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Original Article

Generation of footprint-free, high-quality feline induced pluripotent stem cells using Sendai virus vector

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ABSTRACT

Companion animals, such as felines and canines, could provide an excellent platform for translational research from veterinary to human medicine. However, the use of feline induced pluripotent stems (fiPSCs) of quality in basic or clinical research has not been reported. Here, we generated footprint-free fiPSCs derived from embryonic cells, as well as juvenile feline uterus-derived cells using Sendai virus vector harboring six feline-specific pluripotency-associated genes. The fiPSCs were confirmed to be of high quality with the potential to form teratomas including all three germ layers. Furthermore, our fiPSCs were maintained under feeder-free and chemically-defined conditions using StemFit® AK02N and recombinant laminin 511, iMatrix-511. Further research on fiPSCs could result in their widespread application in veterinary regenerative medicine, which could pave the way for their use in advanced regenerative medicine research for humans.

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1. Introduction

Induced pluripotent stem cells (iPSCs), reprogrammed from somatic cells, share characteristics with embryonic stem cells (ESCs). In humans, iPSCs hold potential for use in regenerative medicine, disease modeling, and drug discovery with clinical research already underway [1–3]. However, the lack of a suitable pre-clinical model represents a challenge, and traditional rodent models may not be adequate owing to genetic and environmental differences from humans [4,5].

Companion animals such as cats, which share human-like complexity of the living environments, genetic diversity, and physiology, often are affected by naturally occurring diseases similar to those humans [6,7]. As a result, veterinary patients exhibit symptoms, natural histories, pathologies, gene associations, molecular phenotypes, environmental risk factors, and responses to medications that are similar to those observed in humans [8]. Companion animals can therefore serve as clinically relevant models for human diseases, facilitating faster clinical research and acting as a critical link between preclinical and clinical research. This translational approach has the potential to simultaneously propel forward both human and veterinary medicine. While cats may represent and provide ready-to-use pre-clinical models [9], iPSC research in cats is lagging considerably behind that in humans.

Previous studies have reported the generation of feline iPSCs (fiPSCs) using retrovirus or lentivirus [10–12], which randomly

Abbreviations: (fiPSCs), feline induced pluripotent stem cells; (ciPSCs), canine iPSCs; (iPSCs), Induced pluripotent stem cells; (ESCs), embryonic stem cells; (MEFs), mouse embryonic fibroblasts; (FBS), fetal bovine serum; (KSR), knockout serum replacement; (SeV), Sendai virus; (EGFP), enhanced green protein fluorescence; (MOI), multiplicity of infection; (LIF), Leukemia inhibitory factor.

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insert exogenous genes into the genome of host cells, posing a risk of tumorigenesis [13]. Additionally, fiPSCs were maintained using mouse embryonic fibroblasts (MEFs) and fetal bovine serum (FBS) or knockout serum replacement (KSR)-containing medium. These xenogeneic components increase the risk of immune rejection in clinical settings and prevent research reproducibility [14]. Reliable approaches for the generation of footprint-free and high-quality fiPSCs, and as well as their maintenance under conditions with reduced xenogeneic components are thus essential for developing further downstream applications.

Sendai virus (SeV) vectors have been effectively used for generating footprint-free, high-quality iPSCs in human and canine cells, as demonstrated in various studies [15,16]. We also found that the use of novel SeV vectors with optimized genetic structure could maximize the reprogramming efficiency [17,18]. Furthermore, we recently demonstrated that the introduction of six species-specific reprogramming genes—*LIN28A*, *NANOG*, *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC*—significantly accelerates cell reprogramming kinetics to pluripotency, when compared to species-promiscuous genes [18]. Extending this approach, the current study focuses on establishing a robust strategy for generating footprint-free fiPSCs using a novel SeV encoding the same six feline-specific factors. Furthermore, we explored fiPSC culture in feeder-free conditions, offering their significant potential for both disease modeling and regenerative medicine.

2. Materials and methods

2.1. Animals and ethical statements

This study was approved by the Ethical Review Committee for Clinical Research of the Veterinary Clinical Center of Osaka Metropolitan University (permission numbers: R5-002) and the Institutional Animal Experiment Committee of Osaka Metropolitan University (permission numbers: 22–73, 22–74, 23–53, 23–56). We performed this study according to the Animal Experimentation Regulations of Osaka Metropolitan University.

2.2. Culture medium composition

The feeder medium (FM) was composed of high-glucose Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beik Haemek, Israel), 2 mM L-glutamine (Nacalai Tesque), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). N2B27 medium consisted of DMEM/Nutrient Mixture F-12 Ham (Nacalai Tesque) supplemented with N2 supplement (1×, Thermo Fisher Scientific, Waltham, MA, USA), B27 supplement (1×, Thermo Fisher Scientific), GlutaMAX (1×, Thermo Fisher Scientific), 0.1 mM minimal essential medium non-essential amino acids (MEM NEAA; Thermo Fisher Scientific), and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Small molecule compounds contained 10 μM Y-27632 (Nacalai Tesque), 0.5 μM PD0325901 (Reprocell, Kanagawa, Japan), 3 μM CHIR99021 (Nacalai Tesque), 0.5 μM A83-01 (Nacalai Tesque), referred to as 4SMs, or 10 μM Forskolin (Nacalai Tesque), and 50 μg/mL L-ascorbic acid (Sigma-Aldrich) in addition to 4SMs, thereby referred to as 6SMs. The embryoid body (EB) medium consisted of 10% FBS (Sigma-Aldrich), 10% KSR (Thermo Fisher Scientific), 1 mM sodium pyruvate (Nacalai Tesque), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM MEM NEAA, and 0.1 mM 2-mercaptoethanol. STAPEL medium consisted of 50% Iscove's Modified Dulbecco's Medium (Nacalai Tesque) and 50% Ham's F-12 nutrient mixture (Nacalai Tesque) supplemented with 5% protein-free hybridoma mixture II (Thermo Fisher Scientific), 0.5% (v/v) bovine serum albumin (A3311; Sigma-Aldrich), 0.5% (v/v) polyvinyl alcohol (Sigma-Aldrich), 0.45 mM α-monothioglycerol (Sigma-Aldrich), insulin-transferrin-selenium-ethanolamine supplement (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 125 ng/mL linoleic acid (Sigma-Aldrich), 125 ng/mL linolenic acid (Sigma-Aldrich), 50 μg/ml L-ascorbic acid (Sigma-Aldrich), 50 μg/ml ascorbic acid 2-phosphate (Fujifilm Wako Pure Chemical Corporation), 1% GlutaMAX (Thermo Fisher Scientific), and SynthChol (Sigma-Aldrich).

2.3. Preparation and culture of feeder cells and feline cells

MEFs were isolated from the fetuses of ICR mice (Japan SLC, Shizuoka, Japan). FEFs were isolated from intrauterine fetuses. The head, visceral tissues, and liver were removed from the fetus. The remaining tissues were cut into small pieces and cultured in FM. MEFs and FEFs were cultured in the FM in a 37°C humidified incubator supplemented with 5% CO_2. Feline uterus-derived cells were isolated from a whole uterus provide from a 6-months-old healthy cat that was not infected by the feline immunodeficiency virus and the feline leukemia virus. The cells were isolated by cutting the uterine tissue into small pieces and homogenizing with trypsin-EDTA (Sigma-Aldrich). Uterus-derived cells were cultured in FM supplemented with 5 ng/mL basic fibroblast growth factor (bFGF, Nacalai Tesque). MEFs, FEFs, and uterus-derived cells were passaged using 0.25% trypsin-EDTA and stored at −80°C using BAMBANKER® (Nippon Zenyaku Kogyo, Fukushima, Japan).

2.4. Reprogramming of feline somatic cells using SeV

FEFs or feline uterus-derived cells were incubated with SeV vector encoding six feline genes at a MOI of 1 (day −1) at 25°C for 2 h and then at 32°C for 16–18 h. The next day, SeV-infected FEFs were reseeded on an MEF-coated dish at a density of 1×10^3 cells/cm^2 in the feeder medium (FM) without L-glutamine (day 0). For selection of SeV-infected cells, these were cultured in the FM without L-glutamine, supplemented with 5 μg/mL puromycin for two days before reseeding, and were then reseeded on an MEF-coated dish at a density of $5–7 \times 10^3$ cells/cm^2. The next day, the medium was changed to N2B27 medium containing small molecule compounds and 10 ng/mL bFGF (Nacalai Tesque). The cells in N2B27 medium were then cultured for 3 days (days 1–4). On day 4, the medium was replaced with StemFit AK02N (Ajinomoto, Tokyo, Japan), and the cells were cultured until primary colonies were picked.

2.5. fiPSCs maintenance

All fiPSCs were maintained using iMatrix-511 (Nippi, Tokyo, Japan) and StemFit AK02N. fiPSCs were passaged as cell clumps using a Pasteur pipette or cell scraper, or as single cells using TrypLE™ Select (Thermo Fisher Scientific, Waltham, MA, USA). For cryopreservation, fiPSCs were dissociated with TrypLE™ Select and stored at −80°C or −276°C using STEM-CELLBANKER® (Nippon Zenyaku Kogyo, Fukushima, Japan).

2.6. siRNA procedure for removing SeV vector

To remove SeV, siRNA was applied one day after passage using RNAi MAX (Thermo Fisher Scientific). siRNA procedure was repeated at every passage until EGFP$^-$ colonies emerged. The removal of SeV was confirmed using qRT-PCR. The sequences of siRNA and primers for SeV are listed in Table S1.

2.7. Alkaline phosphatase staining

fiPSCs were stained with an Alkaline Phosphatase Staining Kit II (REPROCELL, Kanagawa, Japan) according to the manufacturer's instructions.

2.8. In vitro differentiation assay

The *in vitro* differentiation ability of fiPSCs was evaluated as spontaneous differentiation via EB formation. fiPSCs were dissociated into single cells using TrypLE Select and then cultured in Costar® 6-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates (Corning, Inc., Corning, NY, USA) containing EB medium. After seven days, EBs were cultured in EB medium on gelatin-coated slides for seven days, and were then fixed and immunolabeled. Alternatively, dissociated fiPSCs were seeded in Nunclon™ Sphera™ 96-Well U-Shaped-Bottom Microplate (Thermo Fisher Scientific) at a density of 4.0×10^4 cells per well in EB medium and were maintained for 12 days. For endoderm differentiation, EBs were cultured in STAPEL medium [19] supplemented with 100 ng/mL Activin A (Nacalai Tesque) for three days. RNA was then extracted, whereafter qPCR and immunostaining were performed.

2.9. RT-PCR and quantitative RT-PCR

Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Reverse transcription (RT) was performed using random primers and ReverTra Ace (Toyobo, Osaka, Japan). Polymerase chain reaction (PCR) was performed using a Blend Taq Plus (Toyobo). PCR products were resolved on a 2% agarose gel stained with ethidium bromide and observed using an ultraviolet transilluminator (AE-9020; ATTO, Tokyo, Japan).

To quantify mRNA expression levels, PCR was performed using Taq Pro Universal SYBR qPCR Master Mix (Nanjing Vazyme Biotech, Nanjing, China) and a Step One Plus Real-Time PCR System (Thermo Fisher Scientific). *β-ACTIN* was used as a normalization control gene, and relative gene expression levels were calculated via the ΔΔCt method. All primers are listed in Table S1.

2.10. Immunocytochemistry

The cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Tween 20 or Triton-X 100 in PBS (−), and blocked with 10% bovine serum albumin (Nacalai Tesque). The cells were then incubated with primary antibodies at 4°C overnight. Negative control cells were incubated in PBS (−) without primary antibodies. The next day, the cells were washed, incubated with appropriate secondary antibodies at 25°C for 1 h, and mounted using ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific) to label DNA. Immunolabeled cells were observed using a confocal laser-scanning microscope (FV3000; Olympus, Tokyo, Japan). All antibodies are listed in Table S2.

2.11. Flowcytometry

For staining cell surface markers, the cells were labeled in FACS buffer with primary antibody or isotype control for 30 min on ice. FACS buffer comprises PBS (−), 2% FBS, 1 mg/mL sodiumazide (Fujifilm Wako Pure Chemical Corporation), and 0.5 mM EDTA. The cells were washed and labeled with appropriate secondary antibodies in FACS buffer for 15 min on ice. For staining intracellular makers, the cells were fixed in 4% paraformaldehyde, permeabilized with ICC buffer, which comprises PBS (−), 5% FBS, 0.1% Triton-X 100, and 1 mg/mL sodiumazide. The cells were then incubated with primary antibodies or isotype controls in ICC buffer at 4°C overnight. On the next day, the cells were washed and incubated with appropriate secondary antibodies in ICC buffer at 4°C for 30 min. The stained cells were analyzed using CytoFLEX (Beckman Coulter, Brea, CA, USA). All antibodies are listed in Table S3.

2.12. Teratoma formation assay

Approximately 1×10^6 fiPSCs were injected into the testis capsule of NOD/SCID mice. The mice were euthanized by cervical dislocation after three months, and the tumors were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

2.13. Karyotyping analysis

The fiPSCs were incubated with 0.05 μg/mL colcemid (Thermo Fisher Scientific) for 1 h, trypsinized, and incubated with 0.075 M KCl at 37°C for 20 min. The cells were fixed in acetic acid: methanol (1:3), stained with quinacrine mustard and Hoechst 33258, and observed using confocal laser-scanning microscopy (LSM980; Carl Zeiss, Oberkochen, Germany).

2.14. Statistical analysis

Statistical significance was assessed via Tukey–Kramer multiple comparison using SPSS software (SPSS25; SPSS, Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Development of SeV encoding feline pluripotency-associated genes

Recently, a high-quality chromosome-scale assembly of the domestic cat, known as AnAms1.0, became publicly available [20]. We compared the feline pluripotency-associated genes, *LIN28*, *NANOG*, *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* in AnAms 1.0 to the respective reference domestic cat sequences (Felis catus, felCat9) in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/, LIN28: NM_001173445, NANOG: NM_001173442, OCT3/4: NM_001173441, SOX2: NM_001173447, KLF4: NM_001173444, C-MYC: NM_001173446). For LIN28, NANOG, and SOX2, there was only a single nucleotide difference between AnAms1.0 and the reference sequences (Fig. S1). For all the other genes, the sequences were identical. Despite the minor difference in gene sequence, the amino acid sequences for all genes matched between AnAms 1.0 and the reference. Therefore, we built the SeV vector encoding enhanced green protein fluorescence (*EGFP*), puromycin resistance genes, and the six genes above using the reference sequences, according to a previously reported method [18].

3.2. Reprogramming of FEFs using SeV

We previously reported that a small molecule cocktail, consisting of TGFβ, GSK3β, MEK, and Rock inhibitors (4SMs), with the addition of Forskolin and ascorbic acid (6SMs), was adequate for reprogramming canine embryonic and juvenile somatic cells, respectively [18]. We also reported that ciPSCs could be maintained under feeder-free and chemically-defined conditions using StemFit AK02N medium and Lamnin511 E8 fragment (iMatrix-511) [21]. In this study, we first attempted to generate fiPSCs from feline embryonic fibroblasts (FEFs) using a similar approach with some modifications (Fig. 1A).

In both cases, using 4SMs or 6SMs, primary colonies emerged around seven days after seeding and some of them grew enough to be picked up (Fig. 1B). After subculturing and maintaining them with StemFit AK02N and iMatrix-511, we performed a siRNA procedure to remove SeV. Following the siRNA procedure, some cells in the colony became negative for EGFP (Fig. 1D). After repeating the siRNA procedure 2–3 times, we obtained EGFP-negative subclones

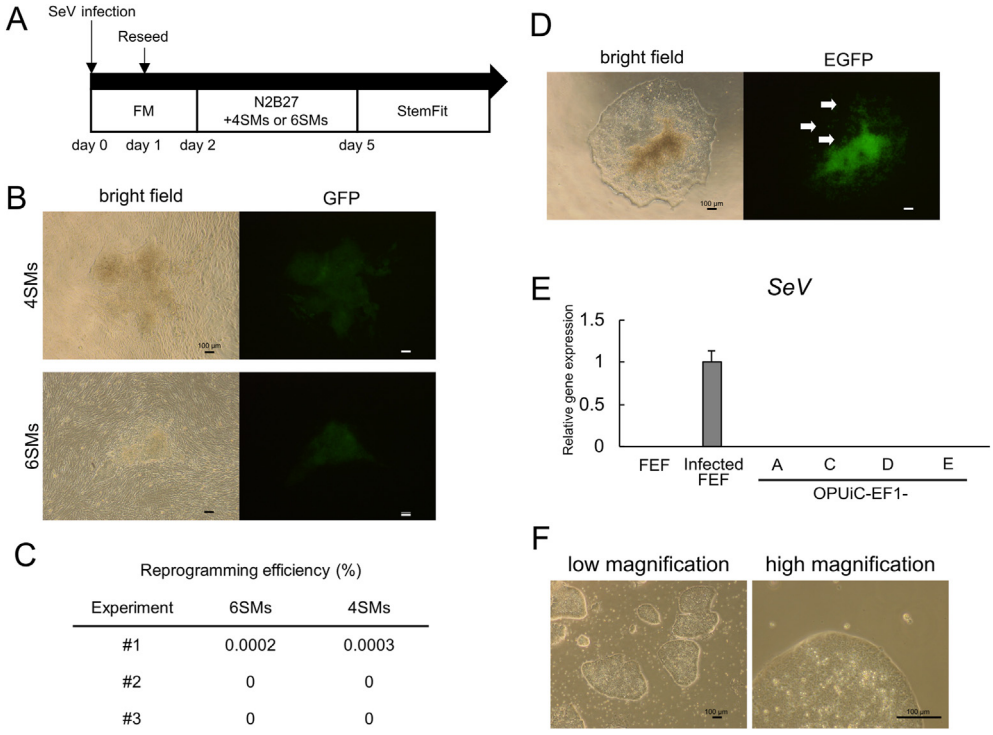

Fig. 1. Reprogramming of FEFs using SeV. (A) Scheme of FEF reprogramming using SeV. FM: Feeder medium. (B) Morphology of primary colonies obtained under 6SMs and 4SMs conditions. Panels on the right show EGFP expression. Black and white scale bar = 100 μm. (C) Reprogramming efficiencies of both 6SMs and 4SMs conditions in respective experiments (n = 3). (D) Morphology of fiPSCs after siRNA treatment. Right panels show EGFP expression. White arrow shows $EGFP^{-}$ undifferentiated cells. Black and white scale bar = 100 μm. (E) qRT-PCR of each fiPSC line for SeV. One fiPSCs generated under 6SMs condition (OPUiC-EF1-A and three fiPSCs generated under 4SMs condition (OPUiC-EF1-C, -D, and -E) did not contain SeV after siRNA treatment. FEFs and infected FEFs as negative and positive controls, respectively. *β-ACTIN* was used as the housekeeping gene. Relative gene expression for each fiPSC line. Data are shown as the mean ± standard deviation (n = 3). (F) Morphology of OPUiC-EF1-C at passage 10. Scale bar = 100 μm.

and successfully generated 5 SeV-free fiPSC lines (OPUiC-EF1-A and -B generated with 6SMs; OPUiC-EF1-C, -D, and -E generated with 4SMs). Although OPUiC-EF-B line could not be cultured stably, the analyzed 4 lines were confirmed negative for SeV using qRT-PCR (Fig. 1E) and successfully maintained under the feeder-free condition. They exhibited morphologies similar to human pluripotent stem cells, even after the withdrawal of exogeneous gene expression (Fig. 1F).

3.3. Characterization of fiPSCs derived from FEFs using SeV

fiPSC lines were positive for alkaline phosphatase (AP) (Fig. 2A), and they also expressed pluripotency markers, as determined via qRT-PCR, immunocytochemistry, and flow cytometry (FCM) (Fig. 2B–D). fiPSCs were positive for OCT3/4, NANOG, SOX2, and SSEA-4 and partially positive for TRA-1-60, while negative for SSEA-1, SSEA-3, and TRA-1-81 (Fig. 2C and D). To assess their capacity for differentiation into all three germ layers *in vitro*, EBs were formed via suspended culture. The ectodermal and mesodermal, but not endodermal, marker expression was detected by qPCR and immunocytochemistry when the fiPSCs were spontaneously differentiated by forming EBs in the EB medium without growth factors (Fig. 2E and F). The endodermal differentiation by adding Activin A could induce the endodermal marker expression (Fig. 2E and F). Furthermore, these fiPSC lines had teratoma-forming capacity after transplantation into immune-deficient mice (Fig. 2G). Additionally, karyotyping of fiPSCs revealed that all fiPSC lines had normal 36+XY karyotypes with matched autosomes (Fig. 2G). The percentage of cells with normal karyotypes was 89%–100% (OPUiC-EF1-A: 12/13, OPUiC-EF1-C: 11/12, OPUiC-EF1-D: 8/9, OPUiC-E1-E: 11/11). However, we could not confirm experimental reproducibility in experiment #2 and #3, as shown in Fig. 1C, suggesting further adaptation of the reprogramming strategy.

3.4. Modification of the FEFs reprogramming method for improving reproducibility

Although we successfully generated footprint-free fiPSCs, we could not confirm the experimental reproducibility (data not shown). Additionally, we observed that the efficiency of SeV infection remained low, despite the multiplicity of infection (MOI) being as high as 5 (Figs. S2A and S2B). Considering the experimental costs, we sought to enhance reproducibility by incorporating a puromycin selection phase for SeV–infected FEFs, instead of increasing the MOI (Fig. 3A). During the selection step, $EGFP^{+}$ cells gradually died under the 4SMs condition, resulting in no primary colonies (data not shown). In contrast, under the 6SMs condition, we could obtain some $EGFP^{+}$ primary colonies with consistent reproducibility. Primary colony morphologies were divided into two types: Type I colonies were flat and tightly packed, with clear borders (Fig. 3B). This morphology suggested that the colonies were completely reprogrammed, similar to those observed in human cells [22]. Type II colonies were dome-shaped in the center and fibroblast-like in the periphery, with indistinct borders (Fig. 3B), indicating that these colonies had undergone partial reprogramming, akin to the process observed in humans [22]. The type I and II colony forming efficiency was 0.0095 ± 0.0018% and 0.023 ± 0.0053%, respectively (n = 3) (Fig. 3B). Furthermore, the type I colonies could be passaged more than ten times, whereas type II colonies could be passaged only a few times. The two fiPSC lines, OPUiC-EF1-F and G, were established from type I colonies. The OPUiC-EF1-F were confirmed the SeV elimination while SeV gene expression were detected in the OPUiC-

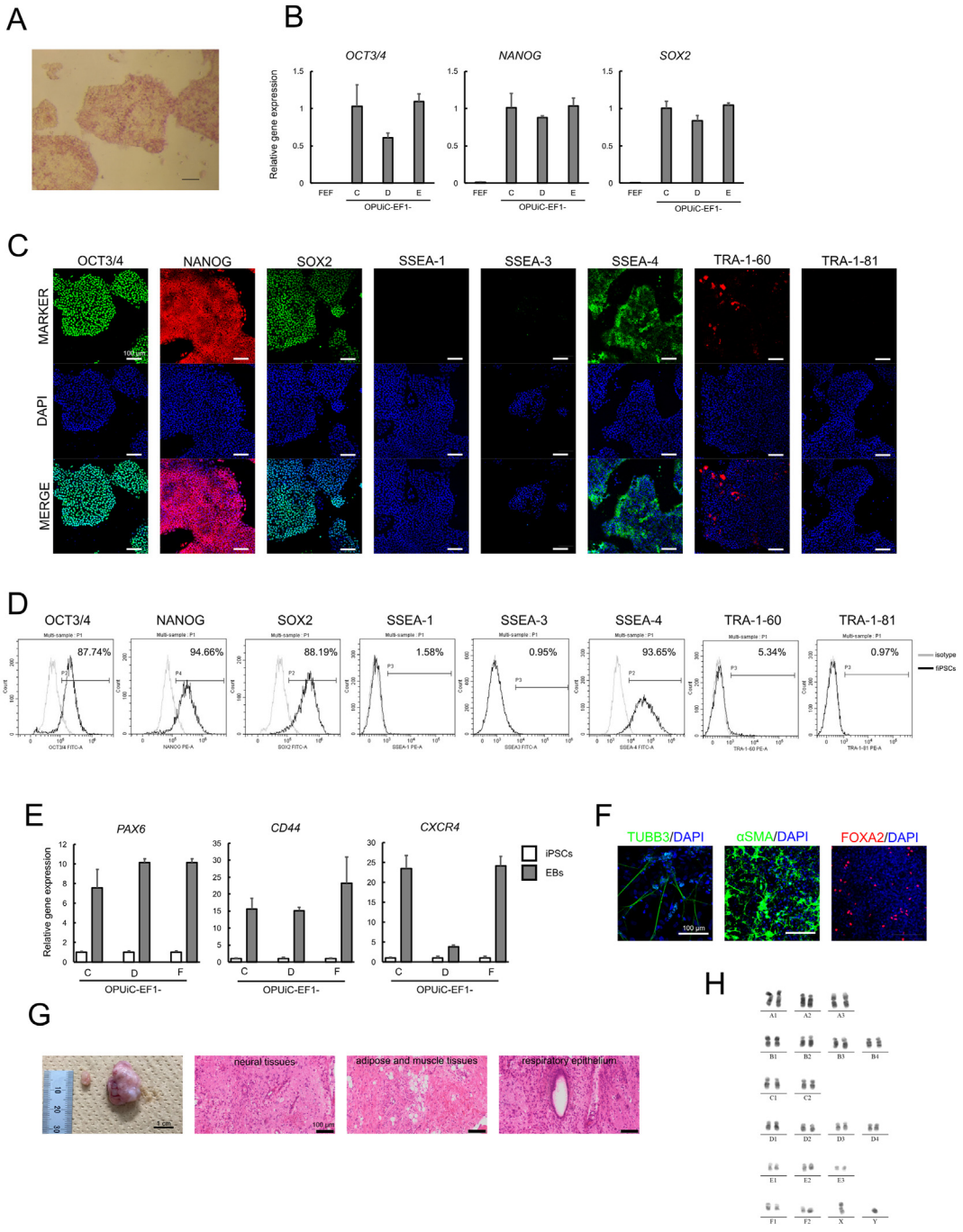

Fig. 2. Characterization of fiPSCs derived from FEFs using SeV. (A) AP staining of OPUiC-EF1-C at passage 10. Scale bar = 100 μm. (B) qRT-PCR of fiPSC lines for feline *OCT3/4*, *NANOG*, and *SOX2*. FEFs are shown as a negative control. *β-ACTIN* was used as the housekeeping gene. Relative gene expression to OPUiC-EF1-C. Data are shown as the mean ± standard deviation (n = 3). (C) Immunocytochemistry of OPUiC-EF1-E at passage 20 for pluripotent markers OCT3/4, NANOG, SOX2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Scale bar = 100 μm. (D) Flow cytometry analysis of OPUiC-EF1-E for pluripotent markers OCT3/4, NANOG, SOX2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Gray lines: isotype control, black lines: fiPSCs. (E) qRT-PCR of EBs derived from fiPSCs for feline differentiation markers. Ectodermal marker: *PAX6*, mesodermal marker: *CD44*, endodermal marker: *CXCR4*. FEFs are shown as negative control. *β-ACTIN* was used as the housekeeping gene. Relative gene expression for each fiPSC line. Data are shown as the mean ± standard deviation (n = 3). (F) Immunocytochemistry for differentiation markers of OPUiC-EF1-E after spontaneous differentiation. Ectodermal marker: TUBB3, mesodermal marker: αSMA, and endodermal marker: FOXA2. Scale bar = 100 μm. (G) Teratoma formation of OPUiC-EF1-E. The image on the left shows the testis with a tumor (left) and normal testis (right). Teratomas contain the three germ layers: ectoderm; neural tissues, mesoderm; adipose, muscle tissues, and endoderm; respiratory epithelium-like cells. Scale bar = 100 μm. (H) Karyotype analysis of OPUiC-EF1-E at passage 10.

EF1-G at low levels (Fig. 3C). The two fiPSCs had almost identical morphology (Fig. 3D), pluripotency marker expression patterns (Fig. 3E–G), and *in vitro* differentiation capacities (Fig. 3H and I) as the fiPSC lines described above. These results confirmed the fiPSCs obtained in the modified method as pluripotent.

3.5. Isolation and reprogramming of juvenile feline uterus-derived cells into fiPSCs

For practical downstream applications of fiPSCs, it is advisable to generate fiPSCs using readily accessible juvenile and adult somatic

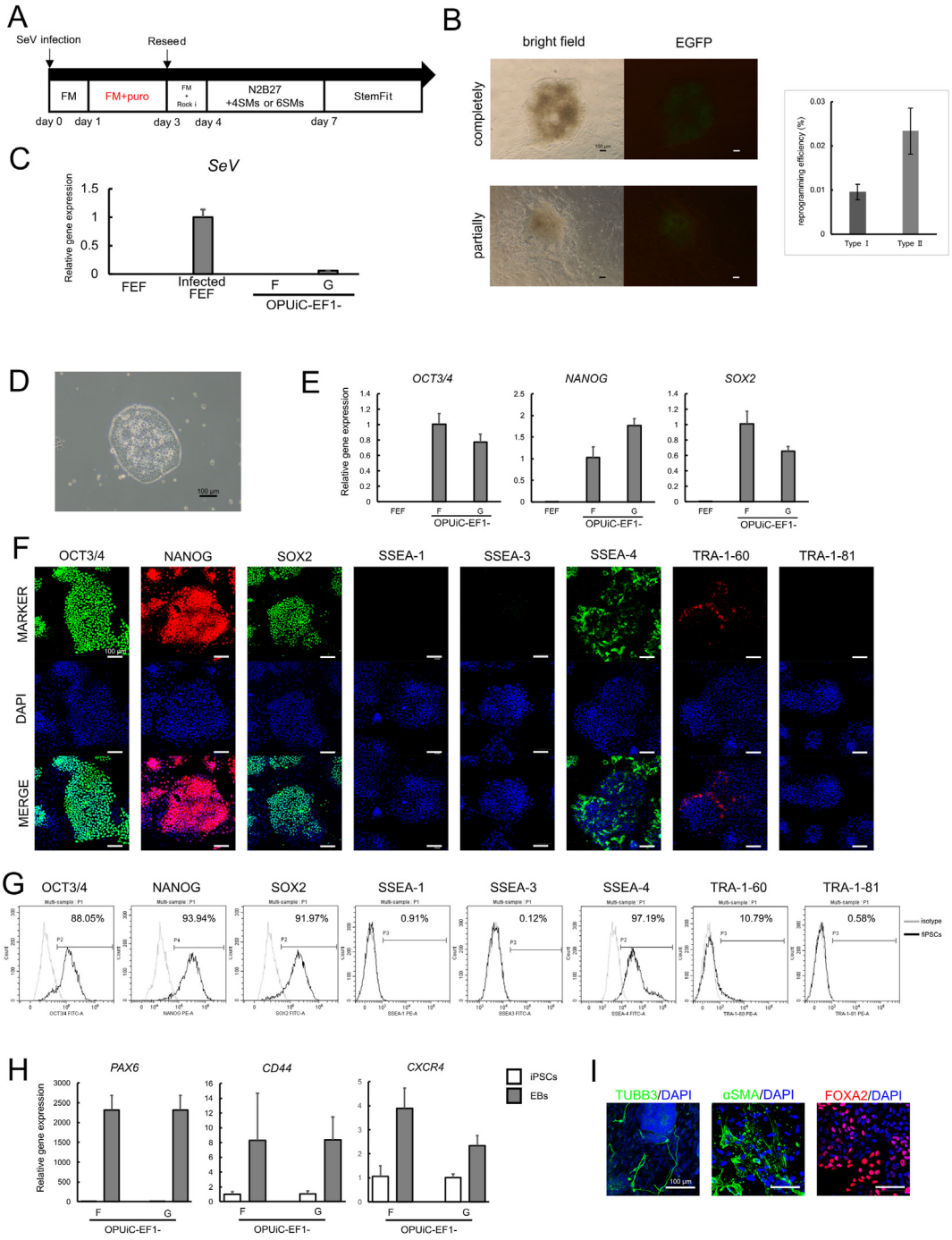

Fig. 3. Modification of the FEF reprogramming method. (A) Scheme of FEF reprogramming including puromycin selection phase. FM: feeder medium, puro: puromycin. (B) Morphologies of two types of primary colonies (Type I: completely reprogrammed-like primary colony, Type II: partially reprogrammed-like primary colony) and each reprogramming efficiency. Right panels of the bright field image show EGFP expression. Data are shown as mean ± standard deviation (n = 3). White and black scale bar = 100 μm. (C) qRT-PCR of fiPSCs for SeV. FEFs and infected FEFs as negative and positive controls, respectively. *β-ACTIN* was used as the housekeeping gene. Relative gene expression for each fiPSC line. Data are shown as the mean ± standard deviation (n = 3). (D) Morphology of OPUiC-EF1-G at passage 15. Scale bar = 100 μm. (E) qRT-PCR of fiPSC lines for feline *OCT3/4*, *NANOG*, and *SOX2*. FEFs are shown as a negative control. *β-ACTIN* was used as the housekeeping gene. Relative gene expression to OPUiC-EF1-F. Data are shown as the mean ± standard deviation (n = 3). (F) Immunocytochemistry of OPUiC-EF1-F at passage 19 for pluripotent markers OCT3/4, NANOG, SOX2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Scale bar = 100 μm. (G) Flow cytometry analysis of OPUiC-EF1-F for pluripotent markers OCT3/4, NANOG, SOX2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Gray lines: isotype control, black lines: fiPSCs. (H) qRT-PCR of EBs derived from fiPSCs for feline differentiation markers. Ectodermal marker: *PAX6*, mesodermal marker: *CD44*, endodermal marker: *CXCR4*. FEFs are shown as a negative control. *β-ACTIN* was used as an internal control. Relative gene expression for each fiPSC line. Data are shown as the mean ± standard deviation (n = 3). (I) Immunocytochemistry for differentiation markers of OPUiC-EF1-G after spontaneous differentiation. Ectodermal marker: TUBB3, mesodermal marker αSMA, and endodermal marker FOXA2. Scale bar = 100 μm.

cells. We focused on juvenile uterus-derived cells as one of the ideal somatic cell sources for fiPSC generation because the feline uterus is removed via sterilization, a procedure commonly performed in the veterinary field and readily available at veterinary clinics or animal shelters. We obtained fibroblast–like cells derived from uterus tissues (Fig. S3A) and attempted to reprogram them using the modified FEF reprogramming strategy. After SeV infection and puromycin selection, most remaining cells were positive for EGFP (Fig. S3B). Around day 28, some primary colonies grew enough to be picked up, exhibiting type I morphologies (Fig. 4A). The primary colonies were obtained with good reproducibility, and the reprogramming efficiency was 0.023 ± 0.0047% (n = 3; Fig. 4A).

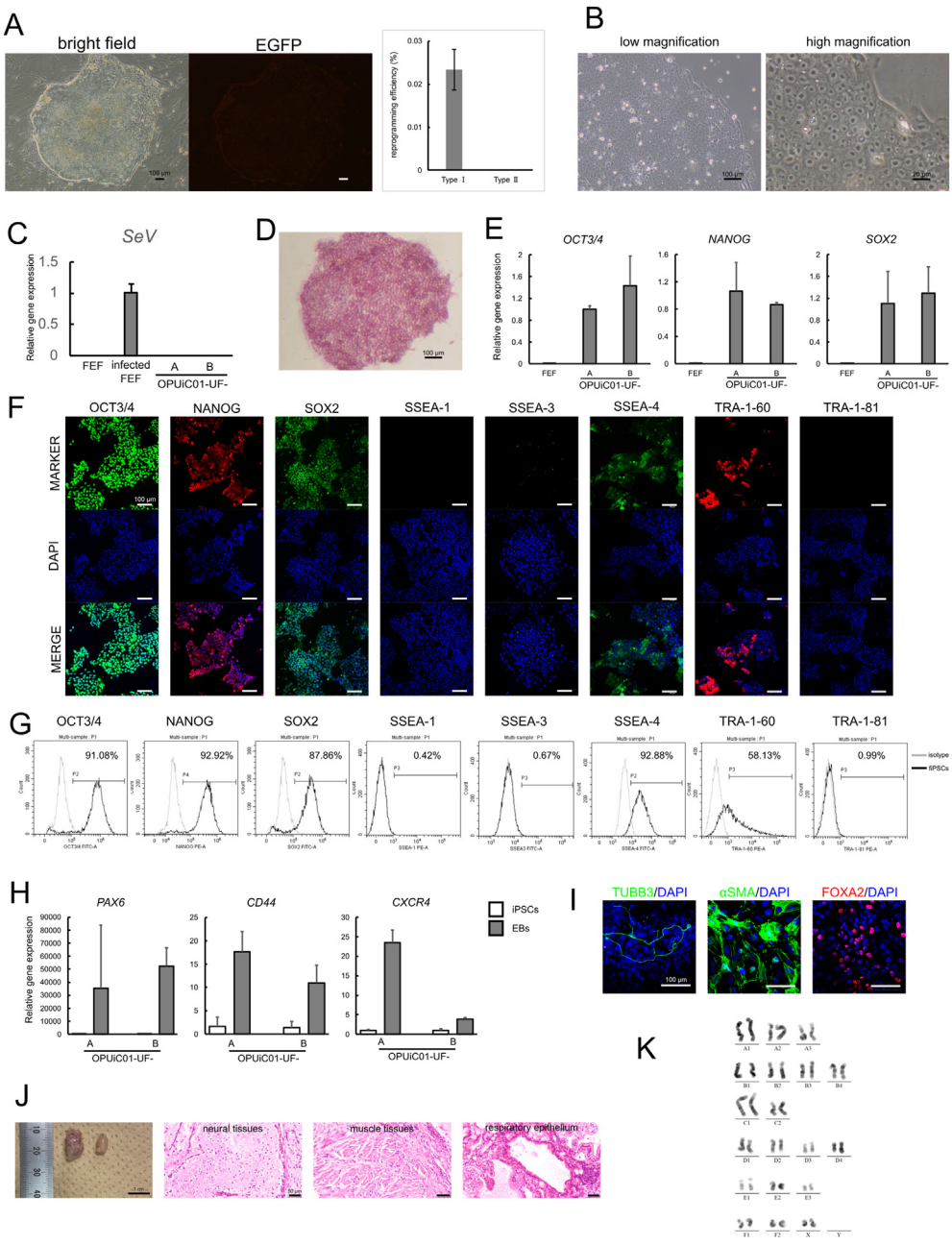

Fig. 4. Isolation and reprogramming of feline uterus-derived cells. (A) Morphology of primary colonies and reprogramming efficiencies. Right panel of the bright field image shows EGFP expression. Type I: completely reprogrammed-like primary colony, Type II: partially reprogrammed-like primary colony. White and black scale bar = 100 μm. (B) Morphology of OPUiC01-UF-A at passage 21. Scale bar = 100 μm. (C) qRT-PCR of fiPSCs for SeV after siRNA treatment. FEFs and infected FEFs as negative and positive controls, respectively. *β-ACTIN* was used as the housekeeping gene. Relative gene expression for each fiPSC line. Data are shown as the mean ± standard deviation (n = 3). (D) AP staining of fiPSCs OPUiC01-UF-A at passage 9. Scale bar = 100 μm. (E) qRT-PCR of fiPSC lines for feline *OCT3/4*, *NANOG*, and *SOX2*. FEFs are shown as negative control. *β-ACTIN* was used as the housekeeping gene. Relative gene expression to OPUiC01-UF-A. Data are shown as the mean ± standard deviation (n = 3). (F) Immunocytochemistry of OPUiC01-UF-A at passage 25 for pluripotent markers OCT3/4, NANOG, SOX2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Scale bar = 100 μm. (G) Flow cytometry analysis of OPUiC01-UF-A for pluripotent markers OCT3/4, NANOG, SOX2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Gray lines: isotype control, black lines: fiPSCs. (H) qRT-PCR of EBs derived from fiPSCs for feline differentiation markers. Ectodermal marker *PAX6*, mesodermal marker *CD44*, endodermal marker *CXCR4*. FEFs are shown as a negative control. *β-ACTIN* was used as the housekeeping gene. Relative gene expression for each fiPSC line. Data are shown as the mean ± standard deviation (n = 3). (I) Immunocytochemistry for differentiation markers of OPUiC01-UF-A after spontaneous differentiation. Ectodermal marker TUBB3, mesodermal marker αSMA, and endodermal marker FOXA2. Scale bar = 100 μm. (J) Teratoma formation of OPUiC01-UF-A. The image on the left shows the testis with a tumor (left) and normal testis (right). Teratomas contain the three germ layers: ectoderm; neural tissues, mesoderm; muscle tissues, and endoderm; respiratory epithelium-like cells. Scale bar = 100 μm. (K) Karyotype analysis of OPUiC01-UF-A at passage 20.

After repeating the siRNA procedure 2–3 times, we generated multiple SeV–negative fiPSCs with iPSC–like morphologies (Fig. 4B). We analyzed two fiPSC lines, OPUiC01-UF-A and -B. SeV was removed from both fiPSC lines (Fig. 4C). Uterus–derived fiPSCs were positive for AP staining (Fig. 4D) and expressed pluripotency genes as determined via qRT-PCR (Fig. 4E). Immunocytochemistry and FCM analyses showed that both fiPSCs expressed the same markers as FEF–derived fiPSCs. However, the rate of TRA-1-60$^+$ cells was higher than that of FEF–derived fiPSCs (Fig. 4F and G). They showed differentiation ability *in vitro* and *in vivo* (Fig. 4H–J). Finally, they had normal 36+XX karyotypes with matched autosomes (Fig. 4K). The percentage of cells with normal karyotypes was 92%–100% (OPUiC01-UF-A: 9/9, OPUiC01-UF-B: 11/12).

4. Discussion

In this study, we successfully generated fiPSCs from embryonic and juvenile somatic cells. The fiPSCs were footprint-free and could be maintained under feeder-free and chemically-defined conditions. Furthermore, they had differentiation capacity both *in vitro* and *in vivo*, retaining normal karyotypes even after multiple passages.

Use in regenerative medicine requires that iPSCs are footprint-free. However, effective methods for generating such fiPSCs have been sparse. Previous studies have established fiPSCs using a retrovirus vector which cause insertions into the host genome [10–12]. In contrast, we utilized SeV, wherein the exogenous genes are expressed without genomic insertion and can be removed from host cells. SeV has been employed to generate footprint-free iPSCs in various species [16,23]. Our study highlights SeV as a powerful tool for establishing footprint-free fiPSCs. Furthermore, we successfully maintained fiPSCs under feeder-free and chemically defined conditions using StemFit AK02N and iMatrix-511, as per the conditions reported for ciPSCs [18,21]. This result eliminates the need to use MEFs or FBS, which requires considerable time and effort and may lead to variations in quality due to technical or significant lot differences [24]. Consequently, this enhances the utility of fiPSC for downstream applications.

Leukemia inhibitory factor (LIF) has been commonly used for fiPSC and feline ES-like cell maintenance [11,25], with a previous report demonstrating that fiPSCs required feline LIF for maintenance [10]. However, our fiPSCs were maintained without LIF, suggesting that it is not essential in this case. The culture system using StemFit AK02N and iMatrix-511 has great potential for maintaining human iPSC survival and proliferation. In humans, high bFGF concentrations play a crucial role in PSC maintenance under feeder-free conditions [26]. Therefore, one of the keys for fiPSC maintenance may be bFGF, as in human PSCs.

The potential for teratoma formation is a critical characteristic when assessing the quality of PSCs [27]. However, to date there have been no reports on the generation of fiPSCs with such potential. Indeed, this study is the first to demonstrate the generation of fiPSCs capable of forming teratomas. This achievement might be attributed to the induction of six feline reprogramming genes by a single SeV and the use of small-molecule cocktails. Recent findings indicate that the use of six reprogramming factors enhances reprogramming kinetics compared to using only four [17]. Moreover, introducing six canine factors reprogrammed canine cells more efficiently than six human factors [18]. Our previous work also showed the addition of small molecule cocktails, particularly 6SMs, positively impacts the reprogramming efficiency and quality of reprogrammed cells [16]. This study validates the efficacy of our reprogramming strategy, which involves the transduction of six feline factors supplemented with 6SMs for fiPSC induction.

Despite employing the abovementioned strategies, we could not confirm reproducibility in FEF reprogramming. This could be due to low infection efficiency and an overgrowth of SeV non-infected fibroblast, which hindered the growth of primary colonies. SeV used in this study contained the puromycin resistance gene. Thus, drug selection improved the purity of SeV-infected cells and resulted in the generation of fiPSCs with high experimental reproducibility.

Interestingly, we found that fiPSCs express SSEA-4, but not SSEA-1, SSEA-3 or TRA-1-81. Notably, FCM analysis revealed significant variation in the rate of TRA-1-60$^+$ cells between fiPSC lines, which indicated that fiPSC lines derived from the uterus were likely to have a higher TRA-1-60$^+$ rate than those derived from FEFs. The expression patterns of SSEA and TRA antigen in feline PSCs remain undefined, as conflicting results have been reported for feline ESCs and iPSCs [11,12,25,28]. These antigens are known to be expressed in a stage- and species-specific manner during embryogenesis [29]. However, their expression pattern in the feline embryo is still unknown. Although we were unable to determine the exact developmental stages of our fiPSCs, the FCM results suggest that fiPSCs derived from the uterus and FEFs are at slightly different developmental stages. Further studies involving feline morula or blastocyst are required to determine their marker expression pattern in cats and identify the developmental stage of newly established fiPSCs.

As a limitation of this study, all primary antibodies used were targeted at human or mouse antigens. Regarding the cross-reactivity of the primary antibodies, we employed the same monoclonal or polyclonal antibody clones for SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, NANOG, and TUBB3 as those used in previous research on feline ESCs or iPSCs [11,12,25,28]. For other markers, the similarity in amino acid sequences of the proteins between cats and humans or mice is high (OCT3/4: 94%, SOX2: 99%, αSMA: 100%, FOXA2: 98%), indicating potential cross-reactivity of these human/mouse-specific antibodies with feline antigens. However, the actual expression of these markers should be further investigated through alternative methods, such as the creation of reporter cell lines. Another limitation was that we used the cells for reprogramming from only one cat. Therefore, the reproducibility of our protocol for reprogramming feline uterus-derived cells should be confirmed in future studies.

Importantly, we generated fiPSCs from FEFs and feline uterus-derived cells. The uterus is frequently removed via sterilization, which is the most common surgical procedure in veterinary medicine. It is therefore easy to obtain the owner's consent for donation. Additionally, uterus-derived cells could be isolated using a simple technique similar to that employed for dermal fibroblasts from the skin and could be efficiently reprogrammed in this study. Thus, uterus-derived cells are accessible and ideal sources for the generation of fiPSCs.

5. Conclusions

We successfully generated footprint-free, high-quality fiPSCs from FEFs and feline uterus-derived cells using SeV encoding feline six factors, *LIN28A*, *NANOG*, *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC*. Furthermore, we showed that fiPSCs could be maintained under feeder-free and chemically-defined conditions using StemFit AK02N and iMatrix-511. This study may facilitate both basic and clinical research in human and veterinary regenerative medicine.

Author contributions

Conception and design, K.K., Ma.T., G.I., M.N., and S.H.; Collection of data, K.K., Ma.T., and K.W.; Assembly of data, K.K., Ma.T., Mi.T., Y.M., K.W., M.O., and M.N.; Data analysis and interpretation, K.K.,

Ma.T., H.S., Mi.T., M.K., Y.M., K.W., and M.N.; Manuscript writing, K.K., Ma.T., and Y.M.; Final approval of the manuscript, K.K., Ma.T., H.S., Mi.T., M.K., Y.M., G.I., K.W., M.O., M.N., K.S., and S.H.; Administrative support, G.I., M.N., K.S., and S.H.; Provision of study material or patients, G.I.; Financial support, G.I., M.N., K.S., and S.H.

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Material availability

There are restrictions to the availability of fiPSC lines and feline six factors-SeV due to the lack of an external centralized repository for their distribution and the need to maintain the stock. We are glad to share them upon reasonable compensation by the requestor for processing and shipping.

Declaration of competing interest

This study was funded by Anicom Specialty Medical Institute Inc. Y.M., G.I., and K.W. are employees of Anicom Specialty Medical Institute Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.08.012.

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