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

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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*).

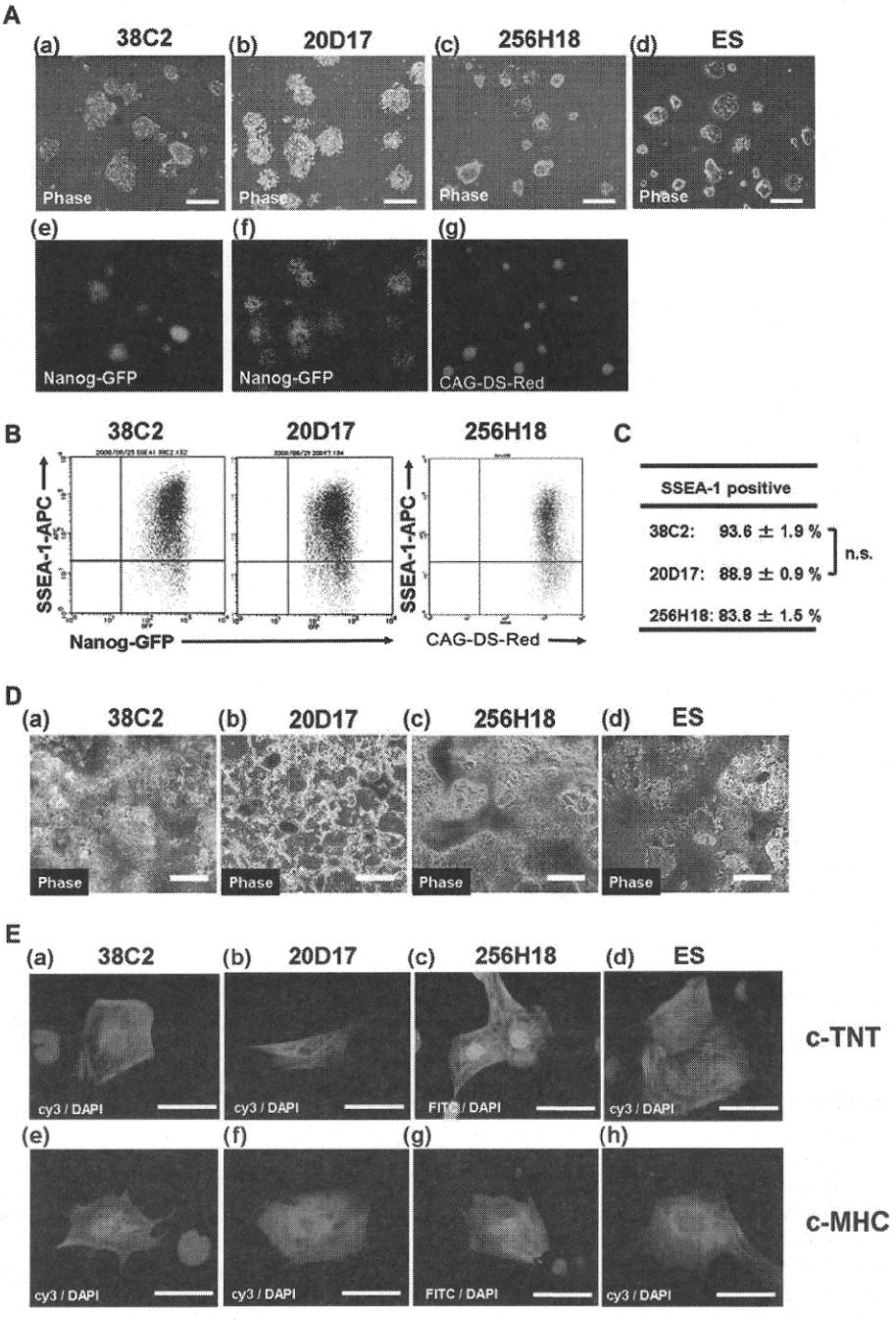

Figure 1 Formation of ES-like colonies from iPS cells and morphological feature of differentiated iPS and ES cells on feeder-free gelatinized plates. (*A*) Photographs of Nanog-iPS (38C2 and 20D17), Myc(-)iPS (256H18), and ES (ht7) cells by phase-contrast (*a–d*), Nanog-GFP fluorescence (e and *f*), 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.

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*).

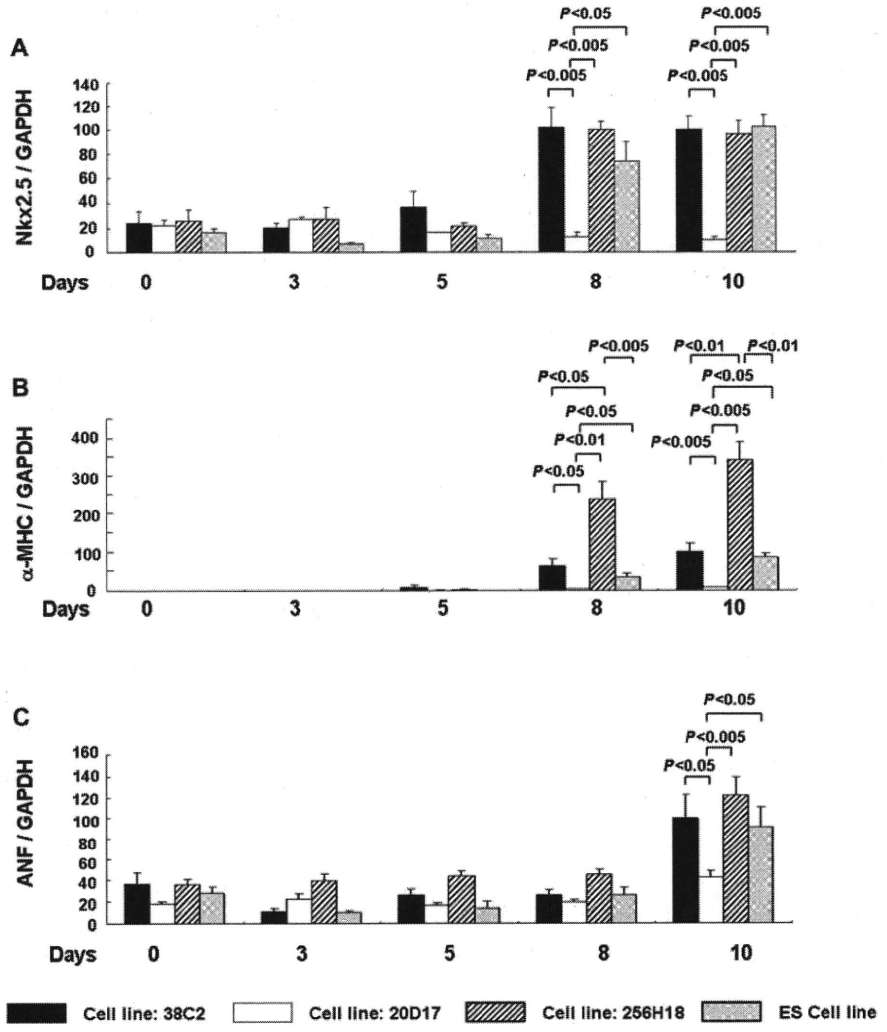

Figure 2 Time-course of the mRNA levels of cardiac-specific genes. Total RNA was extracted from differentiation-induced iPS and ES cells on indicated days, reverse-transcribed into cDNA, and subjected to quantitative RT-PCR. Levels of GAPDH transcripts were used to normalize cDNA levels. The mRNA level 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*).

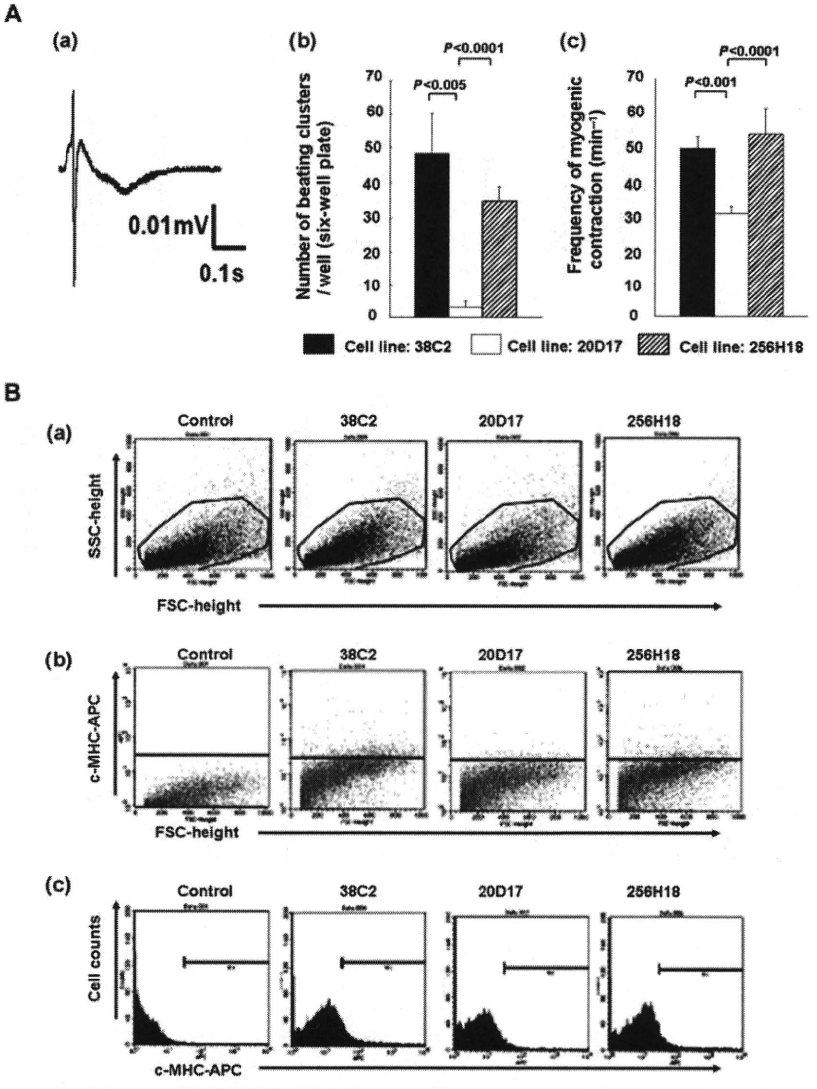

Figure 3 Myocardial characteristics of differentiated iPS cells. (*A-a*) The electrical potentials of beating clusters derived from differentiated 38C2 iPS cells on day 10 were shown. (*A-b*) The number of beating clusters was counted in each well on day 10. The numbers of beating clusters were expressed 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

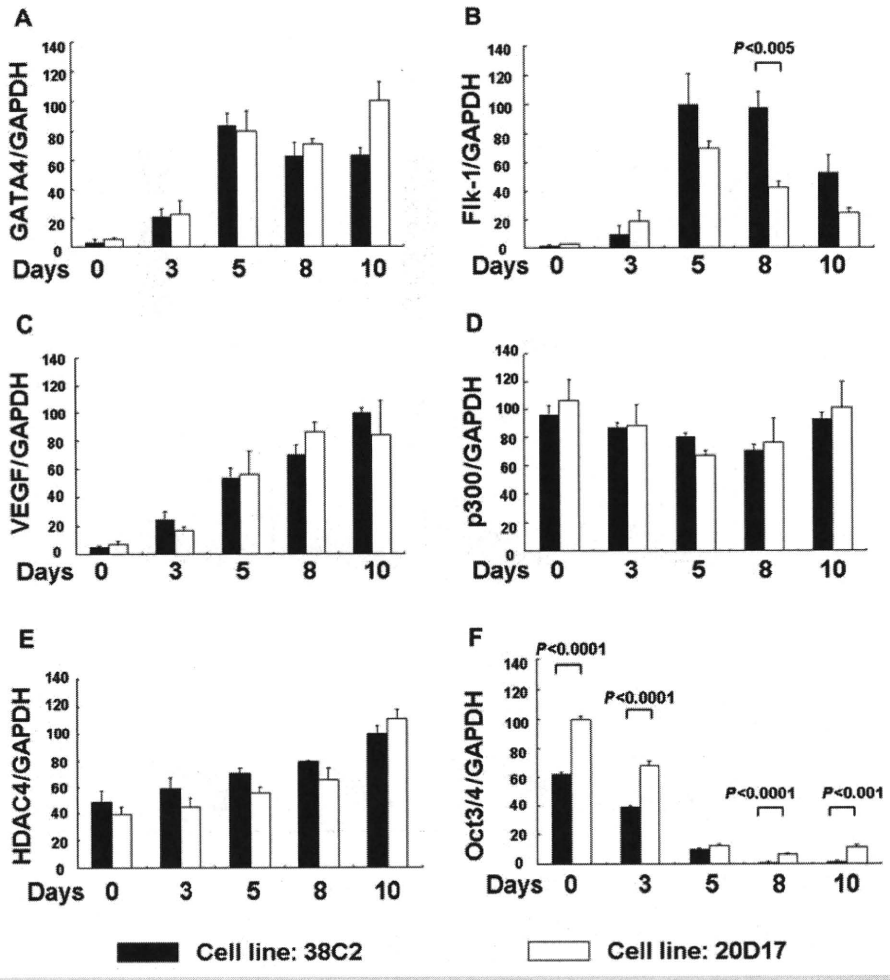

Figure 4 mRNA expression levels of genes involved in cardiogenesis. (*A*) A transcription factor GATA4. (*B*) A lateral mesoderm marker Flk-1. (*C*) A vascular endothelial cell marker VEGF. (*D*) A transcriptional co-activator p300. (*E*) A member of class II HDAC HDAC4. (*F*) An undifferentiated stem 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

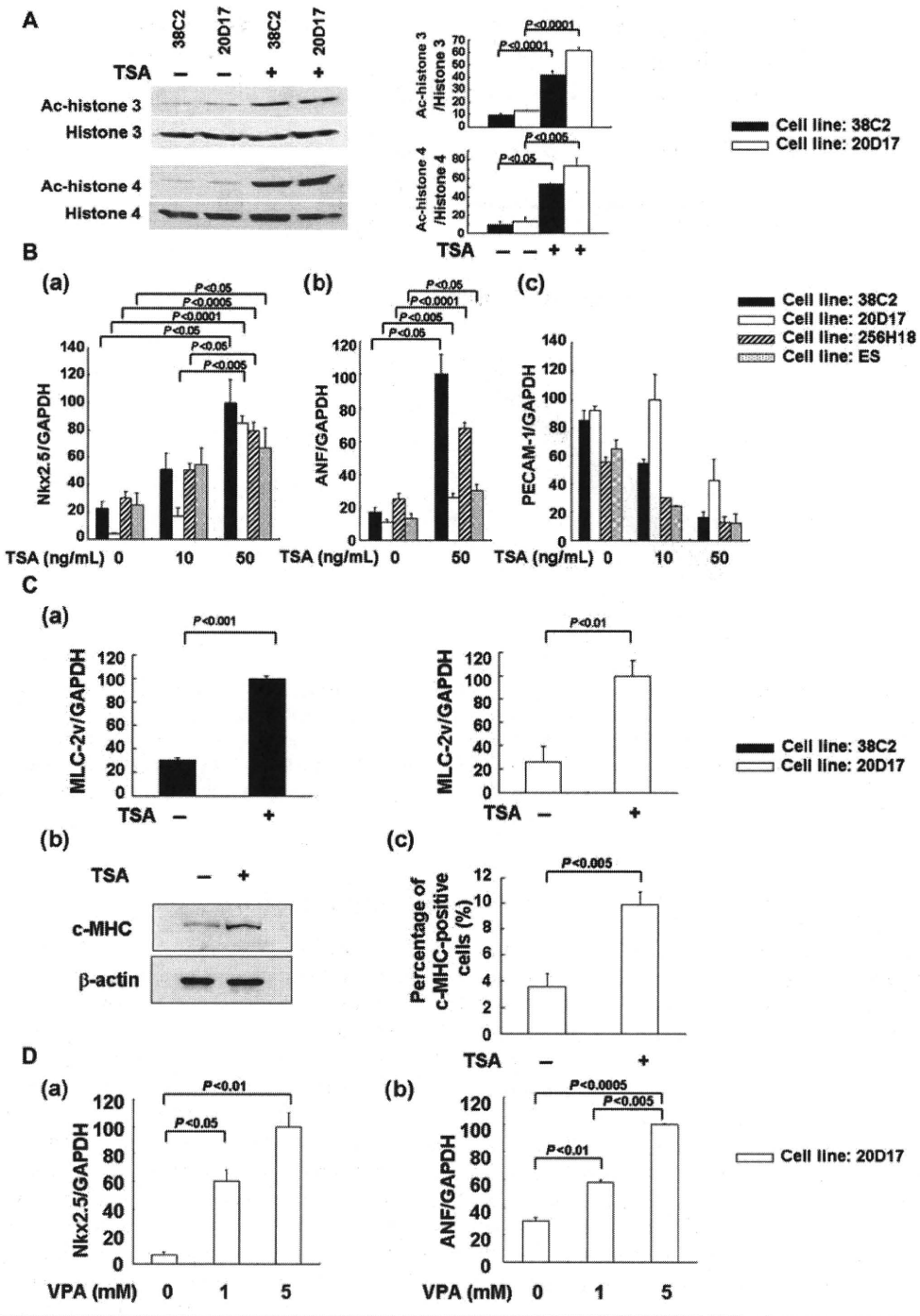

Figure 5 The HDAC inhibitor TSA induces myocardial cell differentiation. (*A*) iPS cells on day 7 were stimulated with TSA (50 ng/mL) or solvent, ethanol, for 24 h. Nuclear extracts were isolated from these cells on day 8 and subjected to western blotting with anti-histone-3/4 and anti-acetylated histone-3/4 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

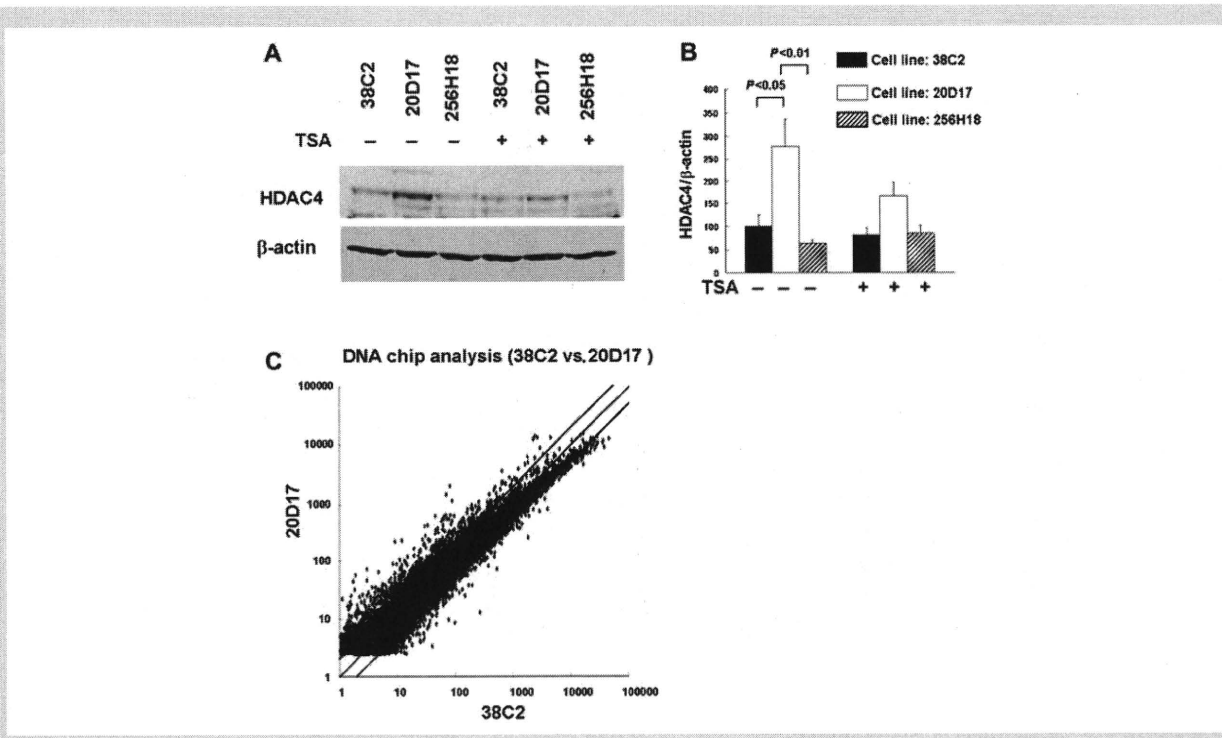

Figure 6 Increased level of HDAC4 protein in the nucleus of 20D17 cells. (*A*) iPS cells on day 7 were stimulated with TSA (10 ng/mL) or its solvent for 24 h. The nuclear extracts were isolated from iPS cells on day 8 and subjected to western blotting. (*B*) The level of the HDAC4 signal was normalized 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*).

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.

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Conflict of interest: none declared.

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Cyclin-Dependent Kinase 9 Forms a Complex With GATA4 and Is Involved in the Differentiation of Mouse ES Cells Into Cardiomyocytes

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The treatment of ES cells with trichostatin A (TSA), an HDAC inhibitor, induces the acetylation of GATA4 as well as histones, and facilitates their differentiation into cardiomyocytes. Recently, we demonstrated that cyclin-dependent kinase 9 (Cdk9), a core component of positive elongation factor-b, is a novel GATA4-binding partner. The present study examined whether Cdk9 forms a complex with GATA4 in mouse ES cells and is involved in their differentiation into cardiomyocytes. Mouse ES cells and Nkx2.5/GFP ES cells, in which green fluorescent protein (GFP) is expressed under the control of the cardiac-specific Nkx2.5 promoter, were induced to differentiate on feeder-free gelatin-coated plates. Immunoprecipitation/Western blotting in nuclear extracts from mouse ES cells demonstrated that Cdk9 as well as cyclin T1 interact with GATA4 during myocardial differentiation. TSA treatment increased Nkx2.5/GFP-positive cells and endogenous mRNA levels of Nkx2.5 and atrial natriuretic factor. To determine the role of Cdk9 in myocardial cell differentiation, we examined the effects of a dominant-negative form of Cdk9 (DN-Cdk9), which loses its kinase activity, and a Cdk9 kinase inhibitor, 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB) on TSA-induced myocardial cell differentiation. The introduction of the DN-Cdk9 inhibited TSA-induced increase in GFP expression in Nkx2.5/GFP ES cells. The administration of DRB into ES cells significantly inhibited TSA-induced increase of endogenous Nkx2.5 mRNA levels in ES cells as well as GFP expression in Nkx2.5/GFP ES cells. These findings demonstrate that Cdk9 is involved in the differentiation of mouse ES cells into cardiomyocytes by interacting with GATA4.

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During embryogenesis, cell type-specific gene expression plays a pivotal role in the determination of cell fate, including differentiation, proliferation, and apoptosis. In contrast to other cell types, cardiac muscle cells are highly organized and their developmental processes require a number of cell type-specific transcription factors (Sachnidis et al., 2003). Among these, the zinc finger protein GATA4, which is one of the pivotal DNA-binding transcription factors involved in myocardial differentiation, is expressed at the earliest stage during heart development (Grepin et al., 1997; Kuo et al., 1997; Molkentin et al., 1997).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs), which are recruited to target genes in association with specific DNA-binding transcription factors, regulate gene expression patterns by affecting the chromatin structure (Johnson and Turner, 1999; Chan and La Thangue, 2001). One of HATs, p300, serves as a transcriptional coactivator of GATA4 and is able to acetylate GATA4 and enhance its DNA-binding activities (Shikama et al., 1997; Johnson and Turner, 1999; Chan and La Thangue, 2001; Yanazume et al., 2003; Kawamura et al., 2005; Takaya et al., 2008). During the differentiation of ES cells into cardiomyocytes, the treatment of ES cells with trichostatin A (TSA), an HDAC inhibitor, induces the acetylation of GATA4 as well as the expression of p300, and facilitates their differentiation into cardiomyocytes (Kawamura et al., 2005).

Positive elongation factor-b (p-TEFb) is a key regulator of the process controlling the processing of RNA polymerase II and possesses a kinase activity that can phosphorylate the carboxy-terminal domain of the largest subunit of RNA polymerase II (Zhu et al., 1997; Shim et al., 2002; Marshall and Grana, 2006). p-TEFb is composed of cyclin-dependent kinase 9 (Cdk9) and one of four C-type cyclin subunits (cyclin T1, cyclin T2a, cyclin T2b, and cyclin K). p-TEFb not only plays an important role in most

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RNA pol II-dependent transcription, but also is recruited to cellular promoters by interacting with a variety of transcription factors (Kanazawa et al., 2000; Barboric et al., 2001; Eberhardy and Farnham, 2002; Simone et al., 2002).

Recently, we demonstrated that by tandem affinity purification and mass spectrometric analyses, Cdk9 is one of the components of the p300/GATA4 complex in HeLa cells as well as cyclin T1 (Sunagawa et al., 2010). The present study examined whether Cdk9 forms a complex with GATA4 and is involved in the differentiation of ES cells into cardiomyocytes.

Materials and Methods

Cell line and cell culture of embryonic stem cells

The 129/Ola-derived ES cell lines we used in the present study were ht7 carrying the Oct3/4-hygromycin^r gene and its derivative (Nkx2.5/GFP ES cells), in which green fluorescent protein (GFP) is knocked into the Nkx2.5 locus (Hidaka et al., 2003). These cells were maintained on gelatinized dishes without feeder cells using culture medium containing Glasgow-modified Eagle's medium (GMEM), 1,000 U/ml leukemia inhibitory factor (LIF, Chemicon International, Billerica, MA), 100 mg/ml hygromycin (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal calf serum, 1 × non-essential amino acids, 1 mmol/L sodium pyruvate, 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 0.1 mmol/L 2-mercaptoethanol. To induce differentiation, 3×10^4 ES cells were seeded in each well of the gelatinized 6-well plates without the formation of embryoid bodies using the above-mentioned medium lacking LIF and hygromycin. Differentiated ES cells were stimulated with TSA (Invitrogen) and/or 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB) (Invitrogen) on day 7 for 24 h.

Immunoprecipitation and Western blotting

Western blotting were performed as previously described (Yanazume et al., 2003) using rabbit polyclonal anti-GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Cdk9 (Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin T1 (Santa Cruz Biotechnology), mouse monoclonal anti-RNA polymerase II (Abcam, San Francisco, CA), mouse monoclonal anti-phosphorylated serine 2 of RNA polymerase II C-terminal domain (Abcam), and mouse monoclonal anti-β-actin (SIGMA, St Louis, MO) antibodies. To detect the physical interaction of GATA4 with Cdk9 in mouse ES cells, nuclear extracts were first immunoprecipitated with a goat polyclonal anti-GATA4 or anti-Cdk9 antibody on day 8, as previously described (Iwanaga et al., 1998; Wada et al., 2002; Hirai et al., 2004). Nuclear extracts were also immunoprecipitated with an anti-IgG antibody as a control. The precipitates were sequentially subjected to Western blotting using rabbit polyclonal anti-Cdk9, rabbit polyclonal anti-GATA4, and rabbit polyclonal anti-cyclinT1 antibodies.

Plasmid constructs

A lentivirus vector, pLenti6/V5-D-TOPO® (Invitrogen), was subcloned to contain the phosphoglycerate kinase (pgk) promoter–enhancer and used as a null expression vector (pLenti-Null) for lentiviral infection. pLenti-DN-Cdk9 was constructed by inserting human Cdk9 cDNA including a point mutation at nucleotide 563, which converts Asp into Asn, into pLenti-Null. pLenti-GFP was constructed by inserting enhanced-GFP cDNA into pLenti-Null.

Lentiviral infection

To generate the lentivirus vectors, 293FT packaging cells (Invitrogen) were seeded at 1.5×10^6 cells per 100-mm dish using Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 0.05 mg/ml streptomycin. On the next day, pLenti6/V5-D-TOPO®-based lentivirus vectors were induced into 293FT cells by the calcium phosphate method. These cells were incubated at 37°C with 5% CO_2 for 8 h. Thereafter, the medium was replaced with GMEM-based medium for the differentiation culture of ES cells. After 12 h at 32°C with 5% CO_2, supernatants containing lentivirus produced by these 293FT cells were passed through a 0.45-μm filter and supplemented with 8 μg/ml polybrene. Thirty thousand ES cells were transferred into each well of 6-well plates including these supernatants and maintained for 12–24 h. Thereafter, the supernatant was replaced with the fresh medium. The day on which ES cells were transferred was designated as day 0. Then, ES cells were differentiated as previously described.

Flow cytometry

For the quantitative analysis of the number of GFP-positive cells, ES cells were dissociated into single cells and analyzed by flow cytometry (BD Biosciences, San Jose, CA) on day 8, as previously described (Hidaka et al., 2003).

Quantitative RT-PCR

Total RNAs from ES cells were isolated using TRIzol® reagent (Invitrogen), reverse transcribed, and amplified as previously described (Yanazume et al., 2003). Quantitative PCR was performed as previously described (Kawamura et al., 2005). Primer sequences of Nkx2.5 (Hidaka et al., 2003), VEGF (Ogawa et al., 2005), β-myosin heavy chain (β-MHC) (Hidaka et al., 2003), Cdk9 (Takaya et al., 2009), and GAPDH (Iwanaga et al., 1998) were described previously.

Statistical analysis

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

Results

Expression patterns of genes involved in myocardial differentiation in ES cells

To investigate patterns regarding the spontaneous differentiation of ES cells, these cells were seeded on gelatin-coated plates with no feeder cells. Differentiation was induced without the formation of EBs by removing LIF and antibiotics from the media. In this system, ES cells exhibited myogenic contractions on day 10. These beating cells were positive for both cardiac troponin T and cardiac myosin heavy chain, and exhibited sarcomere formation and myofibril organization, typical features of cardiomyocytes (Kaichi et al., 2010). During the induction of the spontaneous differentiation of ES cells, we examined the time course of the mRNA levels of genes involved in myocardial differentiation as well as that of Cdk9, a major component of p-TEFb. The day on which we started differentiation induction was designated as day 0. Total RNAs were isolated from ES cells on days 0, 3, 5, and 8 and synthesized cDNAs were subjected to quantitative PCR. We examined the mRNA level of a zinc finger protein, GATA4, and a cardiac-specific transcription factor, Nkx2.5. As shown in Figure 1Aa,b, the levels of both GATA4 and Nkx2.5 increased from days 3 to 5. Then, we examined their mRNA expression levels on day 4. The GATA4 levels increased from days 3 (100 ± 9) to 4 (354 ± 42). Meanwhile, the Nkx2.5 levels were similar between days 3 (100 ± 13) and 4 (97 ± 31). These findings demonstrate that GATA4 mRNA increases from days 3 to 5 and that Nkx2.5 mRNA increases after day 4. Next, we examined the expression of a gene encoding the cardiac structure protein, β-

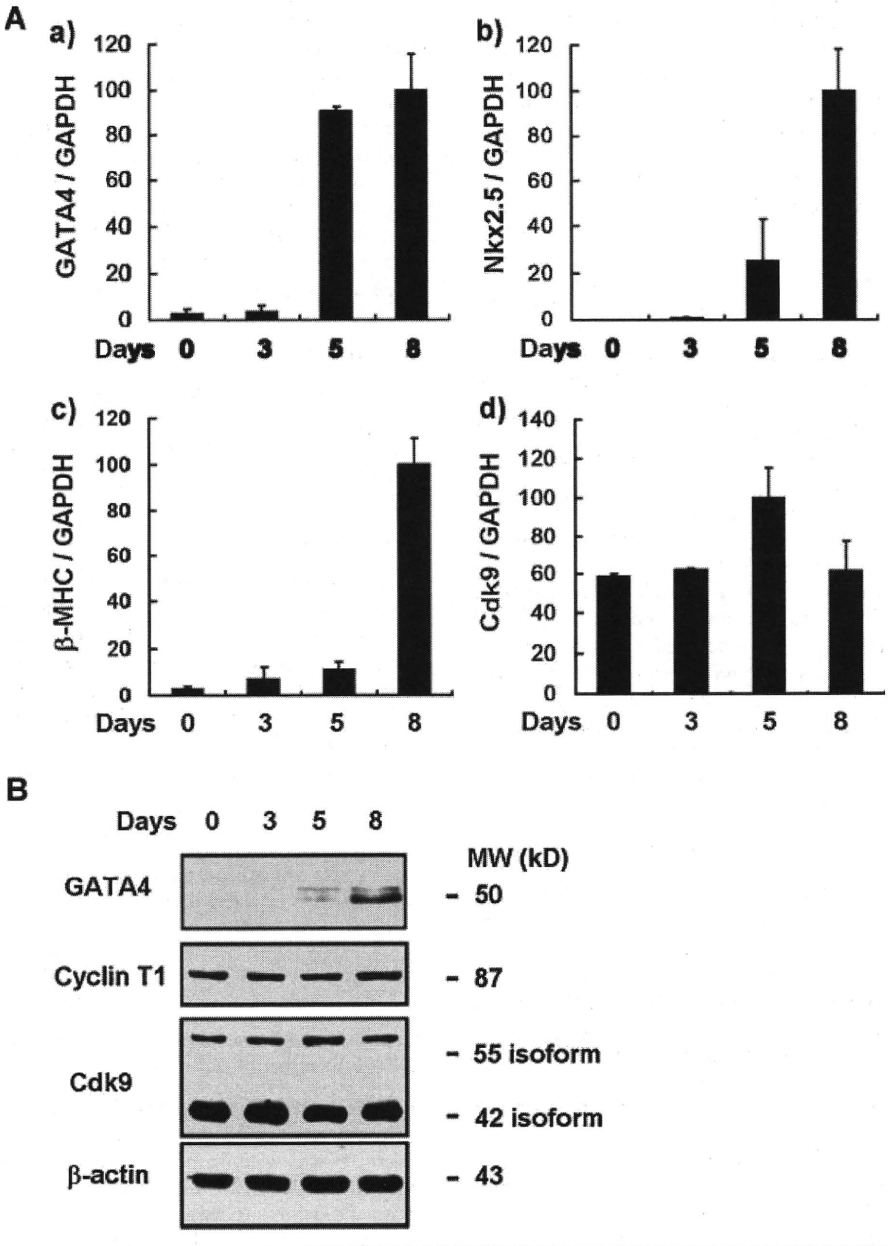

Fig. 1. A: Total RNAs were extracted from ES cells on days 0, 3, 5, and 8, and reverse-transcribed into cDNAs. Using quantitative PCR, expressions of cardiac-specific genes, transcription factors, and Cdk9 were analyzed. Levels of GAPDH transcripts were used to normalize cDNA levels. The maximal mRNA level of specific targets relative to GAPDH at different time points was set at 100. Data are presented as the means ± SE of 3–5 independent experiments. (a) GATA4, (b) Nkx2.5, (c) β-MHC, and (d) Cdk9. B: Nuclear extracts from differentiated ES cells on days 0, 3, 5, and 8 were subjected to Western blotting with anti-GATA4, anti-Cdk9, anti-cyclin T1, and anti-β-actin antibodies at the indicated time points. The photographs are representative. We performed three or more independent experiments and obtained similar results.

myosin heavy chain (β-MHC). A significant level of β-MHC mRNA was detected only on day 8 (Fig. 1Ac). The Cdk9 gene was abundantly expressed at the undifferentiated stage and maintained at almost similar levels throughout the stages of differentiation (Fig. 1Ad).

Then, we examined the protein levels of GATA4, Cdk9, and cyclin T1. Nuclear extracts from ES cells were isolated on days 0, 3, 5, and 8 and subjected to Western blotting. The signal of GATA4 protein was first detected on day 5 (Fig. 1B). In contrast, the signals of Cdk9 and cyclinT1 proteins were already marked at the undifferentiated stage and maintained at relatively constant levels. The expression patterns of these proteins were compatible with those of their endogenous mRNA.

Trichostatin A, an HDAC inhibitor, up-regulates the expression of cardiac-specific genes

ES cells, in which GFP is expressed under the control of the cardiac-specific Nkx2.5 promoter (Nkx2.5/GFP ES cells), were treated with TSA, an HDAC inhibitor, at concentrations of 0, 10, 50, and 100 ng/ml on day 7 for 24 h. These cells were dissociated into single cells by trypsin on day 8 and subjected to analysis by flow cytometry. The rate of Nkx2.5/GFP-positive

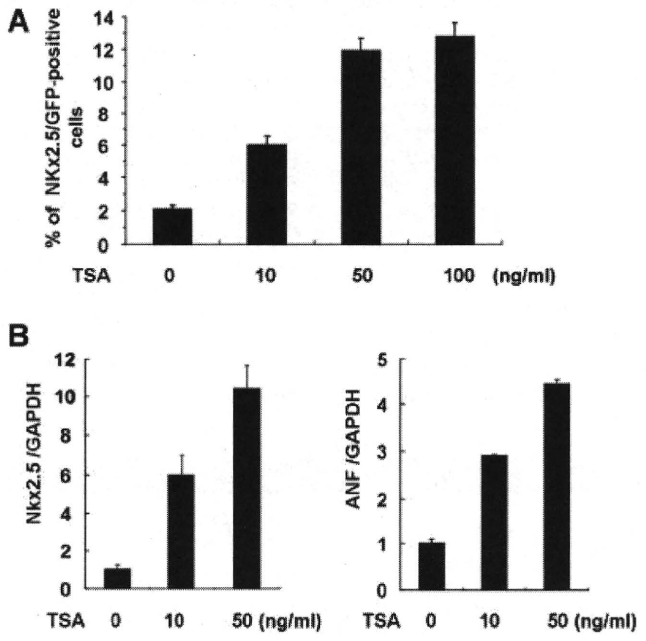

Fig. 2. A: Nkx2.5/GFP ES cells were stimulated with TSA (10, 50, or 100 ng/ml) or a corresponding amount of its solvent, ethanol, on day 7 for 24 h. Differentiated ES cells were dissociated into single cells with trypsin on day 8 and subjected to analysis by flow cytometry. Data represent the rate of Nkx2.5/GFP-positive cells to total extant cells at the indicated TSA concentrations. B: ES cells on day 7 were stimulated with TSA (10 or 50 ng/ml) or its solvent for 24 h. Total RNAs were isolated on day 8 from theses cells, and synthesized cDNAs were subjected to quantitative PCR. Levels of GAPDH transcripts were used to normalize specific cDNA levels. The relative mRNA levels of Nkx2.5 and ANF in ES cells stimulated with 50 ng/ml of TSA were set at 100. Data are presented as the means ± SE of 3–5 independent experiments.

cells to total extant cells before TSA stimulation was around 2%. Treatment with TSA increased Nkx2.5/GFP-positive cells by 3- to 6.5-fold in a concentration-dependent manner (Fig. 2A).

Next, differentiated ES cells were treated with TSA on day 7 for 24 h. Total RNAs were isolated from these cells on day 8, and synthesized cDNAs were subjected to quantitative PCR. Treatment with TSA at the concentrations of 10–50 ng/ml increased the endogenous mRNA level of Nkx2.5 by 6- to 10.5-fold and that of a cardiac-specific peptide hormone, atrial natriuretic factor (ANF), by 3- to 4.5-fold (Fig. 2B).

GATA4 physically interacts with Cdk9/cyclin T1 in ES cells

We demonstrated that Cdk9 forms a physical complex with GATA4 as well as p300 by in vivo GST-binding assay (Sunagawa et al., 2010). To examine the physical interaction of GATA4 with Cdk9 in ES cells, nuclear extracts were isolated on day 8 and subjected to immunoprecipitation/Western blotting (Fig. 3). First, these nuclear extracts were immunoprecipitated with goat polyclonal anti-GATA4 antibody or anti-IgG antibody as a control and sequentially subjected to Western blotting using rabbit polyclonal anti-Cdk9 and rabbit polyclonal anti-GATA4 antibodies. The Cdk9 protein was immunoprecipitated with GATA4. Next, these extracts were immunoprecipitated with goat polyclonal anti-Cdk9 antibody and sequentially subjected to Western blotting using rabbit polyclonal anti-GATA4 and rabbit polyclonal anti-Cdk9 antibodies. The GATA4 protein was also immunoprecipitated with Cdk9. Finally, the precipitates formed by anti-GATA4 antibody and those by anti-Cdk9 antibody were subjected to Western blotting using rabbit polyclonal anti-cyclin T1 antibody. The cyclin T1 protein was immunoprecipitated with GATA4 as well as Cdk9. These results demonstrate that GATA4 physically interacts with the Cdk9/cyclin T1 complex in ES cells.

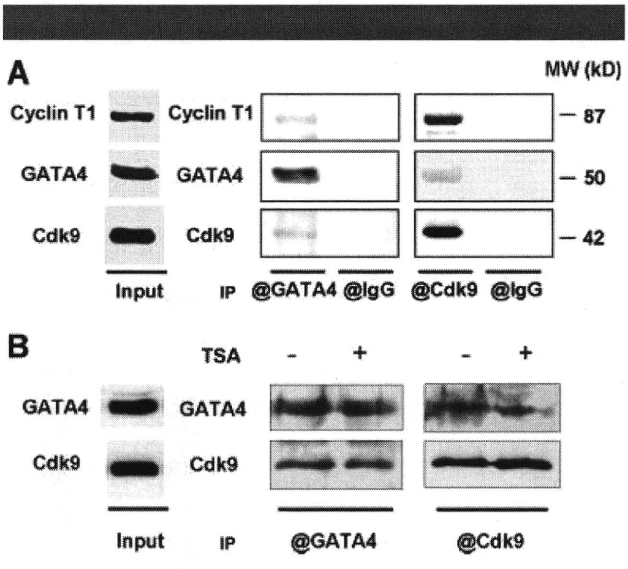

Fig. 3. A: Nuclear extracts from ES cells on day 8 were immunoprecipitated with indicated antibodies and sequentially subjected to Western blotting with anti-GATA4, anti-cyclin T1, and anti-Cdk9 antibodies. The photographs are representative. We performed three independent experiments and obtained similar results. B: Differentiated ES cells were treated with TSA or untreated on day 7 and nuclear extracts were isolated on day 8. These extracts were immunoprecipitated with the indicated antibodies and sequentially subjected to Western blotting with anti-GATA4 and anti-Cdk9 antibodies. The photographs are representative. We performed three or more independent experiments and obtained similar results.

Next, to examine whether TSA treatment alters the interaction of GATA4 with Cdk9, the differentiated ES cells were treated with TSA or untreated on day 7 and the nuclear proteins were isolated on day 8. The nuclear extracts were immunoprecipitated with a goat polyclonal anti-GATA4 or anti-Cdk9 antibody. The precipitates were sequentially subjected to Western blotting using a rabbit polyclonal anti-Cdk9 or anti-GATA4 antibody. The signal levels of GATA4 and Cdk9 were similar between the precipitates treated with TSA and those left untreated (Fig. 3B). These results demonstrate that TSA treatment did not alter the interaction of GATA4 with Cdk9.

A dominant-negative form of Cdk9 inhibited the TSA-induced increase in Nkx2.5/GFP-positive cells

To examine the effect of a dominant-negative form of Cdk9 (DN-Cdk9), which loses its kinase activity, on myocardial differentiation, pLenti-DN-Cdk9 or pLenti-Null was introduced into ES cells at the undifferentiated stage by lentivirus-mediated gene transfer. These cells were induced to differentiate and stimulated with TSA at a concentration of 10 ng/ml on day 7. On the next day, differentiated ES cells were isolated, and the nuclear extracts were subjected to Western blotting (Fig. 4A). The signals of RNA polymerase II protein (RNA pol II a) were similar in DN-Cdk9- and Null-introduced ES cells. However, the signal of the phosphorylated form of RNA polymerase II protein (RNA pol II o) was inhibited by the introduction of pLenti-DN-Cdk9. Treatment with TSA had no effect on the signal levels of both RNA pol II a and RNA pol II o. The signals of the proteins, GATA4, Cdk9, and cyclin T1 were not affected by treatment with TSA or by the introduction of DN-Cdk9.

Next, pLenti-DN-Cdk9 or pLenti-Null was introduced into Nkx2.5/ES cells. Then, these cells were introduced to

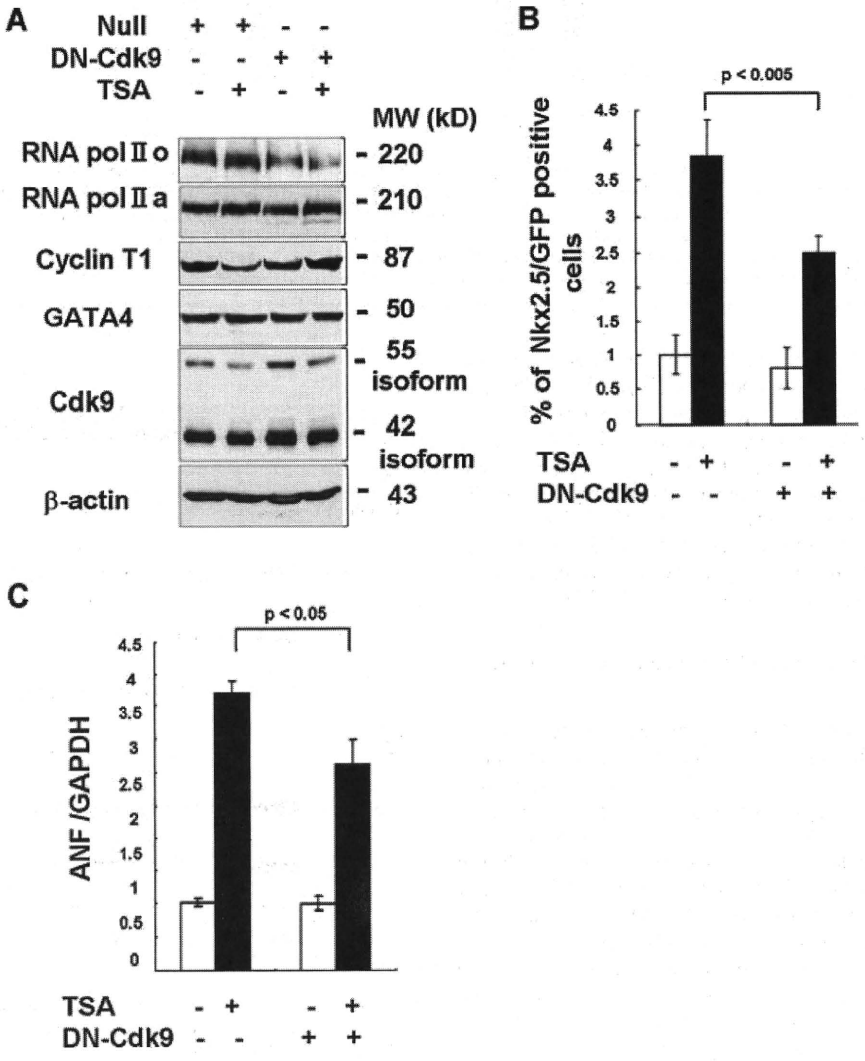

Fig. 4. A: ES cells were transformed with pLenti-Null or pLenti-DN-Cdk9 on day 0. These cells were induced to differentiate and stimulated with TSA (10 ng/ml) or its solvent on day 7 for 24 h. Nuclear extracts were isolated on day 8 and subjected to Western blotting using the indicated antibodies. The photographs are representative. We performed three independent experiments and obtained similar results. B: Nkx2.5/GFP ES cells were transformed with pLenti-Null or pLenti-DN-Cdk9 on day 0, induced to differentiate, and stimulated with TSA or its solvent on day 7 for 24 h. These cells were dissociated into single cells on day 8 and subjected to flow cytometry. These data represent the rate of Nkx2.5/GFP-positive cells to total extant cells and are expressed as means ± SE of three or more independent experiments. C: ES cells were transformed with pLenti-Null or pLenti-DN-Cdk9 on day 0. Then, these cells were induced to differentiate and stimulated with TSA (50 ng/ml) or its solvent on day 7 for 24 h. Total RNAs were isolated by TRIzol® reagent on day 8 and subjected to quantitative RT-PCR.

differentiate and treated with TSA on day 7 for 24 h. On the next day, these cells were dissociated into single cells by trypsin and subjected to flow cytometry. DN-Cdk9 significantly inhibited the TSA-induced increase in Nkx2.5/GFP-positive cells (Fig. 4B).

Furthermore, pLenti-DN-Cdk9 or pLenti-Null was introduced into Nkx2.5/ES cells on day 0. Then, these cells were induced to differentiate and stimulated with TSA on day 7. On day 8, total RNAs were isolated, and the synthesized cDNAs were subjected to quantitative PCR. Treatment with TSA increased the level of ANF mRNA more than 3.5-fold in differentiated ES cells into which pLenti-Null was introduced (Fig. 4C). The introduction of pLenti-DN-Cdk9 into ES cells significantly suppressed the TSA-induced increase in the ANF mRNA level. These results demonstrate that a dominant-negative Cdk9 inhibited TSA-induced ANF expression.

DRB inhibited TSA-induced expression of Nkx2.5

Differentiated ES cells were treated with TSA and/or Cdk9 inhibitor DRB on day 7. On day 8, these cells were isolated and their nuclear extracts were subjected to Western blotting. While the signal levels of RNA pol II a were not affected by treatment with TSA and/or DRB, the signal of RNA pol II o was inhibited by treatment with DRB. The signals of GATA4, Cdk9, and cyclin T1 proteins were not affected by treatment with either agent (Fig. 5A).

Next, Nkx2.5/GFP ES cells were induced to differentiate and treated with TSA and/or DRB on day 7 for 24 h. Then, these cells were dissociated into single cells and subjected to flow cytometry. While DRB did not affect the basal rate of Nkx2.5/GFP-positive cells relative to total extant cells, DRB almost completely inhibited the TSA-induced increase in Nkx2.5/GFP-positive cells (Fig. 5B). ES cells were similarly treated with these agents on day 7 and the total RNAs were isolated by TRIzol® reagent on day 8. The synthesized cDNAs were subjected to quantitative PCR. While treatment with TSA increased the expression of endogenous Nkx2.5 mRNA by more than 12-fold, additional DRB treatment markedly inhibited the TSA-induced increase in the endogenous Nkx2.5 mRNA level (Fig. 5Ca). On the other hand, treatment with TSA did not increase the endogenous mRNA level of VEGF, a maker of vascular endothelial cells. Treatment with DRB did not affect the endogenous mRNA expression of VEGF either (Fig. 5Cb).

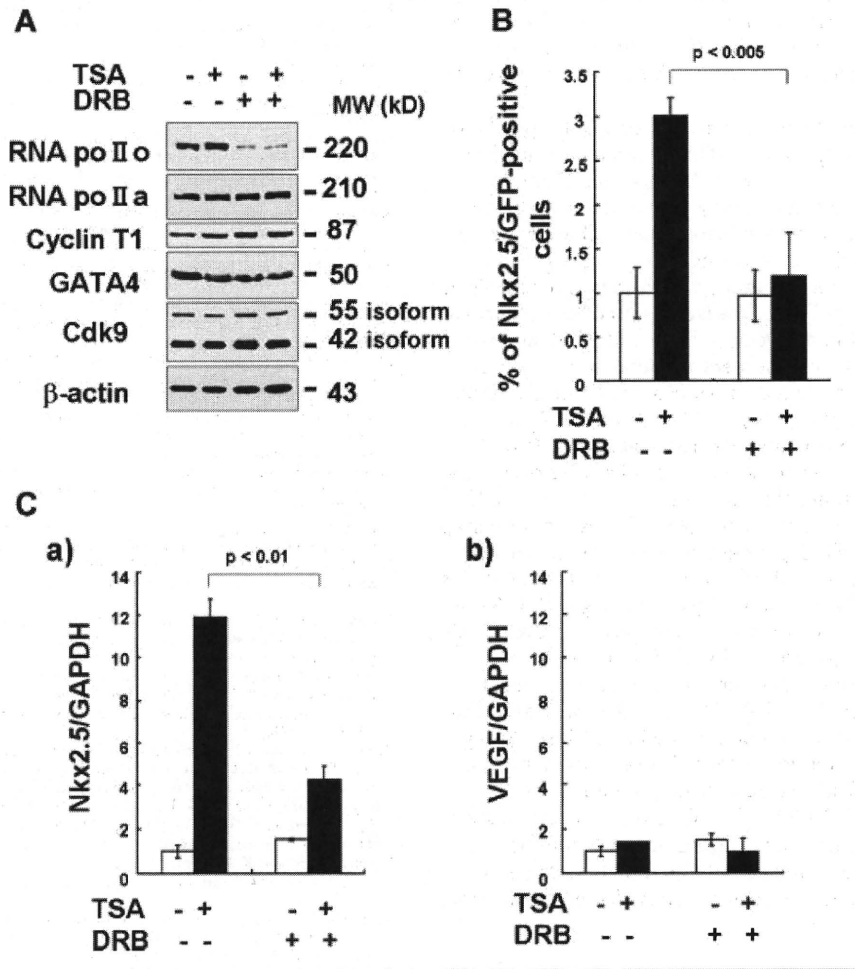

Fig. 5. A: Differentiated ES cells were stimulated with TSA and/or DRB on day 7 for 24 h and collected on day 8. Nuclear extracts from these cells were subjected to Western blotting using the indicated antibodies. The photographs are representative. We performed three independent experiments and obtained similar results. B: Differentiated Nkx2.5/GFP ES cells stimulated with TSA (50 ng/ml) and/or DRB (50 μM) were dissociated into single cells and subjected to flow cytometry. These data show the rate of Nkx2.5/GFP-positive cells to total extant cells. C: Total RNAs from differentiated ES cells treated with TSA (50 ng/ml), and/or DRB (50 μM), or both were isolated, and synthesized cDNAs were subjected to quantitative PCR. Levels of GAPDH transcripts were used to normalize cDNA levels. The mRNA level of Nkx2.5 (a) or VEGF (b) relative to GAPDH in ES cells treated with a solvent, ethanol, was set at 1.0. The data are expressed as means ± SE of three or more independent experiments.

Discussion

In this study, we examined whether Cdk9 forms a complex with GATA4 and is involved in the TSA-induced differentiation of mouse ES cells into cardiomyocytes. To induce the differentiation of ES cells, we used feeder-free gelatin-coated plates without the formation of EBs because it is simple and reproducible. Within this system, we initially examined the expression profiles of genes involved in cardiogenesis, and demonstrated that these profiles were similar to those in experiments involving EB formation (Kawamura et al., 2005). The treatment of ES cells with TSA, an HDAC inhibitor, increased Nkx2.5/GFP-positive cells and endogenous mRNA levels of Nkx2.5 and ANF in a concentration-dependent manner. We also confirmed that TSA increased endogenous mRNA levels of these genes in mouse-induced pluripotent stem cells (Kaichi et al., 2010).

To examine whether Cdk9 is involved in the differentiation of ES cells into cardiomyocytes, we ablated the Cdk9 kinase activity using either DN-Cdk9 or a Cdk9 inhibitor, DRB. The suppression of Cdk9 kinase activity inhibited the TSA-induced expression of the cardiac-specific transcription factor Nkx2.5. While DRB almost completely inhibited TSA-induced myocardial differentiation, inhibition by the introduction of DN-Cdk9 was partial. Cdk9 was abundantly expressed throughout stages of undifferentiation to differentiation, and the efficiency of gene transduction by lentivirus-mediated gene transfer might vary in each transfection. These may have attributed to the discrepancy in the inhibitory effects between DRB and DN-Cdk9.

Studies using either RNA interference or highly specific p-TEFb inhibitors have implicated p-TEFb as an important factor in global transcriptional elongation (Shim et al., 2002). In fact, Cdk9 and cyclinT1 were abundantly expressed from at the undifferentiated stage to differentiated stage. One of the key questions is how p-TEFb is recruited to its target genes in myocardial differentiation. At least, there are two mechanisms for the recruitment of p-TEFb. One is a less specific recruitment through the interaction between p-TEFb and Brd4, and the other is a gene-specific recruitment through an interaction between p-TEFb and transactivators (Kanazawa et al., 2000; Barboric et al., 2001; Eberhardy and Farnham, 2002; Simone et al., 2002). A previous report demonstrated that MyoD recruits p-TEFb onto the promoters and enhancers of muscle-specific genes during skeletal myogenesis (Giacinti et al., 2006). Another reported that recruitment of p-TEFb is a critical step for stimulation of MEF2-dependent transcription in skeletal muscle cells (Nojima et al., 2008). We presented that Cdk9 is recruited onto the ANF promoter by interacting with GATA4/p300 complex in cardiomyocyte hypertrophy, and that Cdk9 kinase activity was required for the p300-induced transcriptional activities, DNA-binding, and acetylation of GATA4 (Sunagawa et al., 2010). We have demonstrated that the Cdk9/cyclin T1 complex combines with GATA4 during myocardial differentiation in ES cells. These findings raise a hypothesis that Cdk9 kinase activity is involved in the TSA-induced activation of GATA4 in ES cells as well as in the pathway of cardiomyocyte hypertrophy. Therefore, it would be interesting to examine the detailed functional association between GATA4 and Cdk9 during myocardial differentiation.

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CASE REPORT

Ring/marker chromosome derived from chromosome 7 in childhood acute megakaryoblastic leukemia with monosomy 7

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Abstract In some cases of childhood acute megakaryoblastic leukemia (AMKL), G-band analysis reveals supernumerary ring/marker chromosomes along with monosomy 7. However, their origin and relevance are poorly understood. We experienced three patients with AMKL, one of whom had Down's syndrome, whose blasts at the first visit exhibited both monosomy 7 and a ring/marker chromosome. For one case, precise molecular-cytogenetic techniques revealed that the ring chromosome was derived from a chromosome 7. It was strongly suggested that the ring chromosome was derived from a chromosome 7 in another case. The ring or one of the 2 marker chromosomes was derived from a chromosome 7 in the other case. All patients responded well to initial induction therapy. While it is not clear whether the ring/marker chromosome 7 affects the long-term prognosis of acute myeloid leukemia with monosomy 7, it may be of prognostic relevance to distinguish pure monosomy 7 from monosomy 7 with a ring/marker chromosome 7. For this purpose, conventional G-banding could be complemented with additional techniques such as spectral karyotyping or fluorescence in situ hybridization, which characterize the aberration in more detail. These methods may be useful for determining the optimal treatment and for elucidating the etiology of AMKL itself.

Keywords Monosomy 7 · Spectral karyotyping · Fluorescence in situ hybridization

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1 Introduction

Acute myeloid leukemia (AML) is a disease of the myeloid compartment of the hematopoietic system and is characterized by the accumulation of undifferentiated blast cells in the peripheral blood and bone marrow. The prognosis of childhood AML has improved significantly over the past decades [1]. Acute megakaryoblastic leukemia (AMKL) is a biologically heterogeneous form of AML that occurs in 10–20% of all cases of AML and has a bimodal age distribution with peaks in early childhood and adulthood [2].

Specific molecular and cytogenetic abnormalities in AML, including AMKL, define distinct pathological features and clinical behavior, in particular the response to therapy and survival. In some AML cases, some or all of chromosome 7 are deleted. AML with monosomy 7 cases is generally considered to have a poor prognosis [3]. This may relate to the possibility that genes on chromosome 7 may play critical roles in controlling the growth and division of cells.

Childhood AMKL can be divided into two groups, namely, Down syndrome (DS)-AMKL and primary AMKL that occurs in the absence of DS (known as non-DS-AMKL). Athale et al. [4] have found that while the two AMKL groups do not differ in terms of microscopic BM findings and the expression of surface antigens, the treatment outcome for patients with non-DS-AMKL is very poor. In contrast, Hama et al. [5] showed that the blasts of

DS-AMKL patients are less mature than those of non-DS-AMKL patients in terms of morphology and immunophenotyping but that the two groups had excellent outcomes. This discrepancy may be resolved by the recent study by Bourquin et al. [6] who showed that DS- and non-DS-AMKL patients have distinct gene expression profiles and that non-DS-AMKL patients can be subdivided into two molecular phenotypes. The meaning of monosomy 7 may be different between DS-AMKL and non-DS-AMKL. Kudo et al. [7] showed that in DS-AMKL, the presence of monosomy 7 is a greater risk factor of adverse outcome. By contrast, the prognostic significance of monosomy 7 in non-DS-AMKL is poorly understood.

In this paper, we describe three cases of childhood AMKL with a ring/marker chromosome 7 in addition to monosomy 7. We discuss the possibility that spectral karyotyping (SKY) and fluorescence in situ hybridization (FISH) analyses performed at the time of diagnosis could be useful for determining the optimal treatment regimen (Table 1).

2 Case reports

2.1 Case #1: DS-AMKL

A boy was diagnosed with constitutional trisomy 21 at birth and underwent surgery for a congenital heart anomaly (atrial and ventricular septal defect). In the neonatal period, he had a history of mild thrombocytopenia, but a transient abnormal myelopoiesis was not evident. At 3 years old, he was admitted with a fever, petechiae, and slowly progressing thrombocytopenia. He had dysmorphic features such as hypertelorism and epicanthal folds. Laboratory tests showed a white blood count of 3300/μl with 1% blasts, Hb of 10.3 g/dl, and platelet counts of 55000/μl. The blood film showed mild anisocytosis, poikilocytosis, and occasional hypogranulated platelets. Upon marrow aspiration, the patient exhibited 'dry tap' and a drop of aspirate revealed that the myeloid and erythroid lineages lacked dysplastic features. A bone marrow biopsy revealed mature megakaryoblast congestion and marked fibrosis. He was diagnosed with AMKL and achieved complete remission (CR) after the first induction therapy, which involved the use of the current JPLSG AML-D05 protocol (AraC 100 mg/m^2 × 7 + VP16 150 mg/m^2 × 3 + THP-ADR 25 mg/m^2 × 2). The patient's blasts were negative for the *FLT3*-internal tandem duplication (ITD). Direct cytogenetic analysis of the bone marrow metaphases revealed three of the 19 cells that were analyzed had 48,XY,−7,add(11)(q23),+21,+21,+r1. SKY was not possible because of insufficient material. Therefore, we employed the FISH technique using a chromosome 7 probe (D7Z1) and found that 0 of 100 cells showed a single D7Z1 signal. Though we could not definitely determine whether FISH probe was on the ring chromosome, add(11)(q23), or

Table 1 Summary of three cases

	Case #1	Case #2	Case #3
Age	3 years 4 months	1 year 0 month	2 years 3 months
Down syndrome	(+)	(−)	(−)
Dry tap	(+)	(+)	(−)
Karyotype of BM cells	48,XY,−**7**,add(11)(q23),+21c, +21,+**r1** [3] 47,XY,+21 [16]	47,XY,−**7**,+21,+**r1** [11] 46,XY [9]	46,X,−X,−2,−**7**,add(17)(q25),del(20)(q11.2),+**r1**,+**mar1**,+**mar2** [20]
Constitutional karyotype	47,XY,+21c [20]	46,XY [20] (BM) 46,XY [100] (buccal mucosa)	46,XX [20]
Surface marker on blast	CD13, CD33, HLA-DR, CD7, CD41a, CD61	CD13, CD33, HLA-DR, CD7, CD41a, CD61	CD13, CD33, HLA-DR, CD7, CD41a, CD61
Trilineage dysplasia	(−)	(−)	(+)
Single signal of chromosome 7 by FISH	0/100 cells	2/100 cells	5/100 cells
SKY	Not done	Refer to Fig. 1	Not done
Induction therapy	JPLSG AML-D05	JPLSG AML-D05	JPLSG AML-05
Grade 3/4 toxicity	Hematopoietic	Hematopoietic	Hematopoietic
CR after first induction	Yes	Yes	Yes
CR duration	13 months	10 months	7 months

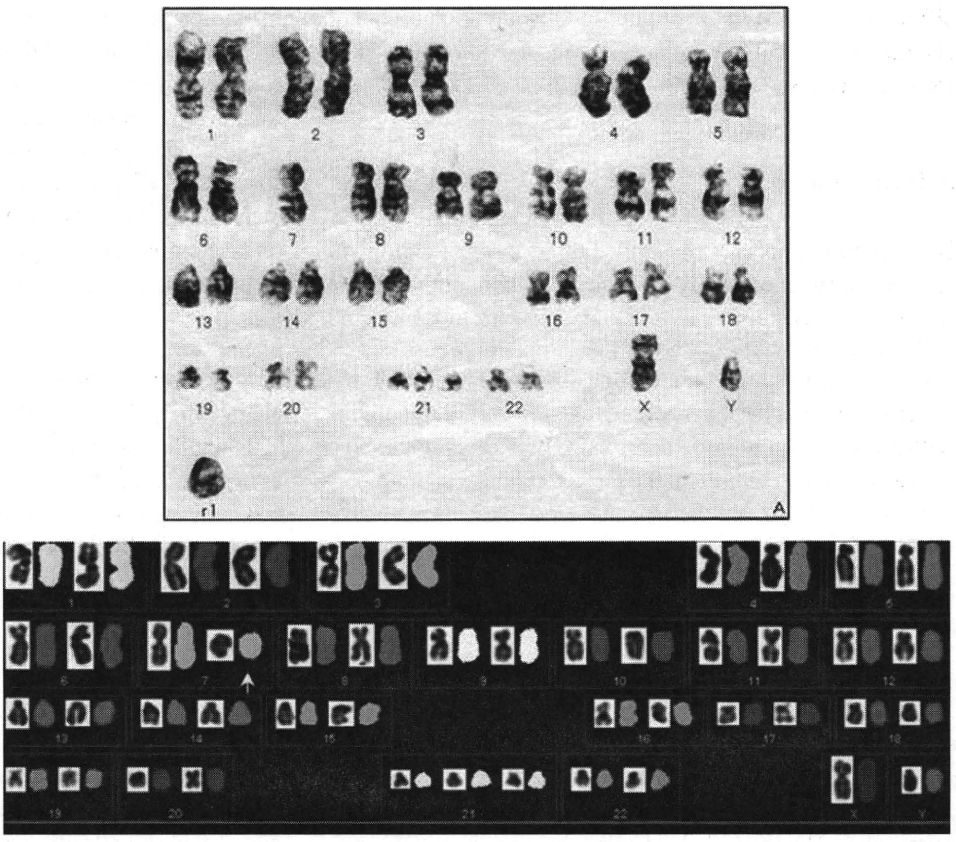

Fig. 1 The results of G-banding and SKY (case #2)

other chromosomes which appeared normal in interphase nuclei, it was strongly suggested that the ring chromosome was derived from a chromosome 7. The CR status of the patient continues to persist for 13 months.

2.2 Case #2: non-DS-AMKL-1

A 1-year-old male who had a past history of hydronephrosis and afebrile convulsion was admitted for further routine laboratory tests to search for an abnormality. Laboratory tests revealed a white blood count of 10900/μl with 4% blasts, Hb of 7.7 g/dl, and platelet counts of 20000/μl. The peripheral blood smear and a drop of bone marrow aspirate had the same features as case #1. Similarly, a bone marrow biopsy revealed mature megakaryoblast congestion and marked fibrosis. The patient was diagnosed with AMKL. His blasts were negative for *FLT3*-ITD. He had no dysmorphic features such as hypertelorism and epicanthal folds. But since 11 of the 20 bone marrow cells that were examined initially by cytogenetics showed 47,XY,−7,+21,+r1, and 9 of 20 cells exhibited 46,XY, it was not possible to completely exclude the possibility of mosaic Down syndrome and so he was treated by the JPLSG AML-D05 protocol. Since buccal cytogenetics then revealed two signals from chromosome 21 by FISH, the patient's treatment regimen was changed to the Japanese AML99 protocol [8] with a moderate dosage. The response to the initial induction therapy was good and the patient continues to have a CR status for 10 months. SKY and FISH analyses revealed that the ring was from a chromosome 7 (Fig. 1).

2.3 Case #3: non-DS-AMKL-2

A 2-year-old female was admitted for further work-up for purpura. The laboratory tests showed a white blood count of 37500/μl with 73% blasts, Hb of 8.3 g/dl, and a platelet count of 13000/μl. A peripheral blood smear and a drop of bone marrow aspirate had the same features as cases #1 and #2 with multilineage dysplasia in the background. The patient was diagnosed with AMKL. Initial bone marrow cytogenetics revealed that all of the 20 cells examined had 46,X,−X,−2,−7,add(17)(q25),del(20)(q11.2),+r1,+mar1, +mar2. However, subsequent FISH technique using a chromosome 7 probe showed that only 5% cells were single positive, which suggests the existence of extra chromosome 7 derivatives. The patient's blasts were negative for *FLT3*-ITD. She was treated by the JPLSG AML-05 protocol (AraC 200 mg/m^2 × 7 + VP16 150 mg/m^2 × 5 + MIT 5 mg/m^2 × 5). Because (1) the response for initial induction therapy was good and (2) her AMKL was considered to be