

—Original Article—

Naïve-like conversion enhances the difference in innate *in vitro* differentiation capacity between rabbit ES cells and iPSCs

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Abstract. Quality evaluation of pluripotent stem cells using appropriate animal models needs to be improved for human regenerative medicine. Previously, we demonstrated that although the *in vitro* neural differentiating capacity of rabbit induced pluripotent stem cells (iPSCs) can be mitigated by improving their baseline level of pluripotency, i.e., by converting them into the so-called “naïve-like” state, the effect after such conversion of rabbit embryonic stem cells (ESCs) remains to be elucidated. Here we found that naïve-like conversion enhanced the differences in innate *in vitro* differentiation capacity between ESCs and iPSCs. Naïve-like rabbit ESCs exhibited several features indicating pluripotency, including the capacity for teratoma formation. They differentiated into mature oligodendrocytes much more effectively (3.3–7.2 times) than naïve-like iPSCs. This suggests an inherent variation in differentiation potential *in vitro* among PSC lines. When naïve-like ESCs were injected into preimplantation rabbit embryos, although they contributed efficiently to forming the inner cell mass of blastocysts, no chimeric pups were obtained. Thus, *in vitro* neural differentiation following naïve-like conversion is a promising option for determining the quality of PSCs without the need to demonstrate chimeric contribution. These results provide an opportunity to evaluate which pluripotent stem cells or treatments are best suited for therapeutic use.

Key words: Embryonic stem cells (ESCs), Induced pluripotent stem cells (iPSCs), Naïve, Neural differentiation, Rabbit
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The generation of induced pluripotent stem cells (iPSCs) from human somatic cells has revolutionized the field of regenerative medicine, because use of these cells is expected to bypass the ethical concerns associated with the generation of human embryonic stem cells (ESCs) and issues of allogeneic immune rejection. However, the safety and efficacy of iPSC-derived cells must be tested rigorously using appropriate animal models and ESCs. We have successfully generated human-type (primed-state) ESCs and iPSCs from the rabbit [1, 2]. The laboratory rabbit (*Oryctolagus cuniculus*) is closer phylogenetically to primates than are rodents [3]. It has a short gestation period (31 days) and shows high fecundity. For these reasons, a number of research groups have long used the rabbit in biomedical research, and it has served as a model for several human diseases [4, 5]. Therefore, rabbit iPSCs have great potential as models for human iPSC research in many technical,

medical and biological fields. They fulfill all the requirements for the acquisition of a fully reprogrammed genomic state, showing strong similarity to ESC counterparts that we have generated recently [2]. However, although the global gene expression profile of the rabbit iPSCs became closer to that of the rabbit ESCs as the number of cell passages increased, a slight but clear difference between the two types of cell lines remained [2]. *In vitro* neural differentiation assays have been used to evaluate whether the differences in their gene expression profiles reflect their differentiation capacities [6]. The *in vitro* neural differentiation capacities of rabbit iPSC lines can vary with the donor cell type, passage number and the target cell type into which they are induced to differentiate. Although the limited early neural differentiation capacity observed in the iPSC lines was improved by continuous passaging, more mature types of neural cells—such as oligodendrocytes—were generated only poorly, even from continuously passaged iPSCs [6].

On the other hand, several attempts to improve the differentiating potential of primed-state pluripotent stem cells (PSCs) have been successful by conversion of naïve-state cells [7–10]. PSCs exist in naïve or primed states, epitomized by mouse ESCs and the developmentally more advanced epiblastic stem cells (EpiSCs) [11]. Primed-state EpiSCs are considered to represent a more advanced “differentiated” state than that shown by naïve-state ESCs. Actually, unlike naïve-state ESCs, primed-state EpiSCs are highly inefficient in repopulating the inner cell mass (ICM) upon aggregation with or

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injection into host blastocysts [12, 13]. Because human and rabbit PSCs so far have only assumed the primed state, their *in vitro* differentiation might be less effective than that of naïve-state PSCs. To overcome the limited neural differentiation capacity observed in primed-state iPSCs, we have established methods for conversion of primed-state iPSCs into a naïve-like state [6]. Indeed, naïve-like converted rabbit iPSCs effectively differentiated into the morphologically mature form of oligodendrocytes with ramified branches, which were not observed even when primed-state ESCs were induced to differentiate. Thus, the limited differentiation capacity of primed-state rabbit iPSCs was successfully improved to a similar or better extent than primed-state rabbit ESCs by naïve-like conversion [6].

Here we evaluated the naïve-like conversion of rabbit ESCs that have shown much better differentiating potential than naïve-like iPSCs. Several characteristics of naïve-like converted rabbit ESCs were assessed, including neural differentiation and the potential for chimeric pup production. Quality evaluation of rabbit PSCs will enable us to identify which type of cell is best suited for each type of human regenerative therapy.

Materials and Methods

Animals

All rabbits and mice were maintained and used for experiments in accordance with the guidelines for animal experimentation of University of Miyazaki, RIKEN Bioresource Center and Tsukuba Primate Research Center after approval by the responsible committees.

Cell culture

The method of establishment of rabbit PSCs used in this study was reported previously [2]. All of the PSCs were derived from Dutch belted rabbits. The PSCs can be divided roughly into three categories: ESCs, liver-derived iPSCs (iPS-L) and stomach-derived iPSCs (iPS-S). All rabbit iPSC lines (iPS-L1, -L2, -L3, -S1, -S2 and -S3) and ESC lines (rdES2-1, rdES4 and rdES6) were maintained by established methods [2]. Briefly, primed-state rabbit PSCs were plated onto mitomycin-C-treated mouse embryonic fibroblast layers at 37 C under 6% CO₂ in air. The culture medium consisted of 78% DMEM/Ham's F-12 supplemented with 20% knockout serum replacement (KSR) (Invitrogen Life Technologies), 1% nonessential amino acids, 0.1 mM β-mercaptoethanol and 8 ng/ml human recombinant basic fibroblast growth factor (bFGF) (Wako Pure Chemical Industries, Osaka, Japan).

Conversion of rabbits cells into the naïve-like state

To convert rabbit ESCs into a naïve-like state, the vector *CSII-EF-hOct3/4-IRES-Venus*, which drives the expression of human OCT3/4 and green fluorescent protein (GFP) under the control of the *EF1α* promoter, was introduced into rabbit ESCs and iPSCs and cultured as previously reported [6]. The culture medium consisted of 38% DMEM/Ham's F-12, 38% Neurobasal, 20% KSR, 1% N2 supplement, 2% B27 supplement, 1% nonessential amino acid (Invitrogen), 0.1 mM β-mercaptoethanol, 10 μM forskolin (Sigma-Aldrich, St. Louis, MO, USA), 5 μM kenpaullone (Calbiochem, San Diego, CA, USA), 3 μM CHIR990021 (Stemgent, Cambridge, MA, USA) and 0.1% human LIF (Wako). PSCs in a naïve-like state were passaged by

incubating the cells with 0.25% trypsin/EDTA for 3 min at 37 C. After termination of the trypsin reaction by serum treatment, the cells were disaggregated mechanically into single cells. These were then resuspended and seeded into fresh culture plates.

RT-PCR analysis

Total RNA was isolated with ISOGEN (Nippon Gene, Toyama, Japan) from cells cultured under appropriate conditions. After DNase treatment to prevent contamination with genomic DNA, the first-strand cDNA was synthesized using a Takara RNA PCR kit (TaKaRa, Shiga, Japan). The synthesized cDNA was amplified by PCR using specific primers (Supplementary Table 1: online only) with a cycle program of 94 C for 3 min and 35 cycles of 94 C for 30 sec, 58 C for 30 sec and 72 C for 30 sec. For quantitative RT-PCR, a LightCycler 96 System (Roche Applied Science, Mannheim, Germany) was used to determine mRNA expression levels using FastStart Essential DNA Green Master (Roche Applied Science) with a program of 95 C for 10 min and 45 cycles of 95 C for 10sec, 60 C for 10 sec and 72 C for 10 sec.

Alkaline phosphatase staining

Rabbit naïve-like-state ES cells were stained using alkaline phosphatase detection kits (Sigma-Aldrich) according to the manufacturer's protocol.

Teratoma formation

To generate teratomas, 1–2 × 10⁶ rabbit cells that had been converted to a naïve-like state were injected under the kidney capsules of 5–8-week-old SCID mice. At 4–8 weeks after transplantation, the teratomas were dissected out and fixed in paraformaldehyde. Paraffin wax sections were stained with hematoxylin and eosin.

In vitro oligodendrocyte differentiation

As previously reported [6], to induce oligodendrocyte differentiation, rabbit PSCs were digested with trypsin, suspended in embryoid body (EB) medium containing 78% DMEM/Ham's F-12 supplemented with 20% KSR, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM β-mercaptoethanol, 1% N-2 supplement (Invitrogen), 4 μM all-*trans*-retinoic acid (Sigma-Aldrich) and 10 μM SB431542 (Tocris Bioscience, Bristol, UK). To achieve single EBs of a uniform size, 1,000 PSCs in a volume of 100 μl were dispensed into each well of low-cell-adhesion 96-well round bottom plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA), tapped gently and cultured at 37 C under 6% CO₂ in air. To obtain differentiated oligodendrocytes, six EBs were transferred to a Matrigel (BD Bioscience, Franklin Lakes, NJ, USA)-coated 4-well Multidish (Nunc) and allowed to attach to the bottoms of the wells. The medium was then changed from EB medium to neural differentiation medium (the same formulation as EB medium, with the addition of 10% KSR). After 10 days, the medium was changed from neural differentiation medium to the culture medium without retinoic acid or SB431542 but with 100 ng/ml Noggin (Wako). The cells were cultured for a further 20 days, with fresh medium added every day.

Immunohistochemistry

Marker protein expression was analyzed by fixing the differentiated cells that had attached to the bottoms of the culture plates in 4% paraformaldehyde for 30 min at room temperature. They were then washed three times (5 min each) with Tris-buffered saline containing 1% bovine serum albumin (BSA; wash buffer). To permeabilize the cells, the cells were treated with 0.1% Triton X-100 in wash buffer for 10 min and then incubated in blocking solution (10% normal donkey serum and 1% BSA in wash buffer) for 30 min. The following primary antibodies were used: anti-O1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CNPase (Sigma-Aldrich). All antibodies were diluted with blocking solution and incubated with samples overnight at 4 °C. The next day, the cells were washed three times with wash buffer and incubated with a secondary antibody at room temperature for 1 h. The cells were washed again and covered with 50% glycerol containing DAPI. The fluorescence signals were detected with a BZ-9000 Series All-in-One Fluorescence Microscope using the BZII image analysis software (Keyence, Osaka, Japan).

Quantification of neural differentiation

The immunopositive cells were quantified using a BZ-9000 Series All-in-One Fluorescence Microscope and the BZII image analysis (Keyence). Immunopositive signals for O1 and merged signals for O1 and CNPase were calculated per unit area of each well. The area of DAPI-labeled nuclei on each image was referred to as the total cell area per unit area of the well. The immunopositive area/DAPI area is indicated as the neural differentiation index. To evaluate the difference in differentiation capacity after naïve-like conversion between ESCs and iPSCs, the relative differentiation index of naïve-like ESCs was divided by that of naïve-like iPSCs.

Evaluation of the chimeric contribution

To evaluate whether the naïve-like ESCs could contribute to the embryos (rabbit or mouse) and pups (rabbit), naïve-like rdES2-1 cells were trypsinized to dissociate into single cells or small clumps. Recipient embryos were recovered from superovulated donor animals at the 8-cell stage following natural mating (for rabbit embryos) or after *in vitro* fertilization (for mouse embryos). Naïve-like rdES2-1 cells ($n = 10\text{--}15$) were injected into the perivitelline spaces of the 8-cell embryos using a Piezo-driven micromanipulator. Two days after injection, the contributions of the injected cells to the ICM of the blastocysts were determined by the presence of GFP fluorescence. From 2–3 h after injection, reconstructed rabbit 8-cell embryos were transferred into the oviducts of pseudopregnant female rabbits that had been treated with 100 IU human chorionic gonadotropin and finger vulval stimulation 3 days before transplantation. Chimerism of the 11.5-day (mouse) and 15.5-day (rabbit) post coitus (dpc) embryos and newborn pups was determined by the presence of GFP and a black coat color.

Statistical analysis

Mean values were compared using one-way analysis of variance using SPSS Statistics ver. 19. Where appropriate, the significance of differences between means was determined with Fisher's exact probability test; $P < 0.05$ was considered significant. All experiments were analyzed in triplicate at least.

Results

Naïve-like conversion of rabbit ES cells

To evaluate the efficacy of naïve-like conversion of rabbit ESCs, which are assumed to have a higher pluripotency than that of iPSCs, three ESC lines derived from Dutch belted rabbits were subjected to naïve-like conversion using a previously reported system [6]. To convert primed-state rabbit ESCs into a naïve-like state, a lentiviral vector was used to introduce a transgene driving *hOCT3/4-GFP* expression under the control of the *EF1 α* promoter. Two or three passages after lentiviral transduction under the naïve-converting culture conditions, the colony morphology of rabbit ESCs changed to be almost indistinguishable from that of mouse ESCs, which showed transduced GFP expression and strong alkaline phosphatase activity (Fig. 1A) (Supplementary Fig. 1: online only). Although these cell lines can be maintained for more than 20 passages by single-cell dissociation with no obvious changes in their karyotype, genetically unmodified ESCs could not be maintained for longer than 2–3 passages even if the same culture conditions were used (data not shown). The mRNA expression levels of endogenous pluripotent stem cell marker genes, *OCT3/4*, *NANOG*, *KLF4*, *SOX2* and *cMYC* were maintained as naïve-like iPSCs after naïve-like conversion of rabbit ESCs (Fig. 1B). Moreover, increased expressions of the candidate genes, *KLF4* and *KLF5*, which are responsible for maintaining a naïve pluripotent state, were also confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 1C; see Supplementary methods). To evaluate the ability to differentiate into tissues representative of all three germ layers *in vivo*, naïve-like converted ESCs were transplanted into severe combined immunodeficient (SCID) mice. At 5–8 weeks after transplantation, teratomas were recovered and analyzed for the existence of three germ layers (Fig. 1D). These results confirmed the successful naïve-like conversion of rabbit ESCs.

In vitro differentiation of naïve-like ESCs into oligodendrocytes

We reported previously that ESCs and iPSCs showed almost the same *in vitro* ability to differentiate into neurons and astrocytes; however, the ability to differentiate into oligodendrocytes of the primed-state rabbit iPSCs was inferior to that of the primed ESCs. On the other hand, naïve-like iPSCs were able to differentiate into mature oligodendrocytes with dendrites, which was not observed even with the primed ESCs when differentiated [6]. It is now clear that *in vitro* differentiation into mature oligodendrocytes is a suitable indicator for assessing the quality of PSCs. Therefore, we used this system to evaluate the differentiation ability of ESCs and iPSCs before and after naïve-like conversion. The ability to differentiate *in vitro* into oligodendrocytes was evaluated among rabbit ESCs (primed and naïve-like states) and iPSCs (primed and naïve-like states; iPS-L and iPS-S cells). To detect oligodendrocytes, immunopositive signals for the anti-O1 antibody that recognizes immature oligodendrocytes and anti-O1 and anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) merged signals for detecting more mature types of oligodendrocytes were used (Fig. 2A and B) (Supplementary Fig. 2A and B: online only). As reported previously [6], although the ability of iPS-S cells to differentiate into oligodendrocytes improved to be equivalent to that of primed-state ESCs by naïve-like conversion, iPS-L cells could not be improved sufficiently even after naïve-like conversion (Fig.

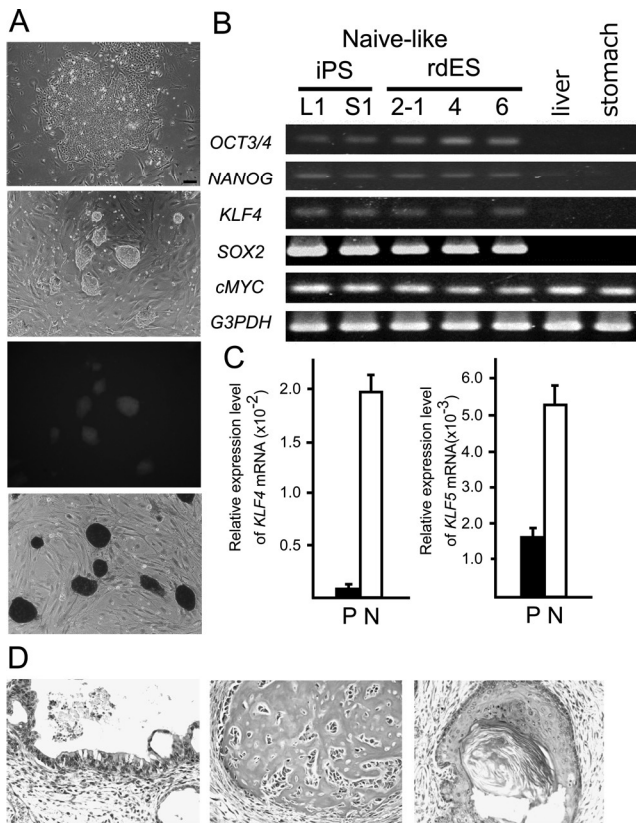


Fig. 1. Characterization of naïve-like rabbit ESCs. (A) Colony morphology of primed-state rabbit ESCs (rdES2-1 cell line) (top panel), naïve-like converted ESCs (upper middle panel), expressing hOCT3/4-IRES-GFP (lower middle panel) and demonstrating alkaline phosphatase activity (bottom panel). Scale bar = 200 μm . (B) RT-PCR analysis of pluripotent marker genes in naïve-like converted liver-derived iPS cells (iPS-L1), stomach-derived iPS cells (iPS-S1), ES cell lines (2-1, 4 and 6) and somatic cells (liver, stomach). (C) Quantitative real-time RT-PCR analysis of *KLF4* and *KLF5* expression in primed (closed bar) and naïve-like (open bar) ESCs (rdES2-1 cells). (D) Naïve-like converted ESCs were able to form teratomas containing tissues originating from the three primary germ layers: epithelium with Goblet cells (left panel; endoderm), bone (middle panel; mesoderm) and epidermis (right panel; ectoderm).

2B). Surprisingly, when naïve-like ESCs were examined, their *in vitro* differentiating capacity increased remarkably to 3.3–7.2 times that of naïve-like iPSCs (Fig. 2A–C). These results also suggested that the relative differentiation efficiencies of ESCs and iPSCs were enhanced by 1.5–2.1 times after naïve-like conversion (Fig. 2D). Interestingly, even though all ESC and iPSC lines were subjected to the same differentiating conditions, naïve-like converted rdES2-1 cells showed the best-developed ramified oligodendrocyte branches among all lines examined (Fig. 2A).

Chimeric rabbit production using naïve-like ESCs

Although naïve-like converted iPSCs have exhibited the capacity to be incorporated into the ICM of blastocysts, no chimeric contributions of these cells to more developed embryos or offspring have

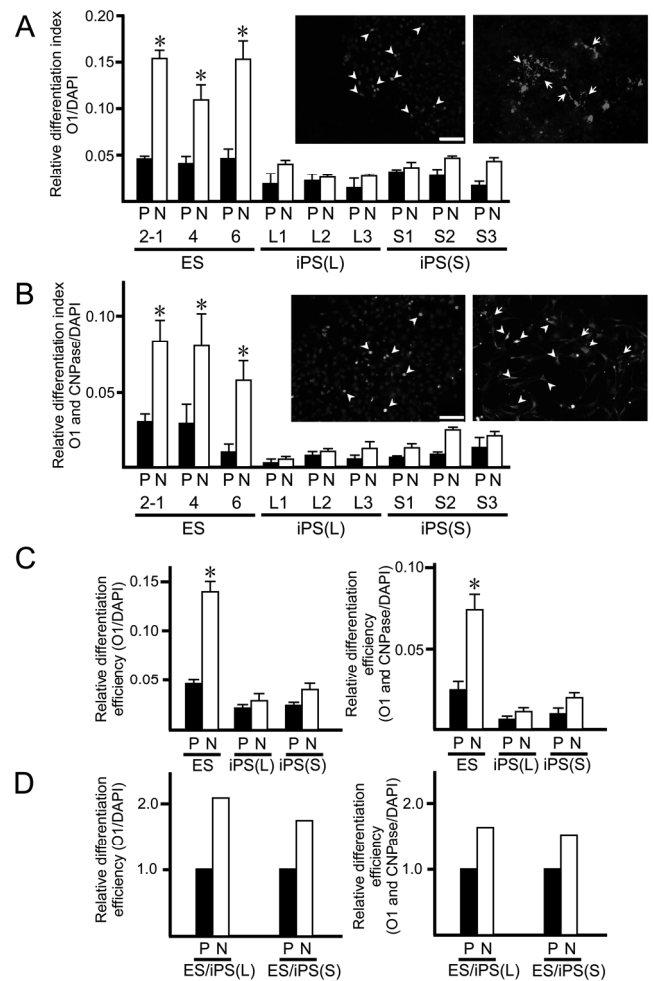


Fig. 2. Evaluation of the capacity for differentiation into oligodendrocytes among primed and naïve-like ESCs/iPSCs. Immunohistochemical detection of oligodendrocytes was evaluated using anti-O1 antibody (red) (A) and anti-O1 and anti-CNPase merged (orange) (B) signals (arrowheads) before (P) and after (N) naïve-like conversion of PSC lines. Mature oligodendrocytes with dendrites (arrows) were derived from naïve-like rdES2-1 cells (right panels). Quantification of the neural differentiation index of primed (P; closed bar) and naïve-like (N; open bar) rabbit ESC lines (rdES2-1, rdES4 and rdES6) and rabbit iPSC lines (liver-derived iPS-L1, iPS-L2 and iPS-L3 cells and stomach-derived iPS-S1, iPS-S2 and iPS-S3 cells). These signals were normalized to DAPI DNA staining signals. Data are shown as the mean \pm SE ($n = 3-7$). * $P < 0.05$. Scale bar = 100 μm . (C) Relative neural differentiation efficiencies of ESC lines and iPSC lines before (P; closed bar) and after (N; open bar) naïve-like conversion. Immunostaining signals for O1 (left graph) and O1/CNPase merged (right graph) from ESCs (rdES2-1, rdES4 and rdES6), liver-derived iPSCs (iPS-L1, iPS-L2 and iPS-L3) and stomach-derived iPSCs (iPS-S1, iPS-S2 and iPS-S3) have been averaged. Data are shown as the mean \pm SE ($n = 9-21$). * $P < 0.05$. (D) The relative differentiation efficiencies before (P; closed bar) and after (N; open bar) naïve-like conversion of ESCs and iPSCs are shown. Immunostaining signals for O1 (left graph) and O1/CNPase merged (right graph) from ES cells (rdES2-1, rdES4 and rdES6) were normalized against liver-derived iPSCs (iPS-L1, iPS-L2 and iPS-L3) or stomach-derived iPSCs (iPS-S1, iPS-S2 and iPS-S3).

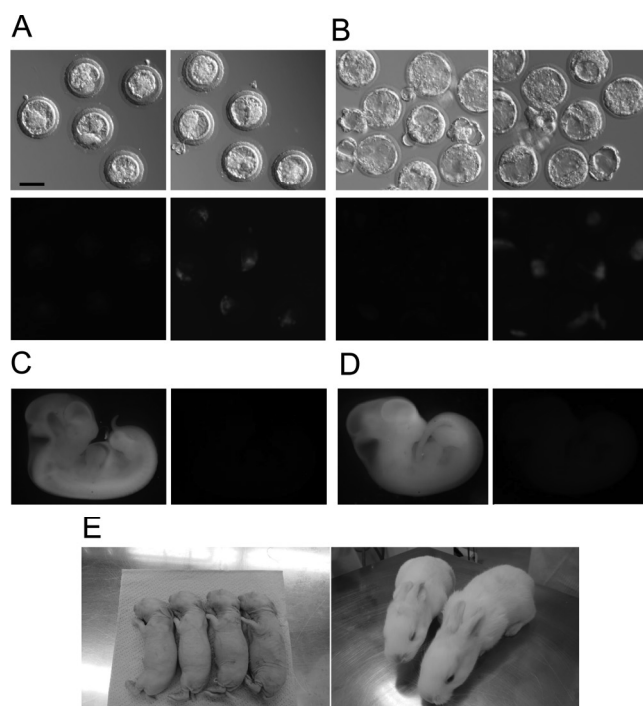


Fig. 3. Chimeric contribution of naïve-like ESCs in reconstructed embryos and pups. (A) EF1 α -OCT3/4-GFP expression in rabbit blastocysts 48 h after an injection into 8-cell rabbit embryos of primed-state rdES2-1 cells (left panels) and naïve-like rdES2-1 cells (right panels). When primed-state ESCs were injected, putatively differentiated ESCs with almost no hOCT3/4-GFP expression did not contribute to the ICM of blastocysts. Scale bar = 100 μ m. (B) EF1 α -OCT3/4-GFP expression in interspecific (rabbit and mouse) blastocysts 48 h after injection into 8-cell mouse embryos of primed-state rdES2-1 cells (left panels) and naïve-like rdES2-1 cells (right panels). (C, D) Detection of chimeric contribution by GFP signals in a reconstructed rabbit embryo (15.5 dpc) (C) and interspecific (rabbit and mouse) embryo (11.5 dpc) (D). No GFP signal was observed (right panels). (E) Detection of chimeric contribution by black coat color. No chimeric contribution was observed in the pups (skin; left panel) or does (hair; right panel).

been examined [6]. Here we examined the chimeric contribution of naïve-like PSCs to developing embryos and pups using naïve-like ESCs with the highest capacity for *in vitro* differentiation (naïve-like rdES2-1 cells). As reported using rabbit iPSCs [6], naïve-like converted ESCs were injected into rabbit 8-cell embryos and cultured *in vitro* to allow the blastocysts to develop. As with the naïve-like iPSCs, the naïve-like ESCs readily colonized the ICMs of the blastocysts, maintaining homogeneous OCT3/4-GFP activity (Fig. 3A). Moreover, these naïve-like converted ESCs were readily incorporated into mouse

blastocysts, forming interspecific chimeric rabbit/mouse embryos (Fig. 3B). Eight days after transplantation (11.5 dpc) into pseudopregnant female mice, embryos were recovered and examined for the presence of rabbit cells. However, no GFP signal was detected (Fig. 3D). The chimeric contributions of naïve-like rabbit ESCs to the developing rabbit embryos were assessed at 11 days after transplantation (15.5 dpc). Although these embryos developed normally, no GFP signal indicating a chimeric contribution was detected (Fig. 3C). Moreover, we could not obtain any evidence for chimeric contribution to these embryos even when using genomic PCR (data not shown). It is possible that the GFP signals indicating this could not be detected because of the disappearance of naïve-like ESCs at earlier stages of development *in vivo*. To convert naïve-like PSCs, we used lentivirus carrying the OCT3/4 and GFP genes under the control of the EF1 α promoter. Although this promoter is known to maintain the expression of transgenes in human ESCs [14], almost all of the GFP signals disappeared after the differentiation of rabbit iPSCs *in vitro* [6]. Because these naïve-like rdES2-1 cells had been established from Dutch belted rabbits (black skin and hair), we injected them into the embryos of Japanese White (JW) rabbits (white skin and hair) to detect any chimeric contribution. Of 105 injected embryos, 78 were transplanted into the oviducts of pseudopregnant JW rabbits. Ten of these (13%) developed to term. Six (8%) live pups were obtained (Table 1). To evaluate any possible chimeric contribution, the coat color (skin and hair) of the offspring was checked. However, none of the examined newborn pups (skin) or does (hair) had a black coat color (Fig. 3E).

Discussion

In this study, we generated naïve-like rabbit ESCs and assessed ability to differentiate *in vitro* into oligodendrocytes by comparing them with rabbit iPSCs. All of the PSC lines showed increased differentiation ability after naïve-like conversion. This highlights the differences in innate capacity among primed-state PSC lines. Although these pluripotent states can be termed “naïve-like” because of the difficulties in confirming a chimeric contribution in reconstructed offspring, our results provide a valuable strategy for the development of cell-based human regenerative medicine. We have already demonstrated that there is a relative differentiation potential of primed-state PSCs according to their origin in the following order: ESCs > stomach-derived iPSCs > liver-derived iPSCs; however, stomach-derived naïve-like iPSCs (not liver-derived naïve-like iPSCs) differentiated at an efficiency equivalent to or better than that of primed-state ESCs [6]. Here we found that naïve-like conversion of rabbit ESCs increased the differentiation capacity to a much greater level (3.3–7.2 times) than naïve-like converted iPSCs. Therefore, one of the most important points in this study is that the capacity for differentiation into oligodendrocytes of all PSCs increased after

Table 1. Development *in vivo* of embryos reconstructed using naïve-like rdES2-1 cells

Donor cells	Embryos injected	Embryos transferred	Pregnant/recipients	Retarded (% per ET)	Term conceptuses (% per ET)	Offspring (% per ET)
Naïve-like rdES2-1 cells	105	78	6/7	6 (7.7)	10 (12.8)	6 (7.7)

naïve-like conversion (Fig. 2A–C). This emphasizes the differences in innate capacity among primed-state PSC lines (Fig. 2D). Moreover, it was obvious that this relative differentiation order observed in primed-state PSCs was maintained even after naïve-like conversion.

There have been several studies on the differences in capacity of various forms of PSCs, and the patterns of global gene expression, DNA methylation and chromatin structures responsible for such variations have been analyzed carefully [15–18]. However, because of the variations between PSC lines, it is difficult to distinguish them from each other after extended culture [16]. Actually, rabbit iPSCs continued to be reprogrammed even after a number of passages, while becoming more similar to ESCs in their gene expression profiles [2]. Furthermore, repeated passages of these iPSCs permitted their differentiation into early neural cell types (neural stem cells, neurons and glial astrocytes) with efficiencies similar to ESCs [6]. Thus, *in vitro* differentiation systems seem to be effective for distinguishing differences between PSC lines [19–21]. Human iPSC lines derived from bone marrow cells, hair keratinocytes and skin fibroblasts were compared using *in vitro* differentiation into cardiomyocytes. All analyzed iPSCs could differentiate into cardiomyocytes, and the functional capacities of those derived from different cell origins were similar. However, bone marrow-derived iPSCs revealed a higher efficiency of cardiac differentiation than keratinocyte- and fibroblast-derived iPSCs [21]. On the other hand, the different potentials between ESCs and iPSCs could be overcome partly by modulating intracellular signaling pathways using chemical treatments, resulting in the efficient generation of desirable cell types, such as neural cells [22]. To understand donor cell specificity in iPSCs, it is very important to reveal the factors responsible for the differences in differentiating capacity between liver- and stomach-derived rabbit iPSCs. They need to be compared with ESCs established from liver cells and stomach cells using somatic cell nuclear transfer [23, 24].

Although the generation or conversion of naïve-like PSCs has been demonstrated using several animal species, the production of chimeric animals with a high contribution of donor cells and germline transmission has not yet been achieved [6, 25, 26]. We could not obtain any evidence of chimeric contribution in the embryos and offspring in this study, even when naïve-like rdES2-1 cells showing the best differentiating ability by *in vitro* assay were injected. These results indicated that we can now easily evaluate the quality of PSCs by neural differentiation following naïve-like conversion without the need to demonstrate chimeric contribution. We found here that the overexpression of *OCT3/4* and our culture environment were insufficient to produce true naïve (ground)-state PSCs. Although there have been several attempts to demonstrate that the overexpression of candidate genes (*KLF2* and *NANOG*) and/or that the treatment of primed-state PSCs with chemical inhibitors could produce naïve-state pluripotency [6, 10, 25, 27, 28], none of these studies confirmed this by chimeric contribution into the offspring or by germline transmission. Other techniques concerning the improved differentiation of mouse EpiSCs into ground-state PSCs have since been reported [29, 30]. These demonstrated the chimeric contribution and germline transmission of primed-state PSCs using modified culture conditions. Such indicators would confirm the derivation of true ground-state PSCs from nonrodent mammalian species. Using the rabbit, we can evaluate germline transmission

easily. Although the derivation of true naïve PSCs could not be demonstrated in the present report, our system provides a powerful potential tool for deriving cells or tissues by *in vitro* differentiation using human iPSC cells.

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