Ff-hiPSCs were generated from skin-derived fibroblasts and blood-derived cells. Although the efficiency of Ff-hiPSC generation was lower than that of iPSCs established on feeder cells, we were able to obtain an adequate number of Ff-hiPSC clones. The cord blood cells appeared to be more potent sources for Ff-hiPSC generation. Almost all Ff-hiPSCs have differentiation potential, and can differentiate into several kinds of somatic-type cells. These findings indicate that the Ff-hiPSCs established under Ff and Xf conditions from several types of somatic cells are similar to the hiPSCs established using the conventional system with feeders.

In conclusion, the present results indicate that hiPSCs with equivalent growth and differentiation potential to feeder-grown hiPSCs can be generated from various human tissue samples under Ff and Xf conditions. Since this method is easy to use, expandable and reproducible, it should prove valuable for generating clinical-grade hiPSCs intended for drug screening and therapeutics, or even basic research applications.

Methods

Production of laminin-511 E8. Recombinant LN511E8 were expressed in 293-F cells (Life Technologies) with 6 × His, HA and FLAG tags at the N-termini of the α5E8, β1E8 and γ1E8 chains, respectively, and were purified using affinity chromatography with Ni-NTA resin and anti-FLAG antibody agarose, as described previously[24]. rLN511E8 was also produced in cGMP-banked Freedom CHO-S cells (Life Technologies) with an N-terminal 6 × His tag on the α5E8 chain (iMatrix-511™, Nippi, Inc.). The cGMP-compatible rLN511E8 was purified using Ni-NTA affinity chromatography and ion-exchange chromatography. cGMP-compatible rLN511E8 was manufactured in a controlled clean room (Nippi and Yokohama Biopharmaceutical Research and Development Center).

Components of StemFit™. In this study, we used newly developed medium manufactured by Ajinomoto Co., Inc., Japan. StemFit™ contains twenty-one amino acids (L-Alanine, L-Arginine, L-Asparagine, L-Aspartic Acid, L-Cysteine, L-Cystine, L-Glutamic Acid, L-Glutamine, Glycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine and L-Valine), ten vitamins (L-Ascorbic Acid, Cobalamin, Biotin, Folic Acid, I-inositol, Niacinamide, D-Calcium Pantothenate, Pyridoxine Hydrochloride, Riboflavin and Thiamine Hydrochloride), five trace minerals (Cupric Sulfate, Ferric Sulfate, Ferric Nitrate, Zinc Sulfate and Sodium selenite) and growth factors, including bFGF. All ingredients are highly defined or purified materials, and none are derived from non-human animal sources. Large-scale manufacturing of StemFit™ has been achieved in well-validated and -qualified factories in Japan.

Feeder-free culture system for hESCs and hiPSCs. The hESCs/iPSCs were cultivated on rLN511E8-coated (0.5 μg/cm^2) cell culture plates with StemFit™ and passaged via dissociation into single cells using TrypLE Select (Life Technologies). Although this approach is similar to a previously published method[12], we separately developed a single cell culture system for hESCs (H9[3] and KhES1[25]) and hiPSCs (201B7[2] and 32R1[18]). Before plating the cells, cell culture dishes or plates were coated with rLN511E8 (0.5 μg/cm^2). The rLN511E8 solution was diluted with PBS(−) and the diluents were transferred to cell culture dishes or plates. The dishes or plates were incubated for one hour in a CO_2 incubator (37°C). The hESCs/iPSCs were dissociated into single cells by treatment with 0.5× TrypLE Select (1× TrypLE Select diluted 1:1 with 0.5 mM EDTA/PBS(−)) for four minutes at 37°C. Since the cells remained attached to the rLN511E8-coated plates, we aspirated the 0.5× TrypLE Select and carefully washed the cells with PBS(−). The cells were scraped under the StemFit™ and dissociated into single cells by pipetting the cells 10 times. The number of dissociated single cells was counted using a Countess Automated Cell Counter (Life Technologies). We typically plated 1.3×10^4 live cells into the rLN511E8-coated wells of a 6-well cell culture plate ($= 1.35 \times 10^3$ live cells/cm^2). A Rock inhibitor (Y-27632, final concentration 10 μM) was used only at the time of plating[26]. The next day, the medium was changed to fresh StemFit™ without Y-27632. The medium was changed every other day. Eight to ten days after plating, the cells reached 80–90% confluency and were ready for the next passage. Frozen stocks of Ff-hiPSC were similarly prepared as single cells and frozen in STEM-CELLBANKER (1×10^6 live cells/mL) (Nippon Zenyaku Kogyo) at −80°C using a standard slow-freezing method.

Establishment of Ff-hiPSCs from fibroblasts and blood cells. Human primary fibroblasts were derived from biopsied skin tissue samples. The fibroblasts were established and expanded with DMEM containing 10% autologous human serum. Using these fibroblasts, iPS cells were generated as described previously[17]. Briefly, following electroporation of reprogramming factors with episomal vectors using the Neon system (Life Technologies), the cells were plated on a non-coated cell culture plate. iPS cells were induced by changing the medium to StemFit™. Twenty to thirty days after plating, iPS cell colonies were observed.

Blood cell-derived iPS cells were generated as described previously[16]. Briefly, mononuclear cells were prepared from peripheral blood using the Ficoll-Paque PREMIUM (GE Healthcare) separation method. The cells were electroporated with episomal vectors using a Nucleofector 4D system (with P3 Primary Cell Kit, Lonza) and plated on rLN511E8-coated cell culture plates. The iPSCs were induced by changing the medium to StemFit™. Twenty to thirty days after plating, iPS cell colonies were observed. A similar method was used to generate Ff-hiPSCs from human cord blood (provided by the RIKEN Bioresource Center Cell Bank). We generated several clones of Ff-hiPSCs from each experiment.

The experimental protocols dealing with human subjects were approved by the institutional review board at our institute (Kyoto University Graduate School and Faculty of Medicine, Ethics Committee). Written informed consent was provided by each donor.

Genomic PCR analysis of the integration of the episomal vectors. Purified genomic DNA was used as a template to detect the remaining episomal vectors in the hiPSCs using a PCR analysis. The episomal vectors were detected using the primer set for EP4. The EP4 primer set was constructed in the EBNA1 region. The Fbx15 gene was amplified with a primer set for Fbx as an internal control. The episomal vector, pCXLE-EGFP, was used as a positive control ("Plasmid"). The primer sets are listed in Supplementary Table 3.

Antibodies used for immunostaining. The antibodies used in this study included anti-Oct3/4 (BD, 611202), -Nanog (R&D, AF1997), -SSEA4 (BD, 560073), -TRA-1-60 (BD, 560071), -Sox17 (R&D, AF1924), -α-smooth muscle actin (SMA) (Dako, M0851), -α-1-fetoprotein (AFP) (Dako, N1501) and -βIII tubulin (Millipore, MAB1637) antibodies.

RNA isolation and reverse transcription. Total RNA purification and RT-PCR were performed as described previously[2,27–29]. The expressions of Nanog, Sox2, Oct3/4, Lin28, L-Myc, c-Myc, Klf4 and G3PDH were detected using the primer sets listed in Supplementary Table 3.

***In vitro* differentiation.** To examine whether Ff-hiPSCs spontaneously differentiate into the three germ layers, the Ff-hiPSCs were cultivated with StemFit™ lacking bFGF (StemFit™-bFGF) for two weeks on rLN511E8-coated cell culture plates. The differentiated cells were immunostained with the indicated antibodies.

Directed differentiation into dopaminergic neurons. Dopaminergic neurons were induced from Ff-hiPSCs under xeno-free conditions. The dissociated Ff-hiPSCs were plated on rLN511E8-coated plates at high density (7.5×10^5 cells/cm^2; Day 0). Glasgow minimum essential medium (GMEM, Life Technologies) was supplemented with 8% Xeno-free knockout serum replacement (Life Technologies), sodium pyruvate (SIGMA), 2-mercapto ethanol (Wako), MEM NEAA (Life Technologies), 500 nM of A-83-01 (Activin inhibitor, Wako), 100 nM of LDN193189 (BMP inhibitor, STEMGENT) and 10 μM Y-27632 (Wako). To induce neural cells with a ventral mesencephalic phenotype, 2 μM Purmorphamine (Wako) and 100 ng/mL of FGF-8 (Wako) were added starting the next day. Starting on day 3, 3 μM CHIR99021 (GSK3β inhibitor, Wako) was also added. On day 12, the first passage was performed with TrypLE CTS (Life Technologies), and the neural cells were replated on a fresh rLN511E8 surface at the same density as on day 0. The basal medium was switched to Neurobasal medium supplemented with Xeno-free B27 (Life Technologies), 200 μM of ascorbic acid (SIGMA), 400 μM of dbc AMP (SIGMA), 2 ng/mL of GDNF (R&D), 20 ng/mL of BDNF (R&D) and 10 μM Y-27632. Throughout the neural induction process, the medium was changed every day. On days 12, 20 and 28, the culture was passaged onto a new rLN511E8 surface. Y-27632 was added to the medium on the day of passage. Immunostaining was performed using anti-TH (Chemicon, AB152), -Foxa2 (Santa Cruz, SC6554) and –Nurr1 (kindly provided by KAN Research Institute) antibodies.

Directed differentiation into blood cells. Ff-hiPSCs were differentiated into blood cells as described previously[30] with some key modifications. Briefly, Ff-hiPSCs were cultivated in Ultra Low Attachment 6-well cell culture plates (Corning) with StemFit™ to prepare EB-like spheres. The spheres were subsequently used for blood cell differentiation according to the established protocol[20].

Directed differentiation into insulin-producing cells. Ff-hiPSCs were differentiated into insulin-producing cells as described previously[21]. Briefly, human Ff-hiPSCs were dissociated and plated on rLN511E8 and cultured with the reported factors required for pancreatic differentiation. The dissociated cells were treated with 100 ng/ml of activin A and 3 μM CHIR99021 for 24 hours, and then were treated with 100 ng/ml of activin A and 1 μM CHIR99021 for four days. Next, the cells were cultured with 1 μM dorsomorphin (Calbiochem, San Diego, CA), 2 μM retinoic acid (Sigma, St. Louis, MO) and 10 μM SB431542 (Sigma) for six days. Subsequently, the cells were cultured with 10 μM forskolin, (Wako), 10 μM dexamethasone (Wako), 5 μM Alk5 inhibitor II (Calbiochem) and 10 mM nicotinamide (StemCell Technologies, Vancouver, BC) for 12 days. Following the culture, the cells were fixed and stained with Hoechst33342 (Life Technologies) and antibodies against insulin (Dako).

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Author contributions

Conceived and designed the experiments: M. Nakagawa, S.S. and S.Y. Performed the experiments: M. Nakagawa, S.S., Y.T., N.T., T.I., K.A., A.M., J.T., T.T., K.O., M. Nishizawa, Y.Y. and K.S. Analyzed the data: M. Nakagawa, S.S. and Y.T. Contributed reagents/materials/analysis tools: M. Nakagawa, S.S., Y.T. and K.S. Wrote the paper: M. Nakagawa.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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Making Steady Progress on Direct Cardiac Reprogramming Toward Clinical Application

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Making Steady Progress on Direct Cardiac Reprogramming Toward Clinical Application

Kenji Miki, Yoshinori Yoshida, Shinya Yamanaka

Reprogramming of Human Fibroblasts Toward a Cardiac Fate
Nam et al
Proc Natl Acad Sci USA. 2013;110:5588–5593.

A new report demonstrates direct cardiac reprogramming in human cells for the first time and points to the possibility of moving this technology toward clinical applications.

Direct reprogramming to somatic cells by forced expression of a combination of lineage-specific transcription factors or micro RNAs (miRNAs) has been demonstrated in a variety of rodent cell types, such as neurons,[1] neural stem cells,[2] hepatocyte-like cells,[3,4] β-cells,[5] and cardiomyocytes.[6–10] Three groups have reported the direct neuronal reprogramming of human fibroblast into neuronal cells.[11–13] Pang et al[11] demonstrated that the same 3 transcription factors used for mouse cells, Brn2, Ascl1, and Mytl1, are insufficient to induce functional neurons from human fetal fibroblasts, but that Brn2, Ascl1, and Mytl1 plus the addition of NeuroD1 can convert human fetal and postnatal fibroblasts into functional neurons. Yoo et al[13] also reported that a combination of neuronal transcription factors and 2 miRNAs, micro RNA (miR)-9/9* and miR-124, can efficiently convert human fibroblasts into functional neurons. These findings suggest that, compared with mouse cells, additional factors might be required to direct the reprogramming of human somatic cells into other lineage cells.

More recently, Nam et al[14] reported the direct reprogramming of human fibroblasts into cardiac-like cells (iCLMs). They first tested whether the same cardiac transcription factors, Gata4, Mef2c, and Tbx5 (GMT) or GMT+Hand2, which were previously reported to be useful for the direct reprogramming of mouse fibroblasts into cardiomyocytes,[6–8,10] could reprogram neonatal human foreskin fibroblasts into iCLMs. However, GMT and GMT+Hand2 both failed to induce cardiac marker expression in human foreskin fibroblasts. To determine the optimal combination of factors for the direct cardiac reprogramming of human fibroblasts, they examined the effects of additional factors, including other cardiac transcription factors and miRNAs, with GMT+Hand2. They found that a combination of Gata4, Hand2, Tbx5, Myocardin, miR-1, and miR-133 (6F) could convert human fibroblasts into iCLMs. The transduction of 6F induced ≈20% of human foreskin fibroblasts, ≈13% of adult human cardiac fibroblasts, and 9.5% of adult human dermal fibroblasts to express cardiac Troponin T 2 weeks later. Furthermore, spontaneous contractions were observed in a small subset of iCLMs derived from adult human cardiac fibroblasts 11 weeks later, but not from either human foreskin fibroblasts or adult human dermal fibroblasts.

In this study, they identified a combination of factors, 6F, that are capable of direct cardiac reprogramming from human fibroblasts. Interestingly, the transduction of Mef2c, which is one of the key factors used to reprogram mouse fibroblasts directly into cardiomyocytes,[6–8,10] with 6F significantly decreased the percentage of cardiac Troponin T^{+} cells. They previously showed that miR-1-1/133a-2 and miR-1-2/133a-1 were down-regulated in the hearts of mice lacking MEF2 expression and a MEF2-dependent upstream enhancer of the miR-1-1/133a-2 has been shown to regulate miR-1-1/133a-2 expression in cardiac and skeletal muscle in vivo.[16,17] Therefore, they mentioned that Mef2c regulates the expression of miR-1 and miR-133,[15] and that these miRNAs probably play an alternative role of Mef2c.

The precise molecular mechanism(s) underlying the direct cardiac reprogramming from fibroblasts is still unknown even in mice, and there is a possibility that there might be a mechanistic difference in the reprogramming process between mouse and human cells.

Regarding the direct reprogramming to somatic cells, the reprogramming efficiency and reproducibility are very important and often controversial subjects. A previous study by Chen et al[18] showed that forced expression of GMT in mouse tail tip fibroblasts and cardiac fibroblasts was insufficient to induce cardiac phenotypes. Nam et al[14] also showed that forced expression of a combination of miR-1, miR-133, miR-208 and miR-499, which were previously reported to be useful for the direct cardiac reprogramming of mouse fibroblasts,[9] failed to induce the expression of cardiac markers in mouse tail-tip fibroblasts. These findings suggest that an accumulation of slight differences among laboratories, such as the culture and isolation methods used for the fibroblasts, the fibroblast lines used or the method of virus production, might cause differences in the reprogramming efficiency and reproducibility. As indicated above, Nam et al[14] first reported the direct cardiac reprogramming of human fibroblasts. In their study, cardiac Troponin T^{+} cells at 2 weeks and calcium transients at 4 weeks after the transduction of 6F were observed in ≈13% and ≈15% of adult human cardiac fibroblasts, respectively. However, spontaneous contractions were observed in only a few iCLMs derived from adult human cardiac fibroblasts. Many other researchers are

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Commentaries serve as a forum in which experts highlight and discuss articles (published elsewhere) that the editors of *Circulation Research* feel are of particular significance to cardiovascular medicine.

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also attempting to reprogram human somatic cells directly into cardiomyocytes, and thus, further improvements to achieve a high reprogramming efficiency are expected, and it is likely that the reproducibility of the method of Nam et al[14] will be demonstrated by another group in the near future.

The reprogrammed iCLMs from human fibroblasts were functionally immature. In the case of mouse cells, Inagawa et al[10] reported that the in vivo transduction of a polycistronic retrovirus vector expressing GMT separated by 2A peptides (3F2A) generated more mature cardiomyocytes compared with the in vivo transduction of GMT. In addition, some studies suggested that the in vivo environment, such as the presence of mechanical force and extracellular matrix and secreted proteins, might be more suitable for direct reprogramming with regard to the reprogramming efficiency, reprogrammed cell survival, and maturation.[5,7,8] The further development of gene delivery systems, such as polycistronic vectors, and the use of the in vivo environment might help to reprogram human fibroblasts into more mature cardiomyocytes.

In addition, the reprogrammed iCLMs from human fibroblasts were heterogeneous. In fact, the expression levels of cardiac and noncardiac genes in the iCLMs varied widely and only a small subset of iCLMs showed spontaneous contraction. It is supposed that partially reprogrammed cells exist in the population of reprogrammed cells, and such partially reprogrammed cells might cause arrhythmias in the heart. The authors mentioned that this heterogeneity of iCLMs is because of variations in the stoichiometry and levels of the expression of factors in individual cells and because of the heterogeneity of the original fibroblasts. In the future, improvements in the reprogramming efficiency and gene introduction methods may reduce the heterogeneity and thereby make the clinical application of this technology more feasible.

Attractive approaches, including direct cardiac reprogramming, have been developed in recent years with the goal of future cardiac regeneration therapy. Human pluripotent stem cells (PSCs), such as embryonic stem cells and induced PSCs, which can efficiently differentiate into cardiomyocytes after the addition of a combination of growth factors and are able to provide large amount of cardiomyocytes, are promising candidate cell sources. The transplantation of cardiomyocytes derived from human PSCs has already been reported to improve the cardiac function in rat and in swine infarction models.[19,20] In addition, Shiba et al[21] reported that human embryonic stem cell–derived cardiomyocytes can electrically couple and suppress arrhythmias in injured guinea pig hearts. However, many problems still remain to be resolved before the clinical application of human PSCs for cardiac disease. In particular, the elimination of undifferentiated PSCs to avoid tumor formation and the preparation of a large amount of cardiomyocytes derived from human PSCs are very important issues. The direct cardiac conversion from somatic cells is also a promising approach for cardiac regeneration therapy and may overcome the risk of tumor formation. Some groups have already reported the in vivo direct cardiac reprogramming of cardiac fibroblasts in the mouse heart.[7–10] In addition, Qian et al[7] and Song et al[8] revealed that the transduction of cardiac transcription factors in vivo improved the cardiac function and reduced fibrosis after myocardial infarction. Before the clinical application of this technology, the full effects of direct cardiac conversion and the safety of the transduction methods using defined factors must be demonstrated in large animal models. In addition, quantitative comparison of cardiomyocytes generated by direct cardiac conversion technology and PSC-derived cardiomyocytes will be required. Although direct cardiac conversion is still a developing technology, this new technology possesses great potential for future cardiac regeneration therapy.

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Disclosures

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Commentaries on Cutting Edge Science

Making Steady Progress on Direct Cardiac Reprogramming Toward Clinical Application

Kenji Miki, Yoshinori Yoshida, Shinya Yamanaka

Reprogramming of Human Fibroblasts Toward a Cardiac Fate
Nam et al
Proc Natl Acad Sci USA. 2013;110:5588–5593.

A new report demonstrates direct cardiac reprogramming in human cells for the first time and points to the possibility of moving this technology toward clinical applications.

Direct reprogramming to somatic cells by forced expression of a combination of lineage-specific transcription factors or micro RNAs (miRNAs) has been demonstrated in a variety of rodent cell types, such as neurons,[1] neural stem cells,[2] hepatocyte-like cells,[3,4] β-cells,[5] and cardiomyocytes.[6–10] Three groups have reported the direct neuronal reprogramming of human fibroblast into neuronal cells.[11–13] Pang et al[11] demonstrated that the same 3 transcription factors used for mouse cells, Brn2, Ascl1, and Mytl1, are insufficient to induce functional neurons from human fetal fibroblasts, but that Brn2, Ascl1, and Mytl1 plus the addition of NenroD1 can convert human fetal and postnatal fibroblasts into functional neurons. Yoo et al[13] also reported that a combination of neuronal transcription factors and 2 miRNAs, micro RNA (miR)-9/9* and miR-124, can efficiently convert human fibroblasts into functional neurons. These findings suggest that, compared with mouse cells, additional factors might be required to direct the reprogramming of human somatic cells into other lineage cells.

More recently, Nam et al[14] reported the direct reprogramming of human fibroblasts into cardiac-like cells (iCLMs). They first tested whether the same cardiac transcription factors, Gata4, Mef2c, and Tbx5 (GMT) or GMT+Hand2, which were previously reported to be useful for the direct reprogramming of mouse fibroblasts into cardiomyocytes,[6–8,10] could reprogram neonatal human foreskin fibroblasts into iCLMs. However, GMT and GMT+Hand2 both failed to induce cardiac marker expression in human foreskin fibroblasts. To determine the optimal combination of factors for the direct cardiac reprogramming of human fibroblasts, they examined the effects of additional factors, including other cardiac transcription factors and miRNAs, with GMT+Hand2. They found that a combination of Gata4, Hand2, Tbx5, Myocardin, miR-1, and miR-133 (6F) could convert human fibroblasts into iCLMs. The transduction of 6F induced ≈20% of human foreskin fibroblasts, ≈13% of adult human cardiac fibroblasts, and 9.5% of adult human dermal fibroblasts to express cardiac Troponin T 2 weeks later. Furthermore, spontaneous contractions were observed in a small subset of iCLMs derived from adult human cardiac fibroblasts 11 weeks later, but not from either human foreskin fibroblasts or adult human dermal fibroblasts.

In this study, they identified a combination of factors, 6F, that are capable of direct cardiac reprogramming from human fibroblasts. Interestingly, the transduction of Mef2c, which is one of the key factors used to reprogram mouse fibroblasts directly into cardiomyocytes,[6–8,10] with 6F significantly decreased the percentage of cardiac Troponin T^+ cells. They previously showed that miR-1-1/133a-2 and miR-1-2/133a-1 were downregulated in the hearts of mice lacking MEF2 expression and a MEF2-dependent upstream enhancer of the miR-1-1/133a-2 has been shown to regulate miR-1-1/133a-2 expression in cardiac and skeletal muscle in vivo.[16,17] Therefore, they mentioned that Mef2c regulates the expression of miR-1 and miR-133,[15] and that these miRNAs probably play an alternative role of Mef2c.

The precise molecular mechanism(s) underlying the direct cardiac reprogramming from fibroblasts is still unknown even in mice, and there is a possibility that there might be a mechanistic difference in the reprogramming process between mouse and human cells.

Regarding the direct reprogramming to somatic cells, the reprogramming efficiency and reproducibility are very important and often controversial subjects. A previous study by Chen et al[18] showed that forced expression of GMT in mouse tail tip fibroblasts and cardiac fibroblasts was insufficient to induce cardiac phenotypes. Nam et al[14] also showed that forced expression of a combination of miR-1, miR-133, miR-208 and miR-499, which were previously reported to be useful for the direct cardiac reprogramming of mouse fibroblasts,[9] failed to induce the expression of cardiac markers in mouse tail-tip fibroblasts. These findings suggest that an accumulation of slight differences among laboratories, such as the culture and isolation methods used for the fibroblasts, the fibroblast lines used or the method of virus production, might cause differences in the reprogramming efficiency and reproducibility. As indicated above, Nam et al[14] first reported the direct cardiac reprogramming of human fibroblasts. In their study, cardiac Troponin T^+ cells at 2 weeks and calcium transients at 4 weeks after the transduction of 6F were observed in ≈13% and ≈15% of adult human cardiac fibroblasts, respectively. However, spontaneous contractions were observed in only a few iCLMs derived from adult human cardiac fibroblasts. Many other researchers are

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also attempting to reprogram human somatic cells directly into cardiomyocytes, and thus, further improvements to achieve a high reprogramming efficiency are expected, and it is likely that the reproducibility of the method of Nam et al[14] will be demonstrated by another group in the near future.

The reprogrammed iCLMs from human fibroblasts were functionally immature. In the case of mouse cells, Inagawa et al[10] reported that the in vivo transduction of a polycistronic retrovirus vector expressing GMT separated by 2A peptides (3F2A) generated more mature cardiomyocytes compared with the in vivo transduction of GMT. In addition, some studies suggested that the in vivo environment, such as the presence of mechanical force and extracellular matrix and secreted proteins, might be more suitable for direct reprogramming with regard to the reprogramming efficiency, reprogrammed cell survival, and maturation.[5,7,8] The further development of gene delivery systems, such as polycistronic vectors, and the use of the in vivo environment might help to reprogram human fibroblasts into more mature cardiomyocytes.

In addition, the reprogrammed iCLMs from human fibroblasts were heterogeneous. In fact, the expression levels of cardiac and noncardiac genes in the iCLMs varied widely and only a small subset of iCLMs showed spontaneous contraction. It is supposed that partially reprogrammed cells exist in the population of reprogrammed cells, and such partially reprogrammed cells might cause arrhythmias in the heart. The authors mentioned that this heterogeneity of iCLMs is because of variations in the stoichiometry and levels of the expression of factors in individual cells and because of the heterogeneity of the original fibroblasts. In the future, improvements in the reprogramming efficiency and gene introduction methods may reduce the heterogeneity and thereby make the clinical application of this technology more feasible.

Attractive approaches, including direct cardiac reprogramming, have been developed in recent years with the goal of future cardiac regeneration therapy. Human pluripotent stem cells (PSCs), such as embryonic stem cells and induced PSCs, which can efficiently differentiate into cardiomyocytes after the addition of a combination of growth factors and are able to provide large amount of cardiomyocytes, are promising candidate cell sources. The transplantation of cardiomyocytes derived from human PSCs has already been reported to improve the cardiac function in rat and in swine infarction models.[19,20] In addition, Shiba et al[21] reported that human embryonic stem cell–derived cardiomyocytes can electrically couple and suppress arrhythmias in injured guinea pig hearts. However, many problems still remain to be resolved before the clinical application of human PSCs for cardiac disease. In particular, the elimination of undifferentiated PSCs to avoid tumor formation and the preparation of a large amount of cardiomyocytes derived from human PSCs are very important issues. The direct cardiac conversion from somatic cells is also a promising approach for cardiac regeneration therapy and may overcome the risk of tumor formation. Some groups have already reported the in vivo direct cardiac reprogramming of cardiac fibroblasts in the mouse heart.[7–10] In addition, Qian et al[7] and Song et al[8] revealed that the transduction of cardiac transcription factors in vivo improved the cardiac function and reduced fibrosis after myocardial infarction. Before the clinical application of this technology, the full effects of direct cardiac conversion and the safety of the transduction methods using defined factors must be demonstrated in large animal models. In addition, quantitative comparison of cardiomyocytes generated by direct cardiac conversion technology and PSC-derived cardiomyocytes will be required. Although direct cardiac conversion is still a developing technology, this new technology possesses great potential for future cardiac regeneration therapy.

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Disclosures

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ORIGINAL ARTICLE
Regenerative Medicine

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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