Transient ectopic expression of the histone demethylase JMJD3 accelerates the differentiation of human pluripotent stem cells

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ABSTRACT

Harnessing epigenetic regulation is crucial for the efficient and proper differentiation of pluripotent stem cells (PSCs) into desired cell types. Histone H3 lysine 27 trimethylation (H3K27me3) functions as a barrier against cell differentiation through the suppression of developmental gene expression in PSCs. Here, we have generated human PSC (hPSC) lines in which genome-wide reduction of H3K27me3 can be induced by ectopic expression of the catalytic domain of the histone demethylase JMJD3 (called JMJD3c). We found that transient, forced demethylation of H3K27me3 alone triggers the upregulation of mesoendodermal genes, even when the culture conditions for the hPSCs are not changed. Furthermore, transient and forced expression of JMJD3c followed by the forced expression of lineage-defining transcription factors enabled the hPSCs to activate tissue-specific genes directly. We have also shown that the introduction of JMJD3c facilitates the differentiation of hPSCs into functional hepatic cells and skeletal muscle cells. These results suggest the utility of the direct manipulation of epigenomes for generating desired cell types from hPSCs for cell transplantation therapy and platforms for drug screenings.

KEY WORDS: Histone demethylase, JMJD3, KDM6B, Human pluripotent stem cells, Hepatocytes, Skeletal muscle

INTRODUCTION

Pluripotent stem cells (PSCs), such as human embryonic stem cells (hESCs) (Thomson et al., 1998) and human induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007; Yu et al., 2007), have the capacity to differentiate into various cell types, accompanied by dynamic alternations in gene expression patterns (Drukker et al., 2012; Fathi et al., 2011; Norman et al., 2013; Yang et al., 2013). hESCs/iPSCs maintain their undifferentiated state by expressing pluripotency-associated genes mediated by lineage-defining transcription factors. We have shown that the forced expression of H3K27me3 alone triggers the upregulation of mesoendodermal genes, even when the culture conditions for the hPSCs are not changed. Furthermore, transient and forced expression of JMJD3c followed by the forced expression of lineage-defining transcription factors enabled the hPSCs to activate tissue-specific genes directly. We have also shown that the introduction of JMJD3c facilitates the differentiation of hPSCs into functional hepatic cells and skeletal muscle cells. These results suggest the utility of the direct manipulation of epigenomes for generating desired cell types from hPSCs for cell transplantation therapy and platforms for drug screenings.
also shown that transient expression of the catalytic domain of JMJD3 dramatically accelerates hPSC differentiation into hepatic cells or muscle cells. These results indicate the utility of H3K27 demethylase in improving the efficiency of hPSC differentiation.

RESULTS

Generation of H3K27me3-deficient hESCs by ectopic JMJD3 expression

The histone lysine demethylases (KDM1-6) possess activity against different substrates (H3K4, H3K9, H3K27 and H3K36), and their specificities have been characterized (Kooistra and Helin, 2012). UTX and JMJD3 (also known as KDM6A and KDM6B, respectively) are specific enzymes that remove di- and tri-methyl-H3K27 (H3K27me2/3). We performed a meta-analysis of previously published transcriptome data (Gifford et al., 2013) and found that among the histone demethylase genes, only JMJD3 was significantly upregulated upon hESC differentiation into the three germ layers (Fig. S1). This finding suggests that increasing levels of JMJD3 are important for inducing H3K27me3 demethylation during differentiation. Thus, we designed an experiment to overexpress JMJD3 transiently in hESCs (SEES3 line, XY) and examined whether demethylation of H3K27me3 occurs in hESCs.

First, we synthesized mRNAs encoding either the full-length JMJD3 (JMJD3f) or the C-terminal region of JMJD3 containing the catalytic domain (JMJD3c) in vitro (Fig. 1A). The 5′ ends of these mRNAs were tagged with hemagglutinin (HA) sequences to allow us to detect the translated proteins. Eight hours after the hESCs were transfected with the synthetic mRNAs, demethylation of H3K27me3 was detected by immunostaining and immunoblotting (Fig. 1B,C). Transfection of the JMJD3c mRNAs induced a more significant reduction in the H3K27me3 levels than in the JMJD3f mRNAs, suggesting that the catalytic domain of JMJD3 is more efficient at demethylating nucleosome histones. The forced expression of the catalytic domain of another H3K27 demethylase, UTX, did not induce a significant reduction in the H3K27me3 levels in hESCs (Fig. S2A,B). Similar results were obtained using another hESC line (H9, XX; data not shown).

As an independent method of manipulating JMJD3 expression, we generated a stable hESC line (SEES3) in which HA-JMJD3c expression was controlled by doxycycline (Dox) treatment (JMJD3c-hESCs) (Fig. 1D). Dox treatment (1 μg/ml) induced HA-JMJD3c expression in essentially all hESCs (Fig. 1E), resulting in a significant reduction in H3K27me3 levels (Fig. 1F). Overexpression of the JMJD3c mutant, which lacks catalytic activity (Fig. 1G), did not induce any changes in H3K27me3 (Fig. 1H), suggesting that the JMJD3c mutant, which lacks catalytic activity (Fig. 1G), did not induce any changes in H3K27me3 (Fig. 1H), suggesting that the H3K27me3 demethylase activity of JMJD3 is necessary and sufficient for the upregulation of developmental genes in hESCs.

More specifically, among the developmental genes that were upregulated by JMJD3c overexpression, genes related to endoderm and mesoderm differentiation, such as SOX17, FOXA2, GATA4/6, EOMES, brachyury (T) and MIXL1, were highly expressed (Fig. 2E). To our surprise, the activation of these genes occurred in the culture conditions used to maintain an undifferentiated state. Typically, the differentiation of hESC/iPSCs into mesoderm/endoderm requires complete changes in the culture conditions using differentiation media, which contain suitable cytokines and growth factors [e.g. activin-A, bone morphogenetic protein (BMP) and fibroblast growth factor (FGF)] (Murry and Keller, 2008). Real-time PCR analyses revealed that the JMJD3c-expressing condition showed expression levels of developmental genes that were comparable to the levels observed under differentiation conditions using cytokines and growth factors (Fig. 2F). More specifically, JMJD3c-expressing hESCs showed a closer similarity to the expression patterns induced by the combination of activin-A, BMP4 and bFGF, suggesting that these cells had begun to differentiate into a mesendodermal lineage. Interestingly, even when the JMJD3c-hESCs were cultured with Dox in neuronal induction medium, the mesodermal genes were strongly activated compared with the neuroectodermal genes (Fig. 2G). We noticed that JMJD3c overexpression itself upregulated BMP2/4, WNT3/3A and CTNNB1 (β-catenin), but not FGF and NODAL genes (Fig. 2H). Transcriptome analysis also revealed that the JMJD3c-upregulated genes included BMP and Wnt signaling-related genes, such as BMPR1B, FZD2 and VANGL1 (Table S1). As BMP and Wnt/β-catenin signaling lead to hPSC differentiation towards the mesodermal lineage (Davidson et al., 2012; Zhang et al., 2008), ectodermal differentiation may be prevented by JMJD3c overexpression through the mesodermal gene regulatory network.

Mesoendodermal genes are upregulated in JMJD3c-hESCs

We noticed that JMJD3c overexpression resulted in morphological changes in hESCs resembling differentiation (Fig. 2A), which was accompanied by reductions in the expression of the pluripotency marker SSEA-4 and alkaline phosphatase activity (Fig. 2B). The differentiating cells proliferated more slowly than the hESCs but did not exhibit increased apoptosis (Fig. S3). Differentiation was observed even under culture conditions that promote the maintenance of an undifferentiated state. To characterize the differentiation, we examined the changes in the transcriptome after JMJD3c overexpression by performing RNA-sequencing (RNA-seq) analyses of Dox-treated and untreated JMJD3c-hESCs. Among the 1377 differentially expressed genes (fold change >2, FPKM >4), 602 genes were upregulated in the Dox-treated JMJD3c-hESCs (Fig. 2C; Table S1). Gene ontology (GO) analysis revealed that these genes were enriched in development and differentiation processes (Fig. 2D). By contrast, the 775 downregulated genes were not enriched in any significant GO terms (Fig. 2D). However, as expected, pluripotency-related genes (e.g. POUSF1, NANOG, SOX2, DNMT3B, TGFβ1, LIN28, ZFP42, DPPA2, KDM4C, TET1, MYC and UTF1) were downregulated in the JMJD3c-overexpressing hESCs (Fig. S4A), and more than half of these genes (SOX2, DNMT3B, DPPA2, KDM4C, TET1, MYC and UTF1) were included in the list of downregulated genes (fold change <0.5) (Table S2). Dox-treated JMJD3c-hESCs upregulated endogenous JMJD3 but not other KDM6 genes (Fig. S4B). The expression of the developmental genes started to increase beginning on Day 1 after Dox treatment and continued to increase (Fig. 2E). These results were accompanied by a decrease in the levels of H3K27me3 after Dox treatment (Fig. 1F). Furthermore, overexpression of the JMJD3c mutant did not induce these changes (Fig. 2E), suggesting that the H3K27me3 demethylase activity of JMJD3 is necessary and sufficient for the upregulation of developmental genes in hESCs.

We next examined the chromatin changes in hESCs after JMJD3c overexpression. Chromatin immunoprecipitation (ChIP) analyses showed that JMJD3c overexpression resulted in a reduction in the amount of H3K27me3 at the promoters of the developmental genes (Fig. 3A). We extended the analyses to genome-wide changes in
H3K27me3 by conducting ChIP-seq analyses and found that JMJD3c overexpression indeed caused a promoter-specific reduction in the H3K27me3 levels (Fig. 3B). There were no significant changes in the H3K27me3 levels at enhancer regions or repetitive elements after JMJD3c overexpression (Fig. S5A). We examined the patterns of the reduced H3K27me3 levels at the promoters marked by H3K27me3 alone and the bivalent promoters (Fig. 3C). Demethylation occurred at both of those promoters. Of the 1907 bivalent genes, 779 (41%) promoters were H3K27me3-demethylated (fold change <0.5) following JMJD3c overexpression (Table S3). When a lower threshold of demethylation levels (fold change <0.66) was used, the number of demethylated bivalent promoters increased (1186 genes, 62%). Furthermore, the reduction in the H3K27me3 levels corresponded to the upregulation of the genes (Fig. 3D). We also noted that in addition to mesoendodermal genes, neuroectodermal genes, such as NEUROG1 and NEUROG3, showed demethylation of H3K27me3 (Table S3). However, these demethylations were not accompanied by upregulation of gene expression (Table S1). Indeed, JMJD3c-mediated H3K27me3-demethylated genes included many neurogenesis-related genes, whereas the JMJD3c-upregulated genes did not (Fig. 3E). On the other hand, JMJD3c significantly upregulated genes associated with the Wnt signaling pathway. These data suggest that the JMJD3c-activated mesoendodermal transcription network inhibits the expression of neuronal genes, even though the chromatin states of the neuronal genes become active. Indeed, an active marker, H3K4me3 enrichment, remained in most bivalent promoters after JMJD3c overexpression (Fig. S5B-D). Moreover, another marker of active chromatin, H3K27 acetylation (H3K27ac), did not show significant changes following JMJD3c overexpression (Fig. S5E).

**Fig. 1. Generation of H3K27me3-deficient hESCs by ectopic JMJD3c expression.** (A) Structures of the JMJD3f and JMJD3c proteins. JMJD3c was designed to contain the JmjC domain (amino acids 1376-1484). HA was added to JMJD3f and JMJD3c and an NLS was added to the N terminus of JMJD3c. (B) hESCs were transfected with the synthetic mRNAs for HA-JMJD3f or HA-JMJD3c and were stained with HA and H3K27me3 antibodies. Non-transfected hESCs were used as a control. The arrowheads indicate the transfected cells. The mean fluorescent intensities of H3K27me3 in the transfected cells were 0.65 for HA-JMJD3f and 0.26 for HA-JMJD3c compared with the non-transfected cells. More than 20 nuclei from three independent experiments were examined. Scale bar: 20 μm. (C) The effects of transfection of the HA-JMJD3c and HA-JMJD3f mRNAs on the H3K27me3 levels were analyzed by immunoblotting. Emerald mRNA (Em) was transfected as a control. The H3 antibody was used as a loading control. The average signal intensities of H3K27me3 from two independent biological replicates were 0.92 for Em, 0.65 for HA-JMJD3f, and 0.36 for HA-JMJD3c compared with the non-transfected cells. (D) Construct used for Tet-On induction of JMJD3c expression in hESCs (JMJD3c-hESCs). pA, polyA signal; PB, piggyBac repeat. (E) JMJD3c-hESCs were stained with X-Gal 3 days after Dox treatment. Scale bar: 500 μm. (F) HA-JMJD3c-induced H3K27me3 demethylation was detected 1 to 3 days after Dox treatment. The average signal intensities of H3K27me3 from two independent biological replicates were 0.70 on Day 1, 0.52 on Day 2, and 0.24 on Day 3 compared with Day 0. (G) A point mutation in JMJD3c (mut) was inserted at aa 1390 for catalytic inactivation. (H) Effects of HA-JMJD3c and the HA-JMJD3c mut on the H3K27me3 levels. The average signal intensities of H3K27me3 from two independent biological replicates were 0.97 for Em, 0.23 for JMJD3c, and 1.0 for the JMJD3c mut compared with the non-transfected cells.
reasoned that the H3K27me3-deficient hESCs could be efficiently differentiated by the forced expression of lineage-defining transcription factors. We transiently overexpressed JMJD3c in hESCs and subsequently expressed one of the transcription factors MYOD1, HNF1A, RUNX2 or SPI1, which are known to regulate the differentiation of myogenic (Tapscott, 2005), hepatogenic (Si-Tayeb et al., 2010), osteogenic (Lian and Stein, 2003) or hematopoietic (Kastner and Chan, 2008) lineages, respectively.
test this hypothesis. In this procedure, we transfected the Dox-treated or untreated JMJD3c-hESCs with synthetic mRNAs encoding each transcription factor (Fig. 4A). Real-time PCR analysis revealed that when the transcription factors were overexpressed in the JMJD3c-expressing hESCs, the relevant differentiation markers [MYOG for myogenesis, AFP for hepatogenesis, COL1A1 for osteogenesis, and CD45 (PTPRC) for hematogenesis] were more significantly upregulated compared with the hESCs that did not express JMJD3c (Fig. 4B). Notably, JMJD3c alone did not change the expression patterns of these tissue-related genes. These results suggest that the combination of JMJD3c and a lineage-defining transcription factor can promote hPSC differentiation towards specific tissues or cell types.

**JMJD3c facilitates hepatocyte differentiation**

As AFP expression was very significantly enhanced by JMJD3c overexpression in the above experiments, we tested the effect of JMJD3c on differentiation into hepatocytes. Hepatocytes can be generated from hPSCs in culture medium supplemented with hepatocyte growth factors (Kajiwara et al., 2012). Using this differentiation medium, we differentiated JMJD3c-hESCs into hepatocytes in vitro. We found that
JMJD3c overexpression accelerated the timing of the expression of hepatic markers (AFP, albumin and CYP1A1) (Fig. 4C). High expression of AFP, albumin and CYP1A1-positive cells was observed in JMJD3c-overexpressing cells on Day 12. Albumin secretion level on Day 12 after differentiation was determined by ELISA. The error bars indicate the s.e.m. from two independent biological replicates. *P<0.05, t-test. (F) qRT-PCR analyses of CYP450 genes in hESCs and untreated and Dox-treated hESC-derived hepatic cells. The expression levels were normalized to GAPDH. The error bars indicate the s.e.m. from three independent biological replicates.
Fig. 5. JMJD3c facilitates the MYOD1-mediated muscle differentiation of hESCs. (A) Schematic of the differentiation protocol. JMJD3c-hESCs were treated with or without Dox on Days 1 to 2 after plating and were transfected with synthetic mRNAs for MYOD1 or Emerald three times on Days 2 and 3. The cells were collected for each experiment on Day 5. (B) qRT-PCR analyses of the expression of myogenic genes in MYOD1-differentiated cells with (+Dox) or without (−Dox) Dox treatment. −, no transfection; Em, Emerald transfection; MYOD1, MYOD1 transfection. The expression levels were normalized to GAPDH. The error bars indicate the s.e.m. from three independent biological replicates. *P < 0.05, t-test. (C-E) ChIP-qPCR analyses of H3K27me3 (C), H3K4me3 (D) and H3K27ac (E) in the MYOG and MEF2C promoters of MYOD1-transfected cells with (+Dox) or without (−Dox) Dox treatment. Three (a-c) and two (a,b) regions of the MYOG and MEF2C promoters were tested, respectively. In human myoblasts, the MYOG (a,b) and MEF2C (a) regions are only enriched for H3K27ac, but not H3K4me3, whereas the MYOG (c) and MEF2C (b) regions are enriched for both markers (see also Fig. S6). T, positive control; SOX1-3, negative control. The error bars indicate the s.e.m. from three independent biological replicates. *P < 0.01, t-test. (F) Immunostaining for MHC in the cells overexpressing JMJD3c (+Dox), MYOD1 or both JMJD3c and MYOD1 (+Dox +MYOD1). Scale bar: 200 μm. (G) The percentage of nuclei contained within the MHC-stained cells out of total cells. The error bars indicate the s.e.m. from three independent biological replicates. *P < 0.01, t-test. (H) MHC immunostaining in the MYOD1-transfected cells overexpressing JMJD3c or the JMJD3c mutant. (I) RNA-seq analyses of upregulated genes in the myogenic cells that were induced from hESCs (ES-iMuscle) and human skeletal myotubes (Myotube). Upregulated genes were determined using ExAtlas (fold-change >4 in induced myogenic cells and >10 in myotubes relative to hESCs, z-value >4).
To characterize the muscle cells generated by overexpressing both JMJD3c and MYOD1, we compared the gene expression patterns of these cells with the expression patterns of human skeletal myotubes. Half of the genes (105 genes) that were upregulated in the generated muscle cells were highly expressed in skeletal myotubes, including genes important for mature muscle cells (Fig. 5I; Table S4). We also found that the combination of JMJD3c and MYOD1 is required for the generation of active chromatin of myogenic genes.

At 4 days post differentiation, JMJD3c/MYOD1-overexpressing hESCs expressed markers of mature muscles: myosin heavy chain (MHC) and myotube-like morphology (Fig. 5F). The percentage of MHC-positive cells was much higher in the JMJD3c/MYOD1-overexpressing hESCs compared with the MYOD1-overexpressing hESCs (Fig. 5G). Notably, forced expression of the JMJD3c mutant and UTX did not induce MYOD1-mediated myogenic differentiation (Fig. 5E). Overexpression of JMJD3c alone (without MYOD1 overexpression) did not induce H3K27ac enrichment in the myogenic promoters (Fig. 5E), suggesting that JMJD3c specifically functions as a H3K27 demethylase for the myogenic differentiation of hPSCs.

To characterize the muscle cells generated by overexpressing both JMJD3c and MYOD1, we compared the gene expression patterns of these cells with the expression patterns of human skeletal myotubes. Half of the genes (105 genes) that were upregulated in the generated muscle cells were highly expressed in skeletal myotubes, including genes important for mature muscle cells (Fig. 5I; Table S4). We also found that further differentiation of the generated muscle cells produced myotube-like cells that exhibited spontaneous contractions (Movie 1).

Finally, to develop a footprint-free system, we attempted to differentiate hESCs into skeletal muscle cells using only synthetic mRNAs for both JMJD3c and MYOD1. The synthetic mRNAs for JMJD3c were transfected into the hESCs twice, followed by three transfections with the MYOD1 synthetic mRNAs (Fig. 6A). Two days after the last transfection of the MYOD1 mRNAs, the majority of hESCs were differentiated into MHC-positive cells (Fig. 6B,C). As a control, we transfected hESCs with synthetic mRNAs for mCherry and MYOD1, which failed to induce myogenic differentiation. Importantly, some MHC-positive cells appeared to be fused cells (Fig. 6D) in which a mature myogenic marker, creatine kinase-M, was highly expressed (Fig. 6E). The fusion capability was confirmed by a cell fusion assay with mouse C2C12 myoblast cells (Fig. 6F). These results suggest that the myotube-like cells induced by the JMJD3c and MYOD1 synthetic mRNAs have the potential to become mature skeletal muscles in vitro. JMJD3c also accelerated MYOD1-mediated differentiation of H9 hESCs and hiPSCs (Fig. 6G), suggesting that JMJD3c generally facilitates the direct conversion of pluripotent cells into terminally differentiated cells.

**DISCUSSION**

Although it has been recognized that epigenetic mechanisms play important roles in the transcriptomic changes observed during differentiation (Gifford et al., 2013; Xie et al., 2013), the epigenetic factors that are primarily responsible for establishing a differentiated state are currently unknown. Previous studies using JMJD3 knockout or knockdown have shown that JMJD3 is required for the activation of mammalian developmental programs (Burgold et al., 2008; Jiang et al., 2013; Ohtani et al., 2013; Satoh et al., 2010; Sen et al., 2008). It has also been shown that JMJD3 functions as an epigenetic barrier during the reprogramming of differentiated cells.
into pluripotent stem cells (Zhao et al., 2013). In this study, we have revealed that the catalytic domain of histone demethylase JMJD3 (JMJD3c) can drive the activation of developmental genes in hESCs in the absence of environmental changes. Moreover, JMJD3c facilitates switching of the hESC gene expression patterns to the patterns of terminally differentiated cells. These results suggest that JMJD3 is a crucial determinant of the differences in the chromatin characteristics between undifferentiated and differentiated mammalian cells.

Forced expression of JMJD3c enabled the hESCs to exit from the pluripotent state to upregulate developmental genes that are associated with H3K27me3 demethylation. The catalytic mutant of JMJD3c failed to induce these phenomena, suggesting that H3K27 demethylation by JMJD3c is directly involved in the transcriptional activity of developmental genes. Notably, overexpression of the JMJD3c domain of UTX (66% sequence homology with JMJD3c) failed to induce H3K27me3 demethylation and hESC differentiation, suggesting that there are functional differences between JMJD3 and UTX. Some reports revealed that UTX regulates gene expression during ESC differentiation independently of demethylase activity (Morales Torres et al., 2013; Wang et al., 2012), indicating that UTX during ESC differentiation independently of demethylase activity JMJD3c. Some reports revealed that UTX regulates gene expression

We have shown that JMJD3c overexpression followed by MYOD1 overexpression significantly increases the efficiency of myogenic differentiation of hPSCs. The myogenic gene program is quickly activated through epigenetic changes only when JMJD3c and MYOD1 are sequentially expressed in hESCs. Interestingly, because H3K27me3 is not enriched at the promoters of myogenic genes (MYOG and MEF2C) in hESCs, the demethylase activity of JMJD3c seems to be indirectly involved in the activation of myogenic genes. The JMJD3c-upregulated mesodermal developmental genes may include the transcriptional co-regulators of MYOD1, enabling the effective activation of myogenic gene expression. Indeed, JMJD3c alone induces the expression of the early myogenic regulators PAX3 and PAX7 in addition to mesodermal differentiation, which could facilitate myogenic terminal differentiation. Alternatively, JMJD3c might cooperate with MYOD1 to activate the myogenic genes by changing the chromatin structures of their regulatory regions. Indeed, when JMJD3c is expressed prior to MYOD1, H3K4me3 and H3K27ac are upregulated at the MYOG and MEF2C promoters. These results suggest that JMJD3c plays key roles in the alteration of the chromatin structures of hPSCs, not only for early differentiation, such as for mesoendodermal differentiation, but also for differentiation into tissue-specific lineages. The SWI/SNF chromatin remodeling component BA60C (SMARCD3) also promotes MYOD-mediated myogenic conversion in hESCs (Albini et al., 2013), supporting the idea that remodeling of the chromatin structure is important for the transcription factor-mediated conversion of pluripotent stem cells into terminally differentiated cells. The detailed mechanisms that promote the ‘directed differentiation’ of hPSCs through epigenetic regulation should be elucidated in the future.

Previous studies have shown that the forced expression of the transcription factors MYOD1 or PAX7 can generate differentiated myogenic cells from hPSCs in vitro (Darabi et al., 2012; Goudenege et al., 2012; Tedesco et al., 2012). In these experiments, the transcription factors were transduced using lentiviral or adenoviral vectors, and those factors were overexpressed after mesodermal or mesenchymal cells were produced from hPSCs. These procedures require multiple differentiation steps and several weeks for complete myogenic differentiation. A recent report showed that when a piggyBac vector system was used to express MYOD1, the transgenic hPSCs could be directly converted into skeletal muscle cells at high efficiency (Tanaka et al., 2013), suggesting that an extremely large amount of MYOD1 is required to activate the myogenic program in PSCs. Indeed, MYOD1-mediated muscle differentiation from hESCs has low reproducibility compared with muscle differentiation from fibroblasts (Albini et al., 2013). In this study, we have reported the efficient (>60%) and rapid (4 days) differentiation of hPSCs with synthetic mRNAs encoding JMJD3c and MYOD1. Because RNA-based protein expression is transient and has no risk of genomic integration and mutagenesis, this approach may be suitable for cell-based therapies in regenerative medicine.

Because JMJD3c overexpression can initiate the transcription program for mesoendodermal lineages, it is conceivable that our method, i.e. the forced expression of JMJD3c and tissue-specific transcription factor(s), can also be applied to differentiation towards specific tissue and cell types other than muscle cells. Indeed, here, we showed that the transient expression of JMJD3c and subsequent expression of HNF1A, RUNX2 or SPI1 activates the differentiation markers for the hepatoblast, osteoblast or hematopoietic lineages, respectively. Moreover, JMJD3c overexpression enhanced hepatic differentiation achieved with an established protocol. These results
suggest that our system has broad utility for various cellular lineages. Although the differentiation potential of hiPSCs holds great promise for therapeutic applications, there are significant variations in the differentiation capacities of hiPSC lines (Boulting et al., 2011), which will limit the utility of iPS cells in regenerative medicine. These variations are thought to be caused by epigenetic differences (Bock et al., 2011; Lister et al., 2011; Ohi et al., 2011). Our results showed less efficient myogenic differentiation in a hiPSC line than that in hESC, which might be due to the epigenetic differences, including DNA methylation and histone modifications, between these cell types. Based on these results, we propose that manipulating H3K27me3 or other modifications with our method allows the alteration of the epigenetic patterns of low-potential iPSC lines and improves their differentiation capacities.

MATERIALS AND METHODS

hPSC culture

SEES-3 hESCs (Akutsu et al., 2015) were obtained from the Center for Regenerative Medicine, National Research Institute for Child Health and Development, Japan. h9 hESCs were obtained from WiCell Research Institute, USA. The hiPSCs were generated from adult human BJ fibroblasts (male) by transfecting them with POU5F1, SOX2, KLF4 and MYC mRNAs. The hESCs/iPSCs were maintained in feeder-free conditions using StemFit AK-03 medium (Ajinomoto) on iMatrix-511 (Nippi)-coated plates. The culture conditions for differentiation are described in the supplementary Materials and Methods section. All experiments were performed in accordance with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Generation of inducible JMJD3c-hESC lines

A full-length human JMJD3 clone was obtained from Addgene (plasmid ID #24167). The point mutation in the catalytic domain was inserted using a PrimeSTAR Mutagenesis Basal Kit (Takara). The HA-tagged JMJD3c and mutant sequences, to which nuclear localization signal (NLS) sequences were added, were cloned into a piggyBac construct containing the Tet-responsive element, IRES-βgeo, and PGK promoter controlling the puromycin resistance gene. SEES-3 hESCs that consistently expressed the reverse Tet-transactivator were co-transfected with these vectors and piggyBac transposase vectors using the GeneJuice transfection reagent (Novagen). Stable clones were generated by puromycin selection. Inducible expression upon Dox treatment was confirmed by X-Gal staining.

Modified RNA synthesis and transfection

The modified mRNAs were synthesized as previously described (Warren et al., 2010). RNA transfections were performed with Lipofectamine 2000 (Invitrogen) or Lipofectamine Messenger Max (Invitrogen), according to the manufacturer’s instructions. For further details, see supplementary Materials and Methods.

Immunostaining and immunoblotting

Antibodies used in this study are listed in Table S5. For immunostaining, the cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT) and permeabilized in 0.5% Triton X-100 in PBS for 10 minutes. The cells were blocked in PBS and 2% bovine serum albumin (BSA) for 10 minutes and incubated with the primary antibodies in a blocking solution (1:500) for 2-3 h at RT or overnight at 4°C. After two washes in PBS, the cells were incubated with Alexa-conjugated secondary antibodies (Invitrogen) in a blocking solution (1:500) for 1 h at RT. Nuclei were counterstained with DAPI (Dako) for 5 min at RT. Immunofluorescence was visualized with an inverted fluorescence microscope IX73 (Olympus). Images were obtained using Olympus cellSens imaging software. The fluorescence intensities were quantified using ImageJ software. More than 20 nuclei from three independent experiments were examined to calculate the average intensities.

For immunoblotting, the cells were lysed with a sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 6% 2-mercaptoethanol and 500 μg/ml urea). The proteins were separated by SDS-PAGE on a 4-15% polyacrylamide gel (Bio-Rad) and were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skimmed milk. The membranes were washed in TBST and then incubated with primary antibodies in TBS/2% BSA (1:1000) overnight at 4°C. The membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) (1:2000) for 1 h at RT. The membranes were washed in TBST, and immunoreactivity was visualized using an ECL Prime Detection Kit (GE Healthcare) and detected using a Luminescent Image Analyzer (LAS-4000; Fujifilm). Immunoblotts were quantified using ImageJ software. The signal intensity levels were normalized to the loading controls, and the average values were calculated from two or three independent experiments.

Alkaline phosphatase staining

Alkaline phosphatase activity was visualized with the BCIP/NBT Substrate System (Dako).

Apoptosis assay

Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (MBL) and analyzed by flow cytometry (FACSAnA II, BD Biosciences).

qRT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen), and cDNAs were generated with random hexamers using the Superscript III First-strand Synthesis Kit (Invitrogen) or the ReverTra Ace kit (Toyobo). Real-time PCR was performed using a SYBR Green PCR system (Takara). The primer sequences used for RT-PCR are listed in Table S6.

cDNA library preparation

Total RNA was extracted using TRIzol solution (Roche) according to the manufacturer’s instructions. The cDNA library was prepared from 500 ng of each total RNA sample for massive parallel sequencing using an NEBNext Poly(A) mRNA Magnetic Isolation Module and an Ultra Directional RNA Library Prep Kit for Illumina (NEB). The cDNA library produced ranged from 400 to 1000 bp, including the adapter sequences.

RNA-seq analysis

RNA-seq was performed with an Illumina MiSeq for 150 single-ended base pairs. The sequence reads were mapped to the human genome (hg19) using TopHat v2.0.13. The expression values for genes were calculated as fragments per kilobase of exon per million mapped reads (FPKM) using Cufflinks v2.1.1. The functional annotation of the genes was performed using DAVID software. Some of the comparisons of gene expression were performed with ExAtlas software (Shaw et al., 2015).

ChiP analysis

The ChiP experiment was performed as previously described (Akiyama et al., 2015). The primer sequences are listed in Table S6.

ChiP-seq analysis

The ChiPed DNA libraries were constructed using an NEBNext ChiP-Seq Library Prep Kit for Illumina (NEB) and were sequenced on an Illumina MiSeq for 150 single-ended base pairs. The sequence reads were mapped to the human genome (hg19) using Bowtie2 (Langmead and Salzberg, 2012). The reads that mapped to the mitochondrial genome were excluded from the subsequent analyses. The chromatin profiles of H3K27me3 and H3K4me3 at the genomic regions were generated with ngs.plot (Shen et al., 2014). The profiles were normalized to the average density of methylation from the transcription end site to +6 kb. Bivalent genes were defined as genes that were enriched both in H3K4me3 and H3K27me3 (ngs.plot_hm values >20 in ±3 kb from the transcription start site). Seventy percent of the bivalent genes defined here overlapped with the bivalent genes defined in previous reports.
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