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Reprogrammed Parthenogenetic ES Cells - New Choice for Regenerative Medicine

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1. Introduction

Regenerative medicine using pluripotent stem cells is indispensable for cell transplantation therapy for the patients; however, the production of human embryonic stem (hES) cells from fertilized embryos or cloned embryos often become the ethical concerns because it needs destruction of viable embryos. On the other hand, the production of induced pluripotent stem (iPS) cells do not need viable embryos; whereas, it is necessary to confirm safety for the use of iPS cells that containing viruses or expression plasmids. Here, we would like to propose parthenogenetic ES (PGES) cells as the 3rd pluripotent stem cells for cell transplantation therapy because the production of PGES cells from fertilization-failure or surplus oocytes overcome both problems (Fig. 1). PGES cells do not need destruction of viable embryos and viruses or expression plasmids for the establishment. Nevertheless, PGES cells have a big hurdle to overcome, namely genomic imprinting.

Mammalian parthenotes cannot develop to term. Mouse parthenogenetic embryos die by day 10 of gestation (Surani et al., 1984; Surani et al., 1986). Most notably, they fail in the trophectoderm and primitive endoderm, which results in failure of the extraembryonic tissues in the whole parthenogenetic conceptus (Surani et al., 1983). Alternatively, viable parthenogenetic chimeras can be produced by normal host embryo rescue, and parthenogenetic cells can give rise to a functional germline (Stevens et al., 1977; Stevens, 1978). In the somatic-lineages of chimeras, parthenogenetic cells are allocated initially randomly in the embryo proper (Clarke et al., 1988a; Clarke et al., 1988b; Thomson & Solter, 1989), but this is followed by a progressive elimination of parthenogenetic cells, most notably between days 13 and 15 of gestation (Fundele et al., 1990). In addition, PG chimeras often show the reduction of body weight. These can be explained by parent-specific epigenetic modification of the genome, genomic imprinting which leads to the altered expressions of imprinted genes in parthenogenetic cells. In general, gene expressions of imprinted genes are greatly dependent on the cytosine-guanine (CpG) methylation status in differentially methylated regions (DMRs) of imprinted genes (Fig. 2). In PG with the two maternal genomes, paternally expressed genes, *Peg1/Mest* (Kaneko-Ishino et al., 1995), *Peg3* (Kuroiwa et al., 1996), *Snrpn* (Barr et al., 1995) and *Igf2* (DeChiara et al., 1991), are silenced; whereas, maternally expressed genes, *Igf2r* (Barlow et al., 1991), *p57^{kip2}* (Hatada & Mukai, 1995) and *H19* (Bartolomei et al., 1991; Ferguson-Smith et al., 1991), are expressed excessively (Fig. 3). Thus, biallelic expression and repression of imprinted genes in parthenogenetic cells could restrict to produce transplantable tissues for regenerative

medicine; however, PGES cell chimeras are more normal in their tissue contribution of donor cells and body weight compared to PG chimeras (Allen et al., 1994). We expected that these phenomena were associated with the more normal epigenotype of PGES cells.

To elucidate the epigenetic mechanisms underlying this, we analyzed DNA methylation status and mRNA expression of imprinted genes in PG and PGES cell chimeras (Horii et al., 2008). Interestingly, the PGES cells showed reprogramming of maternal imprints and acquired more normal pluripotency. In this chapter, we propose that such reprogrammed PGES cells might be utilized for the regenerative medicine.

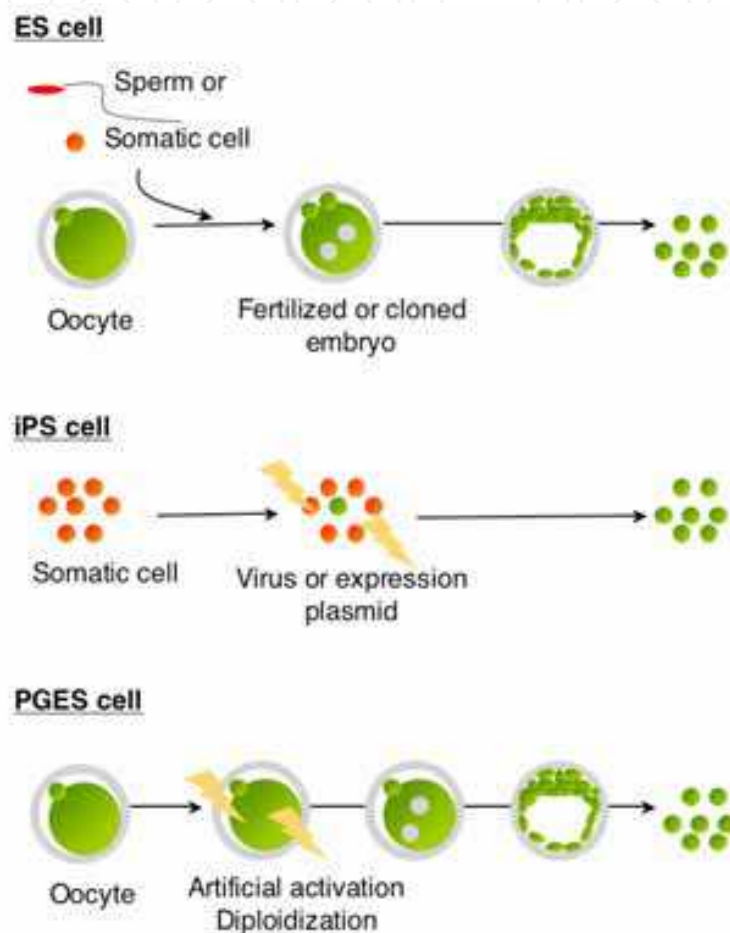


Fig. 1. Production of pluripotent stem cells using various methods.

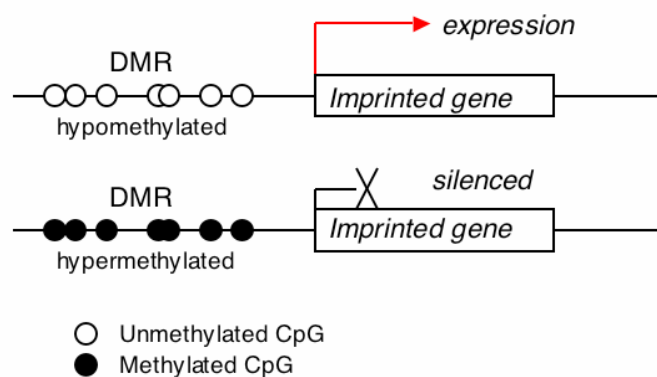


Fig. 2. Representative regulation of gene expression by CpG methylation.

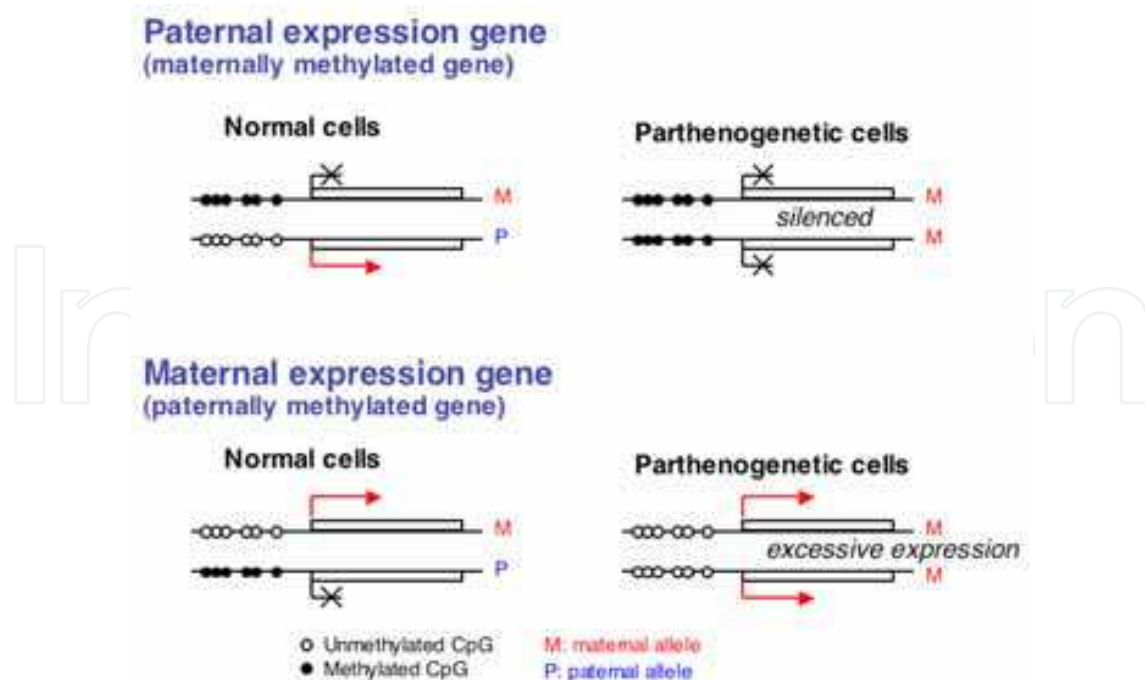


Fig. 3. Genomic imprinting in normal and parthenogenetic cells.

2. Epigenetic reprogramming occurred in PGES cells

2.1 Production of PG and PGES cells

To elucidate the epigenetic difference between PG and PGES cells, we analyzed DNA methylation status and mRNA expression of imprinted genes in PG and PGES cells. Diploid PG were produced as previously described (Horii et al., 2008). Briefly, oocytes at the metaphase stage of the second meiotic division (MII) were collected from the oviducts after human chorionic gonadotrophin (hCG) superovulation, and then cumulus cells were removed by digestion with hyaluronidase in M2 medium. Artificial activation was performed by brief exposure to SrCl₂ and cytochalasin B in Ca²⁺-free M16 embryo culture medium for 6 hours. After activation, the embryos were cultured in M16 medium until developing to the blastocyst stage.

For establishment of PGES cell lines, parthenogenetic blastocysts were cultured for 7 days in Serum-free ES medium, following standard procedures (Horii et al., 2003). After 7 days, ICM outgrowths were harvested in Trypsin/ EDTA, disaggregated by mouth pipetting and plated onto feeder cells in ES medium. Clones resembling ES cells in morphology were then picked and disaggregated a second time. They were then expanded and passaged prior to freezing or use.

2.2 Epigenetic reprogramming occurred in PGES cells

The methylation status of PG and PGES cells was analyzed for the DMRs of maternally methylated imprinted genes *Peg1/Mest*, *Snrpn* and *Igf2r*. To identify methylated CpG sites, sodium bisulfite treatment, by which only unmethylated cytosine residues were changed to thymines, was carried out. PCR amplification for DMRs of *Peg1/Mest*, *Snrpn* and *Igf2r* was carried out on each set of isolated cells as described (Horii et al., 2008). PCR products were subcloned into the TA cloning vector, and positive clones in each sample were sequenced.

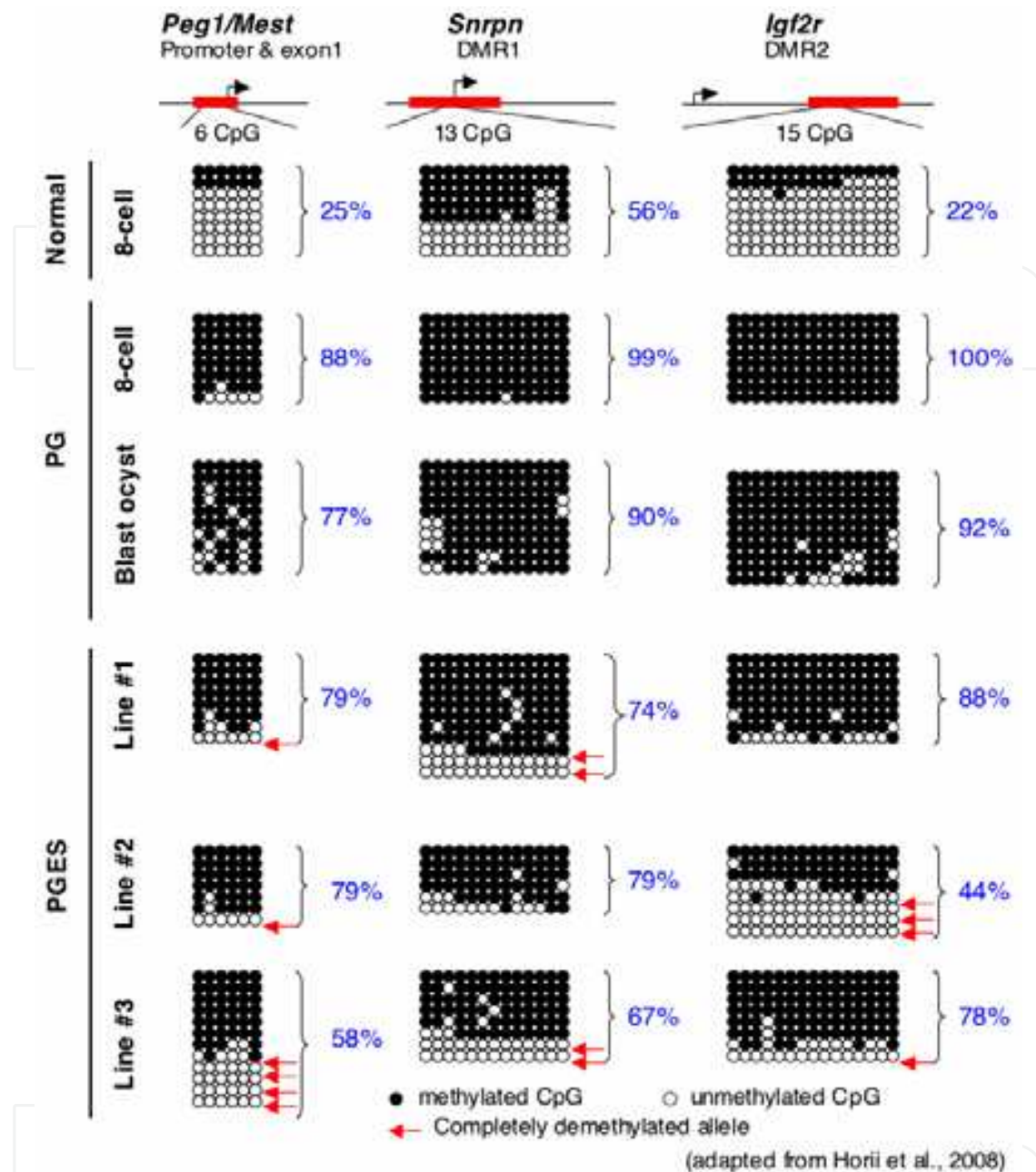


Fig. 4. Epigenetic reprogramming occurred in PGES cells. DNA methylation of normal embryos, PG and PGES cells was analyzed by bisulfite genomic sequencing. The methylation status of maternally methylated imprinted genes, *Peg1/Mest*, *Snrpn* and *Igf2r* is shown schematically. Percentages of methylated CpGs are shown to the right of the sequences.

At the 8-cell stage, normal embryos had both methylated and unmethylated alleles of maternally methylated imprinted genes; whereas, almost all alleles were methylated in PG (Fig. 4). The loss of imprinting was observed at parthenogenetic blastocysts in more 2 days of culture. This partial demethylation also occurred in *in vitro* cultured normal blastocysts as reported previously (Doherty et al., 2000; Mann et al., 2004). Perhaps, parthenogenetic blastocysts also occurs demethylation as well as in normal blastocysts *in vitro*. Anyway, this demethylation was very partial and sparse. On the other hand, completely demethylated

alleles existed in almost all PGES cell samples (Fig. 4; arrows). At this point, loss of imprinting in PGES cells seems to be more progressive than that of PG.

We found that the methylation difference between PG and PGES cells have already exist before differentiation. In the following section, we elucidate whether such a difference affects the pluripotency of PG and PGES cells.

3. Improvement of pluripotency by epigenetic reprogramming

3.1 Production of PG and PGES cell chimeras

Chimeric mice were produced to examine developmental potential and epigenetic status of PG and PGES cells (Fig. 5). Briefly, PG chimeric embryos were produced by aggregating the 4-8 cell stage of GFP+ parthenogenetic embryos with the same stage of normal host embryos. PGES chimeric embryos were produced by introducing GFP+ PGES cells to host embryos. Then, 135 PG chimeric embryos and 338 PGES cell chimeric embryos were

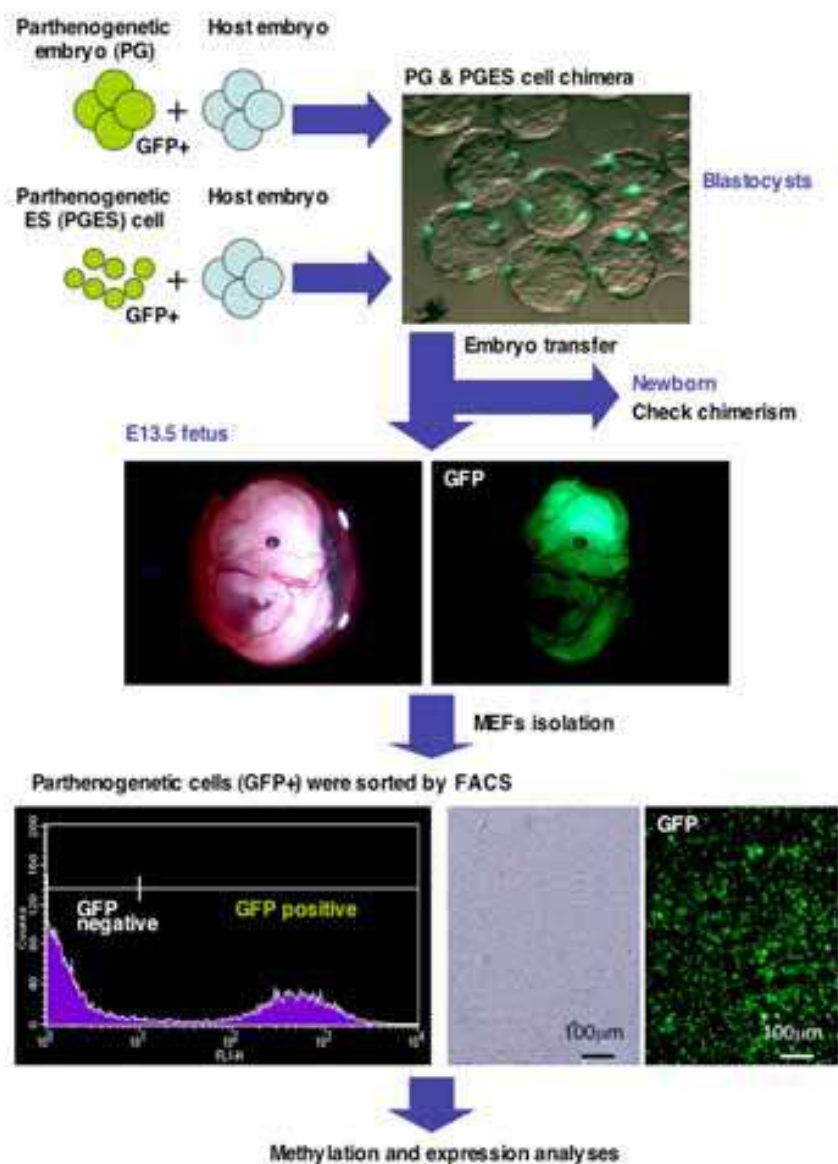


Fig. 5. Production of PG and PGES cell chimeras and analysis for their derivatives.

transferred to the uterine horns of pseudopregnant recipient females, and 18 PG chimeras and 56 PGES cell chimeras were obtained, respectively. In newborns, growth retardation was not observed in PGES chimeras (normal $1.48 \pm 0.21\text{g}$ vs chimera $1.48 \pm 0.37\text{g}$; $P = 0.45$), as previously reported (Allen et al., 1994). Contribution of PGES cells was found in all tissues tested (Fig. 6).

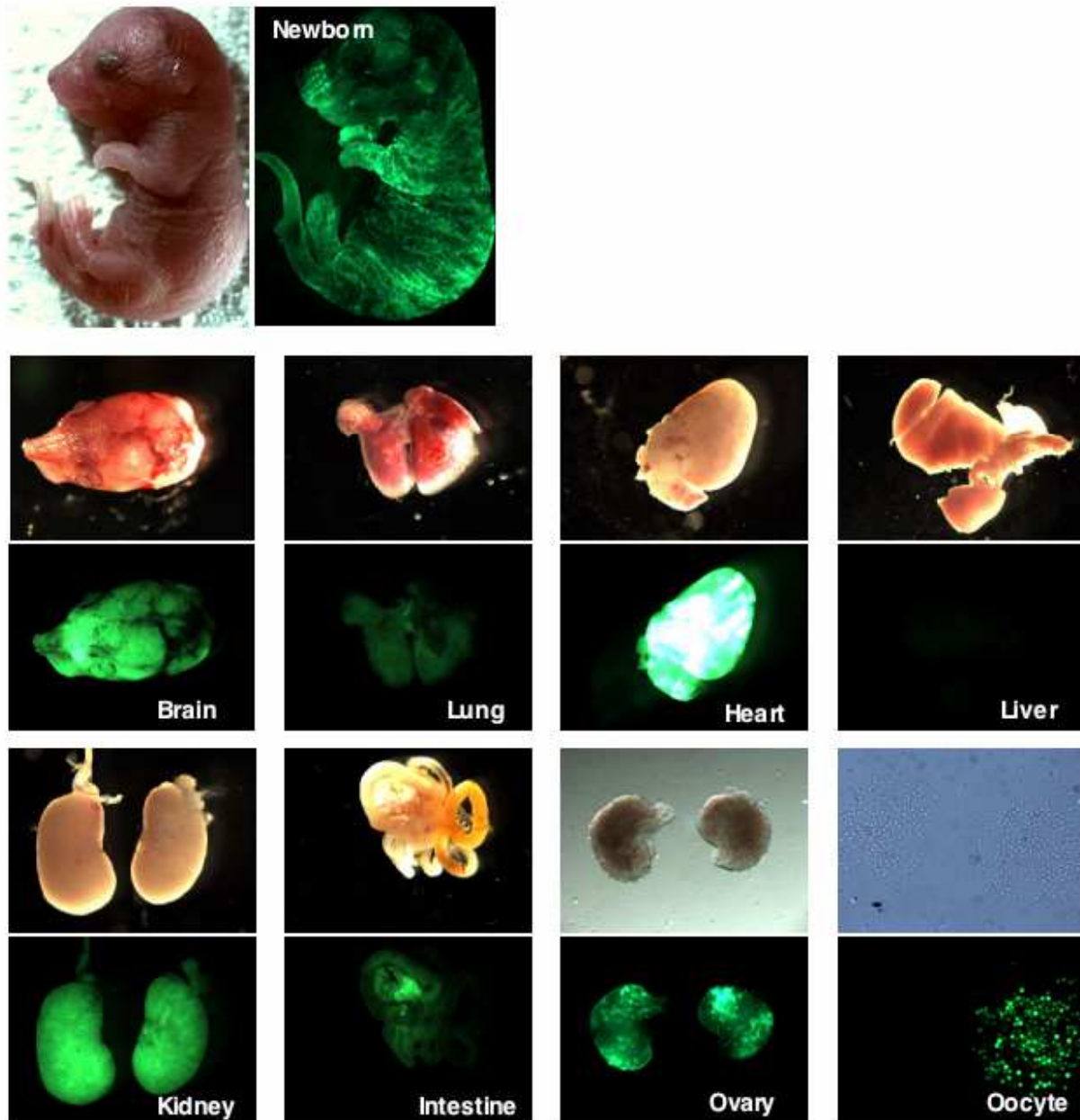


Fig. 6. Various tissue contributions of PGES cells (1 days post partum).

3.2 Epigenetic status of PG- and PGES cell-derived somatic cells

To examine epigenetic status of PG- and PGES cell-derived cells in chimeras, primary mouse embryonic fibroblasts (MEFs) from E13.5 chimeras were isolated, and sorted by fluorescent-activated cell sorter (Fig. 5). In MEFs of PG chimeras, genomic imprinting of donor cells were almost totally maintained (Fig. 7, PG). In contrast, genomic imprinting of donor cells in

PGES cell chimeras were frequently reprogrammed (Fig. 7, PGES #1 and #2). Completely demethylated alleles were included in some PGES cell-derivatives.

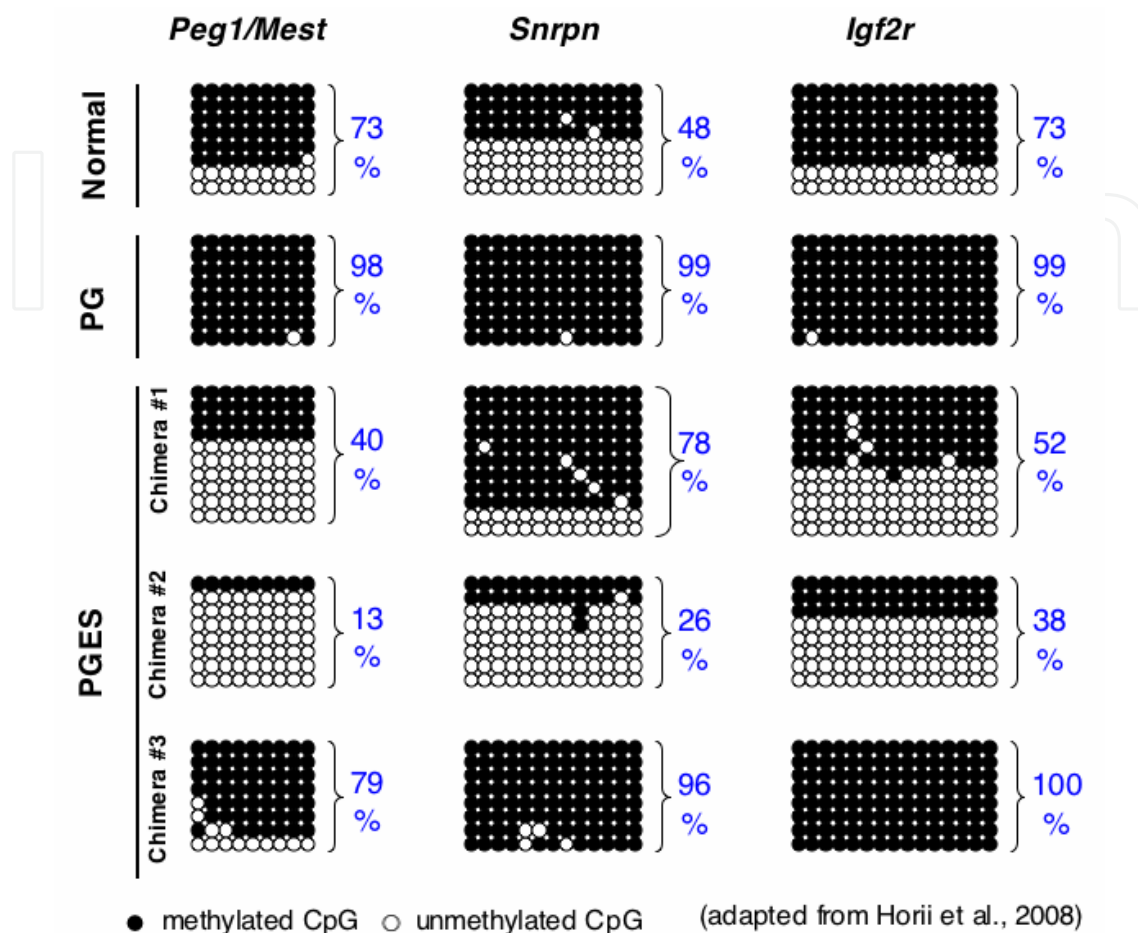


Fig. 7. Epigenetic reprogramming occurred in PGES cell-derived somatic tissues (E13.5 MEFs). DNA methylation of normal, PG-derived and PGES cell-derived MEFs were analyzed by bisulfite genomic sequencing. Percentages of methylated CpGs are shown to the right of the sequences.

Next, quantitative real-time RT-PCR was carried out to clarify whether demethylation is correlated to expression levels of imprinted genes. Maternally methylated imprinted genes, *Peg1/Mest* and *Snrpn* are expressed only from the paternal allele because these expressions are suppressed by DNA methylation in maternal allele. Therefore, in parthenogenetic cells, the loss of imprints leads to the upregulation of *Peg1/Mest* and *Snrpn*. In fact, the average expression level of each gene were upregulated in PGES cell-derivatives (Fig. 8). There were significant correlations ($P < 0.05$) between the methylation status of DMRs and the gene expression level in these two genes (Fig. 9). On the other hand, paternally imprinted genes, *Igf2*, which are regulated by *H19* DMR methylation, were generally unmethylated in both PG and PGES cell-derived cells (data not shown), and the expression level of both genes did not differ between PG and PGES cell-derived cells (Fig. 8). Then, correlations between the demethylation of imprinted genes and the tissue contribution of PGES cell-derived cells were examined. For E13.5 chimeras, there was low correlation between the percentage of methylation and the percentage of chimerism ($R^2 = 0.4719$); however, this correlation was much higher in newborn chimeras ($R^2 = 0.6981$; Fig. 10).

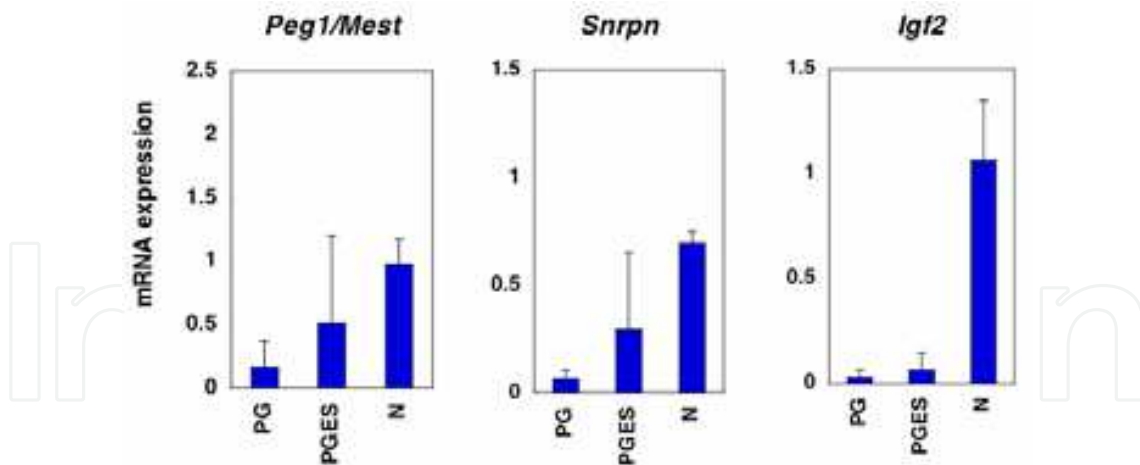


Fig. 8. Expression of imprinted genes in MEFs derived from PG (n = 6), PGES (n = 11) and normal biparental embryos (N; n = 2). Quantitative real-time PCR was performed for *Peg1/Mest*, *Snrpn* and *Igf2* genes. Standard deviations are indicated by bars.

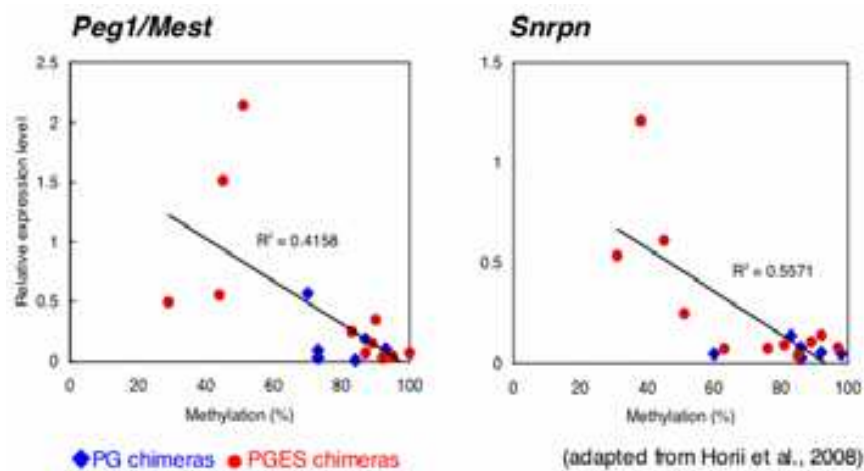


Fig. 9. Epigenetic reprogramming correlated to the gene expression level. The percentage of DMR methylation is plotted against the relative gene expression level for PG chimeras (filled circles; n = 6) and PGES cell chimeras (open circles; n = 11). Significant correlations ($P < 0.05$) were found for these imprinted genes.

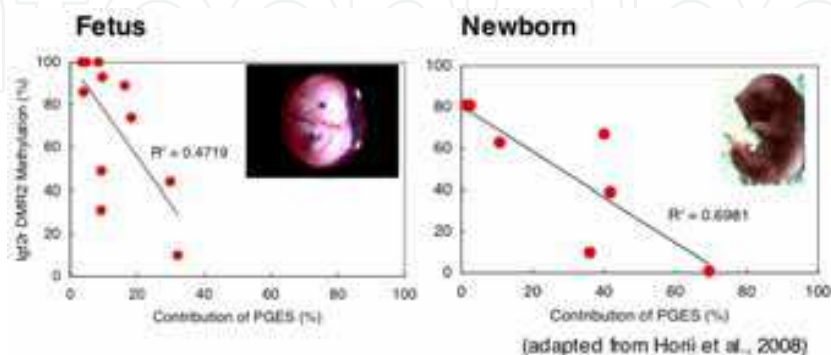


Fig. 10. Epigenetic reprogramming correlated to the pluripotency of PGES cells. Low correlation was observed in E13.5 fetuses (n = 11), whereas higher correlation was observed in newborns (n = 7).

3.3 Improvement of pluripotency by epigenetic reprogramming

In parthenogenetic chimeras, PGES cells resembled PG cells in their pluripotency. However, PGES chimeras are more normal in body weight and tissue contribution than PG chimeras. We postulated that this difference might be caused by the modified expressions of imprinted genes, due to loss of imprinting in PGES cells. To investigate the epigenetic status of parthenogenetic cells in somatic-lineages, we produced parthenogenetic chimeras using PG and PGES cells.

In general, higher overall levels of PGES cells are detected than PG cells in terms of tissue contribution. Furthermore, no significant growth retardation is apparent in PGES chimeras, irrespective of their degree of chimerism or the PGES cell lines used (Allen et al., 1994). Also in our study, growth retardation was not found in PGES chimeras. A phenotypic difference is expected to be caused by the difference in expressions of imprinted genes due to the loss of imprints in PGES derivatives. In some PGES chimeras, the loss of imprints was observed in *Peg1/Mest* and *Snrpn* genes. Especially, the *Peg1/Mest* gene is related to embryonic growth (Lefebvre et al., 1998). Therefore, there is no doubt that these alterations of gene expressions improve the tissue contribution of PGES cells in chimeras. In PG chimeras, progressive elimination of PG cells occurs after day 13 of gestation (Fundele et al., 1990). We found higher positive correlations between demethylation and chimerism in newborns than in E13.5 fetuses, suggesting that demethylated parthenogenetic cells evaded progressive elimination from tissues after day 13 of gestation. Summary of results are shown in Fig. 11.

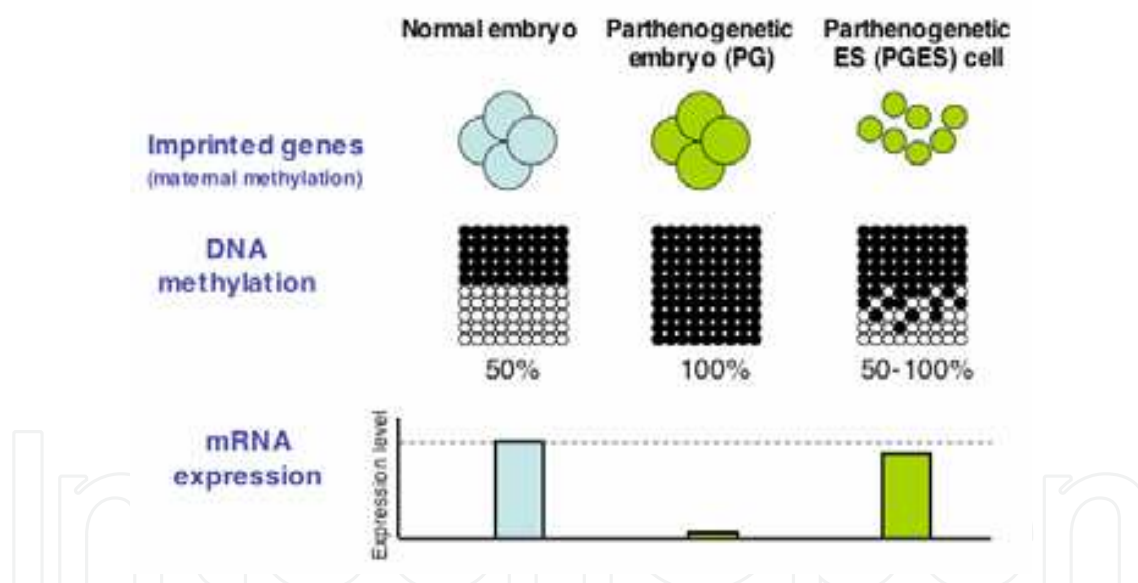


Fig. 11. Summary of epigenetic status of normal embryo, PG and PGES cell.

4. Why epigenetic reprogramming occurred in PGES Cells?

The partial or complete loss of imprints was observed in undifferentiated PGES cells and its derivatives. Why did loss of imprints occur mostly in PGES cells?

A first consideration is that the culture conditions of preimplantation embryos and ES cells sometimes influence the methylation status of genomic imprinting. For example, a sub-optimal culture medium can cause aberrant genomic imprinting of the *Snrpn* and *H19* gene, whereas embryos cultured in potassium simplex optimized medium with added amino acids (KSOM_{AA}) show global gene expression, genomic imprinting and embryo

development resembling that found in *in vivo* developed embryos (Doherty et al., 2000; Mann et al., 2004). Furthermore, long term culture of ES cells also affects the methylation status of imprinted genes and their totipotency (Dean et al., 1998; Horii et al., 2010).

A second consideration is that XX ES cells including PGES cells are more susceptible to demethylation than XO and XY ES cells (Zvetkova et al., 2005). XO and XY ES cells are able to restore the methylation imprints; whereas, XX ES cells are not able to restore them enough. PGES cells have generally two X chromosomes, so that demethylated PGES cells could be demethylated, or progress the demethylation of imprinted genes in chimeras. Then, why XX ES cells including PGES cells show demethylation of imprints? There is a speculation that X chromosome encodes a modifier locus whose product represses *de novo* methyltransferases. The *de novo* methyltransferase, Dnmt3a and Dnmt3b, are known to play a critical role for the restoration of methylation post implantation (Okano et al., 1998; Okano et al., 1999). Cells with two active X chromosomes will overexpress the modifier and therefore have reduced levels of the enzymes. In evidence, the forced expression of Dnmt3a or Dnmt3b restores the DNA methylation of XX ES cells (Zvetkova et al., 2005), suggesting that the expression levels of Dnmt3a and/ or Dnmt3b are not sufficient for PGES cells. On the other hand, once methylation of imprinted genes is completely lost in XX ES cells, the loss is not restored by the forced expression of Dnmt3a or Dnmt3b *in vitro* (Zvetkova et al., 2005). According to the bisulfite genomic sequencing of PG and PGES cells, demethylation was sparse in parthenogenetic blastocysts; whereas, completely demethylated alleles existed in undifferentiated PGES cells (Fig. 4), suggesting that completely demethylated alleles in PGES cells were not able to be remethylated post implantation.

5. Comparison among ES cell, iPS cell and PGES cell.

PGES cells have been proposed as a source of patient-derived therapeutic materials (Cibelli et al., 2002). In addition to normal ES cells and iPS cells, parthenogenesis is another tool for creating pluripotent stem cells. Human PGES cells have already been isolated from human parthenogenetic blastocysts (Mai et al., 2007; Revazova et al., 2007). As described in the introduction, the advantage using PGES cells is that PGES cells do not need destruction of viable biparental embryos like normal ES cells. In addition, PGES cells do not need viruses or expression plasmids for the establishment like iPS cells. The genomic imprinting with uniparental genome sets is the biggest problem for PGES cells; however, we and others clarified that PGES cells partially lost maternally methylated imprints and obtained more normal imprint patterns (Jiang et al., 2007; Horii et al., 2008; Li et al., 2009). In this study, we clarified correlation of expression and methylation of imprinted genes with pluripotency of PGES cells. Even more surprisingly, live parthenogenetic pups were recently produced from reprogrammed PGES cells through tetraploid embryo complementation (Chen et al., 2009). These reports suggest that PGES cells have more normal pluripotency than PG. In contrast, even normal biparental ES cells sometimes obtain abnormal imprinting which influences pluripotency during long-term culture (Dean et al., 1998; Horii et al., 2010). In addition, iPS cells occasionally show aberrant silencing of imprinted genes on chromosome 12qF1 (Stadtfeld et al., 2010). Thus, reprogrammed PGES cells have pluripotency nearly equivalent to normal biparental ES cells or iPS cells. Besides these merits, there are other advantages to use PGES cells for cell transplantation therapy.

For example, normal biparental ES cells derived from fertilized embryos are genetically divergent from any patient requiring tissue transplantation and bring an immune response

resulting in rejection (Drukker & Benvenisty, 2004). The hES cell bank that contains sufficient cell lines with diverse human leukocyte antigen (HLA) genotypes (Taylor et al., 2005; Nakajima et al., 2007) might serve most of the patients in a region; however, this system need numerous numbers of human embryos and might cause ethical issues. On the other hand, PGES cell lines which are homozygous for HLA loci significantly reduce the number of cell lines required for the repository because tissues derived from homozygous PGES cells express only one set of histocompatibility antigens and are more readily matched to patients with less risk of immunological rejection (Fig. 12). Thus, homozygous PGES cells have the potential for cell-based therapy in a significant number of individuals. Although recombination events occur between paired chromosomes in meiosis I (Kim et al., 2007), the successful derivation of stable homozygous PGES cell lines was reported (Lin et al., 2007; Revazova et al., 2008).

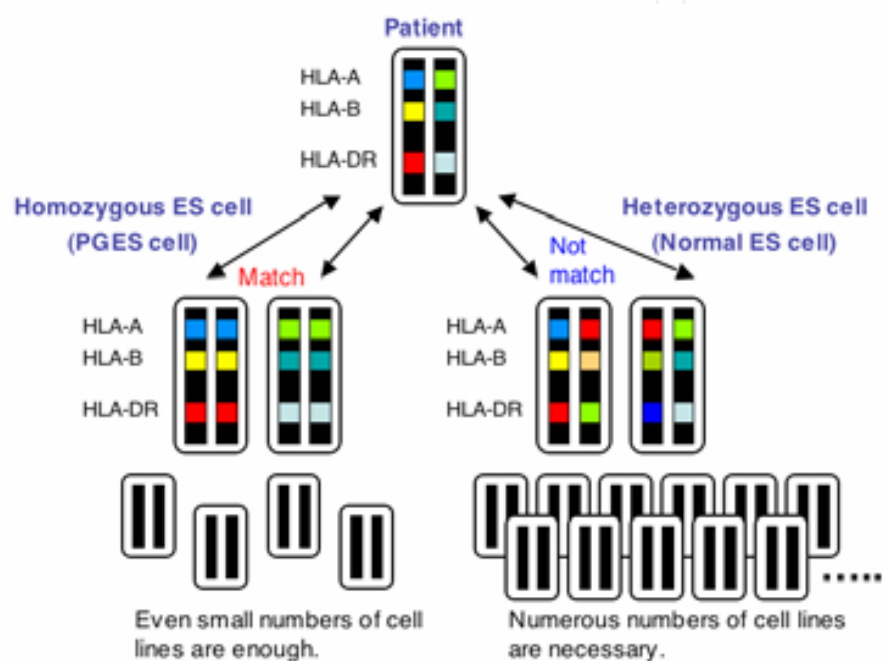


Fig. 12. HLA genotypes in homozygous and heterozygous ES cells. The most important HLA molecules to match for are the HLA class I molecules HLA-A and HLA-B, and the class II molecule HLA-DR.

PGES cells also have a merit in viewpoint of tumorigenicity. Transplanted stem cell-derived tissues occasionally forming tumors becomes a serious problem (Brickman et al., 2002). In many cases, such tumors are teratomas or teratocarcinomas arising from undifferentiated stem cells residing in the differentiated cell population that have not completed the differentiation process. A variety of approaches, such as selective pluripotent apoptotic agents (Bieberich et al., 2004), magnetic and fluorescent activated cell sorting (MACS and FACS; Shibata et al., 2006; Fong et al., 2009) and antibodies against undifferentiated stem cells (Choo et al., 2008; Tan et al., 2009), have been reported to help eliminate tumorigenesis; however, the final obstacle of teratoma formation has not been adequately addressed and remains a major safety hurdle that has to be overcome before tissue transplantations. Interestingly, primary MEFs, whose entire genome is either exclusively paternal (androgenetic) or maternal (parthenogenetic), exhibit dramatically contrasting patterns of growth and tumorigenesis (Hernandez et al., 2003). Parthenogenetic MEFs reach a lower saturation density and senesce; whereas, androgenetic and biparental MEFs increased saturation density, spontaneous

transformation, and formation of tumors. Analysis of individual imprinted genes revealed that *Igf2* regulates transformation and functions as a potent oncogene, converting primary fibroblasts into forming rapidly growing tumors. In our study, high *Igf2* expression was not detected in PGES cell-derived MEFs as well as PG-derived MEFs (Fig. 8), indicating that PGES cells rarely cause tumorigenesis. In addition, *H19*, *p57^{kip2}* and *Igf2r*, which show excessive expression in parthenogenetic cells, are candidate tumor suppressor genes (Hao et al., 1993; Matsuoka et al., 1995; De souza et al., 1995; Yoshimizu et al., 2008). Therefore, expression patterns of oncogenes and tumor suppressor genes in PGES cell-derived tissue could be one of the advantages for cell transplantation therapy (Fig. 13).

Last, summary of comparison among PGES cells and other pluripotent stem cells are shown in Fig. 14.

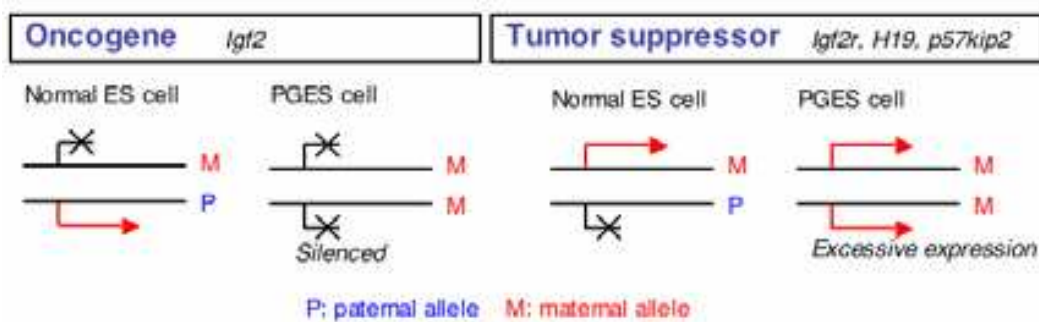


Fig. 13. Gene expression patterns of tumorigenesis related genes in PGES cells.

	PGES cell	ES cell	iPS cell
Ethics	It is not necessary to destroy viable biparental embryos.	It is necessary to destroy viable biparental embryos for establishment.	It is not necessary to destroy viable biparental embryos.
Safety	Viruses or expression plasmids is not necessary for establishment.	Viruses or expression plasmids is not necessary for establishment.	Viruses or expression plasmids is necessary for establishment.
Epigenetics	Genomic imprinting is only maternal origin. → However, PGES cells obtained more normal imprint pattern.	Genomic imprinting is generally normal . → However, aberrant methylation imprints occurred occasionally.	Genomic imprinting is generally normal . → However, aberrant methylation imprints occurred occasionally.
HLA matching	HLA homozygous cell lines are easily obtained.	HLA completely matched cell lines are rarely obtained.	HLA completely matched cell lines are always obtained.
Tumorigenicity	Low frequency of tumorigenesis?	High frequency of tumorigenesis.	High frequency of tumorigenesis.

Fig. 14. Comparison among PGES cells and other pluripotent stem cells.

6. Conclusion

In this study, we clarified epigenetic reprogramming occurred in PGES cells, resulted in nearly normal expression patterns of imprinted genes. These reprogrammed PGES cells could be used for regenerative medicine as the 3rd pluripotent stem cells. In mice, it is reported that uniparental ES cells can differentiate into transplantable hematopoietic progenitors *in vitro* that contribute to long-term hematopoiesis in recipients (Eckardt et al., 2007). The human PGES cells might be utilized for cell transplantation therapy in the near future.

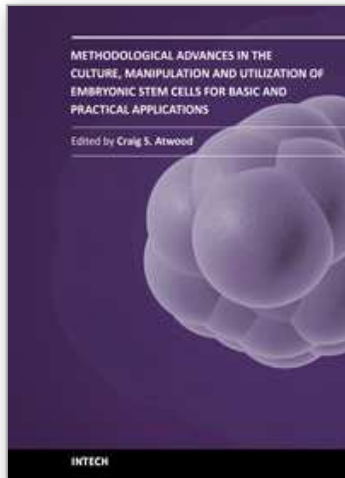
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