genesis 51:763–776 (2013)

ARTICLE

DNA Methylation Profiles Provide a Viable Index for Porcine Pluripotent Stem Cells

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Received 18 December 2012; Revised 25 July 2013; Accepted 27 July 2013

Summary: Porcine induced pluripotent stem cells (iPSCs) provide useful information for translational research. The quality of iPSCs can be assessed by their ability to differentiate into various cell types after chimera formation. However, analysis of chimera formation in pigs is a labor-intensive and costly process, necessitating a simple evaluation method for porcine iPSCs. Our previous study identified mouse embryonic stem cell (ESC)-specific hypomethylated loci (EShypo-T-DMRs), and, in this study, 36 genes selected from these were used to evaluate porcine iPSC lines. Based on the methylation profiles of the 36 genes, the iPSC line, Porco Rosso-4, was found closest to mouse pluripotent stem cells among 5 porcine iPSCs. Moreover, Porco Rosso-4 more efficiently contributed to the inner cell mass (ICM) of blastocysts than the iPSC line showing the lowest reprogramming of the 36 genes (Porco Rosso-622-14), indicating that the DNA methylation profile correlates with efficiency of ICM contribution. Furthermore, factors known to enhance iPSC quality (serum-free medium with PD0325901 and CHIR99021) improved the methylation status at the 36 genes. Thus, the DNA methylation profile of these 36 genes is a viable index for evaluation of porcine iPSCs. genesis 51:763–776. © 2013 Wiley Periodicals, Inc.

Key words: epigenetics; induced pluripotent stem cells; translational research

INTRODUCTION

The use of induced pluripotent stem cells (iPSCs) is expected to dramatically accelerate advances in medical care (Okita and Yamanaka, 2011; Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). In particular, iPSCs may offer novel therapies for previously intractable

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Contract grant sponsor: Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (to Y.H. and H.N.), Contract grant sponsor: Meiji University International Institute for Bio-Resource Research (MUIIBR) (to H.N.); Contract grant sponsor: Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Young Scientists (B) (to J.O.), Contract grant sponsor: Research Project Grant (B) by Institute of Science and Technology Meiji University (to J.O.); Contract grant sponsor: Meiji University Grant for Research by Young Scientist (to Y.A.)

Published online 30 August 2013 in
Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/dvg.22423

conditions, and a wide range of possible applications has been investigated including exploration of the pathogenic mechanisms of refractory diseases and the development of new drugs (Ebert *et al*., 2009; Imaizumi *et al*., 2012; Inoue and Yamanaka, 2011), cell therapy (Montserrat *et al*., 2011; Zhou *et al*., 2011), production of organs and tissues (Kobayashi *et al*., 2010; Usui *et al*., 2012), and generation of germ cells (Hayashi *et al*., 2011, 2012).

Before iPSCs can be used for clinical applications, it is essential that appropriate experiments using animal models are carried out to ensure their effectiveness and safety. In addition to the use of standard laboratory animals such as rodents, investigation of larger animal species, with closer physiological resemblance to humans, will significantly benefit translational research. The pig is one such species and has many similarities in anatomy and physiology to humans (van der Spoel *et al*., 2011; Zhao and Prather, 2011); pigs have often been used in biomedical studies as a large experimental model, which can produce data that can be easily applied to humans (Lunney, 2007; Petters, 1994; Prather *et al*., 2003). Therefore, the generation and evaluation of porcine iPSCs will provide useful information that could help promote clinical application of human iPSCs (Ezashi *et al*., 2009; Fujishiro *et al*., 2013; Montserrat *et al*., 2011; West *et al*., 2010; Wu *et al*., 2009).

The pluripotency of porcine iPSCs can be evaluated by determining their ability to form chimeras (Fujishiro *et al*., 2013; West *et al*., 2010). However, the production of chimeric fetuses and piglets is a labor-intensive and costly process that requires embryo manipulation and transfer. Indeed, few studies have used chimera-forming ability as a means of confirming the pluripotency of porcine iPSCs (Fujishiro *et al*., 2013; West *et al*., 2010, 2011). Therefore, it is essential to develop new methods, either for evaluating the pluripotency of porcine iPSCs, or for pre-screening iPSC lines for use in chimera formation experiments.

Epigenetic regulation, including DNA methylation and histone modifications, is fundamental to tissue- and/or cell-type specific gene expression (Golob *et al*., 2008; Ikegami *et al*., 2009; Lieb *et al*., 2006; Shiota *et al*., 2004). There are a large number of tissue-dependent differentially methylated regions (T-DMRs) in the mammalian genome (Shiota *et al*., 2002; Yagi *et al*., 2008). The DNA methylation status of T-DMRs is determined during embryonic development, and the DNA methylation profile of T-DMRs is distinctive in each cell type (Sakamoto *et al*., 2007; Shiota *et al*., 2002). Mouse ESCs, which are known to be pluripotent stem cells, exhibit unique DNA methylation profiles at T-DMRs. Genes involved in the establishment and maintenance of the pluripotent state, including *Oct3/4* (*Pou5f1*), are hypomethylated in mouse ESCs (Hattori *et al*., 2004; Imamura *et al*., 2006). Genome-wide DNA methylation analyses have identified several hundred mouse ESC-specifically hypomethylated T-DMRs (EShypo-T-DMRs; Sato *et al*., 2010). Furthermore, it has been shown that the DNA methylation profiles of EShypo-T-DMRs in mouse iPSCs with a high efficiency of chimera formation are similar to those of ESCs (Aoi *et al*., 2008; Sato *et al*., 2010). These results indicate that the DNA methylation profile of EShypo-T-DMRs provide a viable index for screening high-quality iPSCs.

We recently generated naïve-like porcine iPSC lines using the four Yamanaka factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*; Fujishiro *et al*., 2013); these factors are also used to generate mouse iPSCs. These porcine iPSC lines exhibit LIF-dependent proliferation abilities similar to those of mouse ESCs/iPSCs (Fujishiro *et al*., 2013); however, the lines also show different characteristics.

In this study, we sought to determine whether the DNA methylation index for mouse EShypo-T-DMRs could be used to evaluate porcine iPSC lines. If this approach is feasible for the evaluation of porcine iPSCs, then the same strategy could be applied to quality enhancement of iPSCs from a wide range of domesticated animal species and ultimately for human iPSCs.

RESULTS

DNA Methylation Profile of 36 Genes Known to be Specifically Hypomethylated in Mouse ESCs in Porcine iPSCs

To analyze DNA methylation profiles of porcine iPSCs, we selected 36 genes that are hypomethylated specifically in mouse ESCs (EShypo-T-DMRs; Sato *et al*., 2010). The 36-gene set was categorized into three groups; those targeted by Oct3/4 (Oct3/4-targets); Klf4, Sox2, or c-Myc (KSM-targets); and genes, which are not targets of these four factors (non-targets; Fig. 1a). In the course of iPSC establishment, activation of target genes of the four Yamanaka factors is required after introduction into somatic cells. Among the four factors, Oct3/4 is of utmost importance since iPSC lines have not been established without *Oct3/4* introduction to date. Thus, target genes of Oct3/4, such as *Sall4* (Tsubooka *et al*., 2009), are thought to have crucial roles in iPSC establishment, and the Oct3/4 target genes (Oct3/4-targets) were separated from the target genes of the other three Yamanaka factors (KSM-targets). Although "non-targets" are genes that are not directly bound by the four Yamanaka factors, their methylation levels in mouse ESCs were lower than those in differentiated tissues/cells (Sato *et al*., 2010), suggesting that the DNA methylation statuses of non-targets can also be useful as an index for evaluation of porcine iPSCs. The classification of target genes was based on ChIP-seq data for several transcription factors, including the four Yamanaka factors (Chen *et al*., 2008). We initially selected 56

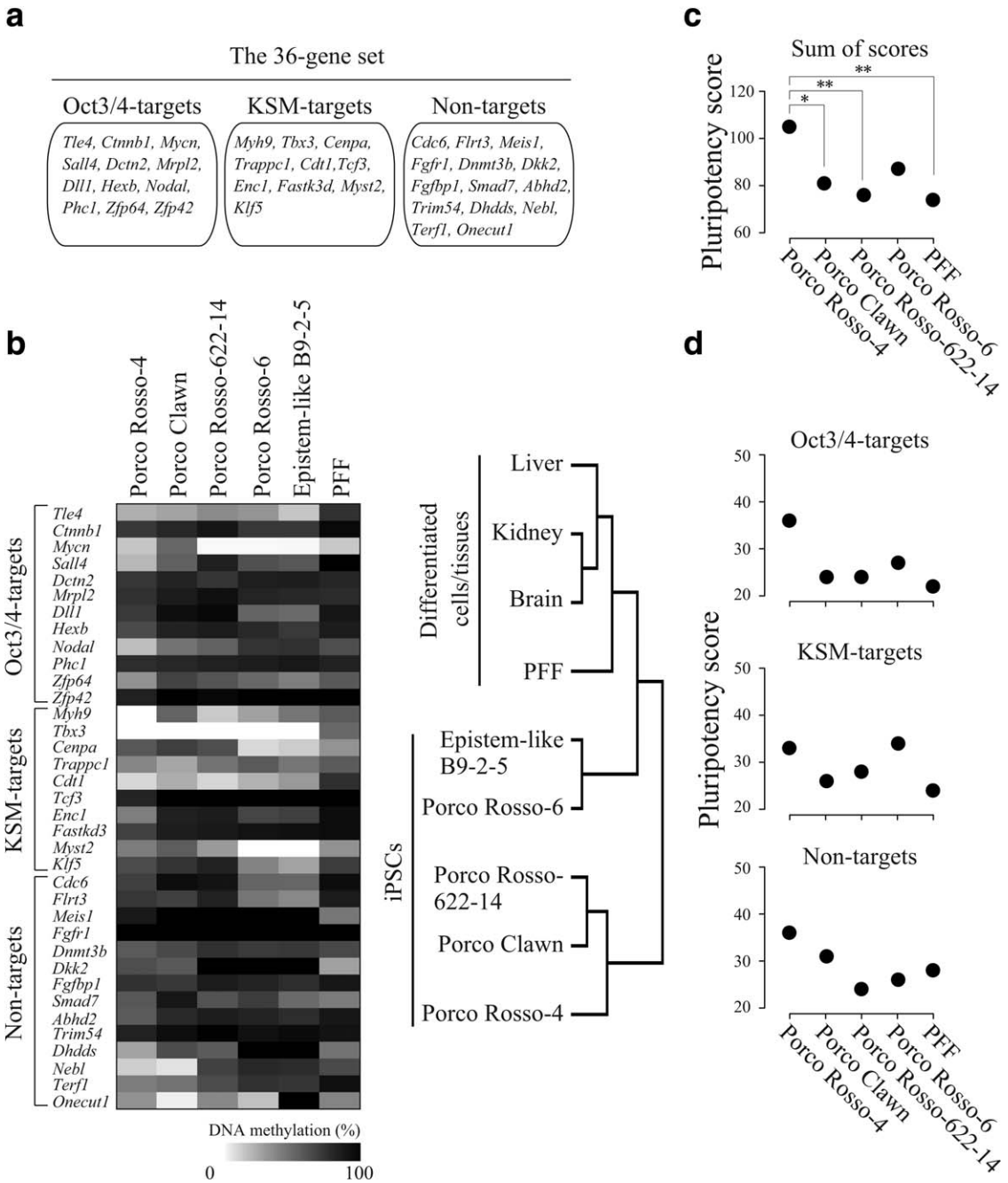


FIG. 1. DNA methylation profiles of porcine iPSCs. (**a**) The 36-gene set analyzed for DNA methylation profiling of porcine iPSCs. The genes were retrieved from EShypo-T-DMRs previously determined in mouse ESCs, and were categorized into three groups: Oct3/4-targets, KSM-targets, and non-targets. (**b**) DNA methylation status of the 36-gene set in five iPSC lines (Porco Rosso-4, Epistem-like B9-2-5, Porco Rosso-6, Porco Rosso-622-14, and Porco Clawn) and porcine fetal fibroblast (PFF) by COBRA assay. The methylation level is represented as a heatmap (left panel). Based on the methylation level determined by COBRA assay, differentiated cell or tissues (brain, liver, kidney, and PFF) and five porcine iPSC lines were clustered according to the similarity of their DNA methylation profiles at the 36 selected genes using the Euclidean distance (right panel). (**c**) Sum of pluripotency scores of the 36 genes for porcine naïve-like iPSCs and PFF. Depending on percentage difference in methylation levels between blastocysts and iPSCs, pluripotency scores for each gene were classified into five categories: $< 20\%$, 20–40%, 40–60%, 60–80%, or $> 80\%$, with pluripotency scores of 5, 4, 3, 2, or 1, respectively. Total scores of the 36-gene set are plotted. Statistical comparison was performed by Wilcoxon test. $^{*}P < 0.05$; $^{**}P < 0.01$. (**d**) Pluripotency scores of porcine iPSCs and PFF for each of the three gene-groups. Total scores of the genes belonging to each group (Oct3/4-targets, KSM-targets, and non-targets) are plotted.

porcine genes orthologous to mouse genes with EShypo-T-DMRs, whose DNA methylation levels were low in mouse ESCs but 30% or more higher in differentiated tissues/cells (Sato *et al.*, 2010). However, 16 of these were found to be hypomethylated in porcine somatic tissues, and further, four genes were hypermethylated in porcine blastocysts, consisting of pluripotent cells (Supporting Information Fig. 1). Therefore, these 20 genes were excluded, and the remaining 36 were used for DNA methylation analyses. Among these 36 genes, several are known to be hypomethylated in mouse ESCs, but to be in a poised state of transcriptional activation, rather than actively expressed. This type of epigenetic regulation has been reported in H3K4me3/H3K27me3-enriched bivalent regions (Meissner *et al.*, 2008; Xu *et al.*, 2007).

We performed DNA methylation analyses of the 36 selected genes in five porcine iPSC lines; Porco Rosso-4, −6, −622-14, Epistem-like B9-2-5, and Porco Clawn. Epistem-like B9-2-5 was cultured in typical non-mouse ESC/iPSC media containing bFGF (Fujishiro *et al.*, 2013), and its colony shape and gene expression patterns were similar to those of mouse epiblast stem cells (EpiSCs; Brons *et al.*, 2007; Tesar *et al.*, 2007). The other four cell lines were cultured with porcine LIF, and their colony shape was similar to that of mouse ESCs (Fujishiro *et al.*, 2013). DNA methylation levels of the 36 genes were examined by combined bisulfite restriction analysis (COBRA) assay (Xiong and Laird, 1997) in iPSCs and porcine fetal fibroblast (PFF) used to produce the iPSC lines (Fig. 1b, left panel). Hierarchical clustering of DNA methylation status was performed on the basis of the 36-gene set for somatic cells/tissues and iPSC lines (Fig. 1b, right panel). Somatic tissues (brain, liver, and kidney) and PFF clustered together, whereas the five iPSC lines clustered separately, indicating that the DNA methylation profile of the 36-gene set could distinguish between differentiated cells/tissues and iPSCs.

Next, we determined a "Pluripotency score" based on comparison of methylation levels of the 36 genes between blastocysts consisting of pluripotent cells and the four naïve-like iPSC lines. Since Epistem-like B9-2-5 is distinguishable from naïve-like iPSC lines based on the mouse EpiSC-like colony shape and bFGF-dependent proliferation (Fujishiro *et al.*, 2013), Epistem-like B9-2-5 was excluded in the following experiments. Scoring was performed for each gene, and the sum of the scores of the 36 genes was plotted (Fig. 1c). In this way, high pluripotency scores are awarded to iPSC lines with DNA methylation profiles close to those of pluripotent cells. Among the four iPSC lines examined, Porco Rosso-4 showed the highest pluripotency score, which was statistically significant when compared with PFF, Porco Rosso-622-14, and Porco Clawn. We further examined the pluripotency score depending on the gene groups (Oct3/4-targets, KSM-targets, or non-targets; Fig. 1d). The Oct3/4-target genes had higher pluripotency scores in the Porco Rosso-4 cell line (36) than the other three cell lines and PFF (22–27). This tendency was also observed for non-target genes. By contrast, the score of KSM-target genes in Porco Rosso-4 was similar to that of Porco Rosso-6. Thus, among the four iPSC lines examined, the DNA methylation profile of Porco Rosso-4 is most similar to that of pluripotent cells. This was consistent with the results of the hierarchical clustering analysis, where the Porco Rosso-4 line was separate from the Epistem-like B9-2-5 line.

We confirmed the DNA methylation status of six gene loci (*Ctnnb1*, *Sall4*, *Tle4*, *Dll1*, *Enc1*, and *Nebl*), whose DNA methylation level was clearly different among the examined iPSC lines (Fig. 1b, left panel), by bisulfite sequencing. All 6 gene loci were hypermethylated in PFF and liver, whereas hypomethylated status was observed in blastocysts (Fig. 2). DNA methylation levels of the Oct3/4-target genes, *Ctnnb1* and *Sall4*, were 24 and 2%, respectively in the Porco Rosso-4 line, which had the highest pluripotency score among the four iPSC lines. However, Porco Rosso-622-14, which had the lowest pluripotency score, exhibited hypermethylation at the *Ctnnb1* (65%) and *Sall4* (61%) loci. This indicates that *Ctnnb1* and *Sall4* are highly demethylated in Porco Rosso-4 but not in Porco Rosso-622-14 cells. At the other four gene loci, partial demethylation was also observed in Porco Rosso-4 but not Porco Rosso-622-14. These bisulfite sequencing results confirm that, in Porco Rosso-4 iPSC line, DNA methylation patterns of the 6 genes, we analyzed changed to the expected direction as pluripotent cells, and Oct3/4-target genes, *Sall4* and *Ctnnb1*, especially underwent demethylation within the entire sequenced regions in the majority of the cell population.

Contribution of Porcine iPSCs to the Inner Cell Mass (ICM) of Blastocysts

Established iPSC lines are intended for use in transplantation and complementation experiments. Considering that the naïve-like iPSC lines do not differ greatly in terms of morphology and marker gene expression (Fujishiro *et al.*, 2013), it is more appropriate to select candidate iPSC lines using different indices. We next investigated contribution of the iPSC lines to the ICM of blastocysts using the aggregation method (Fig. 3a). We performed two independent experiments (Exps. 1 and 2), and found that Porco Rosso-4 cells contributed better to the ICM compared with Porco Rosso-622-14 in both experiments (Fig. 3b). In Exps. 1 and 2, the number of embryos that exhibited ICM contribution of iPSCs statistically differed between Porco Rosso-4 (14/57, 24.6%) and Porco Rosso-622-14 (2/51, 3.9%; Fig. 3c). The sum of pluripotency scores (105) for the three gene groups (Oct3/4-targets, KSM-targets, and non-targets) for Porco

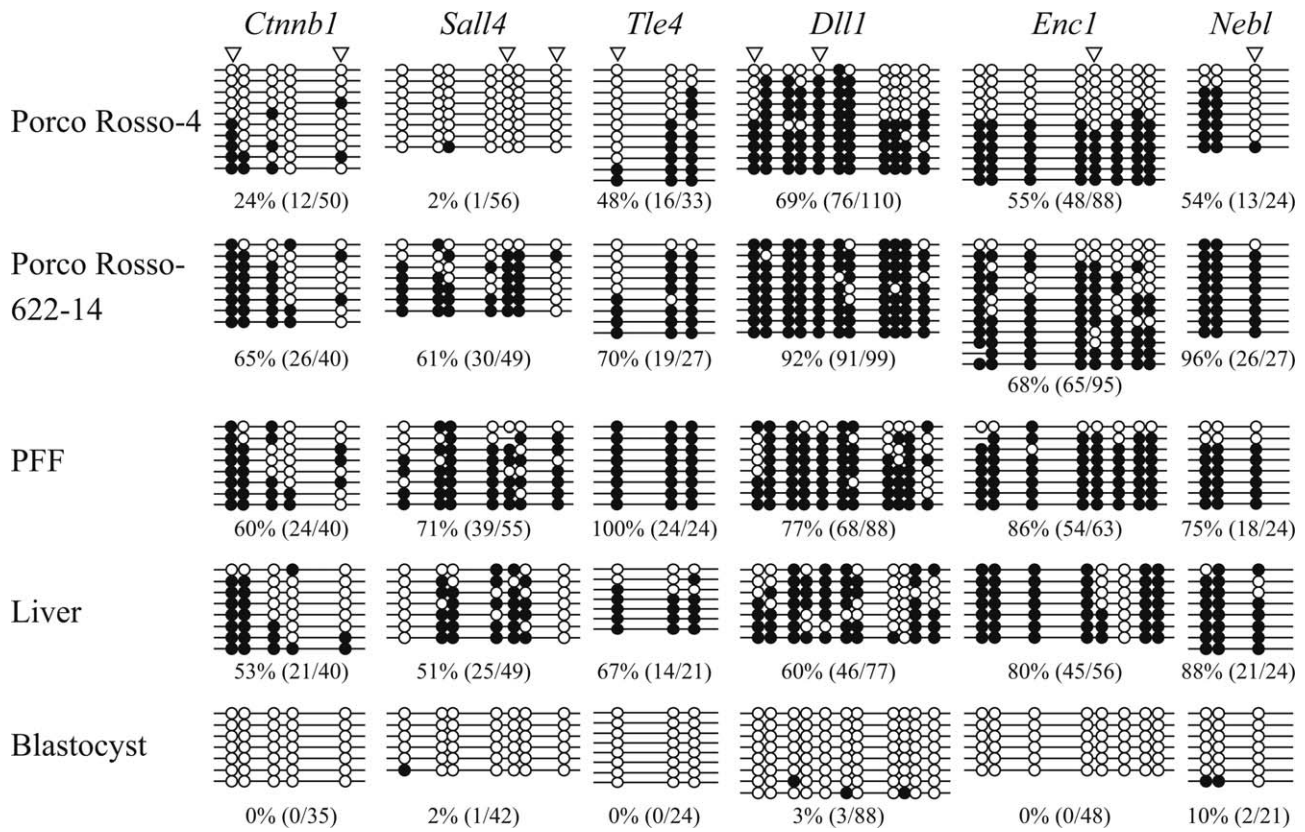


FIG. 2. DNA methylation status of EShypo-T-DMRs in iPSCs (Porco Rosso-4 and Porco Rosso-622-14) analyzed by sodium bisulfite sequencing. Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. Arrowheads indicate CpG sites analyzed by COBRA assay. The methylation level (%) was based on the methylated CpGs/all examined CpGs.

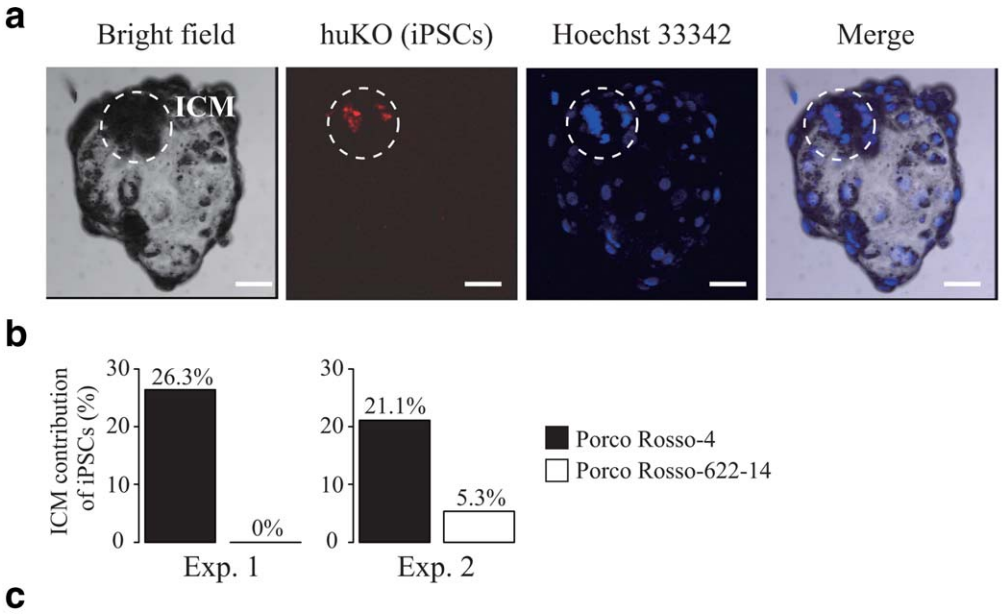
Rosso-4 was higher than that of Porco Rosso-622-14 (76), indicating that a higher pluripotency score coincides with higher efficiency of incorporation of iPSCs into the ICM. Thus, the pluripotency score based on the DNA methylation status of the 36 genes provides a feasible index for evaluating porcine iPSCs.

DNA Methylation Analysis in huKO-Negative Cells

We previously reported that approximately 5% of the cell population in Porco Rosso-4 became negative for humanized Kusabira-Orange (huKO) fluorescence (huKO-negative cells), and their characteristics were closer to those of the expected pluripotent cells (Fujishiro *et al.*, 2013). This implies that high-quality porcine iPSCs were enriched in huKO-negative fraction. To confirm whether the DNA methylation profile of EShypo-T-DMRs is a useful index for screening porcine iPSCs, we analyzed the DNA methylation status of huKO-negative cells. The huKO-negative cells in Porco Rosso-4 were collected by cell sorting, together with cells with high expression of huKO (huKO positive; Fig. 4a). EShypo-T-DMRs (*Dll1* and *Enc1*) were analyzed by bisulfite sequencing, because approximately half of the sequenced clones exhibited obvious hypomethylation in Porco Rosso-4 (Figs. 2 and 4), suggesting that Porco Rosso-4 contains a certain proportion of the cells properly demethylated at these two loci. Based on the bisulfite sequence data (Fig. 4b), the percentage of the cells hypomethylated at these two loci in Porco Rosso-4 was calculated on the basis of the number of unmethylated CpGs in the sequenced clones before and after sorting (Fig. 4c). Before sorting, the percentages of hypomethylated cells at the *Dll1* and *Enc1* loci were estimated as 36% and 50%, respectively. In huKO-negative fraction, 67–80% of the cells were considered as hypomethylated, whereas the proportion of hypomethylated cells in the huKO-positive fraction were lower than that of the cells before sorting, indicating that huKO-negative cells were hypomethylated at these EShypo-T-DMRs as expected from the DNA methylation patterns in mouse ESCs. Thus, the DNA methylation profile based on the EShypo-T-DMRs reflects the characteristics of the expected cells.

Improvement of Pluripotency Scores of Porcine iPSCs by SF + 2i Treatment

It is known that treatment with two signal-transduction inhibitors (2i) of PD0325901, an inhibitor of mitogen-activated protein kinase/extracellular signal-



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Donor cell	n	Developed to blastocyst	Number of * blastocysts analyzed	Contribution to ** ICM	Sum of *** pluripotency score (/180)
Porco Rosso-4	345	280/345 (81.2%)	57	14/57[a] (24.6%)	105
Porco Rosso-622-14	146	123/146 (84.2%)	51	2/51[b] (3.9%)	76
ICM	50	46/50 (92.0%)	46	37/46 (80.4%)	–

ab, $p<0.01$ (chi-square test)
*, The other blastocysts were used for different experiments.
**, Porco Rosso-4, n=38 (Exp. 1) and n=19 (Exp. 2). Porco Rosso-622-14, n=13 (Exp. 1) and n=38 (Exp. 2).
***, Pluripotency score was calculated only for iPSCs.

FIG. 3. Contribution of iPSCs to the ICM of blastocysts. (**a**) Porcine iPSCs (Porco Rosso-4 and Porco Rosso-622-14) were aggregated with porcine parthenogenetic 4- to 8-cell-, or morula-stage embryos. After in vitro culture, contribution of iPSCs to blastocysts was analyzed by fluorescence of the transgene, humanized Kusabira-Orange (huKO). Scale bar = 50 μm. (**b**) The percentage of ICM contribution of Porco Rosso-4 and Porco Rosso-622-14. Aggregation experiments using porcine iPSCs (Porco Rosso-4 and Porco Rosso-622-14) were performed twice independently (Exps. 1 and 2). (**c**) Summary of the contribution of iPSCs to the ICM of blastocysts. Using the ICM cells dissociated from blastocysts as donor cells, most aggregated embryos developed into blastocysts, and donor ICM cells could contribute efficiently to the ICM of the host blastocyst, confirming the contribution of pluripotent cells to the ICM. Statistical comparison was performed by chi-square test.

regulated kinase (ERK) kinase (MEK), and CHIR99021, an inhibitor of GSK3β, is effective for establishment of a naïve state in iPSCs from rodent and human (Buehr *et al.*, 2008; Hanna *et al.*, 2009; Li *et al.*, 2008). Additionally, 2i treatment has been shown to improve the characteristics of porcine iPSCs (Rodríguez *et al.*, 2012). We examined whether the DNA methylation profile of the 36 selected genes is a useful index for evaluation of the changes in characteristics of porcine iPSCs after 2i treatment. We analyzed the DNA methylation status of Porco Clawn cultured in medium with FBS, or in serum-free (SF) or in SF+2i medium, by COBRA assay (Fig. 5a, left panel). Cells treated with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase 1, were also analyzed for DNA methylation status.

In the course of mouse iPSC establishment, promoter regions of pluripotency-related genes, including EShypo-T-DMRs become demethylated (Okita *et al.*, 2007; Sato *et al.*, 2010; Takahashi and Yamanaka, 2006). In this study, we observed that porcine iPSCs cultured under SF conditions exhibited effective demethylation of the Oct3/4-target genes compared with FBS-cultured cells (Fig. 5a, right panel). In addition, the number of demethylated and reprogrammed genes was greatly increased under the SF+2i condition. These tendencies were also observed in the KSM-target and non-target gene groups, suggesting that SF+2i condition induced demethylation of the 36 selected genes. However, 5-aza-dC treatment was not effective, and the number of hypomethylated Oct3/4-target genes

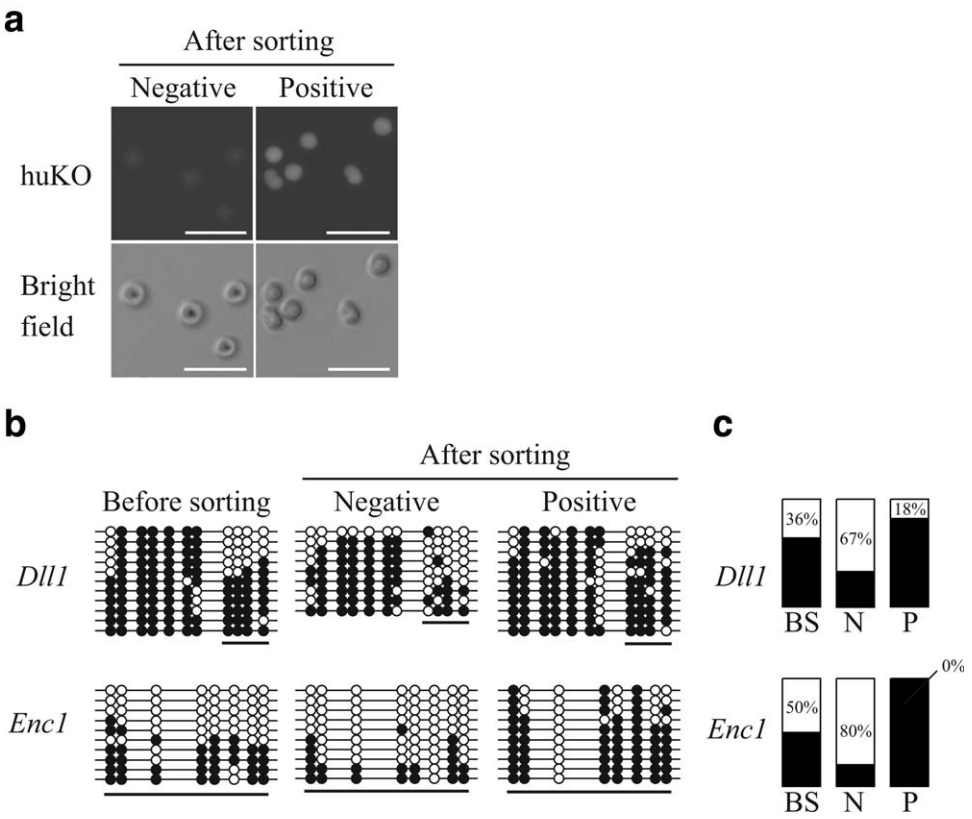

FIG. 4. DNA methylation status of huKO-negative cells after sorting of Porco Rosso-4. (**a**) The huKO-negative and huKO-positive cells of Porco Rosso-4 iPSC line after sorting. Scale bar = 50 μm. (**b**) DNA methylation status of *Dll1* and *Enc1* before and after sorting (huKO-negative and huKO-positive). Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. (**c**) The percentage of (hypomethylated clones)/(all sequenced clones) in the underlined regions of Figure 4b. Hypomethylated clones were defined on the basis of the number of unmethylated CpGs underlined below the circles in each sequenced clone. At the *Dll1* locus, the DNA methylation statuses of four CpGs were clearly different between the bulk Porco Rosso-4 line and PFF in Figure 2, whereas the other CpGs were almost fully methylated in all of the sequenced clones, indicating that the methylation statuses of the four selected CpGs in the underlined region were informative enough for evaluating the ratio of properly reprogrammed cells in huKO-negative and huKO-positive fractions. Thus, sequenced clones with three or more unmethylated CpGs in the four CpGs were designated as hypomethylated at the *Dll1* locus. At the *Enc1* locus, about half of the sequenced clones exhibited six or more unmethylated CpGs within the eight CpGs examined in the bulk Porco Rosso-4 line (Fig. 2). However, the other sequenced clones were almost fully methylated at the eight CpGs. Thus, sequenced clones exhibiting six or more unmethylated CpGs were designated as hypomethylated to strictly evaluate properly reprogrammed cells at the *Enc1* locus. With respect to the other four EShypo-T-DMRs (*Ctnnb1*, *Sall4*, *Tle4*, and *Nebl*) analyzed in Figure 2, the DNA methylation statuses of hypomethylated CpGs in bulk Porco Rosso-4 cells did not differ between huKO-negative and huKO-positive cells (Supporting Information Fig. 2). Black and white bars indicate the ratio of hypermethylated and hypomethylated clones, respectively. BS, before sorting; N, huKO-negative cells; P, huKO-positive cells.

remained unchanged compared with FBS-cultured controls.

Improvement of the DNA methylation status of Porco Clawn cells by culture under SF+2i conditions was confirmed by sodium bisulfite sequencing (Fig. 5b). *Sall4*, *Hexb*, and *Zfp64*, all of which are Oct3/4-targets, were hypermethylated in the presence of FBS, whereas demethylation occurred under the SF+2i conditions. By contrast, demethylation at *Hexb* and *Zfp64* was much less pronounced in the 5-aza-dC-treated cells (Fig. 5b). These results suggest that the SF+2i condition was the most effective of those tested for reprogramming of the 36-gene set. Demethylation also occurred in the SF+2i-treated cells at the *Dll1* and *Enc1* loci (Supporting Information Fig. 3a), whereas a portion of cells remained hypermethylated at the *Dll1* locus (Supporting Information Fig. 3b). In addition, DNA methylation analysis revealed that a proportion of cells still needed to be reprogrammed at the *Enc1* locus even after SF+2i treatment.

To examine whether SF+2i culture conditions are effective in improving pluripotency scores, we cultured Porco Clawn, in addition to several other iPSC lines, under the same conditions (FBS, SF, and SF+2i), and DNA methylation status was analyzed by COBRA assay (Fig. 5c). Of note, the pluripotency scores of all the three gene groups (Oct3/4-targets, KSM-targets, and non-targets) were increased in Porco Rosso-4 cells under SF+2i condition compared with FBS, or SF

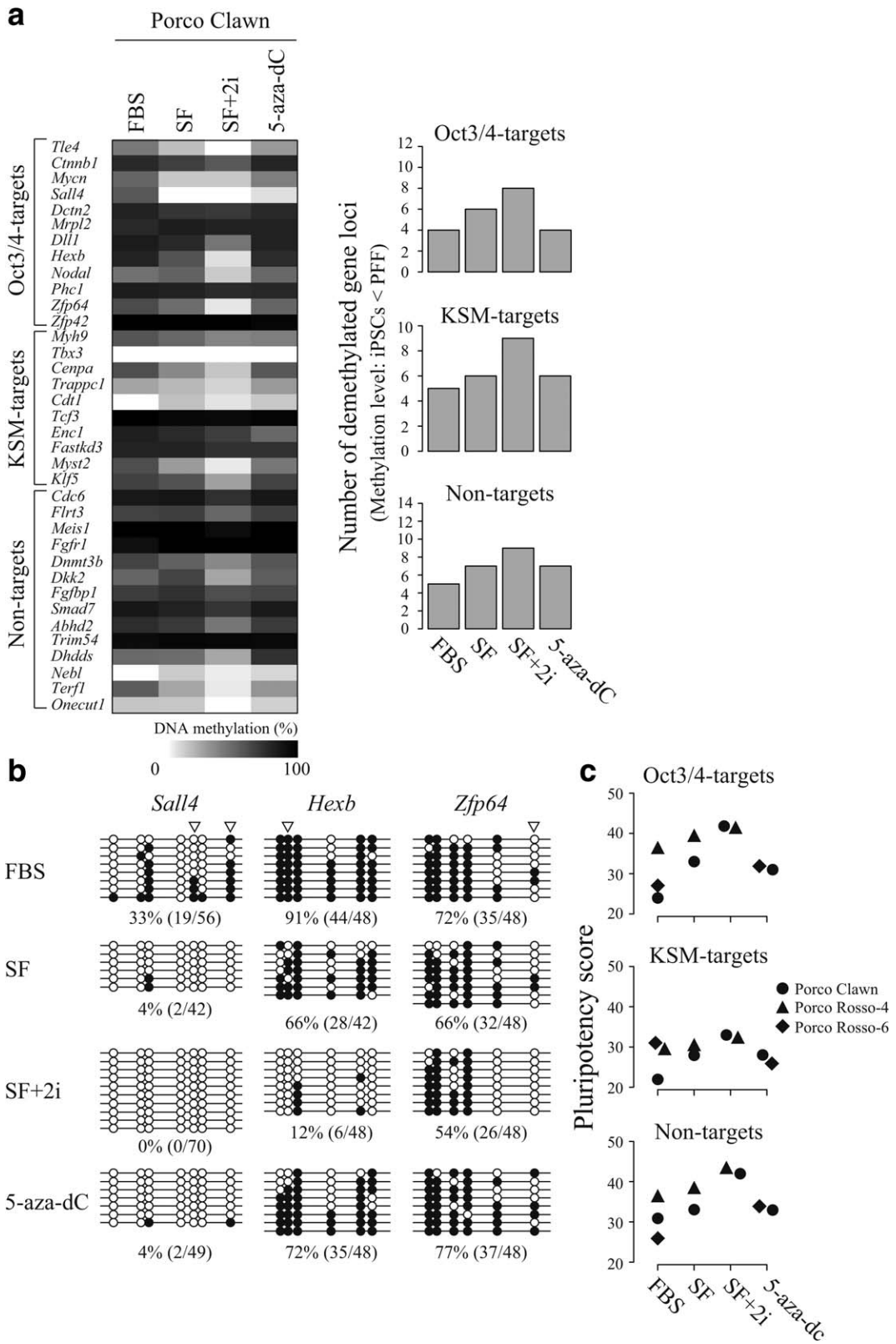

FIG. 5. Continued

conditions. Thus, SF+2i treatment is effective for multiple porcine iPSC lines.

DISCUSSION

The porcine iPSCs analyzed in this study exhibited LIF-dependent self-renewal and a round morphology, similar to mouse ESCs (Fujishiro *et al*., 2013). The expression patterns of several pluripotency-related marker genes, including *Oct3/4*, were also similar among the iPSC lines. As it is intended that these iPSC lines will be used for applications such as chimera formation (Okita *et al*., 2007) and transplantation experiments (Kobayashi *et al*., 2010; Usui *et al*., 2012), it is important that they are able to contribute to the ICM of the blastocyst embryos. In this context, a simple index, other than morphology and marker gene expression, correlating with ICM contribution efficiency is required to allow pre-screening to determine which iPSC lines are appropriate for use in labor- and cost-intensive transplantation experiments. We previously reported that mouse iPSC lines with high chimera-forming ability (Aoi *et al*., 2008) have DNA methylation profiles close to those of ESCs (Sato *et al*., 2010). In this study, we focused on the DNA methylation profile of mouse EShypo-T-DMRs and determined a 36-gene set applicable to porcine iPSC lines. The pluripotency scores, based on the DNA methylation profile of the 36-gene set, were revealed to differ among iPSC lines that were indistinguishable by morphology or marker gene expression. Moreover, the iPSC line with the highest pluripotency score contributed to the ICM as assessed by a chimera formation experiment, whereas the iPSC line with the lowest score contributed much less efficiently to the ICM. Collectively, the DNA methylation profile of the 36-gene set derived from mouse EShypo-T-DMRs correlated with the ability to contribute to the ICM of blastocyst embryos.

In the course of mouse iPSC establishment, reprogramming for activation is important for genes such as endogenous *Oct3/4* and target genes of Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc) (Okita *et al*., 2007; Takahashi and Yamanaka, 2006). In this study, we categorized the 36-gene set into three groups (Oct3/4-targets, KSM-targets, and non-targets), and analyzed DNA methylation status in porcine iPSCs. Among the three groups, differences in pluripotency score were most apparent in the Oct3/4-target genes. The Oct3/4-target group contains genes that are important for the maintenance of pluripotency in stem cells. For example, *Sall4* is an Oct3/4-target gene (Chen *et al*., 2008; Sakaki-Yumoto *et al*., 2006; Tsubooka *et al*., 2009) and was hypomethylated in the porcine iPSC line exhibiting both a high pluripotency score and efficient contribution to ICM. Differences in pluripotency scores among the examined iPSC lines were less significant in the KSM-target group than the Oct3/4-target group. Non-target genes can be thought of as downstream targets of the four Yamanaka factors, thereby non-target genes should be secondarily reprogrammed after activation of the primary targets of the Yamanaka factors. Thus, the pluripotency score of non-target genes was relatively high in iPSCs with high Oct3/4- and KSM-target scores, most likely indicating the activation of the primary target genes. Therefore, although pluripotency scores of Oct3/4-target genes can be considered as the most important index for evaluation of porcine iPSCs, the DNA methylation profiles of all three groups making up the 36 genes are required for evaluation with high accuracy.

For establishing mouse iPSCs, 2i treatment has been reported as effective for obtaining high-quality lines (Buehr *et al*., 2008; Hanna *et al*., 2009; Li *et al*., 2008). The 2i consists of MEK- and GSK3b-inhibitors, both of which prevent differentiation of mouse ESCs. In the porcine iPSC lines we examined, 2i-treatment effectively induced demethylation of the 36-gene set, with a consequent increase in pluripotency scores. Since an increased pluripotency score indicates cells with closer characteristics to those of pluripotent stem cells, such as mouse ESCs, the DNA methylation profile of the 36-gene set is useful for detection of improvements of porcine iPSC lines. In contrast, treatment of 5-aza-dC was less effective for improvement of pluripotency scores, implying that simple DNA demethylation by such compounds is insufficient. Taken together, DNA methylation profiling is useful for evaluation of characteristic changes of porcine iPSCs.

Reprogramming of endogenous pluripotency-related genes such as *Oct3/4* is crucial for stably maintaining iPSCs. By contrast, the first report of mouse iPSCs (Fbx15 iPSCs) observed incomplete demethylation of the endogenous *Oct3/4* region and the cells were not transmitted through the germline (Takahashi and Yamanaka, 2006), whereas superior germline-transmittable iPSC lines (Nanog iPSCs) showed complete demethylation of

FIG. 5. Improvement of DNA methylation status under different culture conditions. (**a**) DNA methylation status of Porco Clawn cells cultured in the presence of FBS, or in serum-free (SF), SF + 2i, or 5-aza-dC conditions was analyzed by COBRA assay; the methylation level is represented as a heatmap (left panel). The number of demethylated genes in each culture condition is shown in the right panel. (**b**) DNA methylation status of three Oct3/4-target genes (*Sall4*, *Hexb*, and *Zfp64*) in Porco Clawn cells cultured under different conditions analyzed by sodium bisulfite sequencing. Open and closed circles indicate unmethylated and methylated CpG dinucleotides, respectively. Arrowheads indicate CpG sites analyzed by COBRA assay. The methylation level (%) was calculated as methylated CpGs/all examined CpGs. (**c**) DNA methylation status in Porco Rosso-4 cells cultured under SF or SF + 2i conditions and in Porco Rosso-6 cultured in the presence of 5-aza-dC was analyzed by COBRA assay. Pluripotency scores of the iPSC lines cultured under each condition were plotted for the three gene-groups (Oct3/4-targets, KSM-targets, and non-targets).

the endogenous pluripotency-related genes including *Oct3/4* (Okita *et al*., 2007). We previously reported that the endogenous *Oct3/4* promoter region was relatively hypermethylated in the same porcine iPSC lines that were examined in this study (Fujishiro *et al*., 2013). However, one iPSC line that we analyzed contained cells showing reprogrammed and hypomethylated status within the whole examined endogenous *Oct3/4* locus, suggesting that a small population of the cells were activated at the endogenous *Oct3/4* locus. In mouse ESCs, a 1.5-fold increase or two-fold decrease in endogenous *Oct3/4* expression led to cellular differentiation (Niwa *et al*., 2000). Therefore, the appropriate expression level of endogenous *Oct3/4* is important for maintenance of pluripotency. In the pig, however, no *bona fide* pluripotent stem cells, such as mouse ESCs, have been described to date; thus, the appropriate expression level of endogenous *Oct3/4* remains to be determined. Based on the bisulfite sequencing data of endogenous *Oct3/4* in our previous study (Fujishiro *et al*., 2013) and several Oct3/4-targets in this study, a proportion of the cells appear to be properly reprogrammed in the iPSC lines we examined. In addition, the finding that huKO-negative cells, which are likely closer to pluripotent cells (Fujishiro *et al*., 2013), exhibited hypomethylation of EShypo-T-DMRs compared with the bulk iPSC line further support the usefulness of DNA methylation profile. Thus, DNA methylation analysis can evaluate the ratio of properly reprogrammed cells in each iPSC line without reference cells such as ESCs in the case of mouse iPSCs.

The porcine iPSC lines examined in this study were likely heterozygous and contained both properly and improperly reprogrammed cells, even after SF/2i-treatment, as shown by the measurement of the DNA methylation patterns of several EShypo-T-DMRs. For aggregation experiments, one iPSC colony large enough to be picked up was dissociated into single cells and aggregated with early embryonic cells. Under the present culture conditions, properly reprogrammed cells such as huKO-negative cells, which we proved to have better characteristics as stem cells (Fujishiro *et al*., 2013), exhibited slower proliferation than improperly reprogrammed cells (data not shown). This implies that visible iPSC colonies, which can be used for aggregation experiments to analyze ICM contribution, contain substantial numbers of improperly reprogrammed cells due to higher proliferation potential than properly reprogrammed cells. Thus, the data demonstrating the ICM contribution of iPSCs in this study may have underestimated the ratio of properly reprogrammed cells that could be deduced from DNA methylation analysis. In this study, we identified several genes whose methylation profiles could be used to evaluate the ratio of properly reprogrammed cells. Thus, we will be able to identify appropriate culture conditions for the maintenance of properly reprogrammed cells by using the DNA methylation profiles of these genes.

In this study, we focused on the DNA methylation profile of the 36-gene set derived from EShypo-T-DMRs and established a new index for evaluation of porcine iPSCs correlating with their efficient contribution to the ICM of blastocysts. We provide proof-of-principle of the concept that mouse EShypo-T-DMR information is applicable to evaluate porcine iPSC lines. The gene set we used in this study is effective on validation of stemness under in vitro culture condition and in blastocyst stage embryos as one aspect of pluripotency. However, the other aspect of pluripotency to have broad lineage potential is also important but cannot be assessed by the gene set used in this study. We previously identified differentiated-tissue-specific hypomethylated loci (Tissuehypo-T-DMRs), which are related to certain specific somatic cell lineages and are hypermethylated in mouse ESCs (Sato *et al*., 2010). By selecting another porcine gene set from Tissuehypo-T-DMRs in addition to the 36-gene set examined in this study, we will be able to perform more strict evaluation of porcine iPSCs in terms of pluripotency of both stemness and broad lineage potential. Since our previous findings indicated that hundreds of mouse EShypo- and Tissuehypo-T-DMRs could be used for evaluation of mouse iPSCs, we anticipate deriving further information about EShypo- and Tissuehypo-T-DMRs in multiple porcine iPSC lines with higher accuracy using next generation sequencer to perform ultra-deep bisulfite sequencing.

In conclusion, information regarding DNA methylation derived from mouse EShypo-T-DMRs is a feasible index for evaluation of porcine iPSCs as a pre-screening tool, distinct from morphology and marker gene expression analysis. Evaluation of porcine iPSC lines by DNA methylation analysis is simple and time/cost-saving. The concept of evaluation of iPSCs by DNA methylation profile is readily applicable to other mammalian cells, including human iPSCs/ESCs, which cannot be evaluated by chimera formation for ethical reasons.

METHODS

Animal Care

All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC-11-1).

Chemicals

Chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Porcine iPSCs

Establishment of porcine iPSCs was described previously (Fujishiro *et al*., 2013). Porcine iPSC lines, Porco

Rosso-4, −6, −622-14, and Epistem-like B9-2-5, expressing humanized Kusabira-Orange (huKO) were derived from PFF of huKO-transgenic pigs (Matsunari *et al*., 2008). Porco Clawn iPSC line was derived from PFF of Clawn miniature pigs (Japan Farm, Kagoshima, Japan). All the iPSC lines were maintained in knockout DMEM medium (Invitrogen, Rockville, MD) containing 15% fetal bovine serum (FBS, Invitrogen) or knockout serum replacement (KSR, Invitrogen), 1% NEAA (Invitrogen), 1% glutamax-L (Invitrogen), 100 μM 2-mercaptoethanol (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin, 10 μM forskolin (Biomol, Farmingdale, NY), and 0.5% porcine LIF with or without 5 μM 5-aza-2′-deoxycytidine (5-aza-dC) for 10 days and two signal-transduction inhibitors (2i) of 0.5 μM PD0325901 and 3 μM CHIR99021 for three weeks on MMC-treated STO cells. huKO-negative and huKO-positive cells of Porco Rosso-4 were sorted by BD FACS Aria (Becton Dickinson, NJ).

COBRA Assay and Sodium Bisulfite Genomic Sequencing

DNA methylation analysis by COBRA assay was performed based on our previous results (Sato *et al*., 2010) focusing on 58 genes that were hypomethylated in mouse ESCs. Genomic DNA was purified using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), and digested with a restriction enzyme *Hind* III (Takara Bio, Shiga, Japan). Digested genomic DNA was purified with a QIAquick Gel Extraction Kit (Qiagen), and bisulfite reactions were performed using the EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA). Bisulfite-treated DNA was amplified with BioTaq HS DNA polymerase (Bioline, London, UK) using specific gene primers (Supporting Information Table 1). Primer sequences are based on the porcine genome assembly, Sscrofa9.2 (November 2009 build) from the UCSC genome database. PCR was performed under the following conditions: 95°C, 10 min; 40 cycles of 95°C, 30 s; 60°C, 30 s; 72°C, 1 min; final extension 72°C, 2 min. Amplified PCR products were digested with *Hpy* CH4 IV (New England BioLabs, Beverly, MA) at 37°C for 3 h, or *Taq* I (Takara Bio) at 65°C for 3 h and analyzed by electrophoresis. DNA methylation levels were calculated using the formula: estimated methylation degree (%) = $100 \times I^{C}/(I^{C} + I^{UC})$, where I^{C} and I^{UC} represent intensities of digested and undigested bands, respectively. The intensities of bands were determined using ImageJ software provided by the National Institutes of Health (http://rsb.info.nih.gov/ij/). COBRA assay was independently performed twice for all samples. PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, WI), and 10 to 16 clones sequenced to determine DNA methylation status.

Preparation of Porcine Parthenogenetic Embryos

Porcine parthenogenetic embryos were generated as reported previously (Matsunari *et al*., 2008). Briefly, porcine ovaries were collected at local abattoirs. In vitro maturated oocytes with expanded cumulus cells were treated with 1 mg/ml of hyaluronidase dissolved in Tyrode's lactose medium containing 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone (HEPES-TL-PVP), and separated from the cumulus cells by gentle pipetting. Oocytes with evenly granulated ooplasm and an extruded first polar body were selected for subsequent experiments. Oocytes were transferred to an activation solution consisting of 0.3 M mannitol (Nacalai Tesque, Kyoto, Japan), 50 μM $CaCl_2$, 100 μM $MgCl_2$, and 0.01% (w/v) PVA, and activated by applying a single direct current pulse (150 V/mm, 100 μsec) using an electrical pulsing machine (LF201; Nepa Gene, Chiba, Japan). Activated oocytes were cultured and treated with 5 μg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body followed by in vitro culture for up to 4 days to obtain parthenogenetic host embryos at 4 to 8-cell- and morula-stages. In vitro culture of parthenogenetic embryos was performed in porcine zygote medium-5 (PZM-5; Research Institute for the Functional Peptides, Yamagata, Japan) under paraffin oil (Kanto Chemical, Tokyo, Japan) in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C.

Production of Aggregated Embryos

Parthenogenetic embryos at 4- to 8-cell (day 3) or morula (day 4) stages were used as host embryos for aggregation of iPSCs. Host 4- to 8-cell embryos or morulae were decompacted by incubation in Ca^{2+}/Mg^{2+}-free Dulbecco phosphate-buffered saline (DPBS; Nissui, Tokyo, Japan) containing 0.1 mM EDTA-2Na and 0.01% (w/v) PVA for 15–20 min, followed by removal of the zona pellucidae by digestion with 0.25% (w/v) pronase in DPBS. Small clumps (~30 cells) of iPSCs were isolated from the feeder layer and kept in a drop of 21 mM Hepes-buffered MEM (Invitrogen) supplemented with 5 mg/ml BSA (Sigma) (MEM-HEPES). Aggregation of iPSC clumps and host embryos was carried out using the micro-well method (Nagy and Rossant, 1993). Each clump of iPSCs was aggregated with host blastomeres isolated from two parthenogenetic embryos at 4- to 8-cell or morula stages.

Some of the parthenogenetic host embryos were aggregated with ICM clumps isolated from parthenogenetic blastocysts on day 6 by immunosurgery as described previously (Nagashima *et al*., 2004). Isolated ICM cells were labeled with fluorescent carbocyanine dye (DiI, Takara Bio) for 30 min, followed by thorough washing with MEM-HEPES.

Embryos produced by the aggregation method were cultured for 48–72 h in PZM-5. Blastocysts at day 6

were observed by confocal microscopy to analyze the incorporation of the donor cells into the ICM. Images of blastocysts placed in a drop of DPBS containing 5 μg/ml Hoechst 33342 were taken using a confocal fluorescence microscope (FV-1000; Olympus, Tokyo, Japan).

ACKNOWLEDGMENTS

The authors acknowledge Dr. Masaki Nagaya for helpful discussions on this manuscript and Mr. Taisuke Matsuda for technical help.

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