—Original Article—

Immortalized prairie vole-derived fibroblasts (VMF-K4DTs) can be transformed into pluripotent stem cells and provide a useful tool with which to determine optimal reprogramming conditions

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Abstract. The cellular conditions required to establish induced pluripotent stem cells (iPSCs), such as the number of reprogramming factors and/or promoter selection, differ among species. The establishment of iPSCs derived from cells of previously unstudied species therefore requires the extensive optimization of programming conditions, including promoter selection and the optimal number of reprogramming factors, through a trial-and-error approach. While the four Yamanaka factors Oct3/4, Sox2, Klf4, and c-Myc are sufficient for iPSC establishment in mice, we reported previously that six reprogramming factors were necessary for the creation of iPSCs from primary prairie vole-derived cells. Further to this study, we now show detailed data describing the optimization protocol we developed in order to obtain iPSCs from immortalized prairie vole-derived fibroblasts. Immortalized cells can be very useful tools in the optimization of cellular reprogramming conditions, as cellular senescence is known to dramatically decrease the efficiency of iPSC establishment. The immortalized prairie vole cells used in this optimization were designated K4DT cells as they contained mutant forms of CDK4, cyclin D, and telomerase reverse transcriptase (TERT). We show that iPSCs derived from these immortalized cells exhibit the transcriptional silencing of exogenous reprogramming factors while maintaining pluripotent cell morphology. There were no observed differences between the iPSCs derived from primary and immortalized prairie vole fibroblasts. Our data suggest that cells that are immortalized with mutant CDK4, cyclin D, and TERT provide a useful tool for the determination of the optimal conditions for iPSC establishment.
Key words: Cellular senescence, Immortalized cells, Induced pluripotent stem cell (iPSC), Pluripotency, Prairie vole
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Pluripotent stem cells maintain the ability to differentiate into cells of any tissue of the body, including germ-line cells. In 2006, Takahashi *et al.* showed that the expression of four reprogramming factors enabled terminally differentiated cells to revert to a pluripotent state, wherein they are referred to as induced pluripotent stem cells (iPSCs) [1]. This method potentially enables terminally differentiated cells derived from various animals to revert into stem cells.

Previously, we showed that embryonic cells from the prairie vole (*Microtus ochrogaster*) require the expression of six reprogram-

ming factors to efficiently transform into iPSCs [2]. In the present study, we present the detailed experimental data describing the optimization of the prairie vole iPSC protocol reported previously. We examined parameters such as promoter selection and the addition of reprogramming factors in order to determine the optimal conditions for reprogramming immortalized adult prairie vole-derived cells, allowing iPSCs to be established.

It is well known that primary cells in culture enter senescence after a threshold number of passages [3, 4], and that this dramatically decreases the efficiency with which they transform into iPSCs [5]. This suggests, therefore, that primary cells must be used at an early passage number when optimizing the reprogramming conditions necessary for the formation of iPSCs from previously unreported species. The accumulation of p16 is a recognized molecular mechanism for the development of cellular senescence. Further, we reported previously that the combined expression of mutated cyclin-dependent kinase 4 (CDK4), cyclin D, and the enzymatic subunit of telomerase reverse transcriptase (TERT) allows the cells of multiple species,

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including cattle, swine, monkeys, and prairie voles, to bypass cellular senescence [6–8]. The amino acid sequences of CDK4 and cyclin D are evolutionarily well conserved across organisms, and thus, the expression of mutated versions of these genes allows senescence signals to be bypassed in the cells of various species. We named the immortalized cells in which these genes were introduced 'K4DT cells' (mutant CD<u>K</u>4, cyclin <u>D</u>, <u>T</u>ERT). In our previous study, we showed that K4DT cells from various animals retain their original chromosomal patterns as well as their original cellular characteristics. Furthermore, as p16 cannot bind to mutant CDK4, K4DT cells do not undergo cellular senescence, and instead continue to proliferate despite an accumulation of p16 protein. This report describes the optimization process that was carried out in order to generate iPSCs from immortalized prairie vole-derived cells. Our data and the resulting cells provide an effective tool by which to optimize reprogramming conditions in various species.

Materials and Methods

Cell culture

In this study, adult prairie vole-derived primary fibroblasts and immortalized fibroblasts were used, as reported previously [6]. Immortalized cells, primary cells, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS (Mediatech, Manassas, VA), and 1% antibiotic/antimycotic mixture (Nacalai Tesque) at 37°C in a humidified atmosphere containing 5% CO_2.

Construction of recombinant lentiviral vectors

The STEMCCA vector, which expresses four reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, *c-Myc*) was kindly provided by Dr. Gustavo Mostoslavsky (Boston University School of Medicine) [9, 10]. The pCDH-CMV-MCS-EF1-copGFP vector (#CD511B-1), used to confirm the activity of the EF1 promoter, was obtained from System Biosciences (Palo Alto, CA). The PL-SIN-EOS-C(3+)-EGFP vector (#21313), which provides the fluorescent signal used to determine successful reprogramming [11], was obtained from Dr. James Ellis via Addgene (Cambridge, MA). The packaging plasmids HIV-gp and pCMV-VSV-G-RSV-Rev were provided by Dr. Hiroyuki Miyoshi (RIKEN BioResource Center, Tsukuba, Japan). Vector schematics are shown in Fig. 1A.

Lentiviral production and infection of prairie vole-derived cells

Recombinant lentiviral STEMCCA, pCDH-CMV-MCS-EF1-copGFP, and PL-SIN-EOS-C(3+)-EGFP vectors were packaged in 293T cells using transient transfection, and viruses were harvested as described previously [12]. Primary adult prairie vole-derived cells or K4DT cells were then seeded at a density of 5×10^4 cells/well in 6-well plates before infection with the recombinant viruses for 2 days, as described previously [12].

Establishment of iPSCs following transfection of the PB-EF1-6F reprogramming vector

The full coding sequences of the six reprogramming factors *Oct3/4, Sox2, Klf4, c-Myc, Lin28,* and *Nanog* were chemically synthesized before being linked by sequences encoding the self-cleaving peptides

P2A and T2A. In the cell, the reprogramming factors are therefore released as individual proteins. This expression cassette containing the six reprogramming factors was then removed from the shuttle vector by restriction digest, and ligated into a pJ547-17 piggyBac transposon-based vector (DNA2.0, Menlo Park, CA, USA). The resulting vector was designated PB-EF1-6F (Fig. 1A).

The PB-EF1-6F vector was mixed with polyethylenimine (PEI, 23966, Polysciences, Warrington, PA, USA) and then the mixture was transfected into 293T cells. After 48 hours, total protein was extracted from both PB-EF1-6F-transfected 293T cells and untransfected control 293T cells, separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting, as described previously [12]. The primary antibodies detecting the various reprogramming factors were as follows: OCT3/4 (1:5000 dilution; sc-32293, Santa Cruz, Dallas, TX, USA), SOX2 (1:10000 dilution; AB5603, Merck Millipore, Darmstadt, Germany), KLF4 (1:500 dilution; 09-821, Merck Millipore), C-MYC (1:2500 dilution; SAB4300319, Sigma Aldrich, St. Louis, MO, USA), LIN28 (1:5000 dilution; 8641, Cell Signaling, Danvers, MA, USA), NANOG (1:5000 dilution; RCAB001P, ReproCELL, Yokohama, Japan), and tubulin (1:1000 dilution; sc-32293, Santa Cruz). In all cases, following incubation with primary antibody, membranes were then probed with horseradish peroxidase-conjugated anti-rabbit IgG at a dilution of 1:2000 (NA934V, GE Healthcare, Chicago, IL, USA). Protein signals were detected using an ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare).

Transfection of the PB-EF1-6F vector into mouse embryonic fibroblasts was performed using Lipofectamine 2000 transfection reagent (11668019; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Several days after lipofection, iPSC colonies were picked and then seeded onto a mouse embryonic fibroblast (MEF) feeder plate. The mouse iPSCs were then cultured in DMEM supplemented with 15% StemSure Serum Replacement reagent (SSR; 197-16775, Wako Pure Chemical, Osaka, Japan), 1% antibiotic/antimycotic mixture, and 1000x human leukemia inhibitory factor (LIF; 125-05603; Wako Pure Chemical).

Transfection of the PB-EF1-6F reprogramming vector into K4DT cells was performed using either the transfection reagent Lipofectamine 2000 or a CUY21EDITII electroporator (BEX, Tokyo, Japan). The transfected K4DT cells were then seeded onto an MEF feeder plate. Cells were cultured in either DMEM/F12 (Wako Pure Chemical) or DMEM media supplemented with 15% SSR, 1% antibiotic/antimycotic mixture, 1000 × human LIF, 4.0 ng/ml fibroblast growth factor (FGF; KHFGF001, DS Pharma Biomedical, Osaka, Japan), 0.75 to 1.5 μM CHIR99021 (Axon1386; Axon Medchem, Groningen, Netherlands), and 0.25 to 0.5 μM PD0325901 (163-24001, Wako Pure Chemical).

Establishment of iPSCs from immortalized prairie vole-derived fibroblasts (VMF-K4DT)

Both EF1 and the CAG promoter (composed of the cytomegalovirus (CMV) early enhancer element and the first intron of the chicken beta-actin gene) are known to have strong transcriptional activity. In order to create an alternative iPSC reprogramming vector, we replaced the EF1 promoter of the piggyBac transposon-based vector PJ547-17 with the CAG promoter, and named the new vector PB-CAG-RFP-
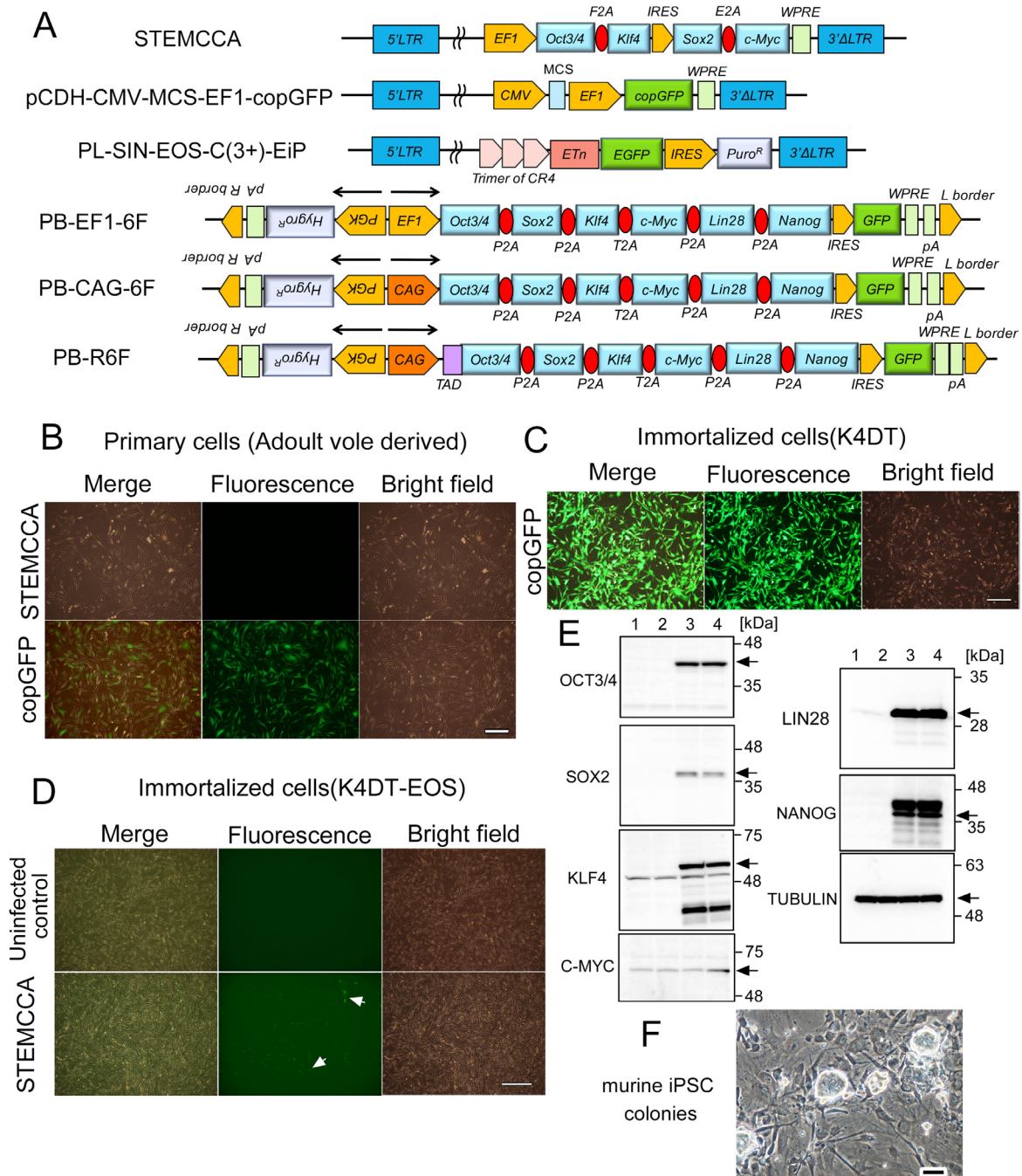
Fig. 1. Reprogramming vector schematics and their expression in primary and immortalized prairie vole cells. (A) Schematics of the six vectors used in this study. EF1: elongation factor 1 synthetic promoter; IRES: internal ribosomal entry site; GFP: green fluorescent protein; LTR: long terminal repeat; CMV: cucumber mosaic virus promoter; MCS: multicloning site; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; CR4: CR4 enhancer; ETn: early transposon element; TAD: transactivation domain; CAG: synthetic promoter composed of the cytomegalovirus (CMV) early enhancer element and the chicken beta-actin promoter; PGK: phosphoglycerate kinase promoter. (B) Infection of primary prairie vole-derived cells with the lentiviral STEMCCA and pCDH-CMV-MCS-EF1-copGFP (copGFP) vectors. Upper panels: STEMCCA vector (negative control). Lower panels: copGFP vector (surrogate marker). Scale bar = 300 μm. (C) Infection of immortalized prairie vole VMF-K4DT cells with pCDH-CMV-MCS-EF1-copGFP (copGFP) vector. Scale bar = 300 μm. (D) Detection of EOS reporter expression in immortalized prairie vole cells following the introduction of four reprogramming factors via the STEMCCA vector. Upper panels: infection-free controls. Lower panels: STEMCCA vector infected cells. Arrows indicate EOS reporter-positive cells. Scale bar = 300 μm. (E) Western blots showing protein expression of the indicated exogenous reprogramming factors, following transfection of 293T cells with the PB-EF1-6F vector. Lanes 1 and 2: infection-free controls; lanes 3 and 4: PB-EF1-6F vector-infected cells. (F) Generation of murine iPSCs. Scale bar = 50 μm.
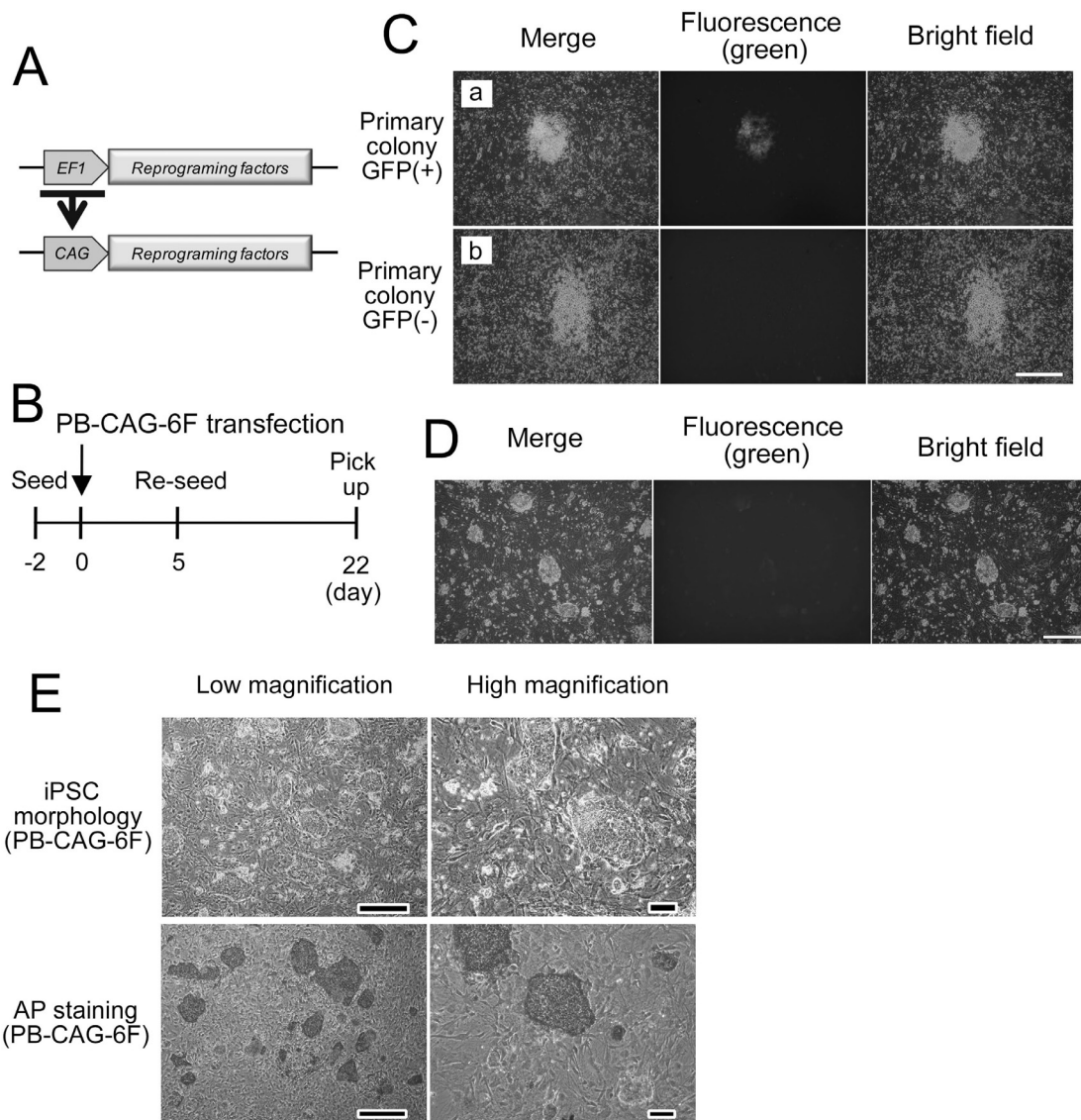
Fig. 2.   Establishment of iPSCs from immortalized prairie vole-derived cells using the PB-CAG-6F reprogramming vector. (A) Schematic representation of the reprogramming vectors, showing the replacement of the EF1 promoter with a CAG promoter for the expression of reprogramming factors. (B) Schematic representation of the experimental time course using the PB-CAG-6F reprogramming vector. (C) Morphology of primary iPSC colonies derived from immortalized prairie vole cells transformed with the PB-CAG-6F reprogramming vector. a: GFP-positive iPSC colony. b: GFP-negative iPSC colony. Scale bar = 300 µm. (D) Low levels of GFP expression in late passage iPSCs derived from immortalized prairie vole cells transformed with the PB-CAG-6F reprogramming vector. Scale bar = 300 µm. (E) Upper panels: morphology of iPSC colonies obtained from immortalized prairie vole cells using the PB-CAG-6F vector. Lower panels: AP staining of iPSCs established from immortalized prairie vole-derived cells using the PB-CAG-6F reprogramming vector. Left panels: low magnification. Scale bar = 500 µm. Right panels: high magnification. Scale bar = 100 µm.

GFP. Using restriction digest followed by ligation we then inserted the expression cassette containing the six reprogramming factors described above into the PB-CAG-RFP-GFP vector, to produce a vector named PB-CAG-6F that contained the CAG promoter and the coding sequences of the reprogramming factors *Oct3/4, Sox2, Klf4, c-Myc, Lin28,* and *Nanog*. Additionally, we created a plasmid, which we named PB-R6F, that was identical to PB-CAG-6F except that the *Oct3/4* gene was fused with the transcriptional activation

domain of MyoD [13] (Fig. 1A). Experimental time courses for the establishment of iPSCs using PB-CAG-6F and PB-R6F are shown in Figs. 2B and 3A, respectively. A detailed description of the composition of the medium used has been reported previously [2].

Staining for alkaline phosphatase activity

iPSCs derived from immortalized prairie vole-derived fibroblasts were fixed in 4% paraformaldehyde in PBS for 3 min. Alkaline

phosphatase (AP) enzymatic activity was assessed using the procedure described in our previous report [12].

Teratoma formation assays

Teratoma formation experiments were approved by the animal committee of Tohoku University (approval number 2013-AgA 003). Firstly, 1×10^6 iPSCs derived from immortalized prairie vole-derived cells transfected with the PB-R6F vector were injected into the testes of severe combined immunodeficiency (SCID) mice. Tumor formation was observed in the testes of 2 out of 3 mice after 12 weeks. The mice were euthanized; then, the tumor nodules were excised and fixed in a 4% paraformaldehyde solution. Slices of the fixed tissue were prepared and stained with hematoxylin and eosin.

Results

Introduction of the reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc into prairie vole-derived cells

Initially, we attempted to establish prairie vole iPSCs by introducing the four reprogramming factors *Oct3/4, Sox2, Klf4,* and *c-Myc* into adult prairie vole-derived primary fibroblasts via the STEMCCA lentiviral vector (Fig. 1A). The STEMCCA vector expresses these proteins using an EF1 promoter. Since the STEMCCA vector does not carry any fluorescence marker genes, and successful vector delivery was estimated by the fluorescence of copGFP expressing self-inactivated type of lentivirus, as a surrogate marker. While the infection efficiency of copGFP expressing lentivirus was enough in primary adult cells (Fig. 1B), no iPSC colonies were obtained from STEMCCA (Fig. 1B) from primary cells.

We reasoned that this inability to isolate iPSC colonies was due to sub-optimal reprogramming conditions, and so we began a series of experiments using immortalized adult prairie vole-derived fibroblasts (VMF-K4DT cells) in order to determine the optimal number of reprogramming factors and the most efficient promoter. Firstly, we evaluated whether the EF1 promoter is active in prairie vole-derived cells by introducing a recombinant lentiviral vector expressing copGFP under the control of an EF1 promoter (pCDH-CMV-MCS-EF1-copGFP; Fig 1A) into VMF-K4DT immortalized cells. Efficient copGFP expression was observed in these cells, indicating that the EF1 promoter has an enough transcriptional activity in prairie vole-derived immortalized cells (Fig. 1C). We then attempted to establish iPSCs from VMF-K4DT immortalized cells using the STEMCCA vector, which expresses four reprogramming factors. Consistent with the observations in primary vole-derived fibroblasts, no iPSC colonies were obtained using four reprogramming factors in immortalized cells (data not shown).

Next, we sought to discover why the expression of these four reprogramming factors was insufficient to produce iPSCs in our system, and we created VMF-K4DT immortalized cells that carried an EOS reporter vector. This vector, named PL-SIN-EOS-C(3+)-EiP, contains an early transposon promoter as well as *Oct4* and *Sox2* enhancer elements (Fig. 1A). Based on previously reported observations, the EOS reporter was expected to express the fluorescent protein EGFP during the early stages of iPSC reprogramming because of elevated transcriptional activity at this time [11]. Interestingly, the immortalized prairie vole-derived cells possessing this EOS reporter, termed

Table 1. Investigating different approaches for the establishment of prairie vole-derived iPSCs using immortalized VMF-K4DT cells

Reprogramming vector	Colony expression	Number of colonies picked	Number of established lines
STEMCCA	No	-	-
PB-EF1-6F	No	-	-
PB-CAG-6F	Yes	18	4
PB-CAG-R6F	Yes	5	2

K4DT-EOS cells, displayed elevated level of green fluorescence following infection with the STEMCCA lentivirus (Fig.1D). These data suggest the occurrence of the enhanced transcriptional activation of endogenous *Oct3/4* and *Sox2* following the introduction of the four reprograming factors, and that the later stages of iPSC reprogramming were not completed because of species-specific differences between mice and prairie voles.

Introduction of six reprogramming factors driven by an EF1 promoter

Previously, our group reported that the expression of six reprogramming factors, *Oct3/4, Sox2, Klf4, c-Myc, Lin28,* and *Nanog*, enabled the creation of pig iPSCs, which partially close to the naïve condition. Furthermore, we observed enhanced expression of *Nanog* as well as active status of the both of X chromosome, indicating that six reprogramming factors are advantageous rather than four [14]. To achieve expression of the six reprogramming factors in VMF-K4DT immortalized cells, we designed the piggyBac transposon-based PB-EF1-6F reprogramming vector, in which expression is driven by an EF1 promoter (Fig. 1A). The expression of the six reprogramming factors was confirmed at the protein level using Western blot analysis, following transfection of the PB-EF1-6F vector into 293T cells (Fig. 1E). Transfection efficiency was assessed by measuring green fluorescence from the internal ribosome entry site (IRES)-GFP locus located downstream of the six reprogramming factors (Supplementary Figure 1A: online only).

Next, the reprogramming ability of the PB-EF1-6F vector expressing the six reprogramming factors was assessed in mouse embryonic fibroblasts. Two to three weeks after transfection, iPSC colonies with three-dimensional morphology were observed (Fig. 1F). Furthermore, stable iPSC lines derived from these mouse iPSC colonies were established with high efficiency, indicating our reprogramming vector is fully functional.

Following these preliminary vector characterization experiments, we introduced the PB-EF1-6F vector into immortalized prairie vole-derived VMF-K4DT cells. Frustratingly, we were still unable to obtain any iPSC colonies from these cells despite using two separate transfection methods, lipofection and electroporation (Table 1).

Creation of iPSCs from immortalized prairie vole-derived cells using the PB-CAG-6F reprogramming vector

Having failed to establish iPSCs from prairie vole-derived cells using the PB-EF1-6F reprogramming vector, we modified the vector by replacing the EF1 promoter into a CAG promoter (Fig. 2A) and

transfected this vector into VMF-K4DT cells. The CAG promoter has been reported to be useful in the establishment of iPSCs [15, 16]. The resulting piggyBac transposon vector, expressing the six reprogramming factors under the control of the CAG promoter, was named PB-CAG-6F (Fig. 1A). A schematic of the experimental time course is shown in Fig. 2B. Approximately three weeks after the introduction of the PB-CAG-6F vector, two kinds of colonies formed on the feeder layer: those positive for green fluorescence protein (GFP), and those negative for GFP (Fig. 2C). As the IRES-GFP locus is located downstream of the reprogramming cassette, the GFP fluorescence intensity is representative of the level of transcription of the six reprogramming factors. Both GFP-positive and -negative colonies were harvested and reseeded onto a feeder layer for further passaging. Notably, while iPSC lines were successfully established from GFP-positive colonies (Fig. 2D), none were obtained from GFP-negative colonies. After sequential passages, the morphology of the iPSC colonies was altered such that they appeared flatter (Fig. 2E).

Although the iPSC colonies established using the PB-CAG-6F reprogramming vector were GFP-positive at early passages, the expression of GFP was significantly reduced in late passage cells (Fig. 2D). One explanation for this is that the CAG promoter has silenced the exogenously introduced expression cassette. To investigate whether established iPSCs retained their pluripotency, we assayed the activity of AP, a pluripotency marker, in these late passage cells. We found that established iPSCs stained positive for AP activity, suggesting that they had indeed retained their pluripotency (Fig. 2E).

Creation of iPSCs from immortalized adult prairie vole-derived fibroblasts using the PB-R6F reprogramming vector

Next, we attempted to establish iPSCs from immortalized adult prairie vole-derived cells using the PB-R6F reprogramming vector; this vector contained the six reprogramming factors described previously with the addition of the M_3O transactivation domain of *MyoD* fused to *Oct3/4* (giving M_3O-*Oct3/4*), which then exhibits enhanced transcriptional activity. A schematic of the experimental time course for iPSC establishment is shown in Fig. 3A. GFP-positive iPSC colonies were visible in the MEF feeder layer approximately 19 days after the introduction of the vector into the immortalized VMF-K4DT cells (Fig. 3B). As seen previously, GFP expression in the iPSCs decreased in later passages (Fig. 3C), which again could potentially be explained by CAG promoter silencing of the exogenous reprogramming cassette. As was observed with the cells carrying the PB-EF1-6F vector, iPSC colonies carrying the PB-R6F vector stained positive for AP, suggesting that they had retained their pluripotency. Importantly, the PB-R6F-transformed iPSC colonies maintained a three-dimensional morphology that was similar to that of colonies derived from primary embryonic prairie vole-derived cells (Fig. 3D).

Differentiation of immortalized prairie vole cell-derived iPSCs

Having shown that we could establish iPSCs from immortalized prairie vole cells using the PB-CAG-6F reprogramming vector, we injected the resulting iPSCs into mouse testes in order to evaluate their differentiation ability. Three to five months later, tumors were formed in the testes (Fig. 4A), and we observed the formation of teratomas in 67% of the three implanted mice. Histological analysis revealed the presence of multiple tissues derived from the endoderm,
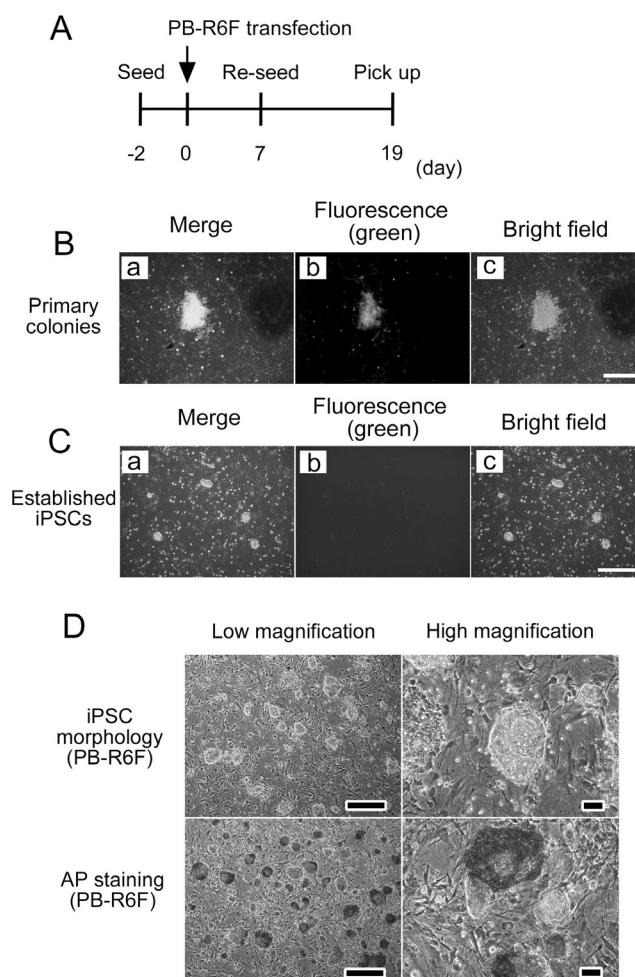

Fig. 3. Establishment of iPSCs from immortalized prairie vole-derived cells using the PB-R6F reprogramming vector. (A) Schematic representation of the experimental time course using the PB-R6F reprogramming vector. (B) Morphology of primary iPSC colonies established from immortalized prairie vole cells using the PB-R6F vector. a: merged images; b: green fluorescent images using GFP; c: bright field images. Scale bar = 300 μm. (C) Morphology of established iPSC colonies generated from immortalized prairie vole cells using the PB-R6F vector. a: merged images; b: green fluorescent images using GFP; c: bright field images. Scale bar = 300 μm. (D) Upper panels: morphology of iPSC colonies established from immortalized prairie vole cells using the PB-R6F vector. Lower panels: AP staining of these iPSC colonies. Left panels: low magnification. Scale bar = 500 μm. Right panels: high magnification. Scale bar = 50 μm.

mesoderm, and ectoderm, confirming that all three germ layers were represented (Fig. 4B), and that the VMF-K4DT-derived iPSCs, established using the PB-CAG-6F vector, retained their pluripotency.

Discussion

In this study, we report the establishment of iPSCs derived from immortalized prairie vole cells using piggyBac transposon-based vectors. Previously, we reported the establishment of iPSCs from
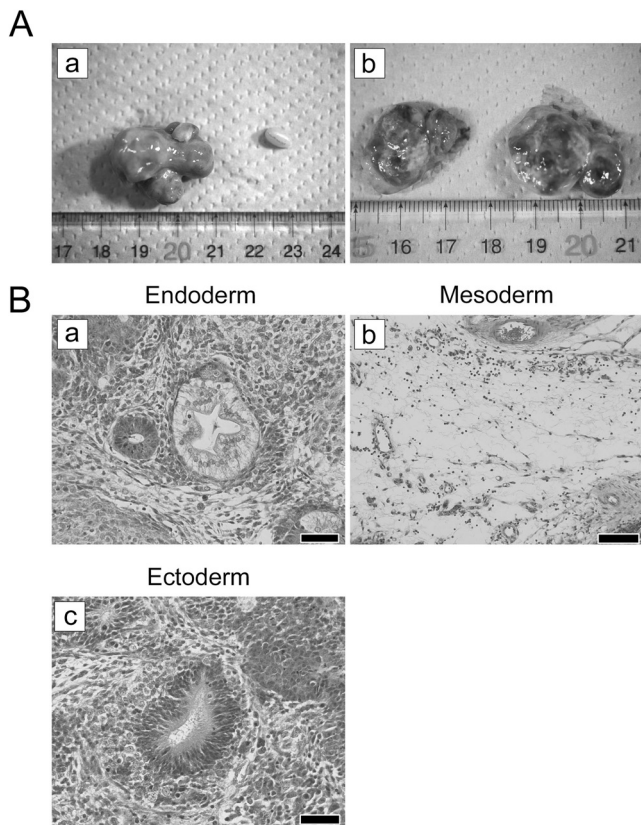
A

B

Endoderm

Mesoderm

Ectoderm

Fig. 4. Formation of teratomas from iPSCs derived from immortalized prairie vole cells transformed with the PB-6F reprogramming vector. (A) Photographs of teratomas derived from iPSCs that were established from immortalized prairie vole cells using the PB-6F vector. a: gross appearance of the teratoma tissue; b: dissected teratoma. (B) Histological analysis of the teratomas shown in (A). a: gastrointestinal tract-like tissue (endoderm, scale bar = 50 μm); b: adipose-like tissue (mesoderm, scale bar = 100 μm); c: neural tube-like tissue (ectoderm, scale bar = 50 μm).

embryonic prairie vole-derived primary cells [2], but this was only made possible by the extensive optimization of experimental conditions using immortalized adult prairie vole cells described in the present study. The prairie vole is a useful animal model with which to analyze social behaviors such as pair bonding [17, 18]. Initially, we attempted to establish iPSCs from both primary adult prairie vole cells and immortalized K4DT cells using the four Yamanaka reprogramming factors, *Oct3/4*, *Klf4*, *Sox2*, and *c-Myc* (Fig.1); however, in both cases, interspecies differences between mice, humans, and prairie voles meant that iPSCs could not be established successfully. Consistent with this, it was previously reported that prairie vole embryonic fibroblast-derived iPSCs, generated using the four Yamanaka reprogramming factors, were established with low efficiency, with only 11 of 2800 primary colonies going on to form an established line [19].

It was therefore necessary to use immortalized cells to optimize the iPSC establishment conditions, including the number of reprogramming factors and the most efficient promoter. These data were

not included in our previous article [2]. The cells used here were immortalized by the expression of mutated CDK4, cyclin D, and TERT. When primary cells are passaged sequentially, they enter senescence and accumulate p16 protein, which in turn binds to CDK4 and negatively regulates the kinase activity of the CDK4-cyclin D complex [20]. In the immortalized cells, the CDK4 R24C mutant does not bind to p16 and thus avoids negative regulation. The amino acid sequences of CDK4 and cyclin D are evolutionarily well conserved, meaning that this immortalization method could potentially be applied to various other species, including pigs, cattle, and monkeys in addition to prairie voles [6–8]. Furthermore, the immortalized prairie vole cells retained both the chromosomal pattern and differentiation ability of the primary cells from which they were derived. Taken together, this suggests that cells immortalized by the expression of mutant CDK4, cyclin D, and TERT provide a useful tool with which to determine the optimal conditions for iPSC establishment.

We successfully obtained iPSCs from immortalized prairie vole cells using two different piggyBac transposon-based vectors that utilize a CAG promoter (Table1). In both cases, the expression of the IRES-GFP locus, located downstream from the reprogramming factor cassette, was lower after sequential passage than in primary colonies; this decrease could potentially be explained by the silencing of the exogenous promoter. The same phenomenon was observed in later passages of iPSCs derived from primary prairie vole cells [2], indicating that immortalized cells had retained the original characteristics of the primary prairie vole cells from which they were derived.

We found that the morphology of late passage iPSC colonies depended on the presence of the M_3O-*Oct3/4* fusion gene. While iPSCs created using the PB-CAG-6F vector, containing native *Oct3/4*, formed flatter colonies, those created using the PB-R6F vector, which contained the M_3O-*Oct3/4* fusion, allowed the establishment of three-dimensional iPSC colonies that were morphologically similar to those derived from primary prairie vole cells [2]. These results confirm the usefulness of immortalized prairie vole fibroblasts in the optimization of the conditions for iPSC establishment. Such an approach reduces the number of animals that need to be sacrificed during this optimization, and thus presents ethical and animal welfare advantages. Furthermore, this method is also less time consuming of the scientist than traditional methods. Finally, using immortalized cells to optimize iPSC reprogramming conditions might prove especially useful when considering rare or endangered animal species, whose primary cells are in limited supply.

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