Regenerative Therapy 28 (2025) 63–72

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Establishment of feline embryonic stem cells from the inner cell mass of blastocysts produced *in vitro*

Takumi Yoshida [a], Masaya Tsukamoto [a], Kazuto Kimura [a], Miyuu Tanaka [b], Mitsuru Kuwamura [b], Shingo Hatoya [a, *]

[a] Department of Advanced Pathobiology, Graduate School of Veterinary Science, Osaka Metropolitan University, Izumisano, Osaka 598-8531, Japan
[b] Department of Integrated Structural Biosciences, Graduate School of Veterinary Science, Osaka Metropolitan University, Izumisano, Osaka 598-8531, Japan

ARTICLE INFO

Article history:
Received 17 July 2024
Received in revised form
12 October 2024
Accepted 17 November 2024

Keywords:
Feline
Embryonic stem cell
Pluripotency
Veterinary regenerative medicine
Artificial reproductive technology
Wildlife conservation

ABSTRACT

Introduction: The rising number of cats as pets and the growing interest in animal welfare have led to an increased need for the latest treatments in feline veterinary medicine. Among these, veterinary regenerative medicine using pluripotent stem cells is gaining significant attention. However, there have been no reports on establishing feline embryonic stem cell (ESC) lines that possess the pluripotent potential and the ability to differentiate into three germ layers.

Methods: In this study, we isolated three inner cell masses from feline *in vitro*-derived blastocysts and subcultured them in a chemically defined medium (StemFit AK02N). We assessed the expression of undifferentiated markers, the ability to differentiate into the three germ layers, and the karyotype structure.

Results: We established three feline ESC lines. Feline ESCs exhibited positive staining for alkaline phosphatase. RT-qPCR analysis revealed that these cells express undifferentiated marker genes *in vitro*. Immunostaining and flow cytometry analysis demonstrated that feline ESCs express undifferentiated marker proteins *in vitro*. In the KSR/FBS medium with or without Activin A, feline ESCs differentiated into all three germ layers (ectoderm, endoderm, and mesoderm), expressing specific marker genes and proteins for each germ layer, as evidenced by RT-qPCR, immunostaining, and flow cytometry. Furthermore, we confirmed that feline ESCs formed teratomas comprising all three germ layers in mouse testes, demonstrating *de novo* pluripotency *in vivo*. We also verified that the feline ESCs maintained a normal karyotype.

Conclusions: We successfully established three feline ESC lines, each possessing pluripotent potential and capable of differentiating into all three germ layers, derived from the inner cell masses of blastocysts produced *in vitro*.

1. Introduction

Embryonic stem cells (ESCs), pluripotent stem cells (PSCs) derived from the inner cell mass (ICM) of preimplantation blastocysts, can proliferate autonomously and differentiate into all lineages. Murine ESCs and human ESCs were established in 1981 [1,2] and 1998 [3], respectively. ESCs can produce germline chimeras when transplanted into blastocysts [4]. Thus, mouse models of numerous diseases can be generated using gene-edited ESCs [5]. Owing to their self-renewal and differentiation ability, ESCs also serve as unlimited sources for drug discovery and *in vitro* organogenesis studies [6]. Therefore, ESCs have been used as tools in various field studies, and the fundamental results obtained from

Abbreviations: AP, alkaline phosphatase; ART, artificial reproductive technologies; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; COC, cumulus-oocyte complexes; EB, embryoid body; EGF, epidermal growth factor; ESCs, Embryonic stem cells; FBS, fetal bovine serum; FEF, feline embryonic fibroblast; ICM, inner cell mass; ICR, Institute of Cancer Research; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; KSR, knockout serum replacement; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; *m*-HTF, modified human tubal fluid; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; PSC, pluripotent stem cells; PFA, paraformaldehyde; RT-qPCR, Quantitative reverse transcription PCR.

* Corresponding author.
E-mail address: hatoya@omu.ac.jp (S. Hatoya).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2024.11.010

these studies have led to the incorporation of human regenerative medicine in practical applications [7–9].

In recent years, the rising number of cats as pets and the growing interest in animal welfare have led to an increased need for advanced treatments in feline veterinary medicine. However, cats suffer from many intractable diseases without established treatments, such as chronic kidney disease. Therefore, feline ESCs must be established for the development of these treatments and the advancement of veterinary regenerative medicine. Cats have a longer lifespan than mice, live in an environment similar to that of humans, and share many genetic diseases with humans [10]. Therefore, research on feline ESCs is also expected to contribute to the development of regenerative medicine in humans.

Furthermore, although domestic cats are being used as animal models for research on artificial reproductive technologies (ART) for endangered wild cats [11,12], the lack of germ cells is limiting this ART research [13]. The establishment of feline ESCs and the subsequent production of sperm and oocytes from these cells might pave the way for the potential development of ESCs with the ability to differentiate into germ cells in wild cats. Additionally, this could provide an almost unlimited supply of germ cells for ART research, thereby solving the germ cell shortage of wild felines.

Despite reports by two groups on the establishment of feline ES-like cells, they could not maintain the undifferentiated state and did not adequately assess the differentiation ability of these cells [14,15]. We hypothesized that the poor quality of blastocysts and inappropriate culture conditions were responsible for the failure to establish feline ESCs. Previously, we found that initially supplementing with bovine serum albumin (BSA) and then switching to fetal bovine serum (FBS) is more suitable for feline embryo development to the blastocyst stage than using BSA alone [16]. In this study, we employed this culture system to create feline blastocysts. Furthermore, we used StemFit AK02N medium to maintain feline ESCs. This medium contained a high concentration of basic fibroblast growth factor (bFGF), the key factor for maintaining human and canine PSCs [17,18].

The objective of this study was to establish feline ESCs. To this end, we isolated feline ICMs from feline blastocysts and maintained ICM outgrowths in the StemFit AK02N medium. Subsequently, we confirmed that the cells retained their undifferentiated state. Differential abilities of feline ESCs were assessed both *in vitro* and *in vivo*. Finally, we examined the karyotype of the generated ESCs to measure their tumorigenic potential.

2. Methods

This study received approval from the Ethical Review Committee for Clinical Research of the Veterinary Clinical Center of Osaka Metropolitan University (approval number: R5-002). The approval covered the collection of ovaries and testes at the local animal clinic and their use in this study. In addition, this study was also approved by the Institutional Animal Experiment Committee of Osaka Metropolitan University (approval numbers: 22–73, 22–74, 23–53, 23–56). All experiments in this study were performed according to the Animal Experimentation Regulations of Osaka Metropolitan University.

Feline ovaries and testes, including the epididymides, were collected from a local veterinary clinic after routine ovariohysterectomy and castration procedures on privately owned cats. The use of these tissues was approved by the local veterinary clinic. Animals were neither operated on, nor sacrificed for the purposes of this study. All cats were privately owned, and consent from each owner was obtained before sample collection.

2.1. Collection of cumulus-oocyte complexes (COCs)

The collected ovaries of a sexually matured domestic cat owned by the owner were preserved in saline solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for 6 h at 4°C. The COCs were then collected and graded from ovaries, as previously described [16]. Briefly, the ovaries were placed in Medium 199 (Thermo Fisher Scientific, Waltham, MA, USA), and the COCs were recovered through incision and puncture using scissors, forceps, and a syringe with a needle. We used oocytes that were completely surrounded by more than four layers of compacted cumulus cells.

2.2. In vitro maturation

The collected COCs were rinsed three times with *in vitro* maturation (IVM) medium, which consisted of Medium 199 supplemented with 0.4% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA), 0.2 μg/mL 17β-estradiol (Sigma-Aldrich), 100 μg/mL gentamycin (Sigma-Aldrich), 137 μg/mL sodium pyruvate, 0.02 IU/mL Follistim® (human recombinant follicle-stimulating hormone; MSD, Tokyo, Japan), and 25 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich). The COCs were then incubated in IVM medium droplets covered with mineral oil (Sigma-Aldrich) at 38.5°C in a humified atmosphere with 5% CO_2 for 28 h.

2.3. Sperm collection and cryopreservation

Spermatozoa from the epididymides of a sexually-mature male cat were used, and spermatozoa cryopreservation was performed as previously described [16] with some modifications. Briefly, the epididymides were cut into small pieces and cultured at 38.5°C in a humidified atmosphere (5% CO_2) in PBS(−). The samples were then filtered through a 20 μm filter (Nipro, Osaka, Japan) and centrifuged for 5 min at 500×*g*. The pelleted sperm were resuspended with EYT-FC solution, which consisted of egg yolk supplemented with 13 μg/mL citric acid (Nacalai Tesque, Kyoto, Japan), 10 μg/mL D-fructose (Nacalai Tesque), 24 μg/mL tris aminomethane (Nacalai Tesque), 1000 IU/mL penicillin (Sigma-Aldrich), and 1 mg/mL streptomycin (Sigma-Aldrich). The sperm was then centrifuged for 5 min at 500×*g*. The pelleted sperm was resuspended in EYT-FC solution and stored at 4°C. After 1 h, an EYT-FC solution containing 14% (v/v) glycerol (Nacalai Tesque) was added to the solution to obtain a final concentration of 1.25×10^7 cells/mL with 7% glycerol. This solution was loaded into 0.25 mL straws (Fujihira, Tokyo, Japan), which were laid horizontally on a rack 4 cm above liquid nitrogen vapor for 10 min, plunged into liquid nitrogen, and then stored in a liquid nitrogen storage tank.

2.4. In vitro fertilization

The straws containing cryopreserved sperm were thawed by soaking in warm water (37°C) for 30 s. The sperm was then released into a modified human tubal fluid (*m*-HTF, Nippon Medical & Chemical Instruments Co. Ltd., Osaka, Japan) and centrifuged for 5 min at 500×*g*. After centrifugation, sperm were suspended in HTF (Nippon Medical & Chemical Instruments Co. Ltd.), centrifuged again for 5 min at 500×*g*, and the concentration was adjusted to 1.5×10^6 cells/mL in *in vitro* fertilization (IVF) medium (HTF with 0.3% BSA). After 28 h of IVM, 5–10 COCs were washed twice in IVF medium and cultured in 100 μL IVF medium droplets covered with mineral oil at 38.5°C in air (5% CO_2) for 18 h.

2.5. In vitro culture of fertilized oocytes

After IVF, a narrow-bore glass pipette was used to remove cumulus cells from the oocytes, and denuded oocytes were used for

in vitro culture (IVC). The oocytes were incubated in 100 μL of IVC I medium for 2 days and then in IVC II medium in a humified atmosphere (5% O_2, 5% CO_2, and 90% N_2). IVC I and II media were composed of Only-One Medium (Nippon Medical & Chemical Instruments) supplemented with 0.3% (w/v) BSA and 5% (v/v) FBS (Biosolutions International, Melbourne, Australia).

2.6. Preparation of feeder cells

Mouse embryonic fibroblasts (MEFs) were isolated from embryonic day 13.5 Institute of Cancer Research (ICR) mouse fetuses (Japan SLC, Shizuoka, Japan). The fetuses were washed with PBS(−), and the head and liver were removed and cut into small pieces. MEFs were passaged by digesting with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) and were treated with 10 mg/mL mitomycin C (Kyowa Kirin, Tokyo, Japan) for 2.5 h at passage 2. After inactivation, MEFs were washed thrice with PBS(−) and seeded onto gelatin-coated tissue culture dishes 1 day before use.

2.7. Derivation and culture of feline ESCs

ICMs were mechanically isolated under a microscope using 31-gauge needles (Dentronics, Tokyo, Japan). Isolated ICMs were individually seeded onto the MEF feeder layer and cultured in StemFit AK02N medium (Ajinomoto, Tokyo, Japan) with 10 μM Y-27632 (Nacalai Tesque). After 5–10 days, putative ESC colonies were formed. These ESC colonies were continuously cultured in StemFit AK02N medium without Y-27632. Subsequent subculturing of ESC lines was performed every 3–5 days mechanically by cutting them into small pieces using a Pasteur pipette with a sharp tip.

2.8. Alkaline phosphatase (AP) staining

AP staining of feline ESCs was performed using the Stemgent™ Alkaline Phosphatase Staining Kit II (REPROCELL, Yokohama, Japan) according to the manufacturer's instructions.

2.9. Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated using a FastGene RNA Premium Kit (Nippon Genetics, Tokyo, Japan) and reverse-transcribed into complementary DNA using random primers and ReverTra Ace (Toyobo, Osaka, Japan). RT-qPCR was performed in triplicate using Taq Pro Universal SYBR qPCR Master Mix (Nanjing Vazyme Biotech, Nanjing, China) and the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific), according to the manufacturer's instructions. All primers used are listed in Table 1. PCR data were analyzed using the Δ/ΔCT method and normalized to β-Actin expression.

2.10. Quantitative analysis of differentiation markers for three germ layers

Feline ESCs were dissociated into single cells, seeded onto Nunclon™ Sphera™ 96-well U-shaped-bottom microplates (Thermo Fisher Scientific), and cultured for 10 days in FBS/knockout serum replacement (KSR) medium [DMEM/Nutrient Mixture F-12 Ham supplemented with 10% FBS, 10% KSR (Thermo Fisher Scientific), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM NEAA, 0.1 mM 2-mercaptoethanol, GlutaMax (1 × ; Thermo Fisher Scientific), and 1 mM sodium pyruvate] with or without 100 ng/mL Activin A (Nacalai Tesque) to form multiple embryoid bodies (EBs). Total RNA isolation, reverse transcription, and qPCR analyses were performed as described above. We used *NESTIN* and *PAX6* as markers for the ectoderm; *SOX17* as a marker for the endoderm; and *CD44*, *ACTA2*, and *DESMIN* as markers for the mesoderm (Table 1).

Table 1
List of primer sequences.

Name		Sequence (5′ → 3′)
β-ACTIN	Forward	AGCCTTCCTTCCTGGGCAT
	Reverse	AGGGCGGTGATCTCCTTCT
OCT3/4	Forward	GGCTGGAGAAGGATGTGGTC
	Reverse	CTCGTTGCGAATAGTCACTGC
SOX2	Forward	AGGATAAGTACACACTGCCCG
	Reverse	ATCATGCTGTAGCTGCCGTT
NANOG	Forward	TCCAGTGCCTGAGATTGATGG
	Reverse	AGGAAGGAAGAGGAGAGACGGT
NESTIN	Forward	AGTTTAGAGGCCAAAGGCGG
	Reverse	CTAAAGACCTCAAGGACCTCTGG
PAX6	Forward	TGCTGGACAATCAAAACGTGTC
	Reverse	GTCTGCCCGTTCAACATCCT
SOX17	Forward	TGTTCGATTCTGGTTGCCCA
	Reverse	AAACACACCCAAGGCGAAAG
CD44	Forward	TGGGTTGTTTGGCATCCAGTGC
	Reverse	CGTTTTCTTCAGTTGGTTCCCAGCC
ACTA2	Forward	AACCCTTCAGCGTTCAGCTT
	Reverse	CCACCATCACTCCCTGATGC
DESMIN	Forward	GGTACAAGTCAAAGGTGTCCGA
	Reverse	TTTCGCAGGTGTAGGACTGG

2.11. Immunocytochemistry

Immunocytochemical analyses were performed for pluripotency and differentiated markers. In brief, feline ESCs cultured in an eight-well glass chamber slide (AGC techno glass, Shizuoka, Japan) coated with MEF for pluripotency and gelatin for differentiated markers were immunolabeled as described below. The cells were fixed in 4% paraformaldehyde (PFA) for 40 min. They were subjected to permeabilization, adjusted based on the localization of the target proteins. For nuclear proteins, cells were simultaneously permeabilized and blocked in 0.1% Triton X-100 and 10% FBS in PBS(−) for 60 min at room temperature. For cytoplasmic and cell surface proteins, the cell was treated with 0.1% Tween 20 in PBS(−) for 5–10 min at room temperature. Following the permeabilization step, the cells were blocked with 10% FBS in PBS(−) for 40 min at room temperature. Subsequently, the primary antibodies (refer to Table 2) were added, followed by incubation for 16 h at 4°C. After washing out the excess primary antibodies, the secondary antibodies (see Table 2) were then added, and the cells were incubated for 1 h at room temperature. After washing the cells, DAPI solution (0.2 μg/mL in PBS(−) with 0.1% BSA) was added for labeling DNA, and the cells were incubated for 2 min at room temperature. Cells were then washed and mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific). Stained samples were examined under a confocal laser microscope (FV3000; Olympus, Tokyo, Japan).

2.12. Flow cytometry

Cells were dissociated through incubation with trypsin and subsequently stained with specific markers, as indicated in Table 2. For cell surface markers, primary antibody staining was conducted in PBS(−) on ice for 30 min. Where necessary, secondary antibody staining was similarly performed for 15 min. For intracellular proteins, staining was executed in cells that had been fixed with 4% PFA in PBS(−). These cells were then permeabilized using 0.1% Triton X-100 in PBS(−) with 5% FBS at 4°C for 45 min. Subsequently, cells were stained with primary antibodies (refer to Table 2) for 45 min, followed by staining with secondary antibodies (see Table 2) for

Table 2
List of antibodies using for immunochemistry and flow cytometory cytometry.

Primary antibodies						
Antibodies	Host	Cat. No.	Clone number	Clonality	Dilution	Company
Normal mouse IgG1	–	Sc-3877	–	–	01:20	Santa cruz biotechnology
Normal rabbit IgG	–	30000-0-AP	–	–	1:200	Proteintech
Normal mouse IgM	–	Sc-3881	–	–	2 μl/test	Santa cruz biotechnology
Anti-NANOG	Rabbit	500-P236	–	Polyclonal	1:500	PeproTech
Anti-OCT3/4	Mouse	Sc-5279	C-10	Monoclonal	1:200	Santa cruz biotechnology
Anti-SOX2	Mouse	Sc-365823	E–4	Monoclonal	1:200	Santa cruz biotechnology
Anti-SSEA-1	Mouse	Sc-21702	480	Monoclonal	1:100	Santa cruz biotechnology
Anti-SSEA-4	Mouse	Sc-21704	813–70	Monoclonal	1:200	Santa cruz biotechnology
Anti-Tra-1-60	Mouse	Sc-21705	TRA-1-60	Monoclonal	1:100	Santa cruz biotechnology
Anti-Tra-1-81	Mouse	Sc-21706	TRA-1-80	Monoclonal	1:100	Santa cruz biotechnology
Anti-TUBB3	Mouse	MAB1637	TU-20	Monoclonal	1:400	Merck
Anti-αSMA	Mouse	M0851	1A4	Monoclonal	1:500	Dako
Anti-SOX17	Goat	AF1924	–	Polyclonal	1:100	R&D systems
Anti-FOXA2	Rabbit	720061	–	Polyclonal	1:250	Thermo Fisher Scientific
Anti-PAX6	Rabbit	901301	–	Polyclonal	1:100	BioLegend
Anti-NESTIN	Mouse	MAB353	Rat-401	Monoclonal	1:100	Merck
Secondary antibodies						
Anti-Goat IgG-Alexa Flour 488	Rabbit	A11008	–	Polyclonal	1:1000	Thermo Fisher Scientific
Anti-mouse IgG-Alexa Fluor 488	Goat	A11029	–	Polyclonal	1:1000	Thermo Fisher Scientific
Anti-Rabbit IgG-Alexa Fluor 546	Goat	A11010	–	Polyclonal	1:1000	Thermo Fisher Scientific
Anti-mouse IgM-Cy3	Goat	AP128C	–	Polyclonal	1:1000	Sigma-Aldrich

30 min. The stained cells were analyzed and sorted using an AriaII flow cytometer (BD Biosciences, CA, USA).

2.13. Teratoma formation assay

To evaluate the teratoma formation properties of ESCs, 1×10^6 feline ESCs were suspended in Matrigel diluted 1:1 in DMEM/Nutrient Mixture F-12 Ham and administered into the left testicular capsules of male nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (all lines: n = 2). Matrigel, diluted 1:1 in DMEM/Nutrient Mixture F-12 Ham, was injected into the right testicular capsules as a control. The mice were euthanized 3 months later; the tumors were fixed in 4% PFA and embedded in paraffin. The paraffin-embedded samples were sliced and stained with hematoxylin and eosin.

2.14. Karyotyping

Feline ESCs were incubated in a medium containing 0.04 μg/mL colcemid (Thermo Fisher Scientific) for 2 h, followed by trypsinization and incubation in 0.075 M KCl at 37°C for 20 min. The cells were fixed in a mixture of acetic acid and methanol (1:3) and then incubated in a solution of quinacrine mustard for 10 min. After rinsing with diluted water, the samples were stained with Hoechst 33258 for 10 min. Analyses were performed under a confocal laser microscope (LSM980; Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. Establishment of feline ESC colonies

In our study, we employed three blastocysts, two hatched and one unhatched, for the generation of feline ESCs (Fig. 1A(i)). Upon coculturing on MEF, each ICM of these blastocysts formed boundary-defined colonies like human ESCs within 5–10 days (Fig. 1A(ii)). These cells exhibited a high nucleus-to-cytoplasm ratio and prominent nucleoli. After subculturing and a freeze-thaw cycle, colonies with morphological characteristics similar to those of the primary colonies were formed (Fig. 1B). These three cell lines were named NCH01, NCH02, and NCH03, respectively.

3.2. Verification of undifferentiated states of feline ESCs

All the cell lines, NCH01, NCH02, and NCH03, showed positive AP staining at passages 3–5 (Fig. 1C (i, iv)). This characteristic was maintained up to passage 30 (Fig. 1C (ii, v)). Moreover, they continued to show positive AP staining after freeze-thaw cycling (Fig. 1C (iii, vi)).

qPCR analysis (Fig. 2A) revealed that all cell lines expressed undifferentiated marker genes (*OCT3/4*, *SOX2*, and *NANOG*) at the mRNA level. On the other hand, MEF served as feeder cells and FEF did not express the undifferentiated marker genes.

Immunostaining (Fig. 2B and Fig. S1) also showed positivity for OCT3/4, SOX2, NANOG, and SSEA-4; a small proportion of cells was positive for Tra-1-60. However, the cells were negative for Tra-1-81 and SSEA-1.

Flow cytometry (Fig. 2C and Fig. S2) confirmed the expression of these proteins, aligning with the immunostaining results. The cells were positive for OCT3/4, SOX2, NANOG, and SSEA-4, with some cells being positive for Tra-1-60. However, most of the cells were negative for Tra-1-81 and SSEA-1.

3.3. Evaluation of the differential ability of feline ESCs

All feline ESC lines formed EBs when cultured in FBS/KSR medium, with or without Activin A. RT-qPCR (Fig. 3A) analysis revealed an increased expression of germ layer-specific marker genes in these EBs compared to their source ESCs, including ectoderm (*NETSIN*, *PAX6*), endoderm (*SOX17*), and mesoderm (*CD44*, *ACTA2*, *DESMIN*) markers. Furthermore, immunostaining (Fig. 3B and Fig. S3) also confirmed the expression of these markers in EBs: PAX6, NESTIN and β-tubulin (TUBB3) for ectoderm; FOXA2 and SOX17 for endoderm; α-SMA for mesoderm.

To assess the *in vivo* differentiation potential of feline ESCs, they were injected into the testes of NOD/SCID mice. All feline ESC lines formed tumors that comprised the three germ layers (ectoderm:

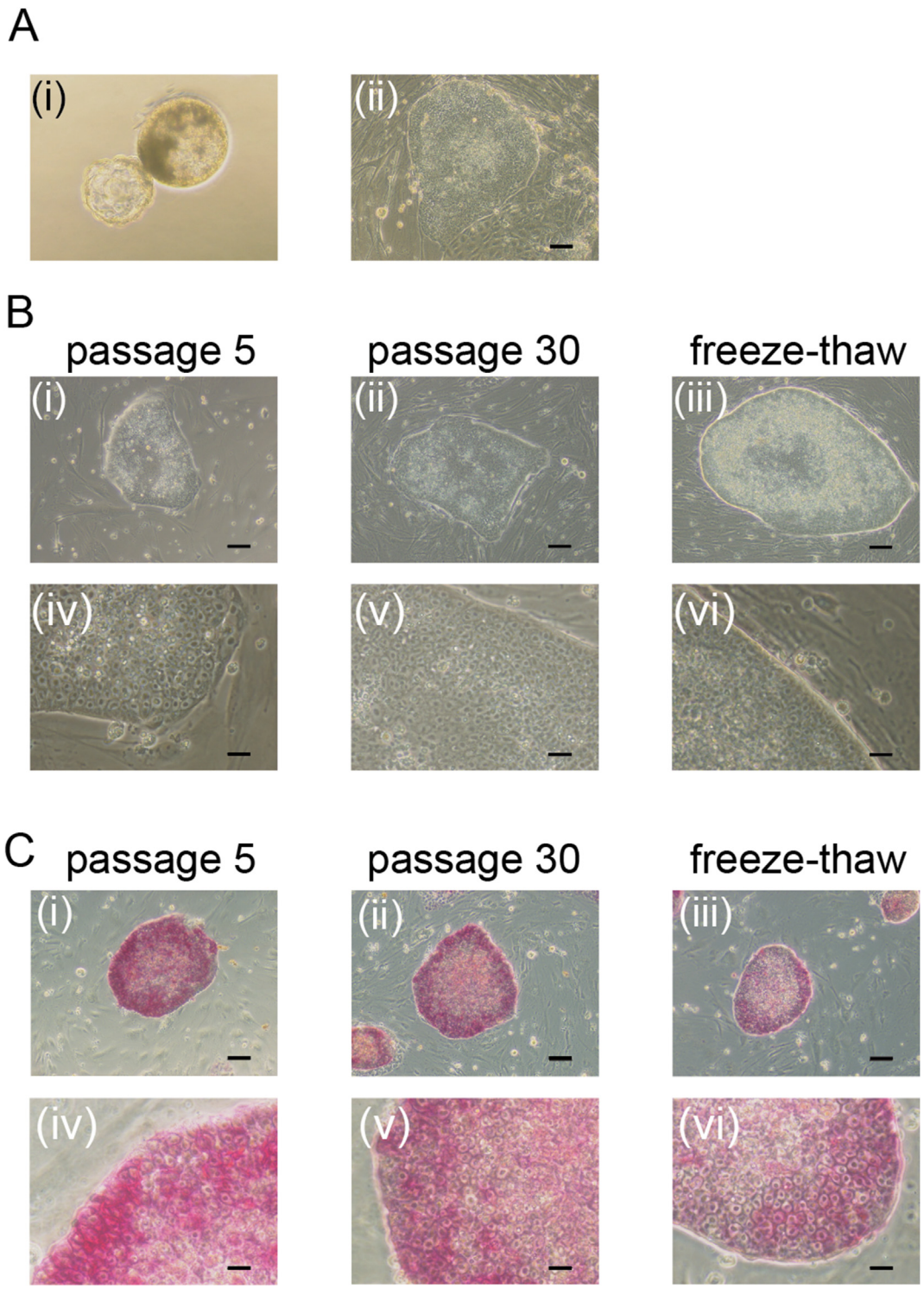

Fig. 1. Morphology of feline *in vitro*-derived blastocyst and feline embryonic stem cells (ESCs). A: Morphology of (i) feline *in vitro*-derived blastocyst and (ii) primary colony of feline ESCs. B: Morphology of feline ESCs at passage 5 (i, iv), at passage 30 (ii, v), and post freeze-thaw (iii, vi). Scale bar (i, ii, iii) = 100 μm; (iv, v, vi) = 20 μm. C: AP staining of feline ESCs at passage 5 (i, iv), at passage 30 (ii, v), and post freeze-thaw (iii, vi). Scale bar (i, ii, iii) = 100 μm; (iv, v, vi) = 20 μm.

epidermis and hair follicle, endoderm: respiratory epithelium, mesoderm: adipose, Fig. 4).

3.4. Karyotyping

All feline ESC lines showed normal karyotypes, with 18 matched pairs of autosomes and XX or XY gonosomes (Fig. 5).

4. Discussion

In this study, we successfully established three feline ESC lines from the ICMs of three *in vitro*-produced blastocysts. While two research groups have previously attempted to establish feline ESCs, these efforts were not entirely successful [14,15]. Here, we used a novel approach that differed from the two previous strategies

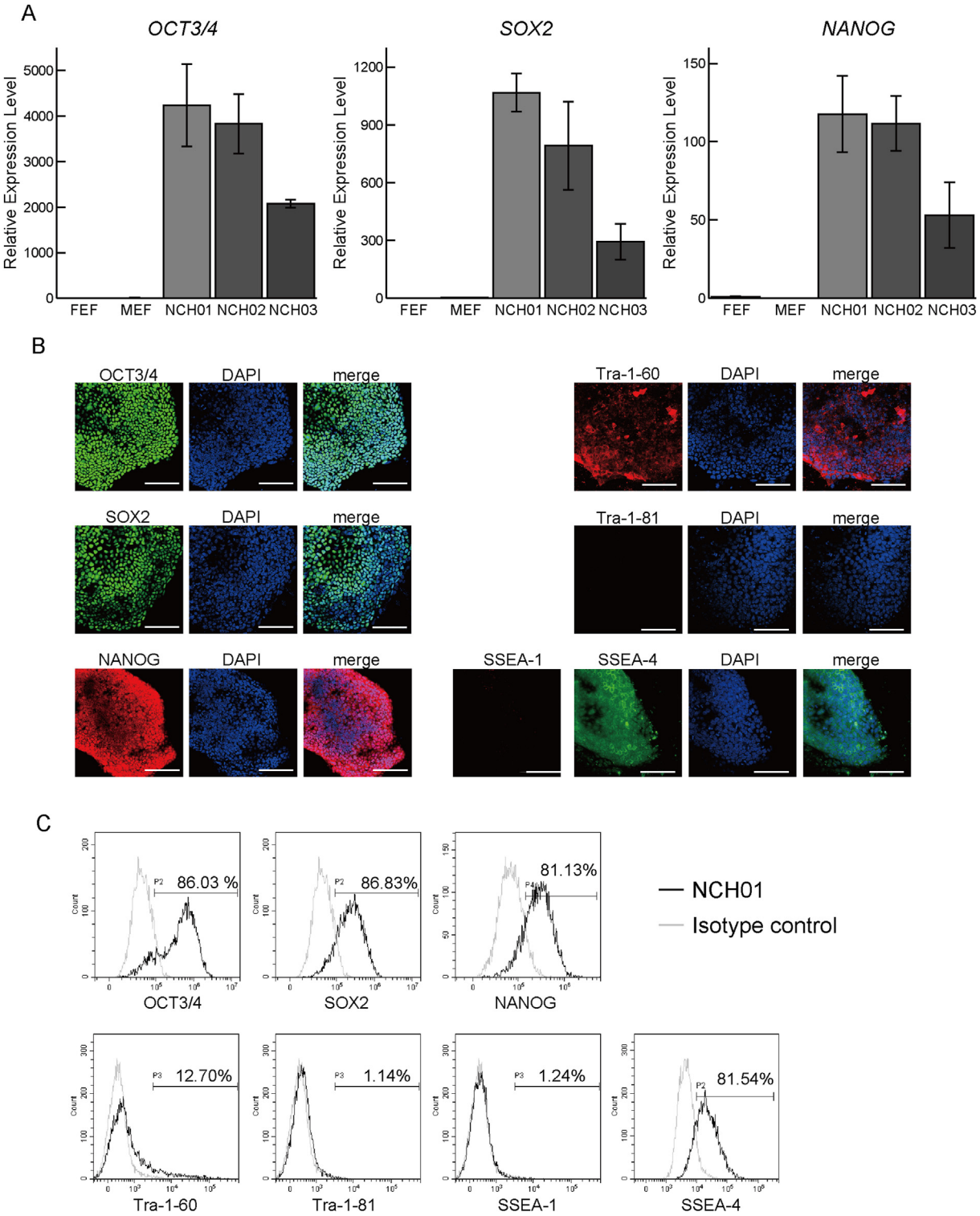

Fig. 2. Assessment of undifferentiated marker genes in feline ESCs using RT-qPCR, immunochemistry, and flow cytometry. A: Expression levels of undifferentiated cell markers (*OCT3/4*, *SOX2, and NANOG*) in mouse embryonic fibroblast (MEF), feline embryonic fibroblast (FEF), feline ESCs at passage 19 (NCH01, NCH02, NCH03) were determined using RT-qPCR. Relative expression levels are presented as ratios relative to the level in FEF. Data are presented as the mean ± SD (n = 3). B: Immunocytochemical detection of undifferentiated marker expression in NCH01 at passage 19 (OCT3/4, SOX2, NANOG, Tra-1-60, Tra-1-81, SSEA-1, and SSEA-4). Scale bar = 100 μm. C: Evaluation of undifferentiated marker expression in NCH01 at passage 21 (black line; OCT3/4, SOX2, NANOG, Tra-1-60, Tra-1-81, SSEA-1, and SSEA-4) using flow cytometry. Isotype controls are shown in each panel (gray line).

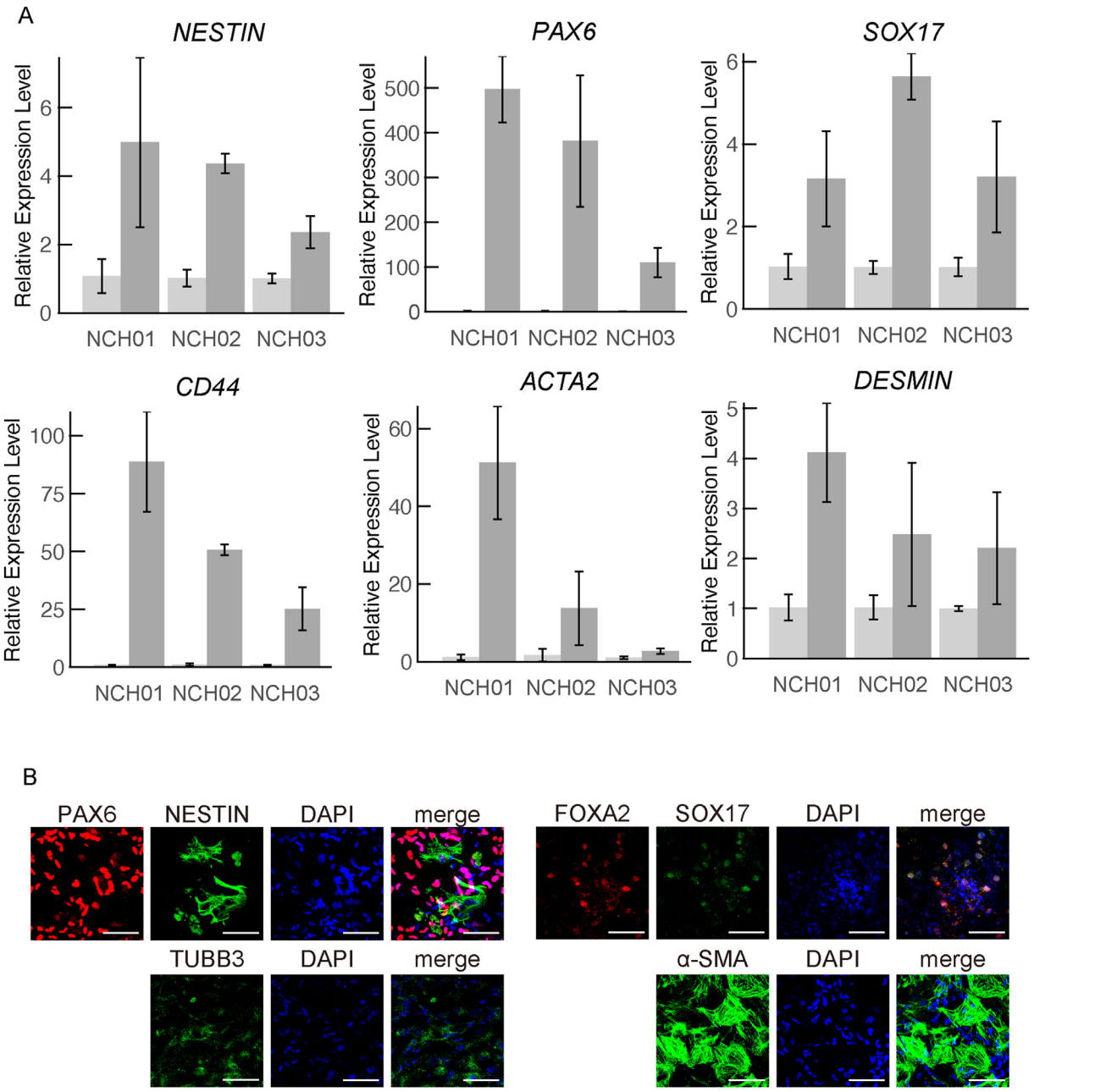

Fig. 3. Evaluation of differentiation ability in feline ESCs using RT-qPCR, immunochemistry. A: Expression levels of differentiation markers in feline ESCs at passage 27 (NCH01, NCH02, NCH03; ectoderm: *NESTIN* and *PAX6*; endoderm: *SOX17*; mesoderm: *CD44*, *ACTA2*, and *DESMIN*). Relative expression levels are presented as ratios relative to the level in feline ESCs. B: Immunocytochemical detection of differentiation markers in ESCs at passage 19 (NCH01; ectoderm: PAX6, NESTIN, and TUBB3; endoderm: FOXA2 and SOX17; mesoderm: α-SMA). Scale bar = 100 μm.

focusing on the IVF method and establishment medium. The quality of blastocysts, crucial for the ESC establishment, largely depends on the culture system used. For instance, Gómez et al. [15] used *in vitro*-derived blastocysts to establish ESCs, and the fertilized oocytes were cultured in an IVF medium containing BSA. However, we previously reported that a combination of BSA supplemented initially, followed by a switch to FBS, was more suitable for feline embryo development up to the blastocyst stage than BSA alone [16]. We utilized this culture system to develop blastocysts and generate feline ESCs. Furthermore, Yu et al. [14] attempted to establish feline ESCs using *in vivo*-derived blastocysts, known for their superior quality and proven effectiveness in enhancing ESC establishment, compared to their *in vitro* counterparts. Despite this, they were unable to establish feline ESCs, possibly due to the use of an unsuitable medium for the establishment and maintenance of feline ESCs [14]. Yu et al. [14] cultured ICM outgrowths without bFGF, while Gómez et al. [15] used a bFGF-containing medium at a concentration of 5 ng/mL. However, bFGF is a key factor in the maintenance of human and canine PSCs [19–21]. Therefore, we used the StemFit AK02N medium containing a high concentration of bFGF and maintained feline ESCs in the undifferentiated state. Based on these findings, we assumed that a high concentration of bFGF was key factor to maintain the pluripotency of feline ESCs. However, StemFit AK02N includes not just a high concentration of bFGF but also a range of other components. It is possible that among these, there are key elements that play a crucial role in

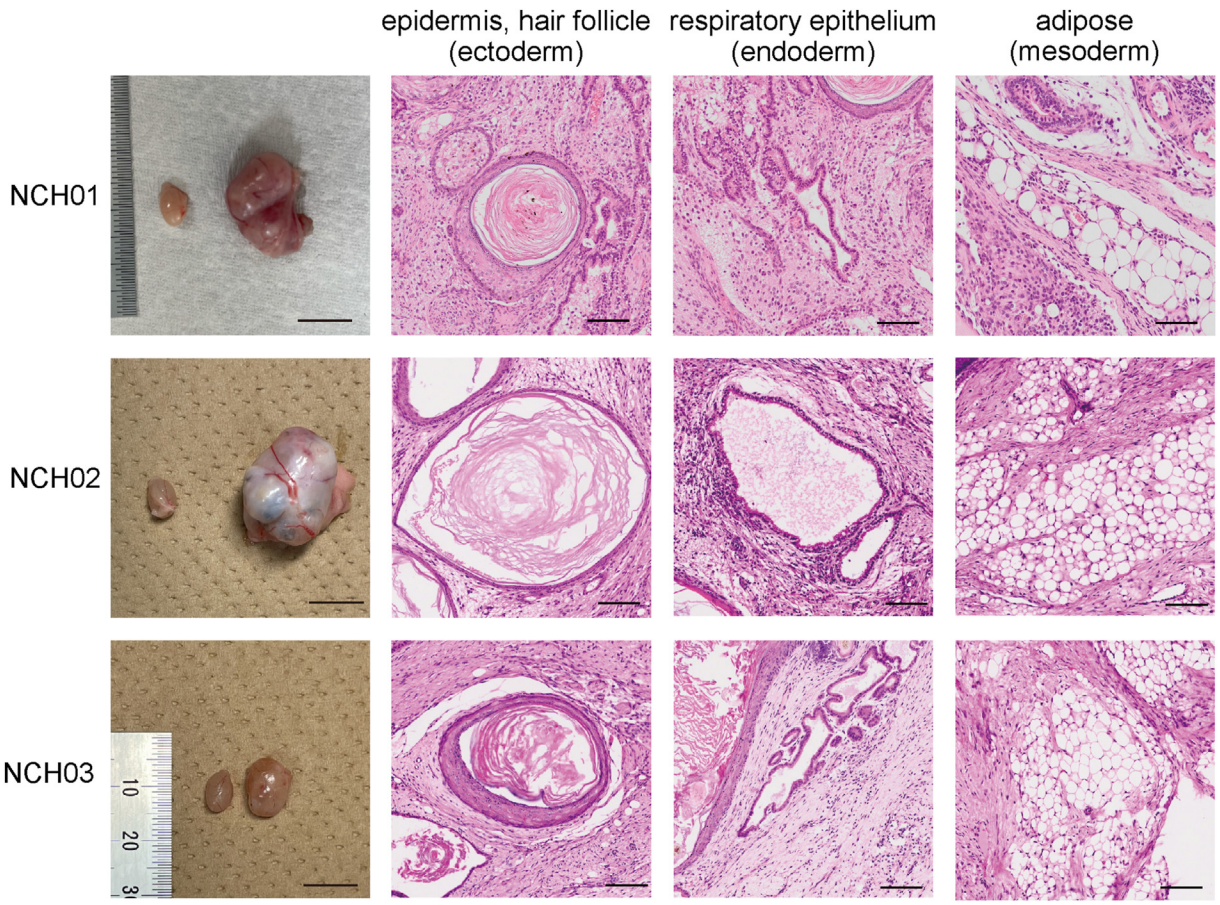

Fig. 4. Teratoma formation by feline ESCs. The left images show the normal testis (left) and tumor-bearing testis (right). Scale bar = 100 mm. Hematoxylin and eosin staining of histological sections of teratoma show the three germ layers: ectoderm (epidermis and hair follicle), endoderm (respiratory epithelium), and mesoderm (adipose). Scale bar = 100 μm.

maintaining the undifferentiated state of feline ESCs, besides the high concentration of bFGF alone. Further research is warranted to identify the essential factors for feline ESC pluripotency.

In this study, we conducted observations of the characteristic morphology and AP staining of feline ESCs. AP staining is a widely recognized method for assessing the undifferentiated status of PSCs in various mammals, including humans [22], mice [23], and dogs [17]. Therefore, this method is considered effective for assessing the pluripotency of feline PSCs. Previous studies have shown that feline ES-like cell colonies initially exhibit AP staining, but lose this activity with spontaneous differentiation [14,15]. All ESC lines showed a flat and compact colony morphology and were positive for AP staining, even after multiple passages or freeze-thaw cycles, signifying a consistent undifferentiated state under our culture conditions.

We verified the expression of pluripotent core markers, including OCT3/4, SOX2, and NANOG. This finding demonstrated that the feline ESCs in this study possessed pluripotent potential. In immunostaining and flow cytometry, we extended our analysis beyond the core markers to include Tra-1-60, Tra-1-81, SSEA-1, and SSEA-4, aiming for a more detailed analysis of undifferentiated states. In their undifferentiated states, PSCs are generally classified into two distinct types: the “naïve” state, predominantly observed in mouse PSCs, and the “primed” state, characteristic of human PSCs [24]. The marker SSEA-1 is specifically associated with the naïve state, while markers such as Tra-1-60, and Tra-1-81, SSEA-4 are uniquely indicative of the primed state [25,26]. This study indicated that feline ESCs expressed or partially-expressed primed markers other than Tra-1-81, did not express a naïve marker, showed flat and compact colony morphology, and demonstrated bFGF- instead of leukemia inhibitory factor (LIF)-dependency, suggesting that these cells exhibited characteristics indicative of a primed state. Considering the existence of intermediate states between naïve and primed states in species such as humans, mice, and horses [27], the feline ESCs generated in this study can be classified as either primed or in an intermediate state. Further comprehensive analyses are needed to elucidate these characteristics.

To date, some findings have been reported concerning the undifferentiated markers of feline ESCs [14,15]. Yu et al. [14] reported that feline ES-like cells were positive for SSEA-1, SSEA-3, and SSEA-4 in immunostaining. In contrast, Gómez et al. [15] noted that feline ES-like cells were positive for SSEA-1 but negative for SSEA-4. These observations differ from our findings. However, both studies noted that the feline ES-like cells underwent differentiation during passaging. Conversely, the feline ESCs established in our study consistently retained their morphological characteristics and AP activity, indicating that they remained in an undifferentiated state. This suggests that our study may offer more insightful information about undifferentiated markers in feline ESCs compared to previous studies. However, the antibodies employed in these previous studies and this study were against rat, mouse, and human proteins. Therefore, continued investigation is key to enhancing the understanding of the specific markers associated with feline undifferentiation.

Moreover, in our study, feline ESCs formed EBs and differentiated into the cells expressing all three germ layers, highlighting the

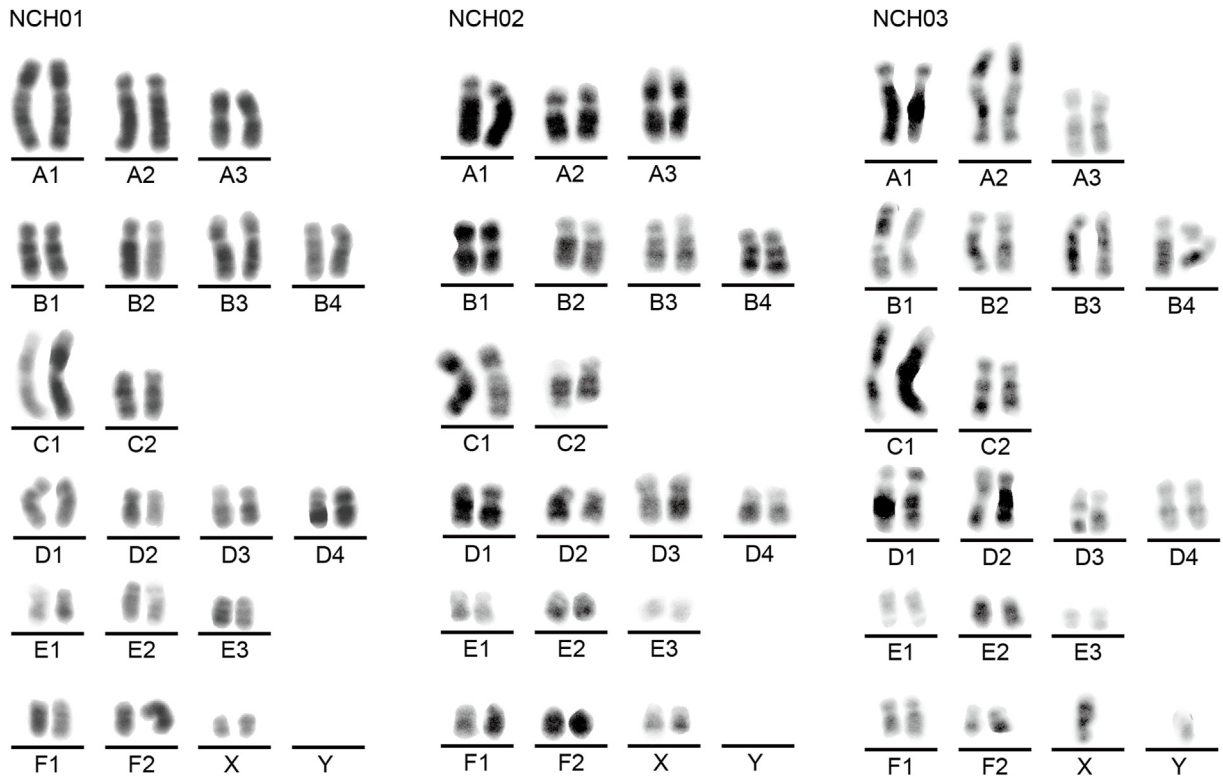

Fig. 5. Karyotype analysis of feline ESCs. Normal: 38, XX with 19 matched pairs of autosomes in line NCH01 and NCH02; 38, XY with 18 matched pairs of autosomes in line NCH03. Chromosomes were classified using the historical nomenclature that employs alphabetical and numerical categories referencing the size and position of the centromere.

potential of our feline ESCs to differentiate into all three germ layers *in vitro*. Prior research by Yu et al. [14] was limited to examining the spontaneous differentiation of feline ES-like cells into beating cardiomyocyte-like cells expressing the mesodermal marker α-SMA without investigating differentiation into ectodermal and endodermal lineages. To the best of our knowledge, this is the first report of feline ESCs differentiating into all three germ layers, as observed in PSCs of other mammals such as humans [3],mice [2], bovines [28], and dogs [26].

In addition to exploring *in vitro* differential ability, we also aimed to determine if feline ESCs could differentiate into various cell types *in vivo* using a teratoma formation assay. This assay is considered the gold standard for assessing differential ability in human and mice [29]. It is similarly employed for evaluating the differential ability *in vivo* in other mammals, including bovines [28], equines [30], and dogs [31]. Although only one report exists on the teratoma formation assay for feline PSCs, hematoma, not teratoma, was formed [32]. In our study, all feline ESC lines successfully formed teratomas. These findings confirm the *in vivo* differentiation capability of our feline ESCs.

Additionally, we assessed the karyotypic structure of these feline ESCs as a measure of tumorigenic potential. PSCs carry inherent risks of tumor formation, and chromosomal aneuploidy is a key indicator of tumorigenic risk. Our results suggested that all feline ESCs examined in this study present a low risk of tumorigenesis, supporting their safety for clinical use.

Although we established three feline ESC lines in this study, several challenges remain for their clinical application, such as developing xeno-free culture systems. We used MEFs as feeder cells for the establishment and maintenance of feline ESCs. Although MEFs secrete beneficial factors, including bFGF, they also produce detrimental factors that can induce cell death and differentiation [33]. Additionally, StemFit AK02N contains partially human-derived components, posing a risk of viral contamination in cultured PSCs [34]. Completely clear, xeno-free culture mediums for human PSCs are currently available, along with feeder-free culture methods that employ a synthetic substrate instead of MEFs [35,36] To advance the clinical application of feline ESCs, a similar xeno-free culture system must be developed for felines.

The outcome of this study is expected to contribute to advances in regenerative veterinary medicine for felines and aid in the generation of reproductive cells in ARTs for endangered wild cats. Recent progress in organoid technology and gene editing has shown potential in elucidating pathophysiology and developing novel medicines that can be applied to feline veterinary medicine. For instance, generating kidney organoids from feline ESCs to model the pathophysiology of chronic kidney disease could lead to the development of new therapeutic agents. Research in humans and mice on generating reproductive cells from pluripotent stem cells is advancing [37–40], with successful reports of generating sperm and oocytes with fertilization and embryonic development capabilities from murine PSCs [41,42]. The current study suggests the possibility of generating sperm and oocytes from feline ESCs, which could help overcome the shortage of reproductive cells—a major challenge in ARTs for feline species. Despite these beneficial outcomes, embryos, the genesis of life, are necessary to establish ESCs. Moreover, creating reproductive cells artificially from ESCs and using them to produce new individuals may also pose a risk of excessive intervention in nature. Therefore, discussion that incorporate public opinion is required on the ethical implications of utilizing embryos and generating reproductive cells.

5. Conclusion

In this study, we established three feline ESC lines. Our observations indicated that these feline ESCs could maintain their

undifferentiated states when cultured in StemFit AK02N medium and exhibited the capability to differentiate into all three germ layers both *in vitro* and *in vivo*. Furthermore, these cells retained their undifferentiated states post freeze-thaw cycles. While additional studies are essential to validate their potential clinical use, our study provides significant insight into veterinary regenerative medicine. Additionally, our results hold the potential to address the critical issue of reproductive cell shortage in ART for wild Felidae.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by JSPS KAKENHI (grant number 22H02525), JST SPRING (grant number JPMJSP2139) and the Sasagawa Scientific Research Grant from The Japanese Science Society (grant number 2022-4070). We would like to thank Editage (www.editage.com) for the English language editing. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.11.010.

References

[1] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981;292:154–6.
[2] Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 1981;78:7634–8.
[3] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.
[4] Mascetti VL, Pedersen RA. Contributions of mammalian chimeras to pluripotent stem cell research. Cell Stem Cell 2016;19:163–75.
[5] Oji A, Noda T, Fujihara Y, Miyata H, Kim YJ, Muto M, et al. CRISPR/Cas9 mediated genome editing in ES cells and its application for chimeric analysis in mice. Sci Rep 2016;6.
[6] Avior Y, Sagi I, Benvenisty N. Pluripotent stem cells in disease modelling and drug discovery. Nat Rev Mol Cell Biol 2016;17:170–82.
[7] Da Cruz L, Fynes K, Georgiadis O, Kerby J, Luo YH, Ahmado A, et al. Phase 1 clinical study of an embryonic stem cell-derived retinal pigment epithelium patch in age-related macular degeneration. Nat Biotechnol 2018;36:328–37.
[8] Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet 2015;385:509–16.
[9] Shroff G. Human embryonic stem cell therapy in chronic spinal cord injury: a retrospective study. Clin Transl Sci 2016;9:168–75.
[10] Lyons LA. Cats — telomere to telomere and nose to tail. Trends Genet 2021;37:865–7.
[11] Tsujimoto Y, Kaneko T, Yoshida T, Kimura K, Inaba T, Sugiura K, et al. Development of feline embryos produced using freeze-dried sperm. Theriogenology 2020;147:71–6.
[12] Yoshida T, Alam ME, Hanafusa K, Tsujimoto Y, Tsukamoto M, Kanegi R, et al. Effects of the preservation medium and storage duration of domestic caovaries on the maturational and developmental competence of oocytes *in vitro*. J Reprod Dev 2022;68:160–4.
[13] Jewgenow K, Zahmel J. Preservation of female genetic resources in feline species. Theriogenology 2020;156:124–9.
[14] Yu X, Jin G, Yin X, Cho S, Jeon J, Lee S, et al. Isolation and characterization of embryonic stem-like cells derived from *in vivo*-produced cat blastocysts. Mol Reprod Dev 2008;75:1426–32.
[15] Gómez MC, Serrano MA, Pope CE, Jenkins JA, Biancardi MN, López M, et al. Derivation of cat embryonic stem-like cells from *in vitro*-produced blastocysts on homologous and heterologous feeder cells. Theriogenology 2010;74:498–515.
[16] Alam ME, Iwata J, Fujiki K, Tsujimoto Y, Kanegi R, Kawate N, et al. Feline embryo development in commercially available human media supplemented with fetal bovine serum. J Vet Med Sci 2019;81:629–35.
[17] Kimura K, Tsukamoto M, Tanaka M, Kuwamura M, Ohtaka M, Nishimura K, et al. Efficient reprogramming of canine peripheral blood mononuclear cells into induced pluripotent stem cells. Stem Cell Dev 2021;30:79–90.
[18] Nakagawa M, Taniguchi Y, Senda S, Takizawa N, Ichisaka T, Asano K, et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci Rep 2014;4:1–7.
[19] Kimura K, Nagakura H, Tsukamoto M, Yoshida T, Shimasaki H, Shishida K, et al. Canine induced pluripotent stem cells can be successfully maintained in weekend-free culture systems. J Vet Med Sci 2024;86(3):247–57.
[20] Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2005;2:185–90.
[21] Levenstein ME, Ludwig TE, Xu R-H, Llanas RA, Van Den Heuvel-Kramer K, Manning D, et al. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cell 2006;24:568–74.
[22] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.
[23] Wobus AM, Holzhausen H, Jäkel P, Schöneich J. Characterization of a pluripotent stem cell line derived from a mouse embryo. Exp Cell Res 1984;152:212–9.
[24] Weinberger L, Ayyash M, Novershtern N, Hanna JH. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. Nat Rev Mol Cell Biol 2016;17:155–69.
[25] Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H, et al. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cell 2002;20:329–37.
[26] Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, et al. Differences between human and mouse embryonic stem cells. Dev Biol 2004;269:360–80.
[27] Yu L, Wei Y, Sun H, Mahdi AK, Pinzon Arteaga CA, Sakurai M, et al. Derivation of intermediate pluripotent stem cells amenable to primordial germ cell specification. Cell Stem Cell 2021;28:550–67.
[28] Zhao L, Wang Z, Zhang J, Yang J, Gao X, Wu B, et al. Characterization of the single-cell derived bovine induced pluripotent stem cells. Tissue Cell 2017;49:521–7.
[29] Müller FJ, Goldmann J, Löser P, Loring JF. A call to standardize teratoma assays used to define human pluripotent cell lines. Cell Stem Cell 2010;6:412–4.
[30] Nagy K, Sung HK, Zhang P, Laflamme S, Vincent P, Agha-Mohammadi S, et al. Induced pluripotent stem cell lines derived from equine fibroblasts. Stem Cell Rev Rep 2011;7:693–702.
[31] Tsukamoto M, Kimura K, Yoshida T, Tanaka M, Kuwamura M, Ayabe T, et al. Generation of canine induced pluripotent stem cells under feeder-free conditions using Sendai virus vector encoding six canine reprogramming factors. Stem Cell Rep 2024;19:141–57.
[32] Kanegi R, Hatoya S, Kimura K, Yodoe K, Nishimura T, Sugiura K, et al. Generation, characterization, and differentiation of induced pluripotent stem-like cells in the domestic cat. J Reprod Dev 2023;69:317–27.
[33] Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci USA 2006;103:6907–12.
[34] Barone PW, Wiebe ME, Leung JC, Hussein ITM, Keumurian FJ, Bouressa J, et al. Viral contamination in biologic manufacture and implications for emerging therapies. Nat Biotechnol 2020;38:563–72.
[35] Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8:424–9.
[36] Irina K, Young C, Larraiane M, Julie J, Michael DW, Robert L. Human embryonic stem cells derived without feeder cells. Lancet 2005;365:1636–41.
[37] Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from *in vitro* primordial germ cell-like cells in mice. Science 2012;338:971–5.
[38] Hwang YS, Suzuki S, Seita Y, Ito J, Sakata Y, Aso H, et al. Reconstitution of prospermatogonial specification *in vitro* from human induced pluripotent stem cells. Nat Commun 2020;11.
[39] Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 2011;146:519–32.
[40] Yamashiro C, Sasaki K, Yabuta Y, Kojima Y, Nakamura T, Okamoto I, et al. Generation of human oogonia from induced pluripotent stem cells *in vitro*. Science 2018;362:356–60.
[41] Zhou Q, Wang M, Yuan Y, Wang X, Fu R, Wan H, et al. Complete meiosis from embryonic stem cell-derived germ cells *in vitro*. Cell Stem Cell 2016;18:330–40.
[42] Yoshino T, Suzuki T, Nagamatsu G, Yabukami H, Ikegaya M, Kishima M, et al. Generation of ovarian follicles from mouse pluripotent stem cells. Science 2021;373.