Cardiovascular Research (2010) **88**, 314–323
doi:10.1093/cvr/cvq189

Cell line-dependent differentiation of induced pluripotent stem cells into cardiomyocytes

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Received 9 October 2009; revised 26 May 2010; accepted 8 June 2010; online publish-ahead-of-print 14 June 2010

Time for primary review: 29 days

Aims Mouse and human fibroblasts can be directly reprogrammed to pluripotency by the ectopic expression of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) to yield induced pluripotent stem (iPS) cells. iPS cells can be generated even without the expression of c-Myc. The present study examined patterns of differentiation of mouse iPS cells into cardiomyocytes in three different cell lines reprogrammed by three or four factors.

Methods and results During the induction of differentiation on feeder-free gelatinized dishes, genes involved in cardiogenesis were expressed as in embryonic stem cells and myogenic contraction occurred in two iPS cell lines. However, in one iPS cell line (20D17) generated by four factors, the expression of cardiac-specific genes and the beating activity were extremely low. Treating iPS cells with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, increased Nkx2.5 expression in all iPS cell lines. While the basal Nkx2.5 expression was very low in 20D17, the TSA-induced increase was the greatest. TSA also induced the expression of contractile proteins in 20D17. Furthermore, we demonstrated the increased mRNA level of Oct3/4 and nuclear protein level of HDAC4 in 20D17 compared with the other two iPS cell lines. DNA microarray analysis identified genes whose expression is up- or down-regulated in 20D17.

Conclusions Mouse iPS cells differentiate into cardiomyocytes in a cell line-dependent manner. TSA induces myocardial differentiation in mouse iPS cells and might be useful to overcome cell line variation in the differentiation efficiency.

Keywords Induced pluripotent stem cells • Cardiomyocytes • Differentiation • Cell culture • An HDAC inhibitor

1. Introduction

Mouse and human fibroblasts can be reprogrammed directly to pluripotency by the ectopic expression of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc)[1–4] to yield induced pluripotent stem (iPS) cells. Recently, iPS cells were generated without the expression of c-Myc.[3] The iPS cells display properties of self-renewal and pluripotency similar to embryonic stem (ES) cells, and yield germ line adult chimeras. This strategy provides an opportunity to generate patient-specific pluripotent stem cells, which will enable us to perform patient-oriented drug screening and the investigation of genetic heart diseases, in addition to cell transplantation therapy. The latest studies have revealed that mouse iPS cells can differentiate into cardiomyocytes by the formation of embryoid bodies (EBs) or through the use of collagen IV-coated dishes and OP9 feeder cells.[5–7] However, it is unknown whether iPS cells can differentiate into cardiomyocytes without the formation of EBs and without using collagen IV-coated dishes or feeder cells. In addition, it should be determined whether the efficiencies of myocardial cell differentiation are uniform or diverse in different iPS cell lines.

Early lateral mesoderm cells express vascular endothelial growth factor (VEGF) receptor-2 (Flk-1), which serves as a marker of progenitors of cardiomyocytes as well as endothelial and haematopoietic cells.[8,9] Transcription factors such as GATA4 and Nkx2.5 expressed in the lateral mesoderm play important roles in the following heart developmental processes.[10–12] The activities of cardiac transcription

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factors are regulated, in part, by histone acetyltransferases and histone deacetylases (HDACs). We previously reported that the treatment of ES cells with trichostatin A (TSA), a specific HDAC inhibitor, induces the acetylation of GATA4 as well as histones and facilitates their differentiation into cardiomyocytes.[13] The present study investigated patterns of differentiation of different lines of iPS cells into cardiomyocytes and their responses to TSA.

2. Methods

2.1 Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The protocol was approved by the ethics committee on the use of laboratory animals in Kyoto University.

2.2 Generation of iPS cells

Mouse iPS cells were established as described previously.[1–3] In brief, we generated Nanog-iPS cell lines (38C2 and 20D17) by introducing Oct3/4, Sox2, Klf4, and c-Myc into mouse embryonic fibroblasts carrying the Nanog-GFP-IRES-Puro^r reporter, and by selecting clones in medium containing puromycin. We generated iPS cell line (Myc(-)iPS) (256H18) by introducing Oct3/4, Sox2, and Klf4 into mouse tail tip fibroblasts isolated from adult *Discosoma* sp. red fluorescent protein (DS-Red)-transgenic mice.

2.3 Culture of iPS and ES cells

An ES cell line (ht7) derived from 129/Ola expresses the hygromycin-resistant gene driven by the Oct3/4 promoter. Three iPS and one ES cell lines were maintained on gelatinized 100 mm dishes in DMEM (Sigma-Aldrich) containing 15% foetal calf serum (FCS; Sigma-Aldrich), 0.1 μmol/L 2-mercaptoethanol (2ME), and 1000 units/mL LIF (Chemicon International). Puromycin was added to the medium maintaining Nanog-iPS cell lines and hygromycin to the medium maintaining an ES cell line. For differentiation, these cells were treated with 0.25% trypsin/ethylenediaminetetraacetic acid (GIBCO), and transferred to gelatinized six-well plates in α-minimum essential medium (GIBCO) supplemented with 10% FCS and 0.05 μmol/L 2ME, at a concentration of 3×10^4 cells/well. The day on which these cells were transferred to six-well plates was designated as day 0. Medium change was performed on days 3, 5, and 8.

2.4 Immunocytochemistry

Differentiated cells were isolated using 0.5% collagenase and transferred to gelatinized dishes on day 8 or 10. These cells were fixed with 4% paraformaldehyde and incubated with an anti-mouse monoclonal cardiac myosin heavy chain (c-MHC) antibody (1:50 dilution; abcam) that reacts with both α- and β-cardiac MHC, an anti-mouse monoclonal cardiac troponin T (c-TNT) antibody (1:50 dilution; abcam). As a secondary antibody, we used donkey anti-mouse IgG antibody (1:200 dilution; Jackson ImmunoResearch Laboratories Inc.) labelled with Cy3 (red) for 38C2 and 20D17 iPS and ES cells, and that labelled with GFP (green) for 256H18 to avoid confusion with colour by expression of the integrated gene.

2.5 Fluorescence-activated cell sorter

Undifferentiated iPS cells were dissociated and stained with an anti-mouse monoclonal stage-specific embryonic antigen-1 (SSEA-1) IgM antibody (Santa Cruz Biotechnology) or a mouse IgM antibody (BD Pharmingen) as an isotype control, followed by incubation with an APC-conjugated anti-mouse Ig multiple antibody (BD Pharmingen). Dead cells were excluded by propidium iodide staining. For intracellular c-MHC and c-TNT staining, differentiated iPS cells were fixed/permeabilized with 100 μL of BD fixation/permeabilization solution per 1×10^6 cells for 20 min at 4°C, and then washed two times with 1 × BD Perm/Wash™ buffer. Then, 100 μL of this buffer containing 1×10^6 iPS cells was incubated with an anti-mouse monoclonal c-MHC antibody (abcam), anti-mouse monoclonal c-TNT antibody (abcam), or a mouse IgG1 antibody (BD Pharmingen) as an isotype control, followed by incubation with an APC-conjugated anti-mouse Ig multiple antibody (BD Pharmingen). Samples were analysed using FACSCalibur or FACSLSR and Cell Quest software (BD Pharmingen).

2.6 Western blotting

Western blotting was performed in nuclear extracts from differentiated iPS cells as previously described[14] using rabbit polyclonal anti-HDAC4 (Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (SIGMA) antibodies. For c-MHC, whole cell lysates were subjected to western blotting using an anti-mouse-c-MHC monoclonal antibody. The levels of signals were estimated using photographs taken with LAS100 plus and by quantification with Multi Gauge V.3.0 (FUJIFILM).

2.7 Detection of histone acetylation

Histones were isolated by acid extraction and subjected to western blotting for histone-3/4 and acetylated histone-3/4 as previously described.[13]

2.8 Quantitative RT-PCR

Total RNAs were isolated using TRIzol® reagent. Quantitative RT-PCR was performed as previously described.[13] Primer sequences were described in Supplementary material online, *Table S1*.

2.9 Electrophysiological examination

iPS clusters showing myogenic contraction were transferred onto the MED-probe dishes (Alpha MED Sciences), and electrical potentials were recorded by using the MED64 System (Panasonic multielectrode system; Alpha MED Science).[15]

2.10 DNA microarray

DNA microarray analysis was carried out on two independent cultures. Total RNA was isolated from two undifferentiated Nanog-iPS cell lines (38C2 and 20D17) using TRIzol® reagent. Cy3 (20D17)- and Cy5 (38C2)-labelled cDNAs were synthesized by reverse transcription from the total RNA using the Amino Allyl MessageAMP II aRNA Amplification Kit (Applied Biosystems). For a DNA microarray, 3D-Gene mouse Oligo Chip 24Ks (Toray Industries Inc.) were used according to the manufacturer's protocol. Arrays were scanned with a ScanArray® Lite (PerkinElmer Inc.) and data were analysed.

2.11 Statistical analysis

The results are presented as means ± SE. Statistical comparisons were performed using unpaired two-tailed Student's *t*-test or ANOVA with Scheffe's test where appropriate, with a probability value ($P < 0.05$) taken to indicate significance.

3. Results

3.1 Different lines of iPS cells predominantly express a stem cell surface marker

We maintained two Nanog-iPS cell lines (38C2 and 20D17) and one Myc(-)iPS cell line (256H18) on feeder-free gelatinized dishes. By our culture system, all iPS cell lines in the undifferentiated stage formed round and compact-shaped colonies with distinct borders, which were morphologically indistinguishable from ES cells (*Figure 1A*).

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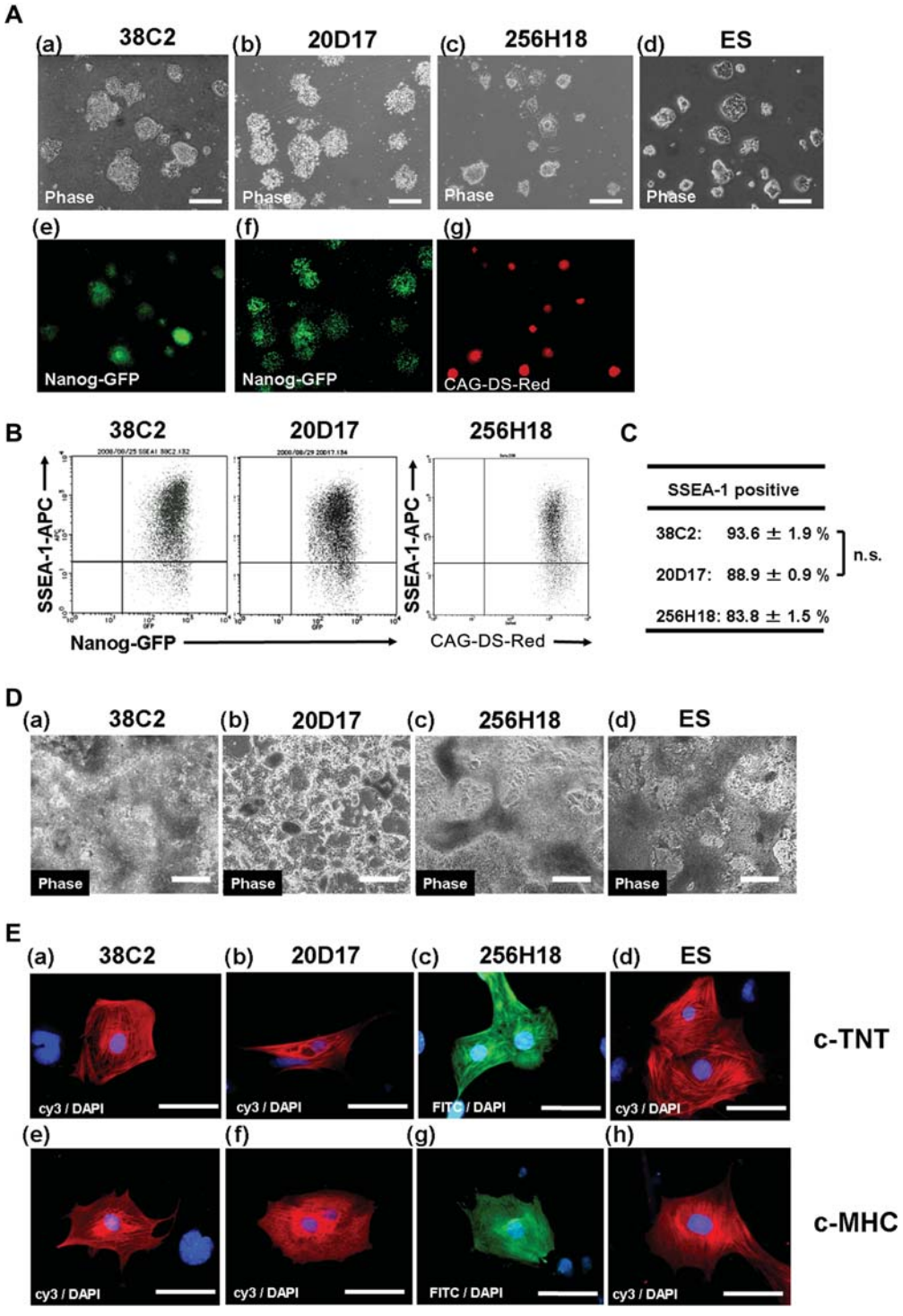

Figure 1 Formation of ES-like colonies from iPS cells and morphological feature of differentiated iPS and ES cells on feeder-free gelatinized plates. (*A*) Photographs of Nanog-iPS (38C2 and 20D17), Myc(-)iPS (256H18), and ES (ht7) cells by phase-contrast (*a*–*d*), Nanog-GFP fluorescence (e and *f*), and CAG-DS-Red fluorescence (*g*). Scale bar 400 μm. (*B*) iPS cells were dissociated into single cells and subjected to flow cytometric analysis. The *X*-axis shows the intensity of GFP or DS-Red fluorescence, and the *Y*-axis shows that of SSEA-1-APC fluorescence. (*C*) The percentages of both SSEA-1- and GFP-positive cells were calculated. Data are presented as the means ± SE of three to five independent experiments. Statistical comparison was performed using ANOVA with Scheffe's test. (*D*) Photographs represent iPS and ES cells induced to differentiate and cultured by day 10: (*a*) a Nanog-iPS cell line 38C2; (*b*) a Nanog-iPS cell line 20D17; (*c*) a Myc(-)iPS cell line 256H18; and (*d*) an ES cell line ht7. Photographs are representative results from more than five independent experiments. Scale bar 400 μm. (*E*) Immunocytochemistry for cardiac contractile proteins in differentiated iPS and ES cell lines were performed: (*a*–*d*) c-TNT and (e–*h*) c-MHC. Scale bar 50 μm.

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Nanog-iPS cells express GFP specifically in the undifferentiated state by the Nanog promoter, whereas Myc(-)iPS cells constitutively express DS-Red under the control of the CAG promoter. Fluorescence-activated cell sorter (FACS) analysis of maintained iPS cells revealed that around 90% of Nanog-iPS cells (38C2 and 20D17) expressed SSEA-1, a stem cell surface marker (*Figure 1B* and *C*, Supplementary material online, *Figure S1*). The percentage of SSEA-1-positive cells was slightly lower in Myc(-)iPS cells (256H18) than that in Nanog-iPS cells, probably due to the fact that Myc(-)iPS cells express no puromycin resistance gene to select undifferentiated cells. The passage number we employed for cultivation in our experiment ranged from 8 to 15. The percentage of SSEA-1-positive cells at the undifferentiated stage was 80% or more in all iPS cell lines. We repeated the FACS analysis of SSEA-1 and found that the percentage was reproducible in this range of passage number.

3.2 iPS cells differentiate into cardiomyocytes on feeder-free gelatinized plates

To investigate patterns regarding the spontaneous differentiation of iPS and ES cells into cardiomyocytes, these cells were seeded on gelatin-coated plates with no feeder cells. Differentiation was induced without the formation of EBs by removing leukaemia inhibitory factor and antibiotics from the media. In 38C2 and 256H18, spontaneous myogenic contractions occurred later than day 8 (Supplementary material online, *Video S1*). However, the 20D17 cell line, one of the Nanog-iPS cell lines, was morphologically quite different from the other iPS cell lines, especially after day 8. The 20D17 iPS cells did not tend to expand over the monolayers and formed round colony-like clusters (*Figure 1D*). Myogenic contractions hardly occurred.

We performed immunocytochemistry to detect iPS cell- and ES cell-derived cardiomyocytes on day 10. We detected cells positive for both c-MHC and c-TNT in all iPS cell lines as well as in ES cells (*Figure 1E*). Positive cells exhibited sarcomere formation and myofibril organization, typical features of cardiomyocytes.

3.3 Expression patterns of cardiac-specific genes in iPS cells

During the induction of the spontaneous differentiation of iPS cells, we examined the time-course regarding the mRNA levels of cardiac-specific genes. We examined genes coding for a cardiac-specific transcription factor Nkx2.5, a cardiac structure protein α-myosin heavy chain (α-MHC), and a cardiac-specific peptide hormone atrial natriuretic factor (ANF). The expression patterns of these genes in 38C2 and 256H18 were almost identical to those in ES cells, whereas the peak of α-MHC was expressed higher in 256H18 than 38C2iPS or ES cells. However, in 20D17, the expressions of these cardiac-specific genes were extremely low. The mRNA levels of these genes were suppressed at the basal level and did not increase even at later stages compared with other cell lines on days 8 and 10 (*Figure 2A–C*). The mRNA levels of the cardiac-specific gene Nkx2.5 in 20D17 did not increase, but rather decreased after day 10. The levels were 100 ± 37 on day 8, 33 ± 1 on day 15, 34 ± 2 on day 20, and 36 ± 4 on day 25. Therefore, the low levels of myocardial differentiation in 20D17 may not be explained by the speed of differentiation.

3.4 Beating activities and intracellular FACS analysis of iPS cells

We measured the electrical potentials of beating clusters derived from differentiated iPS cells on day 10. As assessed by the MED64 system, the spontaneous beating clusters showed extracellular field potentials, a typical feature of cardiomyocytes (*Figure 3A-a*). We measured the number of beating clusters and frequency of beating in all iPS cell lines on day 10. On counting the number of beating clusters, we could easily distinguish one beating cluster from the others due to its clear border and by its difference in frequency and timing of beating. We simultaneously started the differentiation culture of the three iPS cell lines. The beating started on day 8 and increased thereafter. We counted the number of beating clusters per well on day 10 and calculated the mean number in five and six wells. In 20D17, the number of beating clusters was the smallest, and the beating frequency was significantly lower than in 38C2 and 256H18 (see *Figure 3A-b* and *c*, Supplementary material online, *Video S2*). In our system of ES cell differentiation by monolayer culture, the number of beating clusters was small. The mean number of beating clusters in ES cells on day 10 was two per well.

To quantitatively assess the number of cardiomyocytes in the total population, intracellular FACS analyses were performed using an anti-c-MHC antibody on day 10. Cardiac MHC-positive cells were the highest in 256H18 and the lowest in 20D17 (*Figure 3B*). The percentages of c-MHC-positive cells (the results of four and five independent experiments) were $11.4 \pm 3.7\%$ in 38C2, $3.6 \pm 1.5\%$ in 20D17, and $20.2 \pm 4.5\%$ in 256H18 ($P < 0.05$, 20D17 vs. 256H18). These results were compatible with the expression levels of α-MHC mRNA. Furthermore, we performed intracellular FACS analysis with an antibody against c-TNT, a specific protein for cardiac muscle cells. The percentages of c-TNT-positive cells were 6.2% in 38C2, 1.2% in 20D17, and 11.5% in 256H18 (Supplementary material online, *Figure S2*).

3.5 Patterns of genes involved in myocardial differentiation in iPS cells

In order to investigate the mechanisms leading to the discrepancy in the efficiency of myocardial differentiation between the two Nanog-iPS cell lines, which are identical in their genetic background, we first examined a zinc finger transcription factor GATA4, a lateral mesoderm marker, VEGF receptor-2 (Flk-1), and its ligand VEGF. There was no difference in the expression levels of GATA4, Flk-1, and VEGF between the two iPS cell lines by day 5, whereas the expressions of Flk-1 were lower in 20D17 than 38C2 after day 5 (*Figure 4A–C*). Next, we examined the expression of an intrinsic histone acetyltransferase, p300, and a member of class II HDAC, HDAC4. mRNA levels of p300 and HDAC4 did not differ between the two iPS cell lines (*Figure 4D* and *E*).

We also examined the expression of a stem cell marker, Oct3/4. At an undifferentiated stage, the expression was significantly higher in 20D17 than in 38C2. Interestingly, after day 5, the Oct3/4 expression level did not decrease and was maintained in 20D17, whereas the level decreased progressively in 38C2. The expression level of Oct3/4 was higher in 20D17 than in 38C2 on days 8 and 10 (*Figure 4F*).

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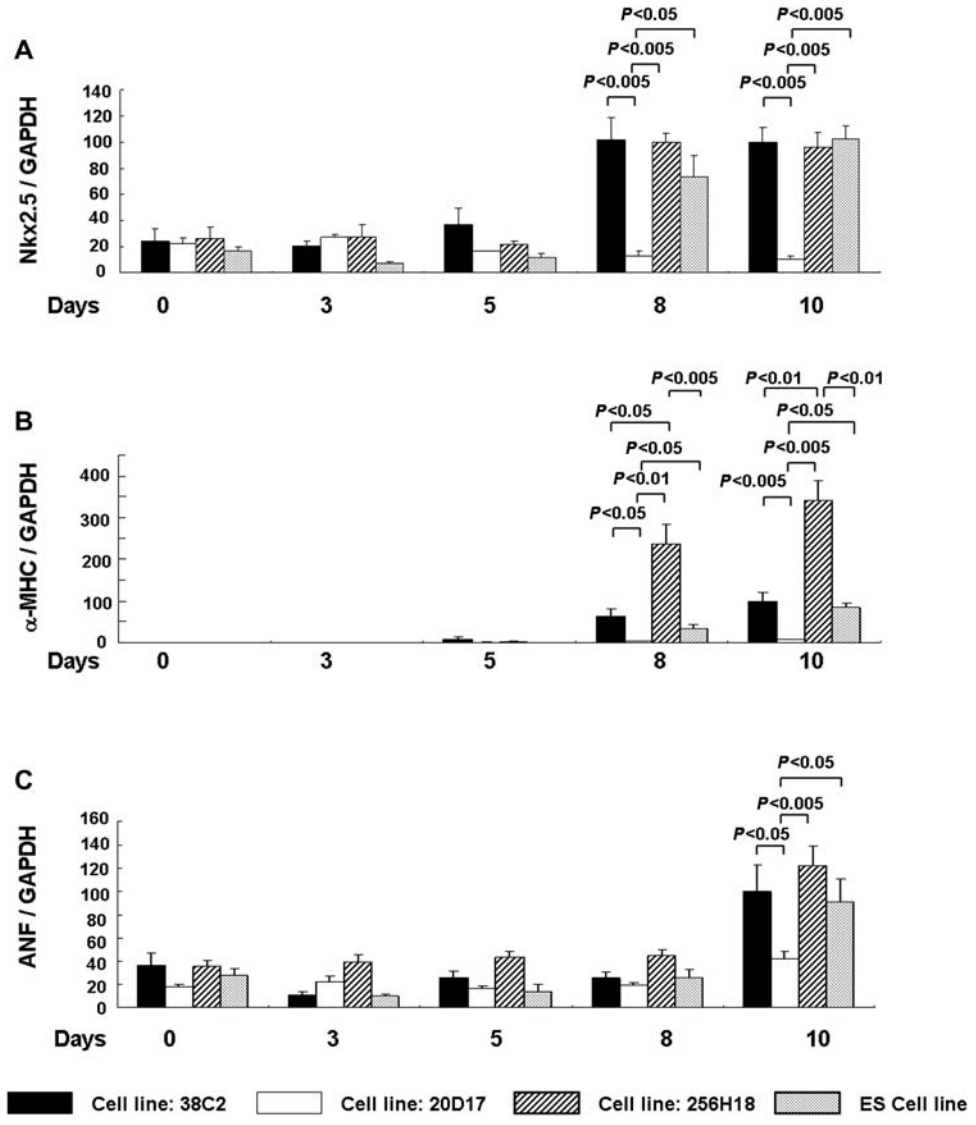

Figure 2 Time-course of the mRNA levels of cardiac-specific genes. Total RNA was extracted from differentiation-induced iPS and ES cells on indicated days, reverse-transcribed into cDNA, and subjected to quantitative RT-PCR. Levels of GAPDH transcripts were used to normalize cDNA levels. The mRNA level of specific targets relative to GAPDH in 38C2 on day 10 was set at 100. Data are presented as the means ± SE of three to five independent experiments. (*A*) Nkx2.5; (*B*) α-MHC; and (*C*) ANF. Statistical comparison was performed using ANOVA with Scheffe's test.

3.6 TSA, an HDAC inhibitor, facilitates myocardial differentiation in iPS cells

We previously reported that TSA treatment of ES cells on day 7 for 24 h acetylated the cardiac-specific transcription factor GATA4, as well as histone tails, and facilitated their myocardial differentiation.[13] We tested whether similar stimulation by TSA facilitates the differentiation of iPS cells into cardiomyocytes. Treatment of iPS cells with 50 ng/mL TSA on day 7 for 24 h significantly induced the acetylation of histone-3/4 (*Figure 5A*). Administration of TSA increased Nkx2.5 expression by three- to five-fold in two iPS cell lines, 38C2 and 256H18, as well as in an ES cell line. Interestingly, in a Nanog-iPS cell line, 20D17, whereas the basal level of Nkx2.5 expression was lowest among iPS cell lines, responses of Nkx2.5 expression to TSA were the greatest. In 20D17, 50 ng/mL TSA increased the expression of Nkx2.5 by 25- to 30-fold (*Figure 5B-a*). TSA also increased the expression level of the ANF gene (*Figure 5B-b*). On the other hand, TSA treatment did not increase the expression level of platelet endothelial cell adhesion molecule-1 (PECAM-1), an endothelial cell-specific marker (*Figure 5B-c*). These findings suggest that the up-regulation of cardiac-restricted markers by TSA cannot be explained solely by an increase in general transcription. We also examined the expression of cardiac contractile proteins. Treatment with TSA by three- to four-fold increased the mRNA level of myosin light chain 2v (MLC-2v) on day 8 in 38C2 and in 20D17 (*Figure 5C-a*). The protein level of c-MHC increased by TSA treatment in 20D17 (*Figure 5C-b*). FACS analysis demonstrated that the percentage of c-MHC-positive cells increased on TSA treatment in 20D17 (*Figure 5C-c*).Treatment with TSA increased the beating frequency in all iPS cell lines (Supplementary material online, *Figure S3*).

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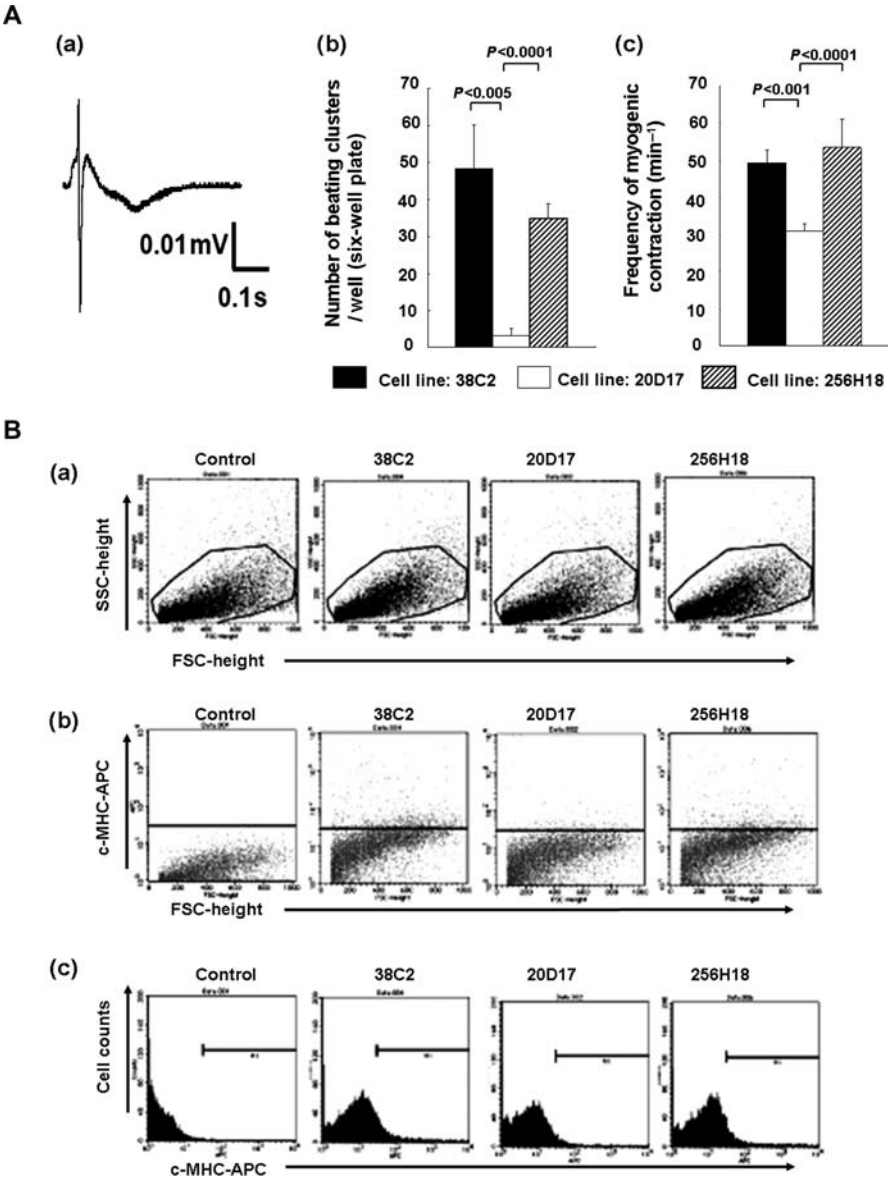

Figure 3 Myocardial characteristics of differentiated iPS cells. (*A-a*) The electrical potentials of beating clusters derived from differentiated 38C2 iPS cells on day 10 were shown. (*A-b*) The number of beating clusters was counted in each well on day 10. The numbers of beating clusters were expressed as means ± SE of five or six wells. (*A-c*) The frequency of myogenic contraction was measured in differentiated iPS cells on day 10. The frequency was expressed as means ± SE of 10 or more beating clusters in each iPS cell line. Statistical comparisons were performed using ANOVA with Scheffe's test. (*B*) Intracellular FACS was performed using c-MHC antibody in differentiated iPS cells on day 10. The provided figures are representative data of four and five independent experiments. (*B-a*) Dot plots with gates. We set a large gate to surround most cells. (*B-b*) The *Y*-axis shows the intensity of APC fluorescence. (*B-c*) The *X*- and *Y*-axes show the intensity of APC fluorescence and the number of APC-positive cells, respectively.

To further examine the role of nuclear acetylation during iPS cell differentiation into cardiomyocytes, we utilized valproic acid (VPA), another HDAC inhibitor. VPA treatment of 20D17 iPS cells for 24 h on day 7 dose-dependently increased the mRNA expression levels of a cardiac-specific transcription factor Nkx2.5 on day 8, and a cardiac-specific peptide hormone ANF on day 9 (*Figure 5D*). Thus, HDAC inhibition by two independent drugs resulted in the induction of cardiac-restricted markers, strongly suggesting the requirement of HDAC inhibition for myocardial differentiation.

We examined the expression levels of Oct3/4 mRNA in 20D17 by quantitative RT-PCR. The relative values were TSA(−): 100 ± 20 and TSA(+): 105 ± 19 on day 8, and TSA(−): 88 ± 7 and TSA(+): 85 ± 21 on day 9. Thus, TSA had no effect on the expression level of Oct3/4. Therefore, the increase in the expression of cardiac-specific genes by TSA treatment may be attributable to mechanisms independent of the Oct3/4 level.

We examined whether residual pluripotent stem cells in the population of differentiated iPS cells increase or decrease on TSA treatment. Differentiated iPS cells from two cell lines 20D17 and 38C2, carrying the Nanog-GFP-IRES-Puro^r reporter gene, were treated or untreated with TSA (50 ng/mL) for 24 h on day 7. On day 8, the differentiation medium was changed to a maintenance medium

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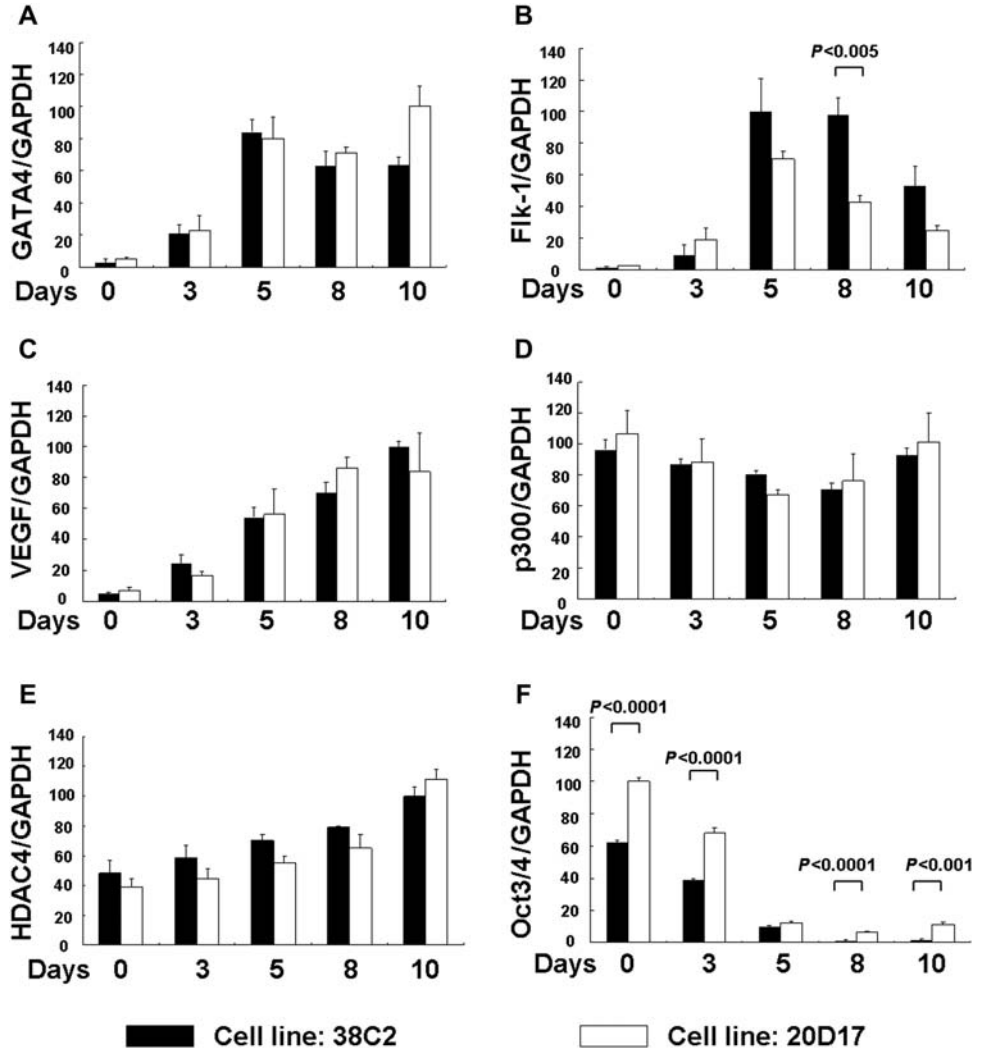

Figure 4 mRNA expression levels of genes involved in cardiogenesis. (*A*) A transcription factor GATA4. (*B*) A lateral mesoderm marker Flk-1. (*C*) A vascular endothelial cell marker VEGF. (*D*) A transcriptional co-activator p300. (*E*) A member of class II HDAC HDAC4. (*F*) An undifferentiated stem cell marker Oct3/4. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests.

including LIF and puromycin. On day 10, the number of GFP-positive colonies was significantly larger in 20D17 than 38C2. On the plates of 20D17 iPS cells treated with TSA for 24 h, both the number and size of these colonies were much smaller than those that were not treated (Supplementary material online, *Figure S4-a–c*). The expression of GFP is controlled by the promoter of Nanog, one of the representative pluripotent stem cell markers. Therefore, these results suggest that TSA decreases possible pluripotent stem cells that reside in the population of differentiated iPS cells.

3.7 Increased level of HDAC4 protein in nucleus of 20D17 cells

In order to investigate the mechanism of the cell line-dependent variation and a TSA-induced increase in cardiac-specific gene expression in iPS cell lines, we examined the expression level of HDAC4. Interestingly, the HDAC4 signal in nuclear extracts from iPS cells isolated on day 8 was the greatest in 20D17 among all iPS cell lines (*Figure 6A* and *B*). As shown in the upper panels of Supplementary material online, *Figure S5*, the HDAC4 protein was located in the nucleus of 20D17 iPS cells. The mRNA expression levels of HDAC4 were similar for the iPS cell lines 20D17 and 38C2. Therefore, the increase in the nuclear HDAC4 protein in 20D17 compared with 38C2 may be attributable to the relatively high levels of HDAC4 in the nucleus rather than in the cytoplasm of 20D17 iPS cells. It has been shown that the nuclear HDAC4 protein suppresses cardiac-specific transcription factors to bind DNA.[16,17] TSA treatment induced the translocation of HDAC4 from the nucleus to the cytoplasm in 20D17 iPS cells (Supplementary material online, *Figure S5*).

We performed DNA microarray analysis of two Nanog-iPS cell lines in order to clarify the important genes to select an iPS cell line, which has a high potential to differentiate into cardiomyocytes, prior to attempting cardiac differentiation. One Nanog-iPS cell line, 38C2, showed a high-level potency of myocardial differentiation, whereas another, 20D17, exhibited the lowest potency of differentiation. We have carried out DNA chip analysis of 20D17 and 38C2 iPS cell lines in two independent cultures. *Figure 6C* demonstrates one representative global gene-expression pattern. In Supplementary

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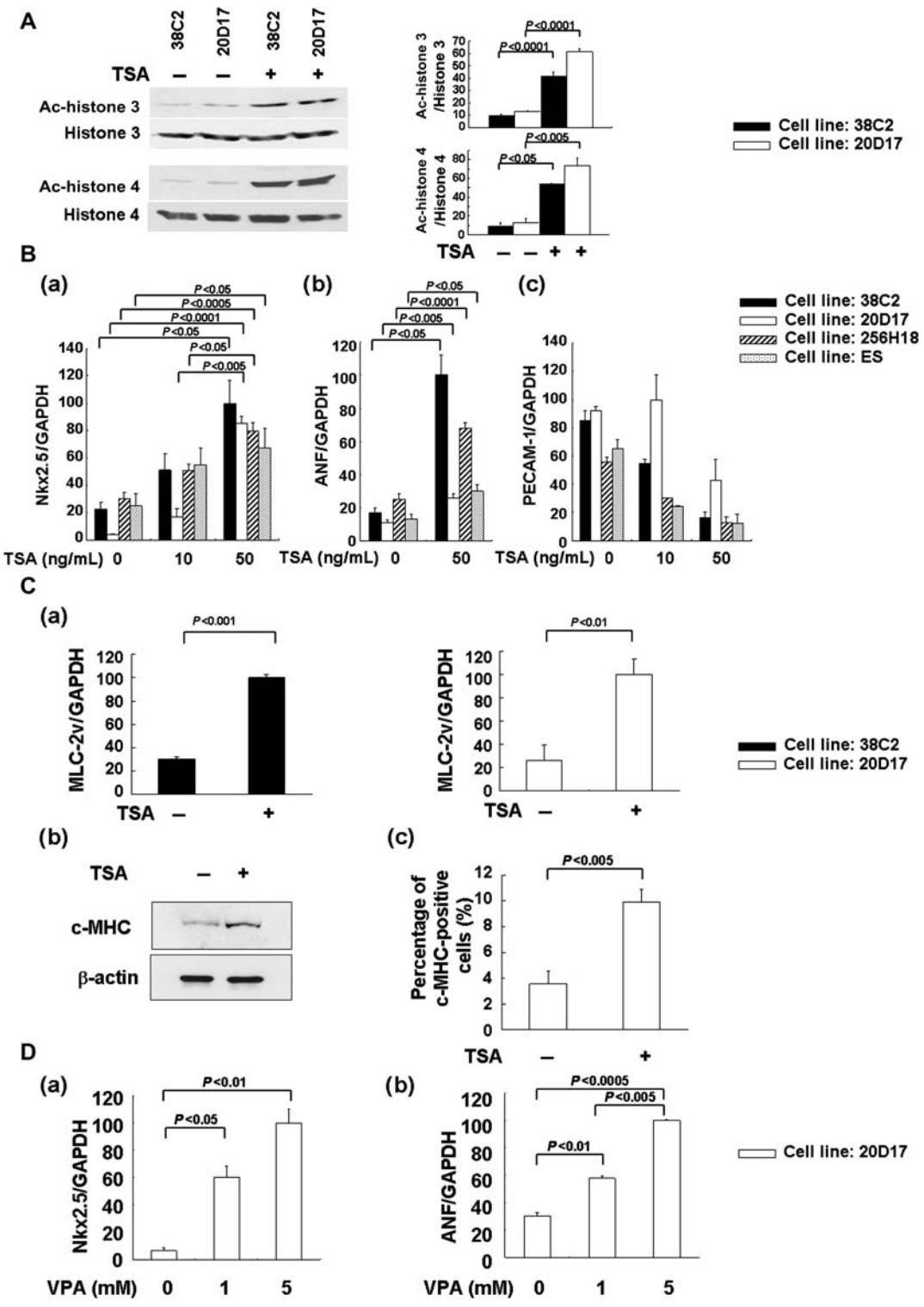

Figure 5 The HDAC inhibitor TSA induces myocardial cell differentiation. (*A*) iPS cells on day 7 were stimulated with TSA (50 ng/mL) or solvent, ethanol, for 24 h. Nuclear extracts were isolated from these cells on day 8 and subjected to western blotting with anti-histone-3/4 and anti-acetylated histone-3/4 antibodies. The levels of acetylated histone-3/4 signals were normalized by histone-3/4 signal. We performed three independent experiments. *Y*-axis units are relative values. The relative level of histone-3/4 without TSA treatment in 38C2 on day 10 was set as 10. (*B*) iPS cells on day 7 were stimulated with TSA (10 and 50 ng/mL) or its solvent for 24 h. Total RNA was isolated on day 8 from these iPS cells, and synthesized cDNA was subjected to quantitative PCR. The amounts of cDNA were measured. Levels of GAPDH transcripts were used to normalize cDNA levels. The maximum level of Nkx2.5 (*a*), ANF (*b*) or PECAM-1 (*c*) mRNA relative to GAPDH mRNA was set at 100. Data are presented as the means ± SE of three to five independent experiments. (*C*) iPS cells on day 7 were stimulated with TSA (10 ng/mL) or its solvent for 24 h. (*C-a*) Total RNA was isolated on day 8 from 38C2 or on day 9 from 20D17. Synthesized cDNA was then subjected to quantitative RT-PCR. The

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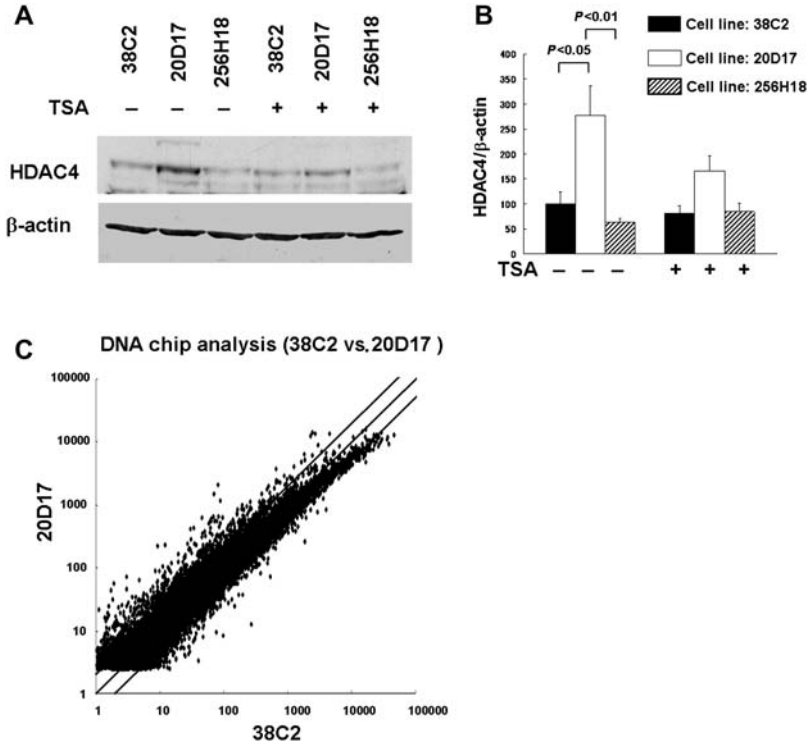

Figure 6 Increased level of HDAC4 protein in the nucleus of 20D17 cells. (*A*) iPS cells on day 7 were stimulated with TSA (10 ng/mL) or its solvent for 24 h. The nuclear extracts were isolated from iPS cells on day 8 and subjected to western blotting. (*B*) The level of the HDAC4 signal was normalized by the β-actin signal. The normalized levels in 38C2 iPS cells treated without TSA were set at 100. Data are presented as the means ± SE of four independent experiments. Statistical comparison was performed using ANOVA with Scheffe's test. (*C*) In two Nanog-iPS cell lines, 38C2 and 20D17, the expression levels of DNA were compared. The representative global gene-expression pattern was shown by scatter plots. (GSE18117: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18117).

material online, *Table S2*, we show the symbols, reference sequences, and descriptions of genes whose values of the log 2 [ratio (Cy3/Cy5)] exceed 1 (20D17 > 38C2) or are below −1 (20D17 < 38C2) in the two experiments. Furthermore, we analysed the data on several genes reported to be expressed in the undifferentiated stage and to regulate myocardial differentiation.[18–21] The expression levels of Wnt11 and FGF4, which induce cardiogenesis,[18–20] were lower in 20D17 than 38C2. It has been reported that endogenous BMP4 expressed during the undifferentiated stem cell stage inhibits cardiogenesis, and that this inhibition is overcome by a BMP inhibitor, noggin.[21] The expression level of BMP4 was higher in 20D17 than 38C2. We also confirmed the gene expression level of BMP4 by quantitative PCR. The relative expression levels of BMP4 were 100 ± 17 in 38C2 and 295 ± 58 in 20D17 on day 0. On day 3, the BMP4 levels decreased to 11 ± 2 in 38C2, whereas the levels remained high (243 ± 18) in 20D17.

4. Discussion

The present study demonstrated that both Nanog-iPS and Myc(-)iPS cells can differentiate into cardiomyocytes on feeder-free gelatin-coated plates. We have chosen this system because it is simple and reproducible. Furthermore, by employing this system, we can exclude variable factors that may possibly affect cardiogenesis when we use feeder cells or a system of EB formation.

The POU transcription factor, Oct3/4, is essential for the initial formation of a pluripotent founder cell population in the mammalian embryo and plays a pivotal role as a master regulator of pluripotency that controls lineage commitment.[22] The present study demonstrated that Oct3/4 expression in undifferentiated iPS cells was increased in the 20D17 compared with the 38C2 line. The expression levels of GATA4 and Flk-1 were similar for 38C2 and 20D17 until day 5. Therefore, iPS cells of the 20D17 line commit to the primitive endoderm

Figure 5 (Continued)
maximum mRNA level of MLC-2v relative to the level of GAPDH was set at 100. Data are presented as the means ± SE of three to five independent experiments. (*C-b*) Whole cell lysates were isolated on day 10 and subjected to western blot analysis using anti-c-MHC antibody. **C-c**, Intracellular FACS was performed as described in the legend to *Figure 4B*, and the percentage of c-MHC-positive cells was calculated. (*D*) The 20D17iPS cells on day 7 were treated with VPA (1 and 5 mM) or remained untreated for 24 h. Total RNA was isolated on day 8 (*a*) or 9 (*b*) from these cells, and synthesized cDNA was subjected to quantitative PCR. The maximum level of Nkx2.5 (*a*) or ANF (*b*) mRNA relative to GAPDH mRNA was set at 100. Data are presented as the means ± SE of three independent experiments. Statistical comparisons were performed by ANOVA with Scheffe's test in (*A*), (*B*), and (*D*), and by unpaired two-tailed Student's *t*-tests in (*C*).

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and mesoderm in a manner similar with those of the 38C2 line. After day 5, however, 20D17 iPS cells did not differentiate into cardiomyocytes. The Oct3/4 level was much higher in 20D17 than 38C2 on days 8 and 10. It has been reported that the over-expression of Oct3/4 triggers the differentiation of cells into primitive endoderm and mesoderm,[22] and that elevated Oct4 at early stages (by day 2) is required for cardiomyogenesis.[23] In contrast, sustained over-expression of Oct3/4 in neural progenitor cells or myoblasts prevents their terminal differentiation.[24,25] The higher Oct3/4 levels at late stages might be attributable to the low efficiency of cardiomyocyte differentiation in 20D17.

A member of class II HDAC, HDAC4, binds to the transcription factors SRF and Mef2C and inhibits the expression of cardiac-specific genes.[16,17] The present study demonstrated that the nuclear protein level of HDAC4 was the highest in 20D17 and the lowest in 256H18 among all iPS cell lines. This difference in the nuclear HDAC4 levels might be involved, in part, in the variation of cardiomyocyte differentiation efficiency in different lines of iPS cells. In addition, by DNA microarray analysis, we showed that expressions of genes involved in cardiogenesis such as Wnt11, FGF4, and BMP4 are up- or down-regulated in 20D17. Further studies are needed regarding precise mechanisms by which these genes modulate cardiomyocyte differentiation in distinct iPS cell lines.

Narazaki *et al*. used iPS cell lines similar to those we employed but did not point out the variation of myocardial differentiation in different iPS cell lines.[5] They selected Flk-1-positive iPS cells and seeded these cells on dishes coated with collagen IV or OP9 feeder cells.[5] As we applied a simple system, myocardial differentiation by our protocol may be more susceptible to differences in gene or protein expression among distinct iPS cell lines.

In the clinical setting, cell line-to-line variation may occur when generating original iPS cells derived from patients with heart diseases. The forced acetylation of histones and transcription factors by HDAC inhibitors in patient-oriented iPS cells would be useful to overcome such variation and efficiently develop cardiomyocytes for the purpose of cell transplantation or drug screening.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgement

We thank M. Kiriyama for their excellent technical assistance.

Conflict of interest: none declared.

Funding

This work was supported in part by the Project for Realization of Regenerative Medicine in the Life Science Program (T.N.), by a grant from the Foundation for Promotion of Research on Adult Vascular Diseases(K.H.), and by a research grant on Publicly Essential Drugs and Medical Devices from The Japan Health Sciences Foundation(K.H.).

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