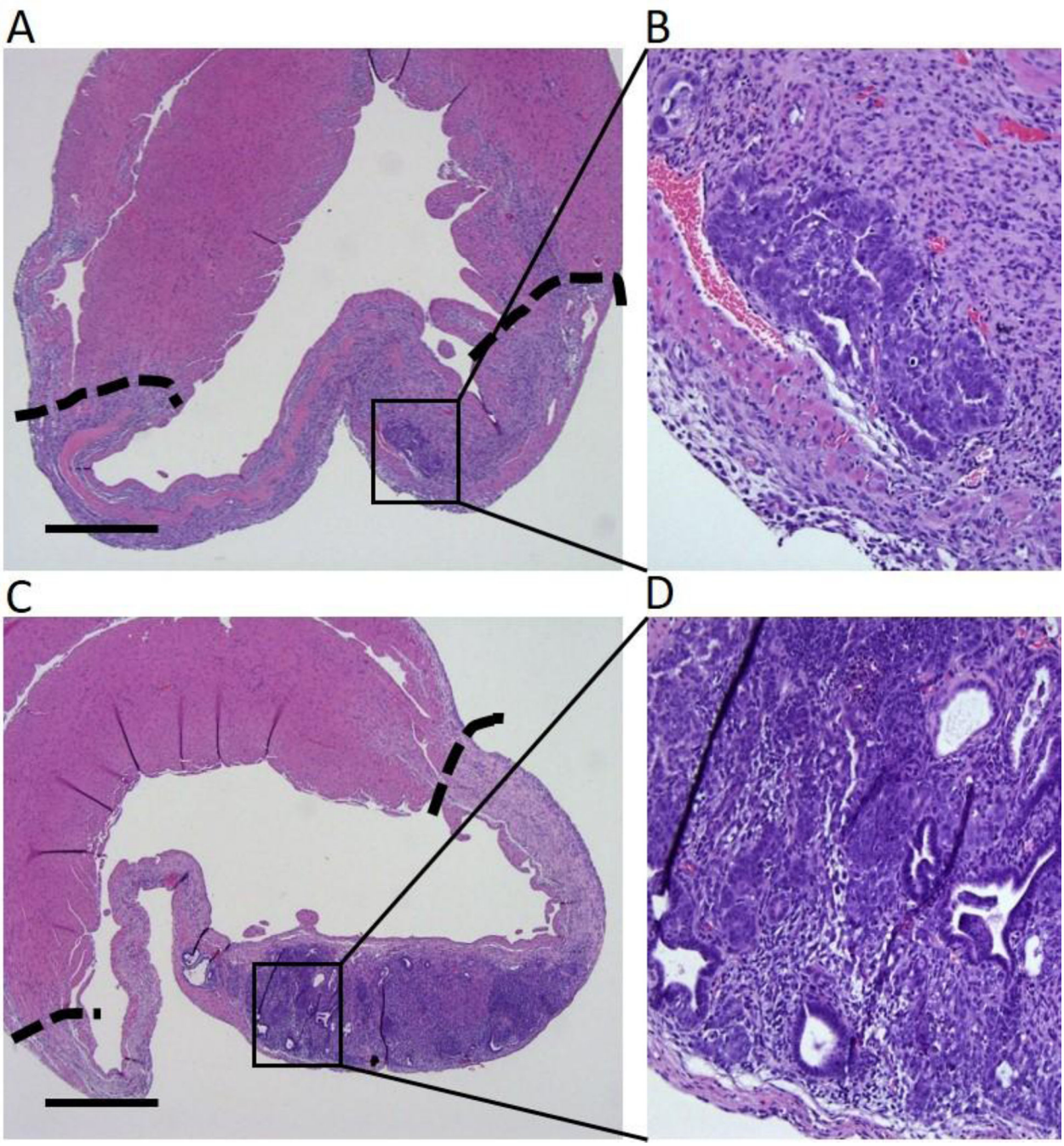

Figure 7. HE staining of AMI hearts 1 and 4 weeks after transplantation of iPS cells derived from cells at day 5 of EB differentiation

(A and B) H&E staining of heart tissue one week after transplantation of iPS-derived EB cells into the AMI. (C and D) H&E staining of heart tissue four weeks after transplantation of iPS-derived EB cells into the AMI. Dotted lines separate healthy and ischemic areas. Scale bar = 100 μM.