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Lab Resource: Stem Cell Line

Genomic imprinting defect in *Zfp57* mutant iPS cell lines

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ABSTRACT

ZFP57 maintains genomic imprinting in mouse embryos and ES cells. To test its roles during iPS reprogramming, we derived iPS clones by utilizing retroviral infection to express reprogramming factors in mouse MEF cells. After analyzing four imprinted regions, we found that parentally derived DNA methylation imprint was largely maintained in the iPS clones with *Zfp57* but missing in those without maternal or zygotic *Zfp57*. Intriguingly, DNA methylation imprint was lost at the *Peg1* and *Peg3* but retained at the *Snrpn* and *Dlk1-Dio3* imprinted regions in the iPS clones without zygotic *Zfp57*. This finding will be pursued in future studies.

Resource table: *Zfp57* mutant iPS cell lines.

Name of stem cell construct	*Zfp57* knockout
Institution	Icahn School of Medicine at Mount Sinai
Person who created resource	Carol M. McDonald, Xiajun Li
Contact person and email	Xiajun Li, xiajun.li@mssm.edu
Date archived/stock date	October 18, 2011
Origin	Mouse MEF cells
Type of resource	Biological reagent: induced pluripotent stem cell (iPS)
Sub-type	cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature (direct URL links and full references)	https://www.stemcell.ucla.edu/creating-ips-cells, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3727693/
Information in public databases	

Resource details

The properties of these isolated iPS clones

The iPS colonies started to form from the infected MEF cells grown on the SNL feeder cells in a week. We found that many iPS colonies also contained some cells that looked like transformed cells. We suspect that it may have been caused by retrovirus infection process or over-expression of reprogramming factors such as MYC during iPS cell derivation. We picked the colonies that displayed the best morphology with fewer "transformed cells" to establish iPS clones. The established iPS clones on feeder cells displayed a similar morphology to undifferentiated ES colonies, as exemplified by one *Zfp57*$^{+/-}$ (M^+Z^+) iPS clone, one *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone and one *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone (Fig. 1A). The genotypes for these iPS clones and parental MEF cells were confirmed by PCR-based genotyping (see Fig. 3B below). They formed embryoid bodies (EBs) when they were grown on non-adherent Petri dish plates (Fig. 1A). Based on semi-quantitative RT-PCR analysis, expression of the endoderm marker *Foxa2* seemed to be increased in EBs compared with iPS clones, in particular in two *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clones and one *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone (Fig. 1C). By contrast, the mesoderm marker *Mlc2a* was expressed in both iPS clones and their EBs (Fig. 1C). We suspect that the expression of *Mlc2a* may reflect the parental origins of these iPS clones as they were derived from MEF cells. Interestingly, the ectoderm marker *Ck18* was highly expressed in the *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone and its EBs although *Ck18* was not much expressed in one *Zfp57*$^{+/-}$ (M^+Z^+) and two *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clones but appeared to be modestly increased in the EB samples derived from these three iPS clones (Fig. 1C). To examine genome integrity of these iPS clones, we performed metaphase chromosome spread for four iPS clones, as exemplified by an image taken for one *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone (Fig. 1B). Then we counted chromosome numbers and the results are summarized in Table 1. We did not find any euploid cells in one *Zfp57*$^{+/-}$ (M^+Z^+) clone (4.2–05) and one *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone (4.3–01). By

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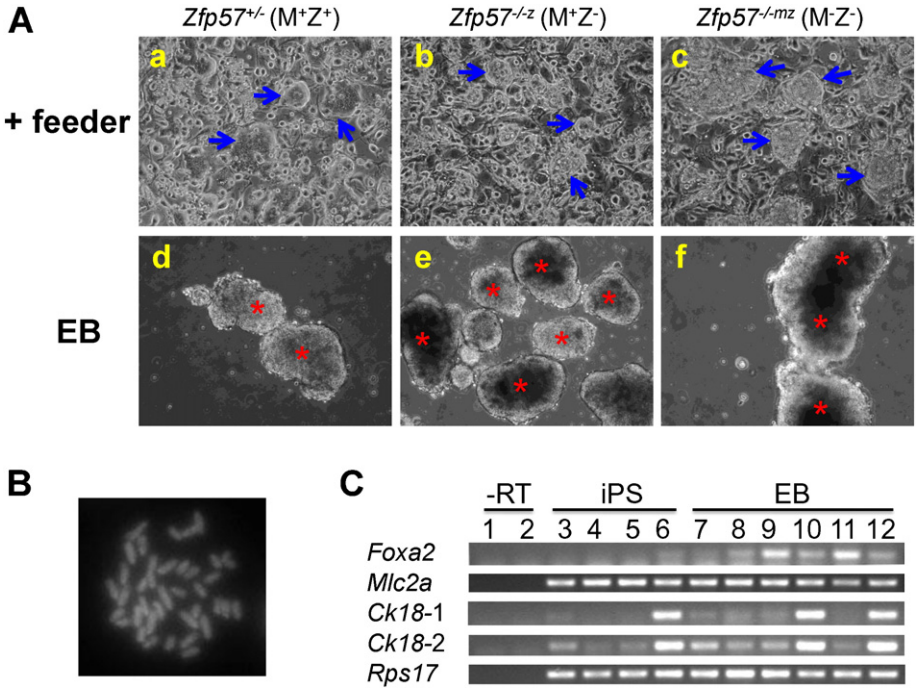

Fig. 1. Derived iPS clones display ES cell-like colonies on feeder cells and formed embryoid bodies (EBs) in suspension culture. A, the iPS clones were cultured on the SNL feeder cells (a, b, c) (McMahon and Bradley, 1990; Takahashi et al., 2007), or grown on non-adherent Petri dish plates for 7–8 days (d, e, f). One *Zfp57*$^{+/-}$ (M^+Z^+) iPS clone (a, d), one *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone (b, e) and one *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone (c, f) are shown here as the examples for the iPS clones derived from the MEF cells after retroviral infection-mediated expression of four reprogramming factors. Blue arrows in a-c, undifferentiated ES-like iPS colonies on top of the SNL feeder cells. Red asterisks in d–f, embryoid bodies (EBs). B, a DAPI-stained metaphase chromosome spread of one cell derived from a *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone. C, semi-quantitative RT-PCR expression analysis of three marker genes in four iPS clones (lanes 3–6) and the EBs derived from these four iPS clones after growing in suspension culture for 8 days (lanes 7–10) or 10 days (lanes 11–12). Lanes 1–2, negative control without reverse transcription (−RT) of the same total RNA samples in lanes 4 and 5, respectively. Lane 3, one *Zfp57*$^{+/-}$ (M^+Z^+) iPS clone grown on the feeder cells. Lanes 4–5, two *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clones grown on the feeder cells. Lane 6, one *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone grown on the feeder cells. Lane 7, day 8 EBs of the *Zfp57*$^{+/-}$ (M^+Z^+) iPS clone. Lanes 8–9, day 8 EBs of two *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clones. Lane 10, day 8 EBs of the *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone. Lane 11, day 10 EBs of one *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone. Lane 12, day 10 EBs of the *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone. *Foxa2*, *Mlc2a* and *Ck18* are the lineage markers for endoderm, mesoderm and ectoderm, respectively. A house-keeping gene *Rps17* was used as the loading control here. 25 cycles of PCR amplification was used for *Rps17*, whereas 35 PCR cycles was applied to *Foxa2* and *Mlc2a*. For *Ck18*, 25 PCR cycles was performed first (*Ck18*–1), followed by additional five cycles of PCR amplification (*Ck18*–2).

contrast, roughly 20% euploid cells were observed in the other *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone (4.3–04) and 35% of the cells in the *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone (7.2–02) were euploid with 40 chromosomes (Table 1).

Expression of pluripotency markers

We analyzed expression of three pluripotency markers (OCT4, NANOG and SOX2) in one *Zfp57*$^{+/-}$ (M^+Z^+) clone (4.2–05), one *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone (4.3–04) and the *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone (7.2–02), together with the control wild-type ES cells (Fig. 2). We observed relatively high expression levels of OCT4 and SOX2 in all three iPS clones that were comparable to those of the wild-type ES cells. By contrast, we only observed high level of NANOG expression in the *Zfp57*$^{-/-mz}$ (M^-Z^-) iPS clone but not in the *Zfp57*$^{-/-z}$ (M^+Z^-) iPS clone. There were a few strong NANOG-positive cells present in the *Zfp57*$^{+/-}$ (M^+Z^+) clone. Since OCT4 and SOX2 are two reprogramming factors used for the derivation of these iPS clones, expression of OCT4 and SOX2 could be either activated from the endogenous loci after reprogramming or expressed from the integrated retroviruses carrying the *Oct4* and *Sox2* transgenes. Further research is needed to distinguish these possibilities.

DNA methylation imprint in iPS clones

Genomic DNA samples were harvested from the control wild-type ES cell, *Zfp57* mutant tail sample, parental MEF cells and derived iPS clones. Their genotypes were confirmed by PCR-based genotyping (Fig. 3B). Due to the presence of trace amount of feeder cells and preferential amplification of the shorter PCR amplicon, a small portion of the PCR product was amplified from the wild-type allele of *Zfp57* in the genomic DNA samples of three *Zfp57*$^{-/-}$ mutant iPS clones (see lanes 11–13 of Fig. 3B). COBRA analysis was performed for these genomic DNA samples. We analyzed DNA methylation imprint at the *Snrpn*, *Peg1*, *Peg3* and *Dlk1-Dio3* imprinted regions (Fig. 3A). Previously, we found that ZFP57 maintains DNA methylation imprint at these four imprinted regions in mouse embryos and ES cells (Li et al., 2008; Zuo et al., 2012). As expected, both methylated and unmethylated DNA products were present at these four imprinted regions in the wild-type ES cells after COBRA (lane 1 of Fig. 3A), whereas only unmethylated DNA was

Table 1
Counting of metaphase chromosome spreads of four iPS clones.

iPS clone	4.2–05	4.3–01	4.3–04	7.2–02
Genotype	*Zfp57*$^{+/-}$ (M^+Z^+)	*Zfp57*$^{-/-z}$ (M^+Z^-)	*Zfp57*$^{-/-z}$ (M^+Z^-)	*Zfp57*$^{-/-mz}$ (M^-Z^-)
# of counted metaphase spreads	20	9	20	20
# of spreads with 40 chromosomes	0	0	4	7
% of euploid cells	0	0	20	35

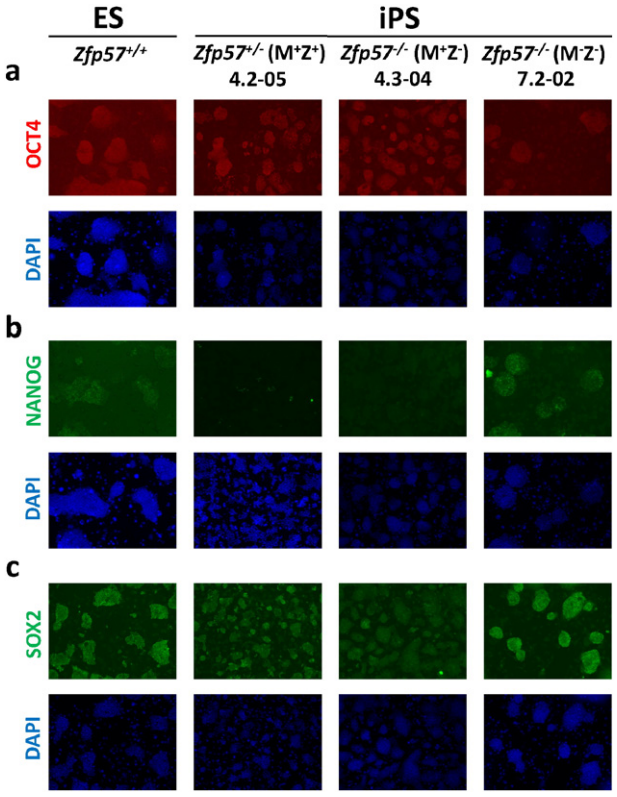

Fig. 2. Immunostaining was performed for pluripotency markers in the iPS clones. Immunostaining was used to analyze the expression of OCT4 (A), NANOG (B) and SOX2 (C) in three iPS clones grown on the feeder cells as well as the control wild-type TC1 ($Zfp57^{+/+}$) ES cells shown in the leftmost column. Blue signal, DAPI staining. 4.2–05, 4.3–04 and 7.2–02 are three iPS clones generated in this study that were derived from the MEF cells that were $Zfp57^{+/-}$ (M^+Z^+), $Zfp57^{-/-z}$ (M^+Z^-) and $Zfp57^{-/-mz}$ (M^-Z^-), respectively. Red signal in A, OCT4 immunostaining. Green signal in B, NANOG immunostaining. Green signal in C, SOX2 immunostaining.

observed in the *Zfp57* mutant tail sample after COBRA (lane 2 of Fig. 3A). DNA methylation imprint was intact in the parental MEF cells derived from $Zfp57^{+/+}$ (M^+Z^+) embryos (lane 3 of Fig. 3A) or parental MEF cells derived from $Zfp57^{+/-}$ (M^+Z^+) embryos (lane 4 of Fig. 3A) containing both maternal and zygotic *Zfp57*. It was partially lost in the parental MEF cells derived from $Zfp57^{-/-z}$ (M^+Z^-) embryos lacking just zygotic *Zfp57* (lane 5 of Fig. 3A), but almost completely missing in the parental MEF cells derived from $Zfp57^{-/-mz}$ (M^-Z^-) embryos without maternal or zygotic *Zfp57* (lane 6 of Fig. 3A). These results are similar to what had been observed in mouse embryos (Li et al., 2008).

We also examined DNA methylation imprint at these four imprinted regions in the established iPS clones (lanes 7–13 of Fig. 3A) derived from these parental MEF cells. Two $Zfp57^{+/+}$ (M^+Z^+) iPS clones also displayed intact DNA methylation imprint at these four imprinted regions (lanes 7–8 of Fig. 3A), similar to that in the parental $Zfp57^{+/+}$ MEF cells in lane 3. Although DNA methylation imprint was maintained at the *Snrpn*, *Peg1* and *Peg3* imprinted regions in both $Zfp57^{+/-}$ (M^+Z^+) iPS clones, it was lost at the *Dlk1-Dio3* imprinted region in one of two $Zfp57^{+/-}$ (M^+Z^+) iPS clones (lanes 9–10 of Fig. 3A). Intriguingly, DNA methylation imprint was lost at the *Snrpn* and *Dlk1-Dio3* imprinted regions in two $Zfp57^{-/-z}$ (M^+Z^-) iPS clones, whereas it was maintained at the *Peg1* and *Peg3* imprinted regions in both $Zfp57^{-/-z}$ (M^+Z^-) iPS clones derived from the MEF cells lacking zygotic *Zfp57* (lanes 11–12 of Fig. 3A). These results are strikingly different from those in the parental MEF cells derived from $Zfp57^{-/-z}$ (M^+Z^-) embryos lacking just the zygotic *Zfp57* (lane 5 of Fig. 3A). This indicates that ZFP57 may be differentially required for the maintenance of DNA methylation imprint at different imprinted regions during iPS cell derivation. As expected, DNA methylation imprint remained absent at these four imprinted regions in one $Zfp57^{-/-mz}$ (M^-Z^-) iPS clone (lane 13 of Fig. 3A), similar to what was observed in the parental MEF cells derived from $Zfp57^{-/-mz}$ (M^-Z^-) embryos without either maternal or zygotic *Zfp57* (lane 6 of Fig. 3A). This result suggests that DNA methylation imprint cannot be re-acquired in iPS cells without ZFP57, similar to what we observed before in ES cells (Zuo et al., 2012).

Taken together, zygotic ZFP57 appears to be essential for the maintenance of DNA methylation imprint at some imprinted regions such as *Snrpn* and *Dlk1-Dio3* imprinted regions during iPS cell derivation even though DNA methylation imprint may be maintained without zygotic ZFP57 (M^+Z^-) at other imprinted regions (e.g. *Peg1* and *Peg3*) during iPS cell derivation from retroviruses-mediated expression of reprogramming factors in MEF cells. Without maternal or zygotic ZFP57 (M^-Z^-), DNA methylation imprint cannot be re-acquired at these four examined imprinted regions in iPS cells. Further research will be needed to gain mechanistic insights into this kind of ZFP57-independent maintenance of DNA methylation imprint at a subset of imprinted regions during iPS cell derivation.

Materials and methods

Generation of MEF cells

Zfp57 is a maternal-zygotic effect gene and displays maternal-zygotic embryonic lethality around midgestation (Li et al., 2008; Shamis et al., 2015). We isolated MEF cells from the live E12.5–E13.5 embryos derived from the cross between $Zfp57^{+/-}$ heterozygous female mice and $Zfp57^{+/-}$ heterozygous female mice or between $Zfp57^{-/-}$ homozygous female mice and $Zfp57^{-/-}$ homozygous male mice. These MEF cells were used for derivation of the iPS clones.

Derivation of iPS clones

We derived the iPS clones that are $Zfp57^{+/+}$, $Zfp57^{+/-}$ or $Zfp57^{-/-z}$ from the MEF cells generated from the cross between $Zfp57^{+/-}$ heterozygous female mice and $Zfp57^{+/-}$ heterozygous female mice. The iPS clones that are $Zfp57^{-/-mz}$ were derived from the MEF cells generated from the cross between $Zfp57^{-/-}$ homozygous female mice and $Zfp57^{-/-}$ homozygous male mice. We followed the protocol described in this original paper to derive iPS clones (Takahashi et al., 2007). First, we transfected phoenix cells by calcium phosphate method with the plasmids pMXs-*Oct4*, pMXs-*Sox2*, pMXs-*Klf4* and pMXs-*Myc* individually. Then we harvested retroviral supernatant 2–3 days after transfection and infected the MEF cells with the equal volume of the retroviral supernatant that expresses four reprogramming factors OCT4, SOX2, KLF4 and MYC, respectively. After three days of culture, 50,000 infected MEF cells were plated onto the SNL feeder cells seeded on a 10-cm dish plate (Takahashi et al., 2007). Next day the medium was replaced with the ES cell growth medium in DMEM supplemented with 15% of fetal bovine serum (FBS). Then the medium was changed every other day until ES cell-like iPS colonies appeared on the plate. These iPS colonies were picked individually and plated on the SNL feeder cells in 24-well plates after trypsin digestion. For the iPS colonies that continued to grow and display ES cell-like morphology on the SNL feeder cells, they were expanded in 6-well plates seeded with the SNL feeder cells to establish the stable iPS cell lines.

Embryoid body (EB) formation for iPS clones

The iPS cells grown on the SNL feeder cells were harvested by trypsin digestion and then added to a non-adherent 10-cm Petri dish plates coated with poly-hema (Sigma). The medium was changed once every 2 days until the floating EBs were harvested for total RNA preparation. The EBs were dissolved in TRIzol reagent (Invitrogen) and total RNA samples were purified according to the manual provided by the manufacturer.

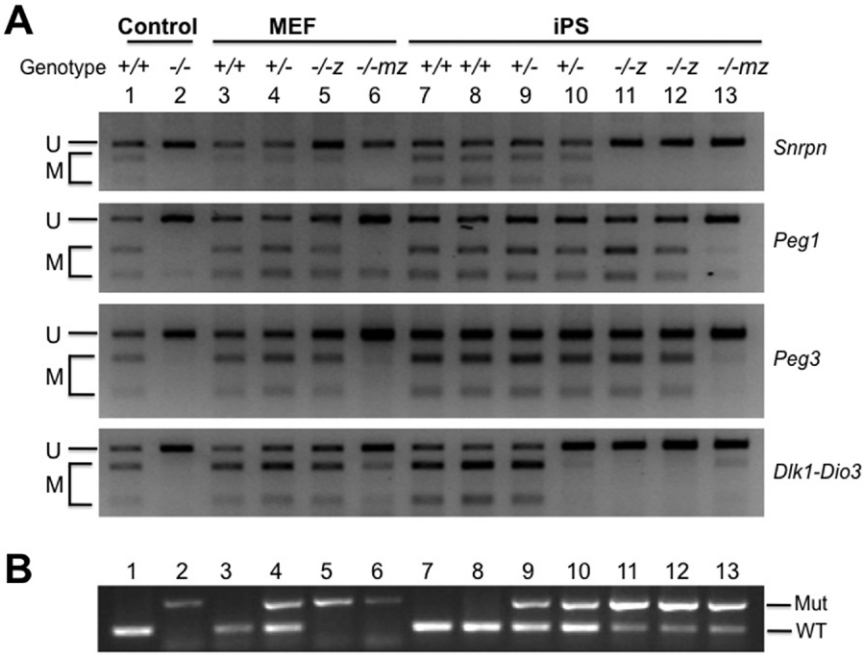

Fig. 3. COBRA analysis was performed for the ICRs at four imprinted regions in the derived iPS clones and parental MEF cells. A, COBRA analysis was carried out for analyzing DNA methylation imprint at the *Snrpn*, *Peg1*, *Peg3* and *Dlk1-Dio3* imprinted regions. Restriction enzyme (RE) digestion and gel electrophoresis were performed for the bisulphite PCR product of the genomic DNA samples. Lane 1, control wild-type ES cells. Lane 2, *Zfp57* mutant mouse tail sample. Lanes 3–6, parental MEF cells. Lanes 7–13, iPS clones. Lane 3, MEF cells derived from $Zfp57^{+/+}$ (M^+Z^+) embryos. Lane 4, MEF cells derived from $Zfp57^{+/-}$ (M^+Z^+) embryos. Lane 5, MEF cells derived from $Zfp57^{-/-z}$ (M^+Z^-) embryos lacking just the zygotic *Zfp57*. Lane 6, MEF cells derived from $Zfp57^{-/-mz}$ (M^-Z^-) embryos without either maternal or zygotic *Zfp57*. Lanes 7–8, two $Zfp57^{+/+}$ (M^+Z^+) iPS clones derived from the MEF cells in Lane 3. Lanes 9–10, two $Zfp57^{+/-}$ (M^+Z^+) iPS clones derived from the MEF cells in Lane 4. Lanes 11–12, two $Zfp57^{-/-z}$ (M^+Z^-) iPS clones derived from the MEF cells in Lane 5. Lane 13, one $Zfp57^{-/-mz}$ (M^-Z^-) iPS clone derived from the MEF cells in Lane 6. U and M, unmethylated (U) and methylated (M) product after RE digestion, respectively. B, PCR-based genotyping was used to confirm the genotypes for the MEF cells and derived iPS clones. Mut and WT, the gel positions for the PCR product of the mutant (Mut) and wild-type (WT) alleles of *Zfp57* that were described in our previously published study (Li et al., 2008).

Counting of the chromosome numbers for iPS clones

Growing iPS clones were used for metaphase chromosome spread. Karyomax (Invitrogen Cat# 15210-040) was added to the iPS cells with a final concentration of Colcemid at 1 μg/ml. After 1-hour incubation, the iPS cells were digested by trypsin and precipitated by centrifugation. The cell pellets were resuspended and gently mixed with 5 ml of ice-cold 0.56% KCl solution in water. After incubation for 6 min at room temperature, the iPS cells were precipitated by centrifugation before being mixed with the fixative solution of acetic acid and methanol (1:3). After precipitation by centrifugation, the cell pellets were resuspended in this fixative solution with DAPI and spotted onto the slides. The slides were dried in the air for 1 h before examination under microscope. The chromosome numbers of good metaphase spreads were counted for four iPS clones and the results are summarized in Table 1.

RT-PCR expression analysis of the lineage marker genes

Total RNA samples were purified from four iPS clones and their EBs after growing in suspension culture for 8 or 10 days. A relatively similar amount of total RNA samples was subjected to reverse transcription (RT) with Transcriptor First Strand cDNA Synthesis Kit (Roche). The anchored-oligo$(dT)_{18}$ primer included in the kit was used to initiate the RT reaction. Then 1 μl of RT product was used for each PCR amplification.

Immunostaining of pluripotency markers

The iPS clones grown on top of the feeder cells in a 24-well plate were directly subjected to immunostaining. The antibodies from Santa Cruz Biotechnology were used for immunostaining against OCT4 (sc-5279), NANOG (sc-376915) and SOX2 (sc-17320). The nuclei were stained with DAPI.

Bisulphite mutagenesis

The genomic DNA samples isolated from these iPS clones were subjected to bisulphite treatment with the EZ DNA Methylation-Gold™ Kit (Zymo Research). The genomic DNA samples after bisulphite mutagenesis were used for DNA methylation analysis of the imprinting control regions (ICR) by COBRA.

Combined Bisulphite Restriction Analysis (COBRA)

The purified bisulphite-treated genomic DNA samples were amplified by PCR with the primers covering a portion of the imprinting control region (ICR) (Zuo et al., 2012). The resultant PCR product was subjected to restriction enzyme digestion targeting the restriction enzyme sites whose presence is dependent on the methylation status at the CpG sites within the restriction enzyme recognition sites. The PCR product after restriction enzyme digestion was separated by agarose gel electrophoresis.

Acknowledgments

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