Original Article

Self-replicating RNA for Cellular Reprogramming in Domestic Cat

Woranop Sukparangsi[1,3*] Nattakorn Suban Na Ayuthaya[1,3] Ratchapon Prakongkaew[1,3]

Santhita Karoon[2,3] Ampika Thongphakdee[2,3]

Abstract

Cat Induced Pluripotent Stem Cells have been previously established using viral vectors integrating into the host genome to deliver reprogramming factors. To aid wild felid conservation using stem cell technology, in this study we utilized non-integrative Venezuelan Equine Encephalitis-based self-replicating RNA (srRNA) to carry the reprogramming factors. This approach induced cat somatic cells towards pluripotency without genomic modification, supporting the future use of srRNA in wild felid cells. The srRNA expressing human OCT4, SOX2, KLF4, CMYC, and GLIS1 (OSKMG) could induce the formation of cat iPSC colonies after 4 rounds of transfections. Different species-specific Leukemia Inhibitory Factor (LIF) could capture the induced cells maintaining pluripotency. A combination of feline and human LIFs, could maintain cat iPSC in a pluripotent state, that was different from those seen in human or mouse embryonic stem cells (ESCs). The cat iPSCs also exhibited spontaneous differentiation towards ectodermal, mesodermal, and endodermal lineages upon the removal of LIF from the culture medium. This work demonstrates the utility of srRNA containing human reprogramming factors to induce reprogramming in somatic cat cells in the presence of serum and LIF. Further investigations into the maintenance of cat iPSC will aid in inducing wild felid stem cells for long-term conservation.

Keywords: cellular reprogramming, domestic cat, felids, induced pluripotent stem cells, self-replicating RNA

[1]*Department of Biology, Faculty of Science, Burapha University, Chon Buri, Thailand*

[2] *Wildlife Reproductive Innovation Center, Animal Conservation and Research Institute, Zoological Park Organization of Thailand under the Royal Patronage of H.M. the King, Bangkok, Thailand*

[3]*Excellent Unit of Wildlife Stem Cells (eWIS), Department of Biology, Faculty of Science, Burapha University, Chon Buri, Thailand*

***Correspondence**: woranop@go.buu.ac.th* (W. Sukparangsi)

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Introduction

Domestic cat Embryonic Stem Cells (cat ESCs) have been attempted to be established from pre-implantation blastocysts (Serrano *et al*., 2006; Yu *et al*., 2008; Yu *et al*., 2009; Gómez *et al*., 2010). Cat ESCs can be maintained in their pluripotent state using modified media containing 2i (CHIR99021, a GSK3β inhibitor and PD0325901, a MEK inhibitor) and Leukemia Inhibitory Factor (LIF) (Zhou, *et al*., 2019). The ESC state of these cells could also be achieved using the addition of fibroblast growth factor-2 (bFGF) to the combination of both feline and human LIFs and 2i in the culture medium, as described by Zhou *et al*. (2019); this combination sustains the expression of NANOG, POU5F1 (OCT4), and SOX2 in the cat ESCs. Cat ESCs propagated in the presence of 2i and LIF exhibit a unique naïve-ground pluripotency that differs from murine 2i supported ESCs (Ying *et al*., 2008). The inclusion of bFGF in cat ESC cultures by Zhou *et al*. (2019) led to results that may benefit from further analysis and additional studies in other felid species to gain a clearer understanding of cat pluripotency. The difficulty of establishing ESCs from embryos of wild felids is widely recognized. The difficulties of obtaining oocytes for IVF and the challenges of collecting embryos from pregnant wild animals make this approach unworkable. However, these difficulties can be largely eliminated by producing induced Pluripotent Stem Cells (iPSCs) from easily harvested somatic cells. Easily accessible skin cells or fibroblasts could be turned into pluripotent stem cells (PSCs) resembling ESCs using this approach.

OCT4 (POU5F1), SOX2, KLF4, and C-MYC (OSKM) are the four original transcription factors that can be used to create iPSCs from mouse and human somatic cells (Takahashi *et al*., 2007; Takahashi and Yamanaka, 2006). Additional reprogramming factors, which have shown promise in several mammalian species representing diverse eutherian orders, such as NANOG, LIN28, GLIS1, KLF2 and KDM4D, can be included in this reprogramming factor cocktail (Maekawa *et al*., 2010; Scoville *et al*., 2017; Wang *et al*., 2019; Yoshimatsu *et al*., 2021; Yu *et al*., 2007). Furthermore, iPSCs have been generated in various mammalian species, including porcine, equine, canine, bovine, galline, caprine, ovine, and feline, using various combinations of cytokines and inhibitors to sustain iPSC self-renewal (Scarfone *et al*., 2020). The introduction of transcription factors necessary for iPSC induction can be accomplished either via vectors that integrate into the genome, such as lentiviral/retroviral vectors, or through non-integrative approaches using Sendai virus, piggyBac transposon, episomal vectors, nonviral minicircle DNA, and CRISPR activator. Non-integrative approaches are essential for creating stem cells intended for clinical applications. Such iPSCs, created via non-integrative approaches, are also regarded as essential tools in wildlife conservation. Producing successful fertile offspring by achieving the differentiation of iPSCs towards germ cells could providing genetic rescue in disappearing species (Thongphakdee *et al*., 2020).

IPSCs from the domestic cat and a number of wild felids have been produced (Dutton *et al*., 2019; Verma *et al*., 2013; Zhou *et al*., 2019). It's important to note that there was a lot of variation in the culture conditions utilised to maintain felid iPSCs, especially with regard to the LIFs and other supplementing techniques used. Despite the fact that both Verma *et al*. (2013) and Dutton *et al*. (2019) depended only on LIF to maintain the iPSCs, Dutton *et al*. (2019) highlighted the importance of feline LIF (FLIF), while mouse LIF (MLIF) was unable to successfully maintain cat iPSCs (Dutton *et al*., 2019; Verma *et al*., 2013). In contrast, Zhou *et al*. (2019) adopted a combination of feline and human LIF and 2i to sustain cat iPSCs, based on their prior expertise with cat ESCs. The research listed above, however, used viral vector-based reprogramming, which involves the introduction of viral genes into host cells. To ensure the practical applicability of these methods in wild felid conservation, non-transgene integrating approaches for cellular reprogramming should be optimized to derive fully reprogrammed felid cells without introducing changes to their genomes.

Self-replicating RNA used for iPSC generation was originally derived from Venezuelan Equine Encephalitis (VEE) Virus genome that was genetically modified to eliminate viral replication competence (Yoshioka *et al*., 2013). Self-replicating RNA (srRNA), is a single-stranded RNA containing four non-structural genes, *nsP1*, *nsP2*, *nsP3*, and *nsP4* encoding the RNA replication complex. Such srRNAs can be used as a valuable tool to deliver reprogramming factors for the generation of iPSCs. Because their propagation does not involve a DNA intermediate, srRNAs can deliver reprogramming factors without any risk of genome modification, creating true integration-free iPSCs. In this respect, the key advantage of srRNA over the use of synthetic mRNAs is that a single srRNA can deliver multiple reprogramming factors at once (Steinle *et al*., 2019). In addition, due to their self-replicative behaviour, srRNA produce reprogramming factors for a longer duration, while mRNA-based methods require repeated transfections over the course of reprogramming. It needs to be pointed out that the introduction of exogenous RNA induces apoptosis due to an innate cellular response, a form of protective immunity. To overcome this, the successful use of srRNA or mRNA-based techniques both require the presence of the B18R protein, a recombinant vaccinia virus-encoded receptor that blocks the production of interferons, inhibiting apoptotic responses (Steinle *et al*., 2019; Yoshioka and Dowdy, 2017; Yoshioka *et al*., 2013). This intervention protects the integrity of introduced RNA vectors after transfection. A similar need for B18R was shown in the reprogramming of human and other mammalian cells (Yoshioka *et al*., 2013; Kim *et al*., 2017; Kim *et al*., 2020).

Given the limited availability of wild felid cells, in particular from endangered and vulnerable felid species, we set out to establish cat iPSCs from differentiated fibroblasts using the non-integrative srRNA approach. The work presented here describes the optimal conditions for the expression of srRNA in cat cells, the necessary components of the induction medium, and characterizes the induced cat iPSCs. The generation of srRNA-based cat iPSCs described in this

study paves the way to establishing a non-integrative approach to produce PSCs from wild endangered felid species for conservation purposes.

Materials and Methods

Animal Ethics: The study was reviewed and approved according to the Animal Care and Use Protocol Non-Housing Animal Procedure and was carried out under Project License IACUC 013/2563, issued by the BUU-IACUC, Burapha University, Thailand. Cryopreserved domestic cat embryonic fibroblasts (derived from an in vitro fertilized (IVF) embryo) were graciously provided by Dr. Ampika Thongphakdee from the Wildlife Reproductive Innovation Center (WRIC), Zoological Park Organization of Thailand (ZPOT). Tissue culture and gene delivery experiments were conducted in the Tissue Culture Facility of the WRIC following the regulations of the ZPOT.

Cell culture: The domestic cat IVF-derived embryo was placed into primary culture under fibroblast medium for a week before cryopreservation. Domestic cat embryonic fibroblasts were cultured in complete fibroblast medium containing Dulbecco's Modified Eagle's Medium (DMEM, high glucose, ThermoFisher), 10% Fetal Bovine Serum (FBS, Sartorius), 1X GlutaMAXTM supplement (ThermoFisher), 1X MEM Non-Essential Amino Acids Solution (ThermoFisher), and 1X Penicillin-Streptomycin Solution (Sartorius). Cells were incubated at 38.0 ºC in a humidified atmosphere containing 5% CO2. Adherent cells were detached using Trypsin-EDTA (Sartorius). The fibroblast cells were cryopreserved in freezing medium containing complete fibroblast medium with 10% DMSO added (Panreac Applichem). Only domestic cat fetal fibroblasts at early (P.0-3) and late (>P.13) passage numbers were used for testing.

Generation of self-replicating RNA: Plasmids based on the self-replicating Venezuelan equine encephalitis (VEE) virus RNA replicon, including the T7-VEE-OKSiM, T7-VEE-OKSiG and T7-VEE-GFP constructs, were originally created by Yoshioka *et al*. (2013). We purchased these vectors from Addgene. Plasmids were linearized with MluI restriction prior to RNA synthesis. Non-modified RNA was synthesized by *in vitro* transcription using the HiScribe™ T7 quick high yield RNA synthesis kit (NEB), according to manufacturer's protocol. The integrity of the produced RNA was stabilized by the addition of a 7-methylguanylate cap structure (Cap 0) to the 5´end of RNA using the Vaccinia Capping System (NEB) and mRNA cap 2'-O-methyltransferase (NEB). Finally, a poly(A) tail was added using E. *coli* Poly(A) Polymerase (NEB). This modified RNA was then treated with Antarctic Phosphatase (NEB) to remove 5´- phosphates from the self-replicating RNA. Between enzymatic steps the RNA was purified by LiCl precipitation. The modified srRNA was dissolved in RNase-free water and stored in -80 ºC.

Generation of domestic cat induced pluripotent stem cells: Domestic cat fibroblasts were seeded at a density of 1 x 10^5 cells per well in a 12-well plate (3 wells for each experiment condition) coated with 1X Attachment Factor Protein (ThermoFisher) and cultured in complete fibroblast medium. After reaching approximately 70% confluency cells were transfected with self-replicating RNA (srRNA) using Lipofectamine™ MessengerMax™ (ThermoFisher) according to a modified Yoshioka and Dowdy (28) method. Briefly, the cells were pre-treated with 200 ng/mL of recombinant viral B18R protein (R&D Systems) for 15 mins before transfection to minimize the cellular immune response. Four srRNA transfections were carried out on days 0, 2, 8, and 14, unless stated otherwise. Fibroblasts were transfected with 1250 ng of T7-VEE-OKS-iM in the experimental groups and 1250 ng of T7-VEE-GFP in the control group on day 0 and day 2, followed by a combination of 625 ng of T7-VEE-OKS-iM and 625 ng of T7-VEE-OKS-iG in the experiment group and 1250 ng of T7-VEE-GFP in the control group on day 8 and day 14. After 3 h of transfection the medium was changed to complete fibroblast medium. This medium also contained 200 ng/mL of recombinant viral B18R protein on day 0. From day 1 to the end of the experiment the medium was changed to cat iPSC induction medium, consisting of Advanced DMEM (ThermoFisher) with 10% EmbryoMax ES Cell Qualified FBS (Merck), 1X GlutaMAX™ (ThermoFisher), 0.1 mM β-mercaptoethanol (ThermoFisher), 0.5x Penicillin-Streptomycin (Peprotech), 200 ng/mL of recombinant viral B18R protein (R&D Systems) and 10 ng/mL of human LIF (HLIF, Peprotech), and/or 10 ng/mL of feline LIF (FLIF, Kingfisher Biotech), unless stated otherwise. Transfected cells were induced in iPSC medium containing the B18R protein. The medium was changed every day until day 9 after the first transfection. On day 16 the induced cells were passaged onto irradiated MEF feeders (ThermoFisher) in a 24 well-plate (5 x 10^3 cells per well). The medium was changed every other day until iPSC colonies appeared. The iPSC colonies were picked within a week after colonies emerged and the trypsinized cells were replated into 96-well plates coated with irradiated mouse embryonic fibroblast (MEF) feeders and cultured in cat iPSC induction medium. The experimental procedure is shown in Fig. 2A. It is noteworthy that we excluded cat feeders (mitomycin-C treated cat fibroblasts) from the experiments due to preliminary tests that did not yield successful cat iPSC colony formation.

Immunofluorescence: Cells were fixed with 4% paraformaldehyde (PFA) for 15 mins at room temperature and washed with DPBS three times. To permeabilize the plasma membrane the fixed cells were treated with 0.1% Triton X-100 in DPBS for 15 mins at room temperature and washed in DPBS for three times with 5 min incubation at each wash. Next, the cells were incubated in DPBS supplemented with 1% BSA for 1 h at room temperature. Primary antibodies including OCT4 (MABD76), SOX2 (AB5603), NANOG (MABD24), and SSEA-1 (MAB4301) (all from Merck) were added at a 1:300 dilution. Antibodies against NESTIN (MAB5326),

ASMA (CBL171), and AFP (ST1673) were used at a dilution of 1:100. Cells were incubated in the presence of the antibodies overnight at 4 ºC and washed three times with DPBS the next day before the addition of the appropriate fluorophore-conjugated secondary antibody, including FITC (Merck, 1:800), Alexa Fluor 488 (Molecular Probe, 1:800), Alexa Fluor 568 (Molecular Probe, 1:800) and Hoechst33342 (ThermoFisher, 1:200). Staining was carried out in the dark for 1 h at room temperature and was followed by three washes in DPBS. Fluorescent micrographs were taken using an Eclipse Ti-S Inverted Research Microscope (Nikon) and digital camera.

RNA extraction, reverse transcription and quantitative real-time PCR: RNA extraction was performed using Total RNA Mini Kit (Blood/Cultured Cell, Geneaid). Complementary DNA (cDNA) was synthesized using iScript™ Reverse Transcription SuperMix (Bio-Rad). All procedures were performed in accordance with the manufacturer's instructions. qRT-PCR analysis was performed using the Luna® universal qPCR master mix (NEB). qRT-PCR reactions contained 5 µL of qPCR Master Mix, 0.25 µL of forward and reverse primer each, 1 µL template DNA, and were adjusted to a final volume of 10 µL with water. Amplification was performed for 40 cycles of denaturation at 95 ºC for 15 s and extension at 60 ºC for 30 s using a CFX96 Touch Real-Time PCR instrument (Bio-Rad). Cq values were recorded for calculating relative mRNA abundance using *ATP5F1* or *GAPDH* as housekeeping genes. The relative expression was calculated in EXCEL using the $2^{-\Delta\Delta CT}$ method. Error bars in qPCR plots represent three technical replicates, unless stated otherwise.

Statistical Analysis: Statistical analyses were perform using Prism (Graphpad) in Figs. 1 and 2. Unpaired t tests with two-stage step-up (Benjamini, Krieger, and Yekutieli) were used. The statistical analyses were performed using Minitab 18.1 (Minitab, Inc.) (Fig.3). The percentage data were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests. Each dataset was tested for normality of distribution using the Anderson–Darling test ($\alpha = 0.05$). All results are expressed as mean ± standard error of mean (SEM) and $p < 0.05$ was considered to be statistically significant.

Table 1 Primer sequences used for qRT-PCR in this study

Gene	Forward primer	Reverse primer
POU5F1/OCT4	CCGAAAGAGAAAGCGAACAAG	GACCACATCCTTCTCCAGC
NANOG	CAGCCCCAGATACAGTTACAG	GCTGGGCACTAAAATACTTGG
SOX2	ATGCACAACTCGGAGATCAG	TTTATAATCCGGGTGCTCCTTC
REX1	AGGCATCTCCTCGTTCATGC	AAACGTTTTCCGCACCCTTC
ESRRB	AGGGTTAGTGGGCTCCAAGT	GCAGAGTTCTGACACGTCCA
FGF5	CGGATGGCAAAGTCAATGGC	TCGGTAAATTTGGCACTTGCAT
GATA6	GTGGACTCTACATGAAGCTCC	CTTTTCCTGGTTTGAATTCCCTC
SOX17	CAGTGACGACCAGAGCCAGC	GCGTTCATCGGCCGCCGGATGC
NCAM1	GACATCACCTGCTACTTCCTG	TTCCTTGGACTCATCTTTCGAG
CDH1	GATAACCAGAACAAGGACCAGG	TCCGAGAATGCCCAGAATG
CDH2	CAGACCAGAGTTCTTACACCAG	GATTCTGTACCTCAACATCCCG
nsP2	TCCACAAAAGCATCTCTCGCCG	TTTGCAACTGCTTCACCCACCC
GAPDH	AAGGCTGAGAACGGGAAAC	CATTTGATGTTGGCGGGATC
ATP5F1	AATCGCCTGGACTATCACATC	TGCTTCTCCACCCAGTTTATC

Results

Induction of cat somatic cell reprogramming using self-replicating RNA: Our aim was to generate cat iPSCs without genetic modification as a model for establishing wild felid iPSC in the future. The necessary reprogramming factors were introduced using the self-replicating RNA replicon system described by Yoshioka *et al*. (2013) and Yoshioka and Dowdy (2017). The VEE replicon vectors were the T7 VEE OKSiM and T7 VEE OKSiG constructs. Both of these carry the same human *OCT4* (O), *SOX2* (S), and *KLF4* (K) coding sequences. However, while the OKSiM replicon carries the human *MYC* (M) gene the OKSMiG construct contains the human *GLIS1* (G) sequence (Fig.1A).

We optimized the experimental strategy of srRNA transfection using the lipoplex approach. First, we tested the requirement of B18R protein (protecting cells from apoptosis caused by innate immunity after the introduction of 'foreign' RNA) for the persistence of the transfected RNA in cat cells. To monitor the efficiency of cellular entry and persistence of srRNA, a vector expressing green fluorescent protein (GFP) (Fig.1A) was utilized. The optimization strategy is summarized in Fig.1B, upper panel. Briefly, cat fibroblast cells were transfected with the *gfp* carrying srRNA using Lipofectamine MessengerMAX. The transfected cells were sub-cultured and maintained in media with or without B18R for 7 days. Widely distributed GFP+ cells became visible in the cultures by day 12, suggesting the effective srRNA replication (Fig.1B). However, in the absence of B18R protein, GFP expressing cells were rapidly lost (Fig.1B). These observations clearly indicated that, the presence of B18R protein in the culture media was necessary for the persistence of srRNA to maintain exogenous gene expression under the VEE replicon promoter.

To initiate the reprogramming of cat fibroblasts, we examined following conditions: the importance of passage number, the number of repeated srRNA transfection rounds, and the need for different cytokines/inhibitors in the induction medium. We first examined the morphology and doubling time of cat FB at early (P.0-3) and late (>P.13) passage numbers. At late passages the cells grew slowly and exhibited clear

sign of senescence in β-galactosidase assays (Fig.1C). In addition, cells from the early passages had significantly shorter doubling times (Fig.1C). We then examined how multiple rounds of repeated transfections affected cell proliferation after transfection with the OKSiM/OKSiG vectors. The cell division cycle of human ESCs is around 15 h while mouse ESCs divide every 10 h (Zaveri and Dhawan, 2018). When cat fibroblasts were induced using four rounds of transfection with reprogramming factors, the resulting cells were much smaller and exhibited epithelial-like appearance, indicating mesenchymal to epithelial transition (MET). In contrast, cells only transfected once retained a more fibroblastic character (Fig.1D). Increasing the number of times cells were transfected with srRNA decreased their doubling time, significantly speeding up cell division (Fig.1E). To confirm the continued presence of self-replicating RNA in T7 VEE OSKiM/T7 VEE OSKiG transfectants that did not contain a reported gene, RT-PCR experiments were conducted. The presence of srRNA was confirmed by amplifying a fragment of the *nsP2* non-structural gene (Fig.1F, upper panel). The intracellular abundance of srRNA was measured by real-time RT-PCR at different time points after transfection. These experiments showed that the abundance of the srRNA after four rounds of transfection was almost five times higher than in cultures subjected to a single transfection (Fig.1F, lower panel).

Next, we examined the impact of human and/or feline LIF on the efficiency of iPSC induction. As Dutton *et al*. (2019) have already demonstrated that mouse LIF was insufficient to support feline iPSCs, we decided not to investigate this option. Human and/or feline LIFs were added to the induction media at the same concentration (10 ng/mL) and the appearance of early changes was monitored in the transfected cells. Although there was some shortening of the doubling time in some of the LIF treated cultures, these differences failed to reach statistical significance ($p > 0.05$) (Fig.1G). When transfected cells were cultured in a medium based on Advanced DMEM (for composition see section 2.4) with or without human LIF, the doubling time shortened significantly compared to cells grown in regular fibroblast medium (Fig.1G). In summary, the induction of reprogramming of somatic cat fibroblasts with self-replicating RNA could be facilitated by the presence of B18R protein, increasing the number of rounds of transfections, and the use of enriched medium based on Advanced DMEM. In contrast, during the early stages of reprogramming, the presence or absence of human or feline LIF had no measurable effect.

SrRNA-based Cat iPSC colony formation and LIF effect to iPSC culture: To achieve the induction of feline cellular reprogramming, we compared non-integrative reprogramming methods using the addition of either feline LIF alone (FLIF protocol) or the combined addition of both human and feline LIF (HFLIF protocol). Both testing conditions included the use of B18R and four rounds of srRNA transfection, as depicted in Fig.2A. Results show that using feline LIF alone (FLIF protocol) was insufficient to produce expandable iPSC colonies. In contrast, when OSKM/G transfected cells were cultured using the HFLIF protocol iPSC colonies appeared a few days after reseeding the transfectants into irradiated MEF containing wells (Fig.2B). Interestingly, adding human LIF to cells initially cultured in feline LIF before reseeding (referred to as F/HFLIF protocol) could rescue iPSC generation, producing numerous colonies (Fig.2B). We observed SSEA1 positive colonies using both the HFLIF and F/HFLIF protocols, indicating the presence of a pluripotent colony signature (Fig.2B). The number of iPSC colonies and reprogramming efficiency were measured as shown in Fig.2C. The HFLIF protocol resulted in significantly higher number of iPSC colonies and reprogramming efficiency than the F/HFLIF protocol, where cultures initially grown in the presence of FLIF and were rescued by the addition of HLIF after seeding. Notably, the appearance of iPSC colonies rescued using the F/HFLIF protocol was also delayed by about a day.

The iPSC colonies produced by the HFLIF and F/HFLIF protocols were picked to generate stable cat iPSC cell lines. All colonies could be expanded but showed different cell/colony morphology, that could be categorized into four types: (i) dome-shaped colonies with naïve iPSC colony morphology similar to murine iPSCs, (ii) flat-shaped colonies representing differentiated colonies or partially reprogrammed cells, (iii) rounded cells without apparent colony formation, representing partially reprogrammed cells, and (iv) fibroblast reversion. Representative examples of the various colony structures are illustrated in Fig.2D. During passage 0, most iPSC clones exhibited dome-shape and flat-shape colonies (Fig.2D right panel). In some colonies, there was a mixture of more than two cell types, indicating the heterogeneity of cell populations. Interestingly, there were more dome-shape colonies exhibiting normal iPSC phenotype created using the F/HFLIF protocol while more flat-shape colonies or partial iPSC clone phenotype appeared under HFLIF conditions (Fig.2D right panel). We continued to passage cat iPSC clones and found that most of the clones from HFLIF condition collapsed, producing rounded cells that no longer formed colonies. However, in some cases, cells became increasingly flat or even reverted back to a fibroblast-like phenotype. Unexpectedly, cat iPSC clones that could be sustained beyond passage 6 were all produced using the F/HFLIF protocol (Fig.2E). This result suggests that there is a timing sensitive point in the LIF response during the reprogramming of cat cells, when LIF is needed to capture the induced cells in their pluripotent state. It is possible that the efficiency of reprogramming could be further improved by fine-tuning the dosage and timing of LIF administration.

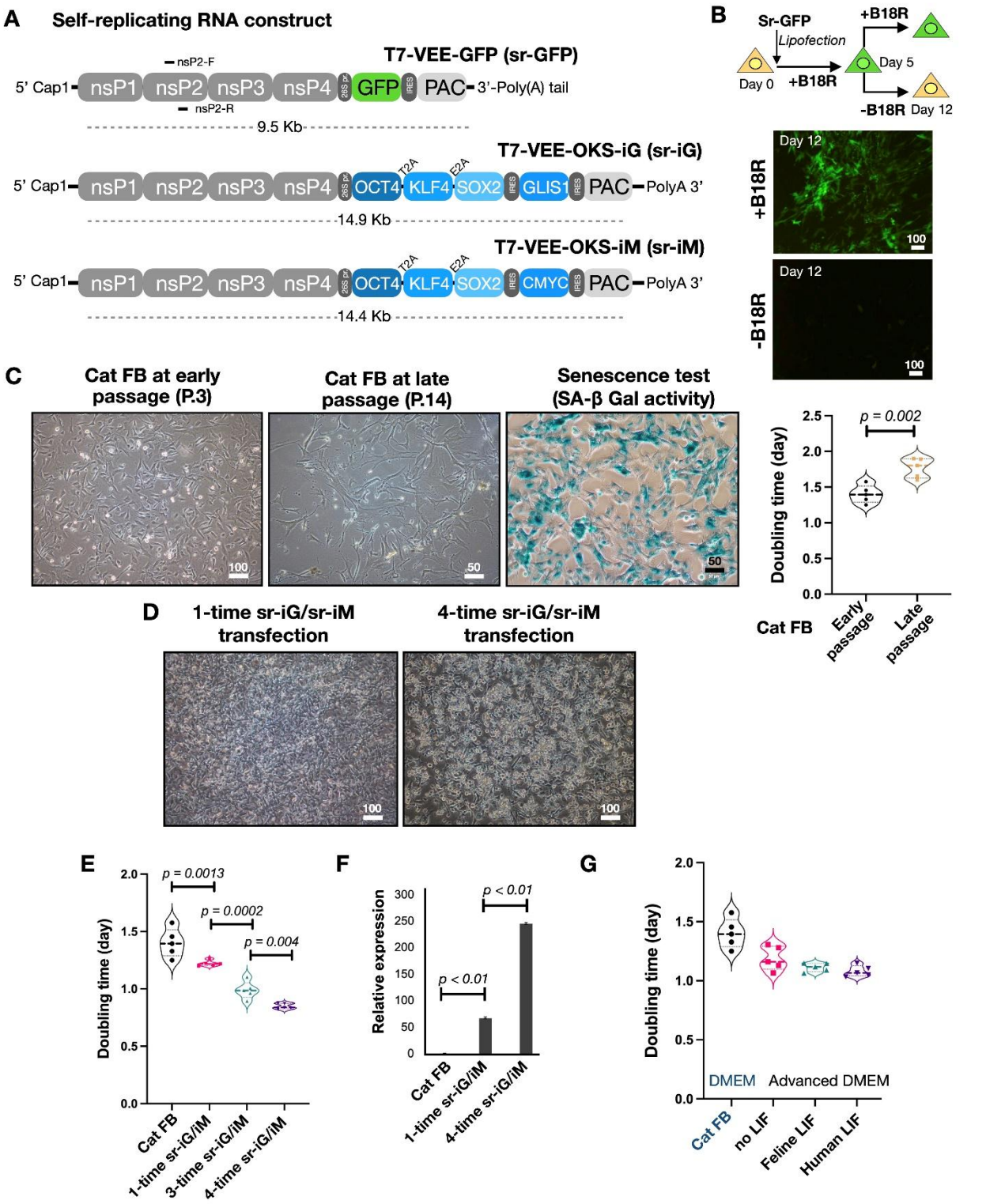


Figure 1 Initiation of reprogramming in somatic cat cells using srRNA. (**A**) Schematic diagram of the self-replicating RNA (srRNA) construct used for iPSC induction. (**B**) Upper panel: Experimental design testing the role of B18R treatment during srRNA transfection and prolonged culture. Lower panel: Representative images of srRNA-GFP transfected cells cultured for 12 days with/without B18R treatment. (**C**) panels from left to right: Brightfield images showing the morphology of cat embryonic fibroblasts from an early and a late passage, and senescence test on the late passage cat fibroblasts (P.15), with violin plots showing doubling time of cells from early passages (P<8) and late passages (P>15). (**D**) Brightfield images showing the morphology of cells transfected once or four-times with srRNA expressing the reprogramming factors according to the experimental designs shown in Fig.1A. (**E**) Violin plots showing the doubling time of cat fibroblasts (cat FB) and cells transfected 1, 3, and 4-times with srRNA. (**F**) Upper panel: RT-PCR demonstrating the presence of self-replicating RNA, detecting *nsP2*, in transfected cells. Lower panel: qRT-PCR quantitating the expression level of the self-replicating RNA by measuring transcript level of nsP2 in the transfected cells compared to fibroblasts. (**G**) A violin plot showing the doubling time of transfected cells cultured in different medium and LIF. Abbreviations: nsP1-4: non-structural gene 1-4; GFP: green fluorescent protein, IRES: internal ribosome entry site, PAC: puromycin resistance gene. All scale bars are in micrometer. P-values in C, E and F were calculated by using unpaired t tests.

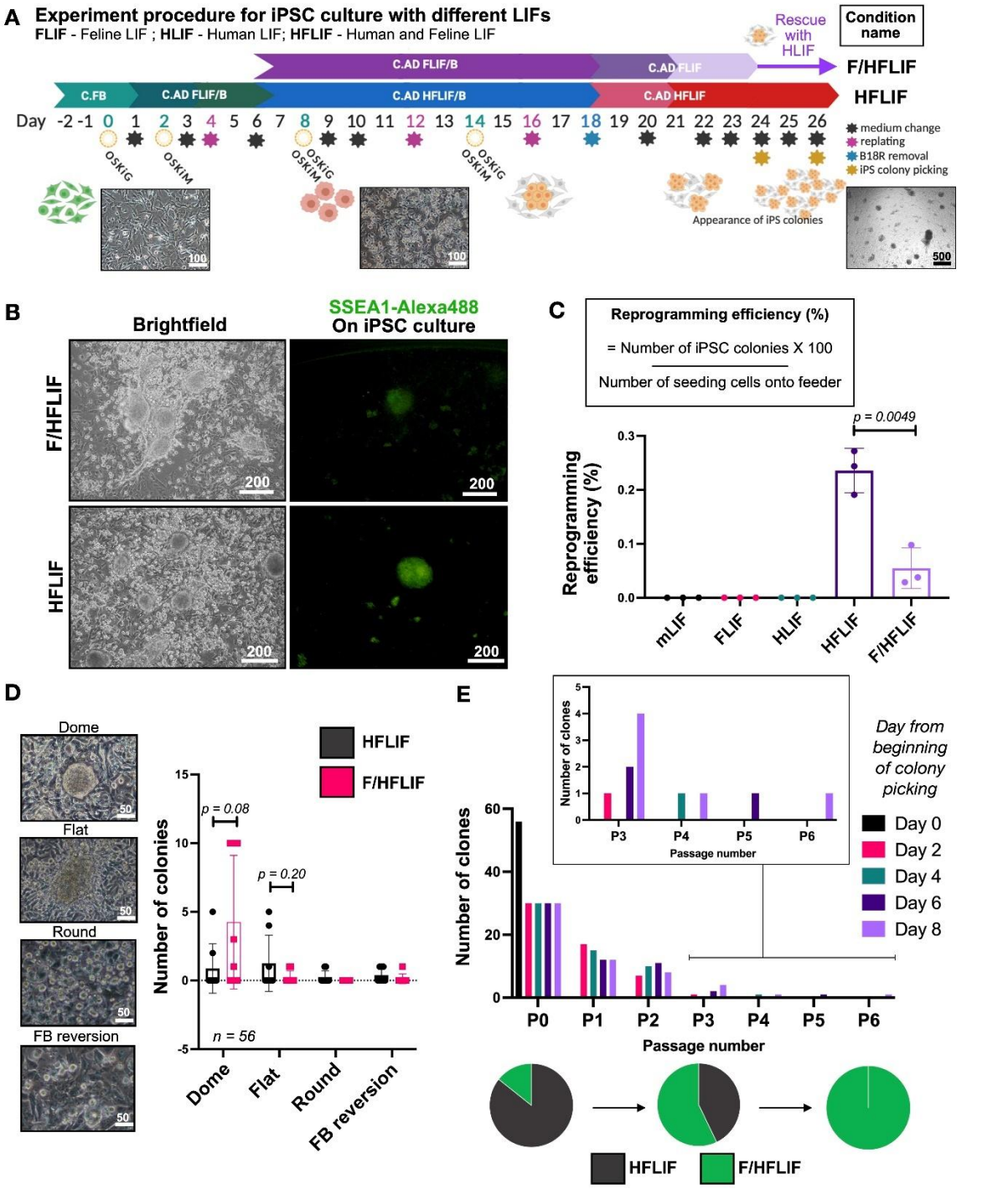


Figure 2 Efficiency gains in the reprogramming strategy using srRNA. (A) Schematic diagram of the timeline of events during cellular reprogramming. Two approaches were tested, referred to as HFLIF and F/HFLIF. In HFLIF cells were cultured in the presence of human LIF and feline LIF continuously after day 6. In the F/HFLIF protocol, the cells were cultured with feline LIF between day 6 to 18, then human LIF was added to the culture from day 18 onwards. The final concentration of both feline and human LIF was 10 ng/mL. (B) Brightfield images of cat iPSCs derived using the F/HFLIF and HFLIF protocols (Left). Fluorescent micrographs showing the expression of the pluripotent stem cell surface marker, SSEA 1, using an-Alexa488-labeled secondary antibody (Right). (C) Reprogramming efficiency using the HFLIF and HFLIF protocols. The calculation of reprogramming efficiency was based on two factors: (1) the number of induced cells seeded onto irradiated MEF feeders (5 x 103 cells per well) and (2) the number of iPSC colonies that appeared and were counted at day 26 after the first transfection. Each data point represents biological replicates. (D) Establishment of cat iPSC clonal cell lines. Left panel: cat iPSC colonies seeded onto irradiated MEF cells showing four distinct patterns: Dome shape, flat shape, round shape without colony formation, and fibroblast reversion (FB reversion). Right panel: Box plots showing the frequency of colony morphology found in culture after seeding (n=56 clones). (E) Progression of stable cat iPSC clonal cell lines from 56 colonies. Circular graphs underneath represent the proportion of expandable iPSC clones derived using the HFLIF versus F/HFLIF protocol. All scale bars are in micrometer. P-values for the experiments shown in C and D were calculated by using unpaired t tests.

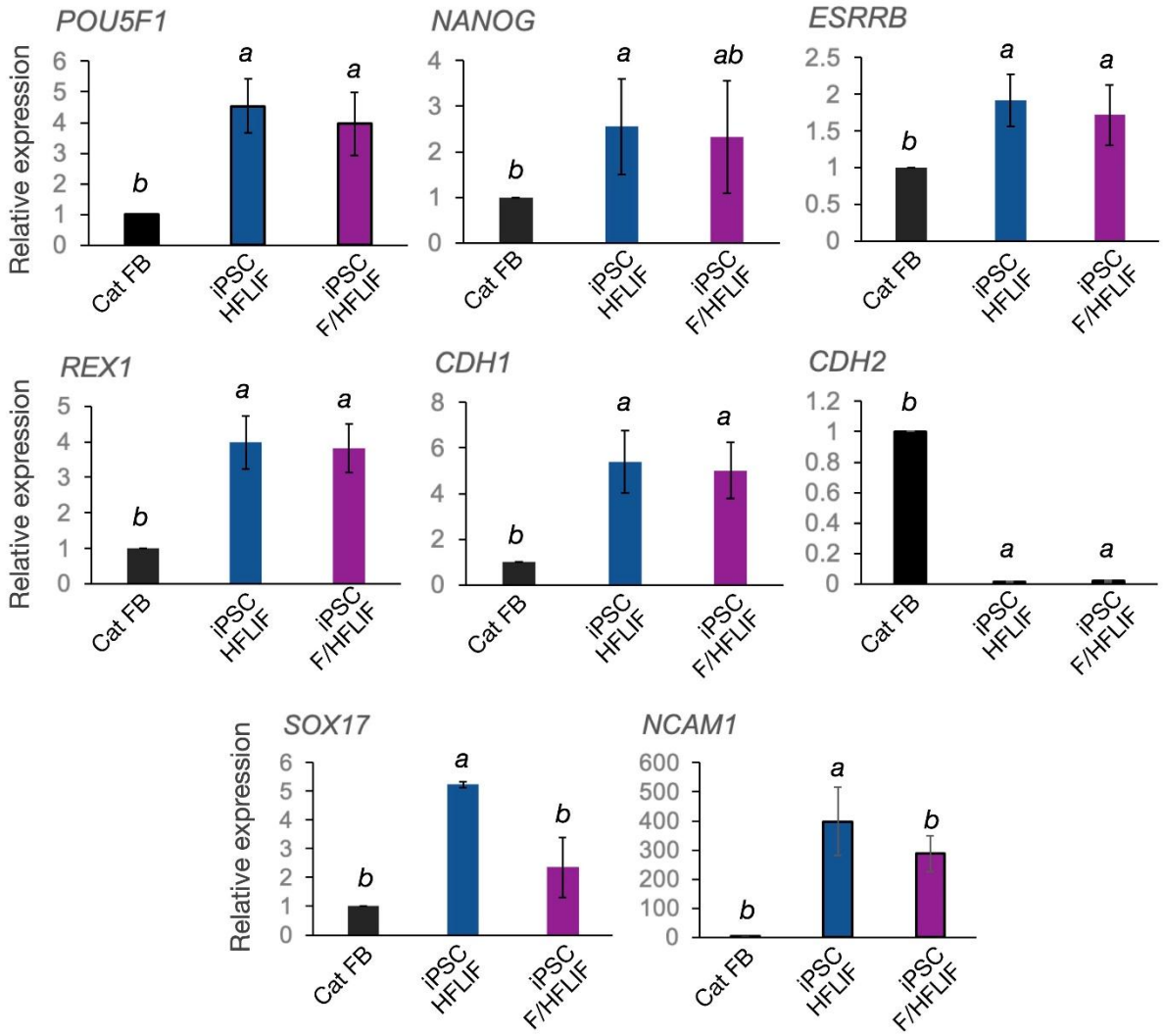
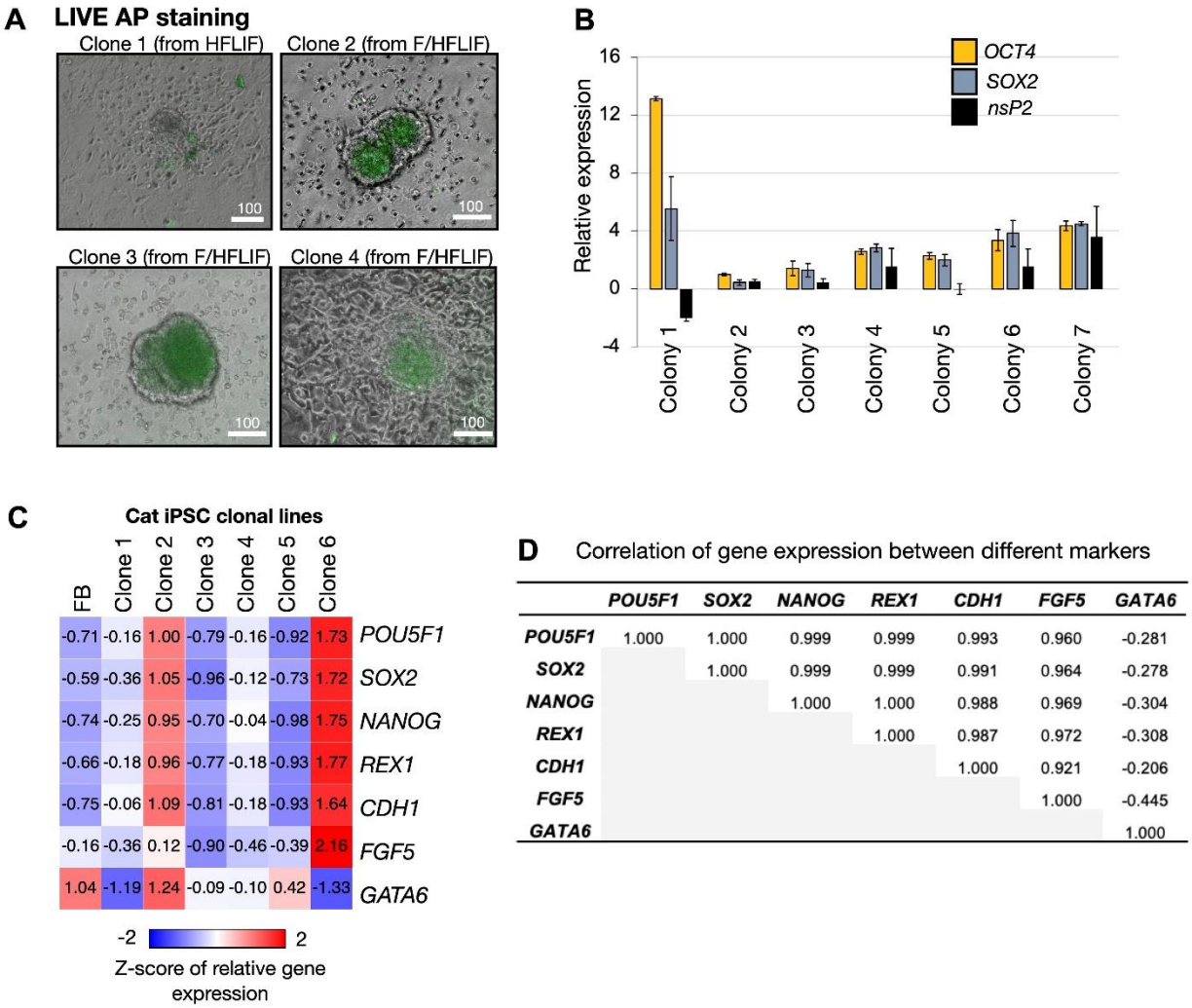


Figure 3 Gene expression analysis of cat iPSC cultures cultured using different LIF conditions. Relative expression of the pluripotency *markers POU5F1, NANOG, ESRRB*, and *REX1*, markers of differentiation, *SOX17* as endoderm marker and *NCAM1* as neuroectoderm marker, and cell adhesion molecules *CDH1* and *CDH2* as detected by real-time RT-PCR of the reprogrammed cells using the iPSC culture conditions described in Fig.2. ATP5F1 was used a housekeeping gene for normalization. The HFLIF and F/HFLIF protocols are described in Fig.2. Gene expression was quantitated using the $2^{-\Delta\Delta Ct}$ method. Data shown as mean ± standard deviation. Letters a-c indicate significant differences at $p<0.05$ based on grouping information using the Tukey Method and 95% confidence.

Gene expression analysis of srRNA-induced cat iPSC cultures shows a unique signature of pluripotency: To elucidate the nature of the heterogeneity in cultured cat iPSCs, we performed a panel of quantitative RT-PCR tests to assess the transcript abundance of various markers of pluripotency and differentiation. The comparison of fibroblasts and iPSC cultures derived using the HFLIF and F/HFLIF protocols showed that *POU5F1* (*OCT4*), *REX1*, and *ESRRB* abundance was significantly higher under both conditions of iPSC derivation. *NANOG* was significantly higher in iPSCs generated using the HFLIF protocol and slightly lower when iPSCs were produced under F/HFLIF conditions. We then analyzed cell adhesion molecules, which can be used as markers of pluripotency in stem cells. It has been shown that murine iPSCs generally expressed E-cadherin (encoded by *CDH1*) while fibroblasts strongly expressed N-cadherin (encoded by *CDH2*) (Redmer *et al*., 2011). We also observed the strong expression of *CDH1* (encoding E-cadherin) and a reciprocally low level of *CDH2* (encoding N-cadherin) in cat iPSCs irrespective of the conditions used for their induction. In contrast, fibroblasts exhibited strong *CDH2* expression. To establish the degree of differentiation between iPSCs derived under various conditions, we tested the expression of the neuroectoderm marker *NCAM1* and *SOX17*, an endoderm marker. Unexpectedly, both of these markers were expressed higher in iPSCs induced using the HFLIF protocol. Based on the expression of these differentiation markers, it can be concluded that cat iPSC cultures did not represent a pure population of pluripotent cells but showed a degree of differentiation bias toward different lineages. We believe that this indicates spontaneous differentiation of unstable pluripotent cells that was more likely to happen in cells derived under the HFLIF protocol (Fig.3).

Cat iPSC clones exhibit unique pluripotency-related gene expression patterns: Next, we maintained cat iPSC clones for longer periods to assess their ability to retain pluripotency over time. In these experiments, alkaline phosphatase activity was used to monitor the presence of pluripotent cells derived under the various iPSC culture conditions described in the previous section. Clones derived using the F/HFLIF protocol consisted of colonies with clear AP activity in the center, while cells on the periphery showed no evidence of AP production. In contrast, in HFLIF clones the AP signal was absent from the center of the colonies but small clusters of AP positive cells could be seen near the edge (Fig.4A). We next asked if the instability of cat iPSC colonies resulted from the prolonged production of reprogramming factors by measuring the presence of srRNA. In these experiments the expression of the *nsP2* non-structural protein RNA and the abundance of *OCT4* and *SOX2* mRNAs were assessed simultaneously. Interestingly, colonies with detectable *nsP2* all expressed lower levels of *OCT4* and *SOX2* while in colonies where the RNA of the non-structural protein was no longer detectable higher levels of *OCT4* and *SOX2* were seen (Fig.4B). To clarify this situation, further additional iPSC clones were established and the expression of early embryonic markers was assessed by qRT-PCR. The clones exhibited a high degree of divergence (Fig.4C). Most of them consisted of partially reprogrammed cells expressing low levels of pluripotency markers. However, there were two clones showing an abundant expression of key markers associated with pluripotent cells, including *POU5F1*, *SOX2*, *NANOG*, *REX1*, and *CDH1*. However, there was divergence in the pattern of other early embryonic markers. Clone 2 expressed low levels of *FGF5* but showed high levels of *GATA6* while clone 6 showed the opposite pattern, with high *FGF5* but low *GATA6* expression (Fig.4C). To detect any bias in the expression of early embryo markers in iPSC clones, the relative abundance of different markers was calculated (Fig.4D). This approach showed that the expression of all key pluripotency markers (*POU5F1*, *SOX2*, *NANOG*) showed correlation. Furthermore, the expression of *REX1-FGF5* and *CDH1*, both hallmarks of a naïve-primed state, showed correlation with the expression levels of pluripotency markers. However, the expression of *GATA6* did not follow this pattern. This indicates that cat iPSCs induced by reprogramming factors delivered via srRNA can show distinct levels of pluripotency that differs from murine or human iPSCs.

SrRNA-derived cat iPSC clones can differentiate into all three germ layers: After detecting the expression of *OCT4* and *NANOG* at mRNA level in iPSC cultures and clonal lines, we confirmed the presence of OCT4 and NANOG proteins using immunofluorescence to visualize the localization of these core transcription factors (Fig.5). We found strong OCT4 staining in iPSC colonies but the expression was more heterogeneous in cells in those colonies showing signs of differentiation. The expression of OCT4 followed the same pattern, with stronger staining in colonies showing typical iPSC-like morphology and weaker expression in non-colony forming cells (Fig.5A). To investigate the capacity of the established cat iPSCs to differentiate into all three germ layers, and assess bias within the cat iPSC clones, iPSC clones were investigated by immunofluorescence after culturing them in the absence of LIF. After 7 days in culture without LIF, an increasing number of cells started to express the neuroectodermal marker NESTIN, the mesodermal marker alpha smooth muscle actin (ASMA), and the endodermal marker alpha-fetoprotein (AFP) (Fig.5B). These findings indicated that cat iPSCs derived by the use of srRNA contained pluripotent cells with the potential to differentiate towards all three germ cell layers.



	POU5F1	SOX2	NANOG	REX1	CDH1	FGF5	GATA6
POU5F1	1.000	1.000	0.999	0.999	0.993	0.960	-0.281
SOX2		1.000	0.999	0.999	0.991	0.964	-0.278
NANOG			1.000	1.000	0.988	0.969	-0.304
REX1				1.000	0.987	0.972	-0.308
CDH1					1.000	0.921	-0.206
FGF5						1.000	-0.445
GATA6							1.000

Figure 4 Cat iPSC clones exhibit diverse phenotypes. (**A**) Merged images of brightfield and fluorescent alkaline phosphatase staining (green) of cat iPSC clones from different cultures. (**B**) Relative expression ($2^{-\Delta\Delta Ct}$ method) of pluripotency markers (*OCT4* and *SOX2*) and self-replicating RNA (detection by *nsP2*) in different iPSC clones. (**C**) qRT-PCR analysis of cat iPSC clones shown as a heat map. The values in the heat map represent relative gene expression (as z-score calculated using Morpheus). Expression of early embryo markers for iPSC phenotype analysis included genes specific for pluripotency (*POU5F1*, *SOX2*, *NANOG* and *REX1*), primed state (*FGF5*), cell adhesion (*CDH1*) and primitive endoderm marker (*GATA6*) in pluripotent cells. (**D**) Correlation of gene expression patterns shown in C.

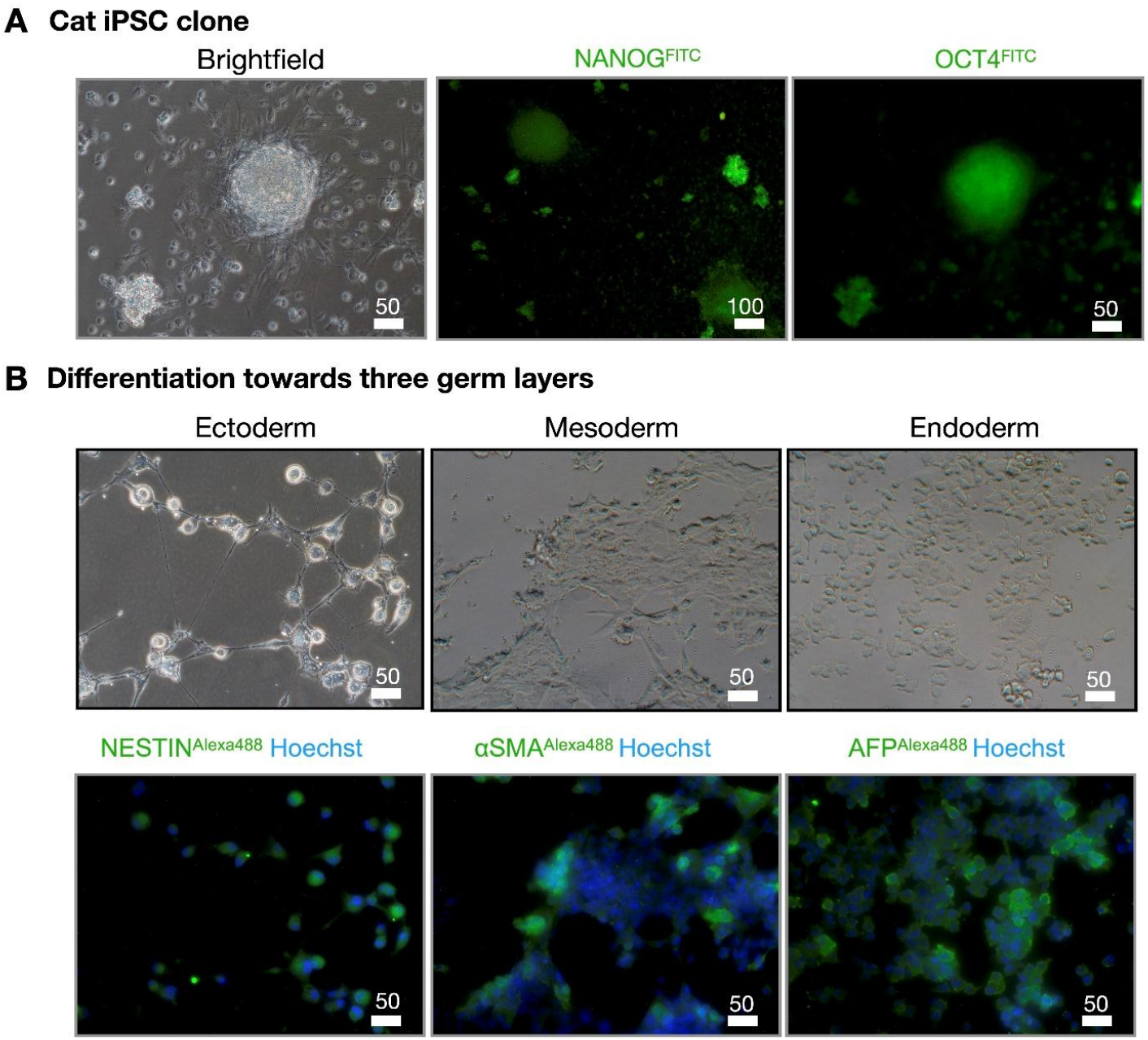


Figure 5 Cat iPSC clones express core pluripotency markers and can differentiate towards three germ layer cells. (A) Representative cat iPSCs at passage 6 were stained with nuclear stain Hoechst33342 and pluripotency markers including OCT4 and NANOG detected with a FITC-labeled secondary antibody (green). (B) Cat iPSCs were cultured in the absence of LIF for 7 days and stained with antibodies detecting NESTIN, ASMA and AFP to detect ectodermal, mesodermal and endodermal differentiation, respectively. All scale bars are in micrometer.

Discussion

Feline cellular reprogramming is a promising novel strategy to protect wild felid species from extinction (Thongphakdee *et al*., 2020). In the work presented here we explored the potential utility of non-transgene integrating srRNA, based on the Venezuelan Equine Encephalitis replicon (Bailly *et al*., 2022), in reprogramming somatic cells in domestic cats. This approach avoids any risk of modifying the host genome, resulting in a strategy that could be applied in the conservation of wild felid species. The first step in the optimization of this approach was the liposome-based transfection of a GFP expressing srRNA construct that enabled the easy monitoring of the expression of the transgene. These transfectants showed that the srRNA system works effectively in cat cells, suggesting that this approach is likely to be applicable in other wild felids after some optimization. The maintenance of srRNA within cat cells requires the presence of B18R to inhibit the production of interferons and apoptosis, while also protecting the

integrity of introduced RNA vectors after transfection, as demonstrated in other mammalian cell reprogramming studies (Kim *et al*., 2020; Kim *et al*., 2017; Yoshioka *et al*., 2013).

Several previous studies delivered reprogramming factors using a mixture of individual vectors, carrying one factor each. This approach resulted in great variability in the expression levels of the introduced factors, leading to the production of non-uniform iPSC populations. An srRNA replicon can carry all reprogramming factors in a single polycistronic construct. The uniform changes in cell morphology seen in our studies clearly demonstrate the superiority of delivering all factors using a single vector. Furthermore, the continued expression of all reprogramming genes from a single srRNA allowed sufficient time for these factors to induce pluripotency in a more reproducible way than mRNA transduction. These findings are consistent with the observations reported by Steinle *et al*. (2019). In the reprogramming of human cells, a single transfection of srRNA was sufficient to establish iPSCs (Steinle *et al*., 2019; Yoshioka *et al*., 2013). However, for the reprogramming of domestic cat cells, multiple rounds of srRNA transfection were necessary. We were able to reliably achieve morphological changes consistent with epithelial transition (MET) after four rounds of srRNA transfections. The resulting cells also showed a high cell division rate, another sign of early reprogramming (Li *et al*., 2010; Ruiz *et al*., 2011). The requirement of multiple srRNA transfection in the cat fibroblasts may reflect the need for higher doses of reprogramming factors in cat cells. The need for an optimal stoichiometry of reprogramming factors in achieving successful iPSC induction has been previously demonstrated (Wen *et al*., 2016). The rate of cell proliferation by induced cat fibroblasts was dependent upon the medium used. A switch from DMEM to Advanced DMEM medium was a strategy Yoshioka *et al*. (2013) also used in inducing iPSCs. Fortunately, the human reprogramming factors OCT4, SOX2, KLF4, CMYC, and GLIS1 appear sufficiently conserved between the two species to effectively reprogram the cat cells. The addition of exogenous NANOG was not required for the induction of cat iPSC colony formation in this study. However, not using NANOG for the induction of iPSCs in our experiments might explain the spontaneous differentiation of the resulting iPSC clones (Dutton *et al*., 2019; Verma *et al*., 2013; Zhou *et al*., 2019). This requirement of NANOG addition was shown previously by Verma *et al*. (2013) in wild cats, and by Dutton *et al*. (2019) and Zhou *et al*. (2019) in the domestic cat.

The induction of pluripotency in domestic cat cells using srRNA also required the addition of LIF to the culture media. The specificity of LIF from different species can affect to the outcome of reprogramming. Mouse LIF, as shown by Dutton *et al*. (2019), could not induce the formation of iPSCs by cat cells. In our hands, the use of feline LIF alone was insufficient to induce iPSC-like colony formation. This finding is at variance with the observations reported by Dutton *et al*. (2019). Similar to our experience, Zhou *et al*. (2019) also used a combination of human and feline LIF to induce iPSCs in cats. The need for this combination is somewhat unexpected since the sequence of human and feline LIF is 97% homologous. In comparison, murine LIF shows much more divergence. The need for the addition of both human and feline LIF probably shows some similarity to the need for a double dose of LIF that was sufficient to induce colony formation in cat cells using other induction strategies. The ability of rescuing iPSC formation by the addition of human LIF at the stage of seeding cells onto irradiated feeders suggests that perhaps LIF effected the formation of iPSC colonies at a late stage of reprogramming.

LIF/STAT3 is a critical pathway in maintaining pluripotency in murine embryonic stem cells (ESCs) (Matsuda *et al*., 1999; Niwa *et al*., 1998; Raz *et al*., 1999; Ying *et al*., 2008). It promotes the expression of core genes in the pluripotency circuit and suppresses the expression of differentiation-related genes (Tang and Tian, 2013; Zhang *et al*., 2018). The action of LIF in cat iPSCs might be analogous to the murine LIF-STAT3 pathway, where it is essential for maintaining pluripotency and self-renewal (Tang *et al*., 2012; Yang *et al*., 2010). However, other pathways might also be involved as our cat iPSC clones exhibited a uniquely ambiguous signature mixing features of naïve, primed, and ground state pluripotency. Cat iPSC clones also exhibited variable expression of the three germ layers markers, indicating their heterogeneity and clonal variation. Most of the clones had a tendency for spontaneous differentiation, while a few clones expressed a signature compatible with true pluripotency. Importantly, our cat iPSC clones could differentiate into all three germ layer cell lineages in LIF removal condition. In addition, cells induced using the HFLIF protocol tended to differentiate towards the neurogenic pathways, as suggested by their *NCAM1* expression. This is probably due to the prolonged activation of STAT3 under continuous LIF treatment. This effect has been previously demonstrated in STAT3-mediated neurogenesis (Su *et al*., 2020). Variation in gene expression of cat reprogrammed cells was not surprising, as it has been reported in human and mouse models that maintaining the expression of these pluripotent genes poses a formidable challenge. This challenge is based on the heterogeneity of induced pluripotent stem cells (iPSCs), which can result from various factors such as the origin of the donor, genetic variation, and the reprogramming process. The reprogramming process can be influenced by stochastic phase versus hierarchic phase (O'Malley *et al*., 2013; Buganim *et al*., 2012; Kim *et al*., 2010; Kim *et al*., 2011; Rouhani *et al*., 2014). Understanding and overcoming these challenges are essential to improve the efficiency and stability of cat iPSC generation using non-integrative approaches.

In sum, our results demonstrate the feasibility of using a self-replicating replicon as a new tool for delivering exogenous genes into felid cells. This approach could be successfully utilized for the acquisition of pluripotency in feline cells, as summarized in Fig.6. Because wild felid cells are hard to obtain due to limited availability of animals, tissue types, and age of animals accessible for biopsy, our research at this stage used the domestic cat as a model species. Establishing a strategy for non-transgene integrating felid cellular reprogramming in cats is

likely to be applicable for the reprogramming of somatic cells of endangered wild felids. The work described here defines the details for the optimal induction, culture, and maintenance of iPSCs in a feline model using srRNA to deliver the necessary factors. It also addresses some concerns about clonal stability and variation. Further refinements of this technique will open the possibility of generating genome modification free iPSCs from endangered wild felids, providing valuable cells that can be cryopreserved in biobanks. These, in turn, can ensure the secure long-term existence of threatened wild felid species. The capacity of iPSCs to differentiate into any cell type raises the potential to generate a whole animal body to create viable offspring. The combination of ARTs and iPSC technology can help re-establish endangered wild felids in captivity. This, together with zoological organization and governmental policies can provide a route for communities to re-introduce wild felids, or other wild animals, back to their natural habitats.

Conflicts of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contribution: Designed experiments, W.S. and A.T. designed the experiments, S.K. and A.T. derived the cat fibroblasts, W.S. and N.S. produced the self-replicating RNA and induced cat reprogramming, W.S. performed immunofluorescence and real-time PCR, N.S. conducted the statistical analysis. All authors have read and agreed to the submitted version of the manuscript. The authors confirm that the data supporting the findings are available within the article.

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