



Ultrastructural Maturation of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes in a Long-Term Culture

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Background: In the short- to mid-term, cardiomyocytes generated from human-induced pluripotent stem cells (hiPSC-CMs) have been reported to be less mature than those of adult hearts. However, the maturation process in a long-term culture remains unknown.

Methods and Results: A hiPSC clone generated from a healthy control was differentiated into CMs through embryoid body (EB) formation. The ultrastructural characteristics and gene expressions of spontaneously contracting EBs were analyzed through 1-year of culture after cardiac differentiation was initiated. The 14-day-old EBs contained a low number of myofibrils, which lacked alignment, and immature high-density Z-bands lacking A-, H-, I-, and M-bands. Through the long-term culture up to 180 days, the myofibrils became more tightly packed and formed parallel arrays accompanied by the appearance of mature Z-, A-, H-, and I-bands, but not M-bands. Notably, M-bands were finally detected in 360-day-old EBs. The expression levels of the M-band-specific genes in hiPSC-CMs remained lower in comparison with those in the adult heart. Immunocytochemistry indicated increasing number of MLC2v-positive/MLC2a-negative cells with decreasing number of MLC2v/MLC2a double-positive cells, indicating maturing of ventricular-type CMs.

Conclusions: The structural maturation process of hiPSC-CMs through 1-year of culture revealed ultrastructural sarcomeric changes accompanied by delayed formation of M-bands. Our study provides new insight into the maturation process of hiPSC-CMs. (*Circ J* 2013; **77**: 1307–1314)

Key Words: Cardiomyocytes; Induced pluripotent stem cells; Ultrastructure

Induced pluripotent stem cells (iPSC) can differentiate into functional cardiomyocytes (CMs), and are a powerful model for regenerative therapy and investigating the mechanisms underlying inherited cardiac diseases.^{1–5} Although several studies have shown that iPSC-derived CMs (iPSC-CMs) have molecular, structural and functional properties resembling those of adult CMs,^{6–9} they have proved to be less mature than adult and fetal CMs.^{10–12} Thus, there is limited information about the electrophysiological and biochemical properties of iPSC-CMs, and the ultrastructural maturation process has not been investigated fully.

The ultrastructural features of human iPSC-CMs (hiPSC-

CMs) at around 30 days after cardiac differentiation have been described as being similar to those of adult CMs showing myofibrillar bundles with transverse Z-bands.^{4,13,14} However, in those reports, hiPSC-CMs still remain embryonic in phenotype, lacking a mature sarcomeric structure with M-bands and a variable degree of myofibrillar organization. It is unknown whether hiPSC-CMs can develop the adult CM-like ultrastructure in vitro. Immaturity of the hiPSC-CMs may hamper their application for studying cardiac diseases, drug development, and regenerative medicine, and could affect functional properties and drug responses in vitro and increase the risk of abnormal growth in vivo. Therefore, it is crucial to elucidate the

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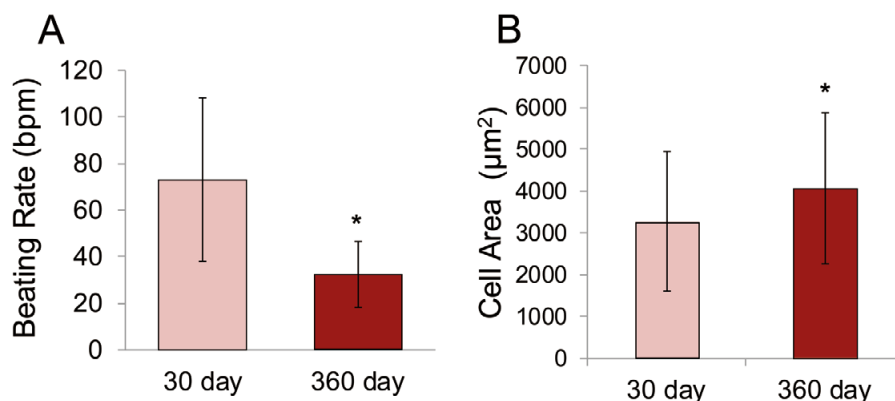


Figure 1. Comparison of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) at 30 and 360 days after cardiac differentiation. **(A)** The rate of beating (beats/min [bpm]) of embryoid bodies (EBs) at 30 days was significantly higher than that of 360-day EBs (73.2±34.9 bpm vs. 32.2±14.2 bpm, $P<0.0001$). **(B)** Size of hiPSC-CMs increased significantly after long-term culture (3,277.4±1,679.5 μm^2 vs. 4,067.9±1,814.6 μm^2 , $P=0.01$). * $P<0.05$ vs. 30-day.

maturation process and establish a protocol for creating homogeneous mature iPSC-CMs.

In this study, we investigated the ultrastructural, immunocytological, and gene expression changes of hiPSC-CMs in a long-term 2D culture. Here, we first report that mature sarcomeric structures with M-bands were detected only in 360-day hiPSC-CMs, which might be associated with lower expression levels of M-band-specific proteins compared with adult heart cells.

Methods

Culture of hiPSC and CM Differentiation

The hiPSC line 201B7 was retrovirally transfected with Oct3/4, SOX-2, Klf4, and c-Myc.^{1,9} These lines displayed all the defining parameters¹ and the hiPSCs were maintained as described.¹⁵

We differentiated hiPSC-CMs as embryoid bodies (EBs).^{16,17} In brief, hiPSCs aggregated to form EBs, and were cultured in suspension for 8 days. On day 8, the EBs were plated onto fibronectin-coated dishes and for the first 20 days, we followed the protocol as described previously.^{16,17} Cultures were maintained in a 5% CO₂, 5% O₂, 90% N₂ environment for the first 12 days and then transferred into a 5% CO₂/air environment for the remainder of the culture period. At 20 days after cardiac differentiation, EBs were maintained in culture DMEM/F12 supplemented with 2% fetal bovine serum, 2 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 0.1 mmol/L β -mercaptoethanol, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin.³ The medium was renewed every 2–3 days.

Immunocytochemistry

For immunostaining, single cells were isolated from microdissected 30- and 360-day-old beating EBs using collagenase B (Roche) and trypsin EDTA (Nacalai Tesque). The cells were plated onto fibronectin-coated dishes for 3 days to allow attachment. The cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 (Nacalai Tesque). The samples were stained with the following primary antibodies: rabbit polyclonal anti-cardiac troponin I (cTnI) (1:200; Santa Cruz), mouse monoclonal anti-myosin light chain 2a (MLC2a) (1:200; Synaptic Systems), rabbit polyclonal anti-myosin light

chain 2v (MLC2v) (1:100; Proteintech Group), mouse monoclonal anti- β III tubulin (1:100, Promega), mouse monoclonal anti-fibroblast (1:100, Acris Antibodies), and mouse monoclonal anti-human smooth muscle actin (1:100, Dako). We used the appropriate secondary antibodies: donkey anti-rabbit Alexafluor 594 (1:500, Invitrogen) and donkey anti-mouse Alexafluor 488 (1:500, Invitrogen). The nuclei were stained with DAPI (1:2000, Wako). The specimens were observed under a fluorescence microscope, Biozero BZ-9000 (Keyence), and the areas of cTnI-positive cells were calculated using a BZ-II analyzer (Keyence).

Transmission Electron Microscopy (TEM)

TEM was performed on 14-, 30-, 60-, 90-, 180-, and 360-day old EBs derived from hiPSC-CMs. EBs were microdissected and fixed for 1 h in 2% glutaraldehyde at 4°C in phosphate-buffered saline (PBS). All sections were treated with OsO₄ (1% for 1 min, and 0.5% for 20 min at 4°C) in PBS, dehydrated in ethanol and propylene oxide, and embedded in Luveak 812 (Nacalai Tesque). Ultrathin sections were cut with an ultramicrotome (Leica, Heidelberg, Germany) and observed with TEM (H-7650; Hitachi). All stages of EBs were examined in triplicate.

Analysis of mRNA Expression by Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen) from 20 to 30 EBs microdissected from 30-, 90-, 180-, and 360-day-old hiPSC-CMs, and treated with TURBO DNA-free Kit (Applied Biosystems). Total RNA from human heart tissue (left ventricle, left atrium, and fetal heart) was also reverse transcribed into complementary DNA (cDNA) for comparison. cDNA was synthesized from 1 μg of total RNA, in a total volume of 20 μl , using oligo (dT)₁₈ primer with Transcriptor First Strand cDNA Synthesis Kit (Roche). The PCR-related primers are detailed in Table S1. The real-time qPCR was performed using power SYBR Green PCR Master Mix (Applied Biosystems) for 6 samples. The expression of genes of interest was normalized to that of *GAPDH*. Relative quantification was calculated according to the $\Delta\Delta\text{C}_\text{T}$ method. The changes in gene expression levels were compared with those of hiPSC-

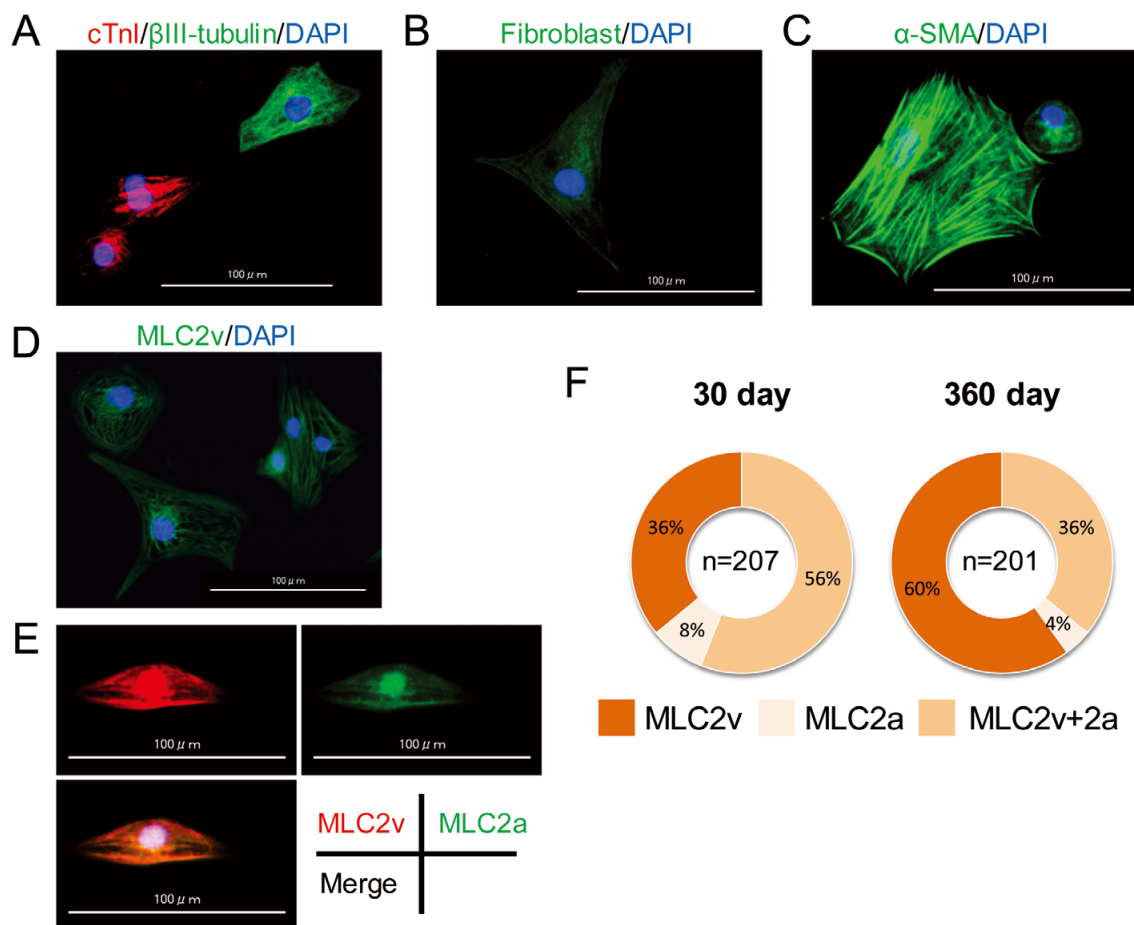


Figure 2. Immunostaining of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) at 30 days after cardiac differentiation. (A–E) Immunostaining of single cells isolated from microdissected beating embryoid bodies (EBs) at 30 days using the following antibodies: (A) cTnI (red), β III-tubulin (green), and DAPI (blue); (B) fibroblast (green) and DAPI (blue); (C) α -SMA (green) and DAPI (blue); (D) MLC2v (green) and DAPI (blue); (E) MLC2v (red) and MLC2a (green). (F) hiPSC-CM expressing both MLC2v and MLC2a. (F) Properties of MLC2v-positive/MLC2a-negative, MLC2v-negative/MLC2a-positive, and MLC2v/MLC2a double-positive cells at 30 days and 360 days. Among the MLC2v- or MLC2a-positive cells, 36% of 30-day hiPSC-CMs were MLC2v-positive/MLC2a-negative mature ventricular CMs and 56% were MLC2v/MLC2a double-positive immature ventricular CMs. At day 360, MLC2v-positive/MLC2a-negative mature CMs increased to 60%, whereas MLC2v/MLC2a double-positive immature ventricular CMs decreased to 36%.

CMs at 30-day differentiation. The fold change is expressed as mean $\pm$ SEM.

Statistical Analysis

All values are presented as mean $\pm$ SEM. Statistical significance was evaluated by Student's t-test for 2 groups or 1-way analysis of variance followed by Tukey test for comparisons of multiple groups. Differences with $P < 0.05$ were considered statistically significant.

Results

Long-Term Maintenance of hiPSC-CMs

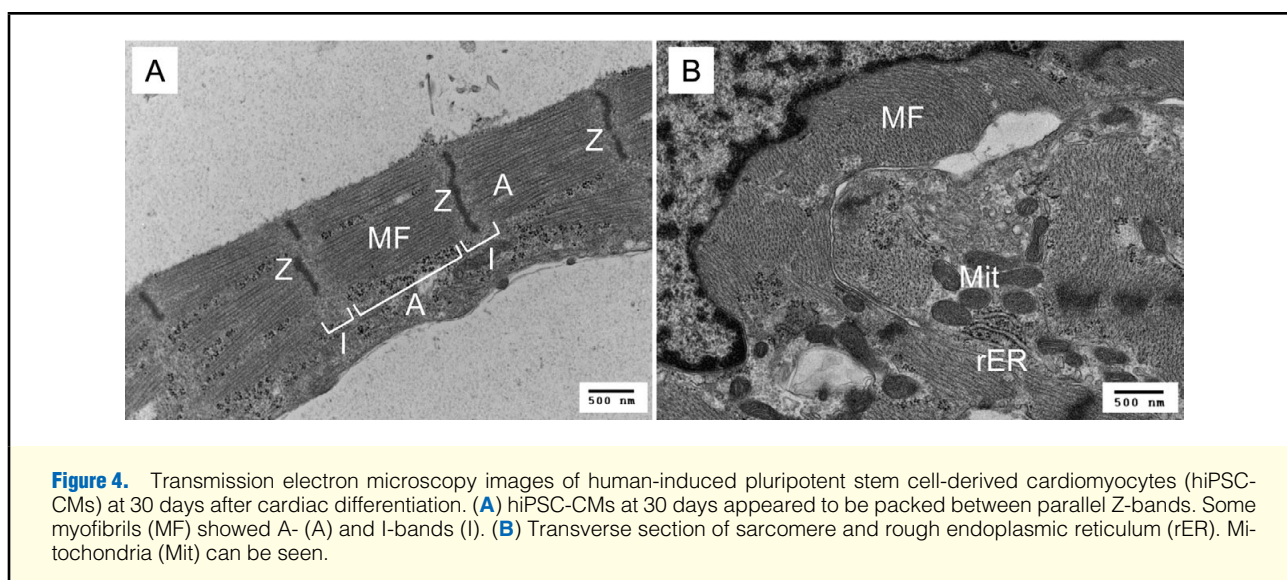
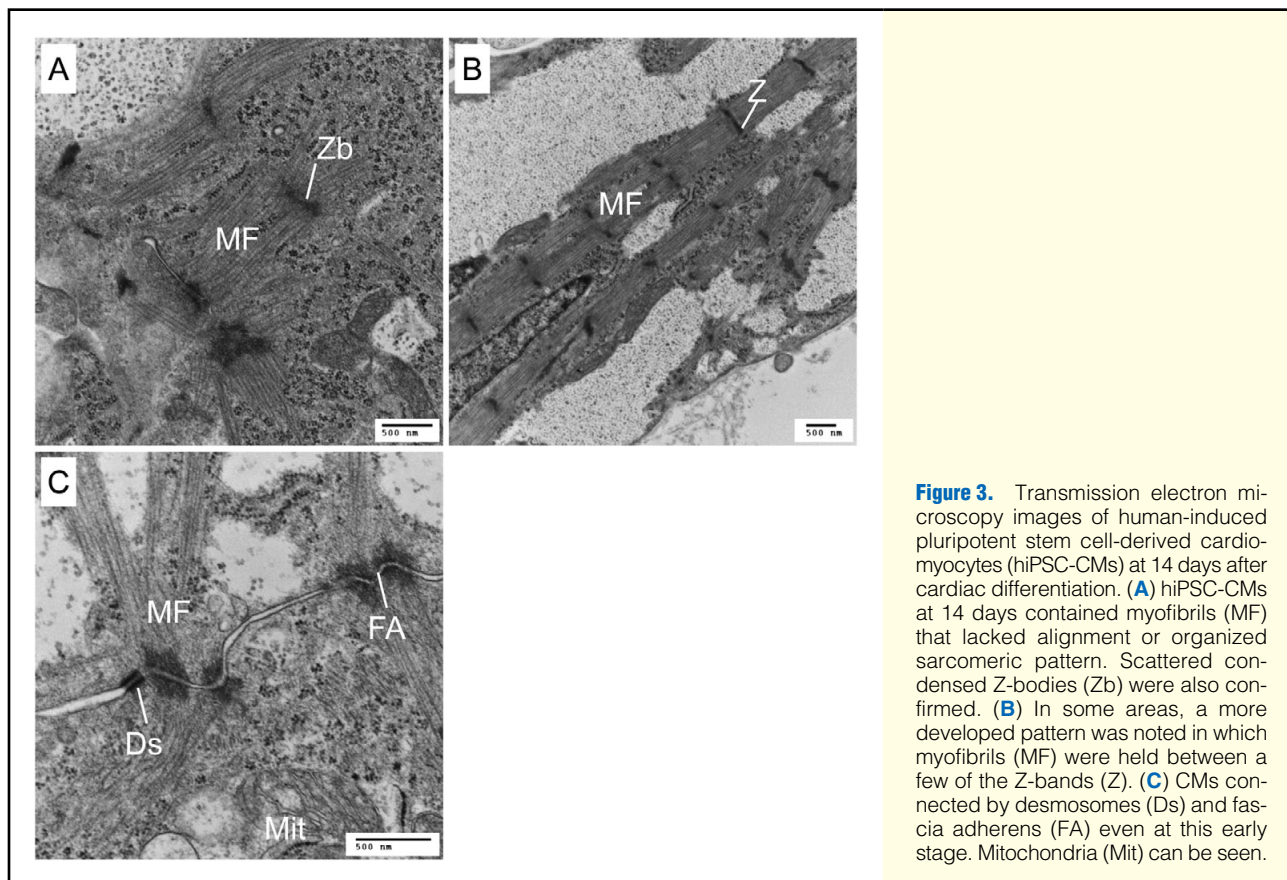
Areas of spontaneous beating became visible as early as day 8 after differentiation, and kept beating for more than 360 days (Movie S1). The beating rate of 30-day-old EBs ($n=42$) was significantly higher than that of 360-day-old EBs ($n=41$; 73.2 ± 34.9 beats/min vs. 32.2 ± 14.2 beats/min, $P < 0.0001$) (Figure 1A).

The size of dispersed hiPSC-CMs increased significantly after long-term culture as measured by their cell area ($3,277.4 \pm 1,679.5 \mu\text{m}^2$ vs. $4,067.9 \pm 1,814.6 \mu\text{m}^2$, $P=0.01$) (Figure 1B).

Immunostaining Analysis of Beating EBs at 30 and 360 Days After Cardiac Differentiation

Immunostaining of single cells isolated by microdissected beating EBs detected cells positive not only for cTnI, MLC2v, and MLC2a, but also β III-tubulin, fibroblasts, and α -SMA, suggesting the existence of neural cells, fibroblast-like cells, and vascular smooth muscle cells in the beating EBs as well as CMs (Figures 2A–E).

Among randomly selected single cells isolated from 30- ($n=213$) and 360-day-old ($n=191$) beating EBs, 61% and 64%, respectively, were positive for cTnI. Double immunostaining with anti-MLC2v and anti-MLC2a antibodies revealed that among the MLC2v- or MLC2a-positive cells, 36% were MLC2v-positive/MLC2a-negative, 8% were MLC2v-negative/



MLC2a-positive, and 56% were MLC2v/MLC2a double-positive CMs at 30-day differentiation. By day 360, MLC2v-positive/MLC2a-negative CMs increased to 60%, whereas MLC2v/MLC2a double-positive immature ventricular CMs decreased to 36% (**Figure 2F**).

Ultrastructural Analysis of hiPSC-CMs at 14-, 30-, 60-, 90-, 180-, and 360-Day Differentiation

hiPSC-CMs at 14-day differentiation contained myofibrils that lacked alignment or organized sarcomeric pattern, and were distributed diffusely in the cytoplasm in a disorganized fashion. Scattered patterns of condensed Z-bodies were also confirmed. However, in some areas, a more developed pattern was

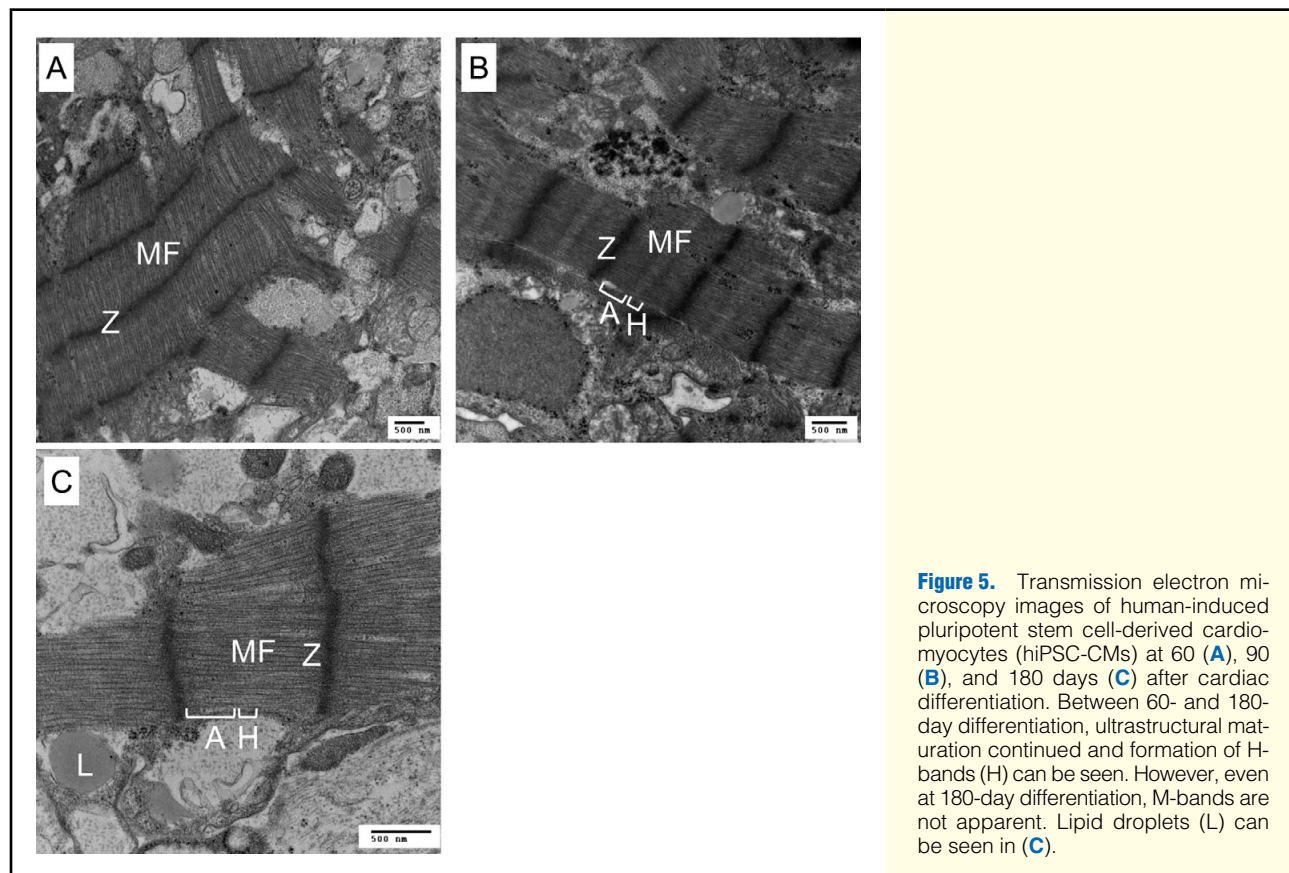


Figure 5. Transmission electron microscopy images of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) at 60 (A), 90 (B), and 180 days (C) after cardiac differentiation. Between 60- and 180-day differentiation, ultrastructural maturation continued and formation of H-bands (H) can be seen. However, even at 180-day differentiation, M-bands are not apparent. Lipid droplets (L) can be seen in (C).

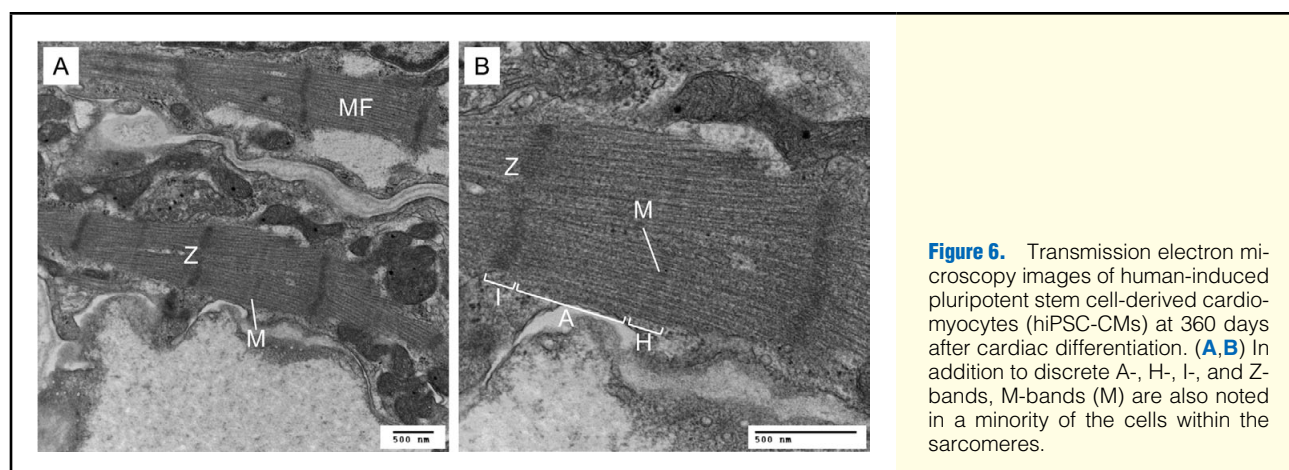


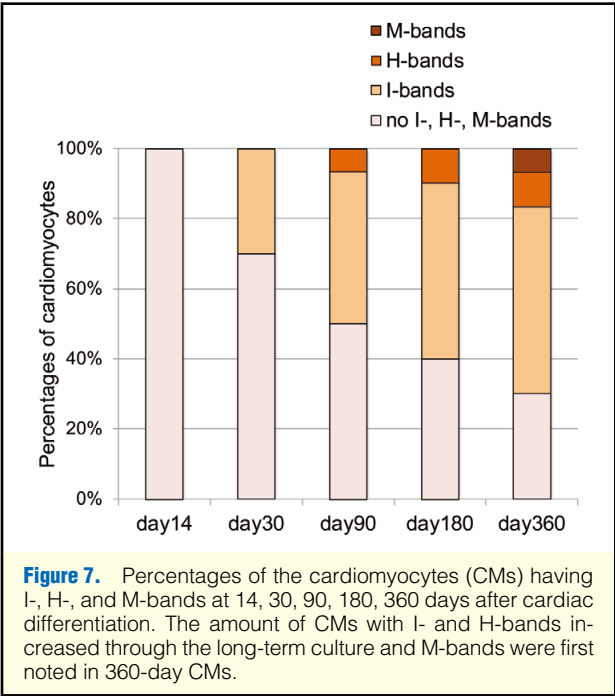
Figure 6. Transmission electron microscopy images of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) at 360 days after cardiac differentiation. (A,B) In addition to discrete A-, H-, I-, and Z-bands, M-bands (M) are also noted in a minority of the cells within the sarcomeres.

noted in which myofibrils were held between a few of the Z-bands (Figure 3). However, A-, H-, I-, and M-bands were not recognized. CMs were connected by desmosomes and fascia adherens at this early stage.

At 30-day differentiation, nascent myofibrils decreased and appeared to be packed between Z-bands. Parallel Z-bands were demonstrated to confine the myofibrils in the typical sarcomeric pattern. Some myofibrils showed A- and I-bands. However, they still lacked the formation of H-, and M-bands (Figure 4). Mitochondria and rough endoplasmic reticulum were also noted, as previously reported.¹³

Between 60- and 90-day differentiation, ultrastructural maturation continued and formation of H-bands could be observed. However, even at 180-day differentiation, M-bands could not be detected (Figure 5).

Finally, at 360-day differentiation, in addition to discrete A-, H-, I-, and Z-bands, M-bands were first noted in a minority of the cells within the sarcomeres (Figure 6). Myofibrils appeared to be tightly packed and distributed in an oriented fashion. The amount of sarcomeric structure in a single CM continued to increase, but was still scarce compared with an adult CM. Even at this stage, different degrees of organization

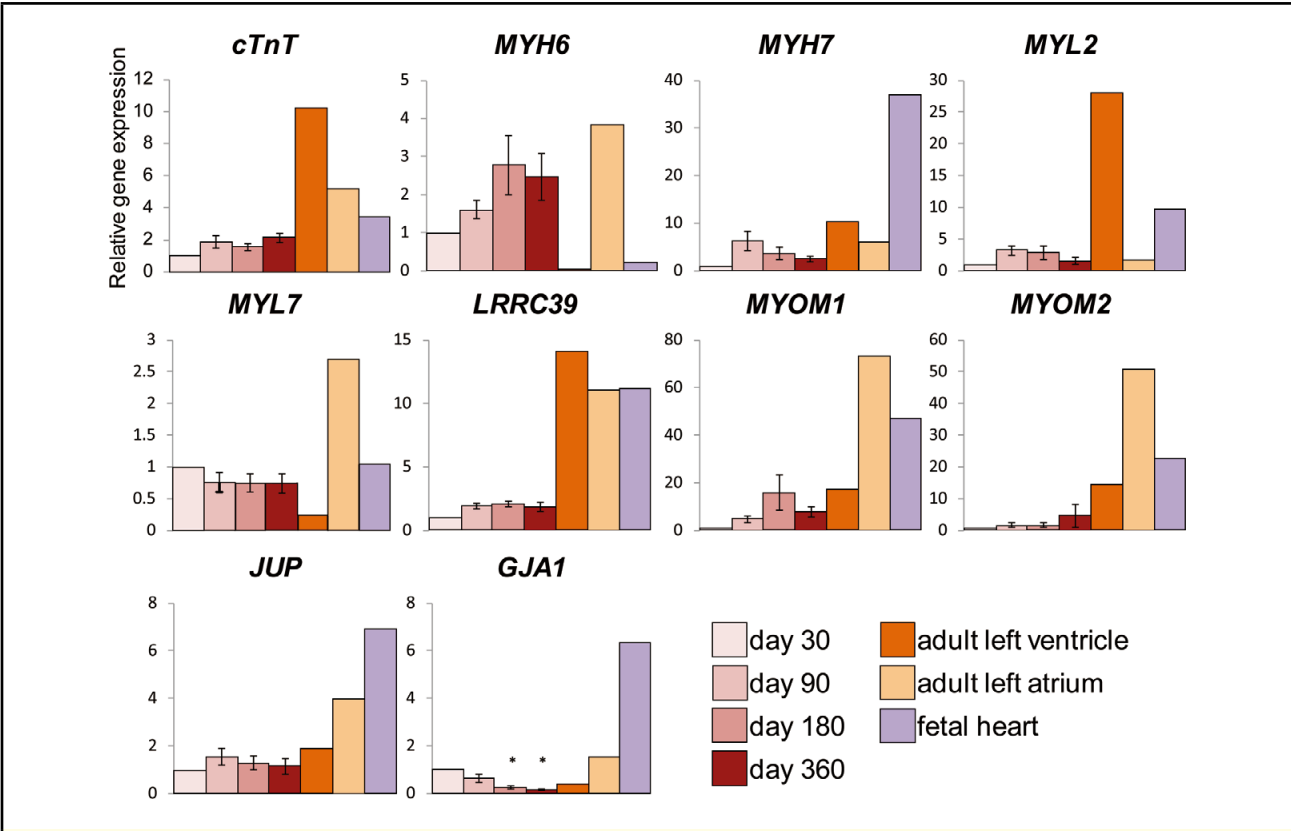


existed simultaneously in the same EB.

We evaluated 30 CMs with sarcomeres on randomly selected electron micrographs to assess the maturation process of sarcomeres quantitatively. **Figure 7** shows the percentages of CMs having I-, H-, and M-bands at 14-, 30-, 90-, 180-, 360-day differentiation.

Expression of Cardiac-Specific Genes

Leucine-rich repeat-containing protein 39 (*LRRC39*), myomesin 1 (*MYOM1*), and 2 (*MYOM2*), components of M-bands,¹⁸ increased at 360-day differentiation compared with 30-day differentiation, supporting the observation of M-band formation in 360-day hiPSC-CMs (**Figure 8**). However, the expression levels of the M-band-specific proteins in the hiPSC-CMs were lower compared with those of the adult heart. The expression of cardiac troponin-T (*cTnT*), myosin heavy chain 6 (*MYH6*), myosin heavy chain 7 (*MYH7*), and myosin regulatory light chain 2 (*MYL2*) also increased after the 1-year culture. However, the expression levels of cardiac-specific genes in the hiPSC-CMs were also considerably lower than those in the adult heart left ventricle or left atrium, and in the fetal heart. The expression levels of gap junction α -1 protein were significantly decreased in 180-day and 360-day hiPSC-CMs compared with 30-day hiPSC-CMs.



Discussion

In this study, we demonstrated that hiPSC-CMs continue to mature through a 1-year culture. This is the first report of the feasibility of 1-year 2D culture of hiPSC-CMs and description of the sarcomeric maturation process represented by the emergence of M-bands and the increase in the cardiac-specific gene expressions.

So far, the reported ultrastructure of hiPSC-CMs has been immature and their maturation process remained unknown.^{4,13,14} Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) are reported to follow a roughly similar maturation process to that reported both in vivo and in an in-vitro murine ES model.^{19–24} The hiPSC-CMs in the present study showed a similar maturation process to that of hESC-CMs.²⁵ At first, narrow, diffusely distributed, and frequently not well aligned myofibrils, resembling those of hiPSC-CMs at 14 days, developed into sarcomeres with clear band patterns including the Z-, I-, and A-bands, responding to hiPSC-CMs at between 30 and 90 days, and ultimately resulted in the generation of well-designed sarcomeres with A-, H-, I-, and M-bands. The ultrastructural findings of hiPSC-CMs in the literature now available relate to around 30 days of differentiation, and only Z- and I-bands have been visible.^{4,13,14} In our study, the 30-day hiPSC-CMs similarly showed only Z- and I-bands, not H- or M-bands. Notably, we are the first to find that only 360-day hiPSC-CMs, not 180-day hiPSC-CMs, show a mature sarcomeric structure with M-bands. However, even at 360-day differentiation, different degrees of organization patterns existed simultaneously in the same EB and homogeneous maturation was not confirmed. Our 1-year culture system was able to confirm more mature sarcomeric structures than previously reported, but still not that of adult CMs. It is reported that human CMs derived from fetal hearts do not achieve full ultrastructural maturity and that myofibrillar development continues throughout the entire fetal period.²² The insufficient maturation of hiPSC-CMs after long-term culture could be explained by several factors. In vitro culturing conditions lack the presence of adjacent non-myocyte proliferating cells, which play an important role in the maturation of CMs via paracrine and humoral signals in vivo. In addition, the CMs grown in the absence of hemodynamic workload typical of in vivo working CMs are reported to lack appropriate ultrastructural development.²⁶ The differences between in vitro and in vivo conditions, such as the absence of humoral factors and organized mechanical and electrical stress in vitro, might result in delayed ultrastructural maturation.

In electron micrographs of the sarcomere, the M-band appears as a series of parallel electron-dense lines in the central zone of the A-band. The M-band has been reported to play a role not only in mechanical stability in the activated sarcomere, such as reducing the intrinsic instability of thick filaments and helping titin to maintain order in sarcomeres, but also in the biomechanical conditions in contracting muscle such as stress sensing.²⁷ M-band formation was confirmed in the latest stage and has been considered the endpoint of myofibrillar maturation.^{18,21} The lower expression levels of the M-band-specific proteins in the hiPSC-CMs compared with the adult heart might be associated with the delayed appearance of M-bands. Maturation of iPSC-CMs is critical for their application in regenerative medicine, as well as for investigating the mechanisms underlying inherited cardiac diseases. Techniques to promote the maturation of ESC-CMs, such as 3D culture methodology,²⁸ electric stimulation,²⁹ and coculture with non-cardiomyocytes³⁰ may be applicable to iPSC-

CMs to overcome the problem, although it has not been fully investigated in hiPSC-CMs. Improved methods are needed to produce homogeneous, mature iPSC-CMs.

In addition to ultrastructural maturation, there was a significant increase in the size of hiPSC-CMs after long-term culture, supporting the process of morphological maturation. Also, the lower rate of beating of 360-day hiPSC-CMs compared with 30-day hiPSC-CMs suggested electrophysiological maturation, because it has been reported that the resting membrane potential becomes progressively more negative in the developing atrial and ventricular myocytes, which correlates with an increasing presence of I_{K1} , and ultimately, the fetal atrial and ventricular myocytes exhibit stable resting membrane potentials with little automaticity.³¹

Changes in the expression patterns of MLC2v and MLC2a occur during the maturation process.³² hiPSC-CMs were thought to be immature and similar to human fetal CMs because of the presence of a number of MLC2v/MLC2a double-positive CMs.³³ Our immunostaining analysis demonstrated that the percentage of MLC2v/MLC2a double-positive hiPSC-CMs decreased after long-term culture, accompanied by an increase in MLC2v-positive/MLC2a-negative hiPSC-CMs, suggesting maturing of the ventricular-type CMs.

This study also showed for the first time, changes in the expression levels of cardiac-specific genes and genes related to intercalated discs throughout the 1-year culture. The cardiac-specific genes tended to increase during 1-year culture, supporting the maturation process of hiPSC-CMs. The connexin (gap junction proteins) are reported to be more abundant in the neonate than the adult.³⁴ The significant decrease in *GJA1* expression levels in 180- and 360-day hiPSC-CMs compared with 30-day hiPSC-CMs also suggested maturation of hiPSC-CMs.

Study Limitations

We used microdissected beating EBs for the gene expression studies. The fact that EBs contain CMs at various stages of differentiation, as well as non-CMs, might obscure the results of the gene expression studies. We conducted immunostaining analysis of single cells from microdissected beating EBs 3 days after enzymatic dispersion, which might allow non-CMs to increase and affect the results of the percentage of CMs in the beating EBs.

Conclusions

The current study demonstrated developmental changes in the ultrastructural, immunocytological, and gene expression properties of hiPSC-CMs. Our results confirmed mature sarcomeric structure with M-band formation in long-term culture of hiPSC-CMs for the first time, which provides a new insight into the maturation process of hiPSC-CMs. For application of homogeneous mature hiPSC-CMs in regenerative medicine and in vitro modeling of human cardiac diseases, further maturation of cardiac cells will be needed.

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Disclosures

None.

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Supplementary Files

Supplementary File 1

Table S1. Primer Sequences Used for Real-Time qPCR Analysis

Supplementary File 2

Movie S1. 360-day-old beating embryoid bodies.

Please find supplementary file(s);
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