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Theriogenology 77 (2012) 220–228

Theriogenology

www.theriojournal.com

Inducing pluripotency in somatic cells from the snow leopard (*Panthera uncia*), an endangered felid

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Received 27 June 2011; received in revised form 7 September 2011; accepted 22 September 2011

Abstract

Induced pluripotency is a new approach to produce embryonic stem-like cells from somatic cells that provides a unique means to understand both pluripotency and lineage assignment. To investigate whether this technology could be applied to endangered species, where the limited availability of gametes makes production and research on embryonic stem cells difficult, we attempted generation of induced pluripotent stem (iPS) cells from snow leopard (*Panthera uncia*) fibroblasts by retroviral transfection with Moloney-based retroviral vectors (pMXs) encoding four factors (*OCT4, SOX2, KLF4* and *cMYC*). This resulted in the formation of small colonies of cells, which could not be maintained beyond four passages (P4). However, addition of *NANOG*, to the transfection cocktail produced stable iPS cell colonies, which formed as early as D3. Colonies of cells were selected at D5 and expanded *in vitro*. The resulting cell line was positive for alkaline phosphatase (AP), OCT4, NANOG, and Stage-Specific embryonic Antigen-4 (SSEA-4) at P14. RT-PCR also confirmed that endogenous *OCT4* and *NANOG* were expressed by snow leopard iPS cells from P4. All five human transgenes were transcribed at P4, but *OCT4*, *SOX2* and *NANOG* transgenes were silenced as early as P14; therefore, reprogramming of the endogenous pluripotent genes had occurred. When injected into immune-deficient mice, snow leopard iPS cells formed teratomas containing tissues representative of the three germ layers. In conclusion, this was apparently the first derivation of iPS cells from the endangered snow leopard and the first report on induced pluripotency in felid species. Addition of NANOG to the reprogramming cocktail was essential for derivation of iPS lines in this felid. The iPS cells provided a unique source of pluripotent cells with utility in conservation through cryopreservation of genetics, as a source of reprogrammed donor cells for nuclear transfer or for directed differentiation to gametes in the future.
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Keywords: Snow leopard; Induced pluripotent stem cells; Conservation; Teratoma

1. Introduction

Gene banking of cells and tissues using cryopreservation is an important and useful approach for genetic preservation of valuable domestic cat breeds and for conservation management of endangered wild feline species [1]. Although, cryopreservation of gametes is the most useful method of supporting endangered species breeding programs [2,3], collection of gametes from these species for assisted reproductive technology (ART) is often difficult. Recent advances in embryonic stem (ES) cell technology have provided an alternative approach, since ES cells can differentiate to gametes *in vivo* and therefore have the potential to provide a source

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0093-691X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2011.09.022

of gametes for *in vitro* for embryo production. In addition, they can also be used as a donor cell for nuclear transfer (NT) and can be readily cryopreserved for gene banking [4].

The snow leopard (*Panthera uncia*) is a large cat that lives in the mountain ranges of Central Asia, between 3,000 and 5,500 m (9800 and 18,000 ft) above sea level [5]. Although the secretive nature of the snow leopard makes an accurate population census difficult, estimates suggest that only between 3,500 and 7,000 snow leopards still exist, making them an endangered species with numbers on the decline [6].

Endangered felid species are often difficult to breed both in captivity and under natural conditions. One of the most important reasons for infertility or subfertility is decreased genetic diversity caused by inbreeding, due to genetic bottle-necks because of geographic isolation and population contraction [7]. Consequently, there has been increasing interest in techniques for maintaining genetic diversity of endangered wild felids [8].

Pluripotent stem cells differentiate into all the cell types in the body, while retaining the capacity for indefinite self-renewal [9]. These cells have great potential for application in regenerative medicine, assisted reproductive technologies, development of new biotechnologies, and drug development [10]. Pluripotent stem cells have traditionally been derived from embryos, which are destroyed in the process, raising ethical and moral concerns for the derivation of stem cell lines in humans and also in endangered species. For species in which embryos are particularly difficult to obtain, such as endangered species, this approach also faces logistical concerns, as the supply of embryos in wild felids for isolation of ES cells is limited. Induced pluripotent stem (iPS) cells, which are derived from somatic tissue are a potentially useful alternative to ES cells.

Production of iPS cells was first reported by Takahashi and Yamanaka [11] using viral transduction of mouse fibroblasts to screen a combination of 24 candidate genes with putative roles in pluripotency. They found that four transcription factors (*OCT3/4, SOX2, KLF4* and *cMYC*) were required to reprogram mouse embryonic fibroblasts (mEFs) and adult tail tip fibroblasts to iPS cells, that were almost indistinguishable in morphology from mouse embryonic stem (mES) cells [12,13]. Subsequently, iPS cells have been derived from the somatic cells of rodents, primates, dogs, sheep, horses, pigs and cattle [14-23], but there are no reports of iPS cells from any felid or endangered species.

To investigate whether this technology could be applied to endangered species, we attempted generation of iPS cells from snow leopard ear fibroblasts by retroviral transfection with Moloney-based retroviral vectors (pMXs) encoding either four (*OCT4, SOX2, KLF4* and *cMYC*) or five (*OCT4, SOX2, KLF4, cMYC* and *NANOG*) human transcription factors. Our hypothesis was that inclusion of *NANOG* to the cocktail, which is critical for pluripotency in large animals [24], would be required to generate snow leopard iPS cells. Our aim was to derive and characterize iPS cells from snow leopard fibroblasts using retroviral vectors and to examine their differentiation potential both *in vitro* and *in vivo.*

2. Materials and methods

2.1. Animals

Animal handling and experiments conformed to the code of practice of the Australian National Health and Medical Research Council (2004) and were approved by Monash University Animal Experimentation Ethics Committee.

2.2. Isolation of snow leopard ear fibroblasts

Tissue samples were collected from the ear pinnae of snow leopard, which had died of natural causes or were euthanized due to health-related problems identified by a zoo veterinarian. All samples were donated by Mogo Zoo (Australia).

Adult dermal fibroblasts cell lines were derived from the tissue samples using standard isolation and culture techniques [23]. A small sample of ear tissue (~5 mm^2) was minced using sterile surgical instruments and plated into six well-dishes with Fibroblast Plating (FP) medium, containing Dulbecco's Modified Eagle's Medium, high glucose with penicillin/streptomycin (Invitrogen, Mulgrave, Vic, Australia) and 10% (v/v) fetal bovine serum (GIBCO, Melbourne, Vic, Australia), the dispersed tissue was cultured at 38.5 °C in 6% CO_2 in air for 7 d [25] and fibroblast outgrowths from the tissue explants were then transferred to two T175 cm^2 flasks for expansion.

2.3. Feeder layer preparation

The mEF were isolated from fetuses collected from mice on D13.5 post-coitum and used as a feeder layer for the iPS cells as previously described [26]. The mEFs were cultured in DMEM (high glucose, Invitro-

gen) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 2 mM GlutaMAX, 0.5% penicillin/streptomycin and 1 mM non-essential amino acids. Feeder cells were treated with 10 μg ml^{-1} mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 3 h to arrest mitosis, then washed in PBS, and replated at a density of 40,000 cells/cm^2 in organ tissue culture dishes.

2.4. Induction of pluripotency

Moloney-based retroviral vectors (pMXs) containing the coding sequences of GFP, human *OCT4, SOX2, KLF4, cMYC* and *NANOG* genes were obtained from Addgene (Cambridge, MA, USA). The retroviral vector, pMX-GFP, was transfected into PLAT-A packaging cells stably expressing *gag*, *pol*, and *env* genes (Jomar Biosciences, Kensington, SA, Australia) for the production of amphotropic virus. Then, 9 μg of each of the vectors described above was cotransfected to Plat A cells by Fugene 6 (Roche, Dee Why, NSW, Australia) according to manufacturer's instruction. Virus-containing supernatants were collected 48 and 72 h post-transfection and filtered through a 0.45 μm pore-size filter and supplemented with 4 μg/ml of Polybrene (Sigma). Snow leopard fibroblasts were plated 24 h before infection at a density of 4×10^4 cells/cm^2. Equal parts of the five transcription factors-containing retroviral supernatants were added to the plated snow leopard fibroblasts. Two rounds of infection were performed 24 h apart. The culture medium was changed to mouse embryonic stem cell medium at D4 post-infection. The iPS cell colonies were selected at D5 with distinct boundaries. Snow leopard fibroblasts cells were infected with GFP-containing retroviral supernatant to monitor the transduction efficiency. The overall efficiency of reprogramming was calculated by the number of colonies formed/number of cells plated.

2.5. Cell culture

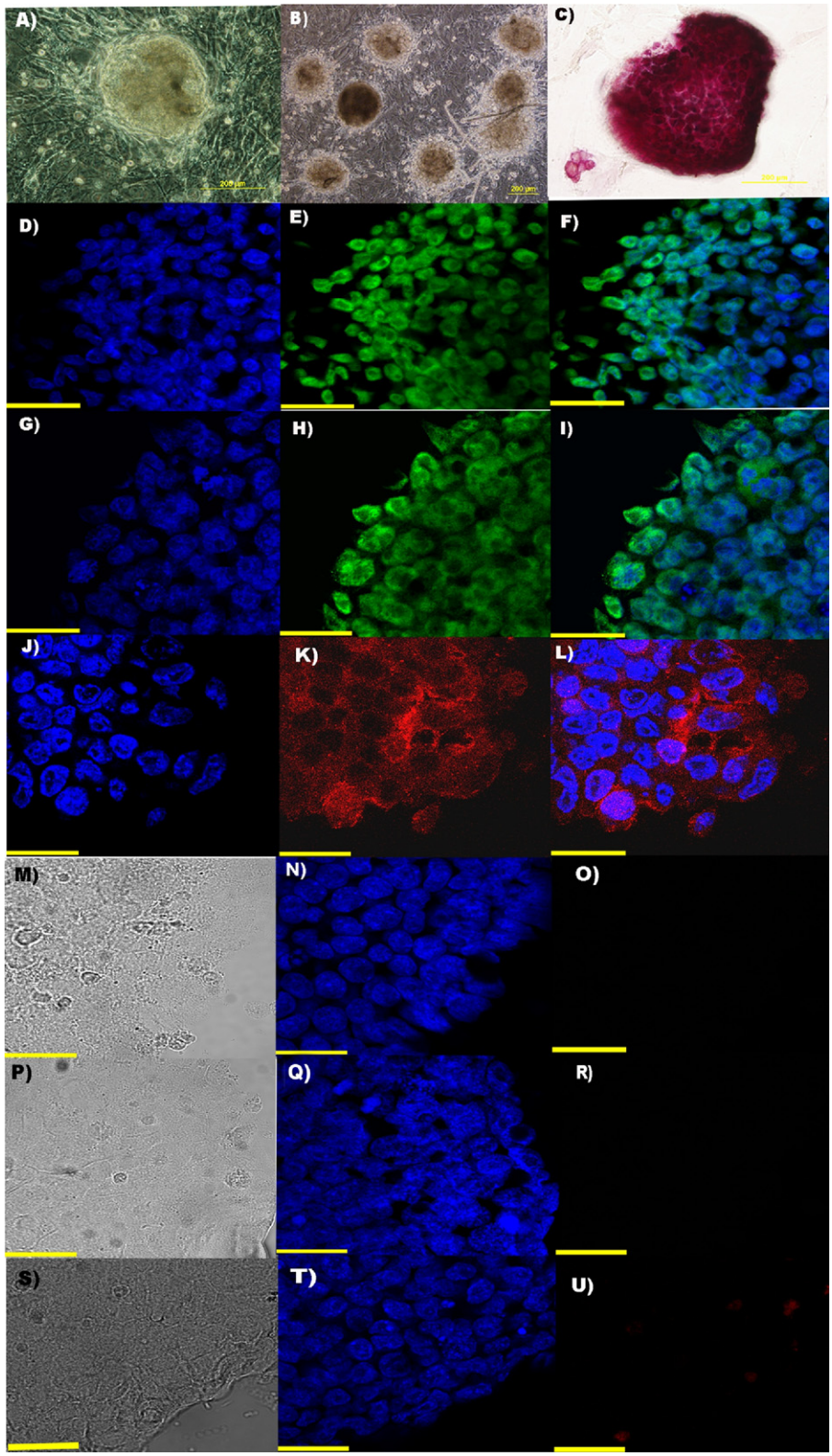
Mitotically inactivated mEF were plated in six-cm organ culture dishes. The FP medium was replaced with mES cell medium containing DMEM (high glucose, Invitrogen) supplemented with 0.1 mM 2-beta mercaptoethanol (β-ME), 1M non-essential amino acids (NEAA), 2 mM GlutaMAX, 20% (v/v) HyClone serum, 0.5% penicillin/streptomycin, 10^3 U ml^{-1} murine leukemia inhibitory factor (ESGRO); (Invitrogen) after double infection. Medium was changed every day and every third to fourth day the snow leopard iPS cell colonies were passaged on to fresh feeder layers. These cells were cultured in 6% CO_2 at 38.5 °C.

2.6. Immunocytochemistry

Subconfluent cells were tested for alkaline phosphatase (AP) activity and examined immunocytochemically, using antibodies against markers typically expressed by pluripotent cells. AP staining was performed using a diagnostic alkaline phosphatase substrate kit according to the manufacturer's specification (SK-5300, Vector Laboratories, Inc., USA). For immunocytochemistry, colonies were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature (RT) and washed three times with PBS pH 7.5. Cells examined for OCT4 and NANOG were blocked with PBS pH 7.5 supplemented with 1% (w/v) BSA, 5% (v/v) goat serum, and 0.1% (v/v) Tween. Those tested for SSEA-4 were blocked with 1% (w/v) BSA, 5% (v/v) goat serum in PBS pH 7.5 for 60 min at RT. Mouse anti-human OCT4 IgG (SC-5279, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-mouse SSEA-4 IgG (MC 813–70, Millipore, Melbourne, Vic, Australia) and rabbit polyclonal IgG to NANOG IgG (AB 80892, Abcam, Waterloo, NSW, Australia) were used as the primary antibodies at 1:100 dilution in PBS containing 5% (v/v) goat serum in an overnight incubation at 4 °C. The next day, the cells were washed three times with PBS and a

Table 1
Comparative analysis on the number of colonies between four factors and five factors and their survival in culture until Passage 4 (P4). Total number of cells plated per dish was 40,000.

Experiment	4 factors (O,S,K,M) 5 factors (O,S,K,M,N)	Transduction efficiency (%)	No. colonies formed	Efficiency	Survival after P4
1	4 factors	96	12	0.000300	0/5
	5 factors	96	21	0.000525	4/5
2	4 factors	95	12	0.000300	0/5
	5 factors	95	20	0.000500	4/5
3	4 factors	98	13	0.000325	0/5
	5 factors	98	21	21/40,000 = 0.000525	Colonies pooled


A)
B)
C)
D)
E)
F)
G)
H)
I)
J)
K)
L)
M)
N)
O)
P)
Q)
R)
S)
T)
U)

secondary antibody was added in PBS containing 5% (v/v) goat serum for 1 h at RT (OCT4: anti-IgG Alexa 488 1:500, NANOG: anti-IgG Alexa 488 1:500, and SSEA-4: anti-IgG Alexa 594 1:500). In addition, control cell-lines with the primary antibodies excluded (negative control) were maintained. Cells were subsequently washed three times with PBS, mounted in Vectashield + DAPI (Abacus) and cover slipped.

2.7. Microscopy

Cells were examined and images were captured with phase-contrast and bright-field microscopy using a Nikon Elite (2000) microscope or an Olympus IX71 microscope. Fluorescent images for immunocytochemistry were captured using confocal microscopy at MicroImaging facility of Monash Institute of Medical Research.

2.8. RT-PCR

Total RNA was extracted from snow leopard iPS cells at P4, P14 and P36, and also from human embryonic stem cells (hES), mES, mEF and snow leopard ear fibroblasts (sLF) using RNAeasykit (Qiagen, Doncastor, Vic, Australia) according to the manufacturer's instructions. The resulting total RNA was subjected to DNase1 treatment using DNA-free kit (Qiagen) to digest any contaminating genomic DNA. cDNA was synthesized using the Superscript lll reverse transcriptase kit (Invitrogen), and subjected to PCR amplification with the relevant primer pairs. We used GAPDH expression as a control for the amount of template in each reaction and the presence of contaminating genomic DNA. PCR was carried out using HotStart Taq DNA polymerase under the following conditions: 95 °C for 10 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, with final extension at 72 °C for 10 min (*OCT4* and *GAPDH*) or 95 °C for 10 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with final extension at 72 °C for 10 min (for *NANOG*) (Suppl. Table 1; online version only) [28].

For human transgenes gene expression, (Suppl. Table 2; online version only) the RT-PCR reaction was carried out under the following conditions: 95 °C for 5 min, 35 cycles of 94 °C for 30 s, (*OCT4*), 47 °C (*NANOG*), 47 °C (*SOX2*), 48 °C (*KLF4*) and 47 °C (*cMYC*) for 30 s and 72 °C for 1 min, with final extension at 72 °C for 5 min [27]. The amplified cDNAs were separated on 1.5% (w/v) agarose gels, and the bands were visualized by ethidium bromide staining.

2.9. Freeze-thawing

Snow leopard iPS cells were cryopreserved in the freezing medium composed of 90% (v/v) FBS and 10% (v/v) DMSO (Sigma) using a conventional slow freezing method for mouse cells [30]. To test the survival ability of these cells after cryopreservation, the iPS cells were thawed at 37 °C water bath for 1 min and then washed with iPS medium by centrifuging at 200 g for 2 min. Snow leopard iPS cells were then replated onto fresh feeder layers with iPS cell medium and cultured in a 6% CO_2 incubator at 38.5 °C.

2.10. Teratoma formation

Snow leopard iPS cells were harvested at P18 from six cm dishes using 0.05% (w/v) trypsin-EDTA. A suspension of 2×10^6 cells suspended in MES medium, were injected subcutaneously into the thigh muscle of a 5 wk old severe combined immunodeficient (SCID) male mouse. At 10 wk after injection, the mouse was euthanized and the resulting teratoma was excised and fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin by the Histology Laboratory core facility at Monash Institute of Medical Research. Stained sections were then examined by a USA Board certified veterinary pathologist at Gribbles Pathology (Melbourne, Vic, Australia).

2.11. Transgenes genomic PCR

To confirm that the teratoma formed in SCID mice was derived from snow leopard iPS cells and not from mouse cells, genomic DNA was extracted from snow leopard iPS cells at P18, teratoma and mouse cells (mES) and compared. Primers for the human transgenes, *OCT4, SOX2, KLF4* and *cMYC* were used to detect the presence of the respective transgenes in the

Fig. 1. Morphology and characterization of snow leopard iPS cells at P14. (A) The formation of an iPS colony at Day 3 post-infection; (B) morphology of the snow leopard iPS colonies at P1; (C) Alkaline phosphatase staining; Immunofluorescence staining of snow leopard iPS at P14 with appropriate phase images; confocal images of (D, E, F) NANOG green fluorescence, DAPI, merged; (G, H, I) OCT4; green fluorescence, DAPI, merged (J, K, L) SSEA-4; red fluorescence, DAPI, merged. The negative controls are NANOG bright field, DAPI, fluorescence (M, N, O), OCT4; bright field, DAPI, fluorescence (P, Q, R) and SSEA-4; bright field, DAPI, fluorescence (S, T, U). The scale bar for all confocal images (D–U) is 25 μm.

genomic DNA. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). Amplification of integrated transgenes was performed using gene-specific primers (Suppl. Table 2) and GoTaq Green Master Mix (Bio-Rad, Gladville, NSW, Australia). The PCR reaction included an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 45 s and extension at 72 °C for 75 s, followed by final extension at 72 °C for 5 min. Amplicons were separated through a 1% agarose gel at 100V for 1 h.

2.12. Karyotype analysis

Karyotype analysis of the snow leopard iPS cells at Passage 14 was performed at the Cytogenetics Department, Monash Medical Centre using standard techniques [23]. A minimum of 60 metaphase spreads were counted to check for normal chromosomes number and morphology.

3. Results

3.1. Generation of snow leopard iPS cells

Transduction efficiency of the retroviral transfection using pMX-GFP transgene expression, averaging 96% from three repeated experiments, are shown in Table 1. The reprogramming efficiency for initial colony formation following five factor induction was 0.000517%, compared with 0.000308% for four factor induction. Only five factor induction resulted in colony survival (80%) beyond P4. Three day post-infection, the appearance of compact colonies was noted (Fig. 1A). The colonies that were disaggregated at D5 and transferred to mitomycin C-inactivated mEF showed well-developed secondary colonies (Fig. 1B), with the cells exhibiting a high nuclear-to-cytoplasm ratio with prominent nucleoli, consistent with typical ES morphology. These colonies stained positive for alkaline phosphatase activity (Fig. 1C).

Snow leopard iPS cells proliferated consistently, requiring subculture at a 1:10 ratio every 3 to 4 d throughout the 36 passages reported in this study.

3.2. Immunocytochemistry

The snow leopard iPS cells reacted positively for antibodies against NANOG (Fig. 1D, E, F), OCT4 (Fig. 1G, H, I) and SSEA-4 (Fig. 1J, K, L), respectively, and negatively with ear fibroblasts cells at P14, NANOG (Fig. 1M, N, O), OCT4 (Fig. 1P, Q, R) and SSEA-4 (Fig 1S, T, U).

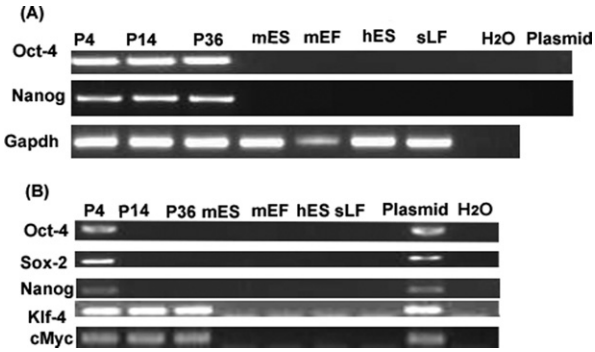


Fig. 2. RT-PCR of cat (Specific) endogenous genes and human transgenes. (A) Gene expression of endogenous cat *OCT4* (286 bp), *NANOG* (246 bp) and *GAPDH* (271 bp) and (B) human transgenes expression of pMX-*OCT4*, pMX-*SOX2*, pMX-*NANOG*, pMX-*KLF4* and pMX-*cMYC* on snow leopard iPS cells at P4, P14, P36, mES (Mouse Embryonic Stem Cells), mEF (Mouse Embryonic Fibroblasts), hES (Human Embryonic Stem Cells), sLF (Snow Leopard Fibroblasts).

3.3. Expression of pluripotent genes in snow leopard iPS cells

At P4, P14 and P36, RT-PCR analysis demonstrated the expression of endogenous *OCT4* and *NANOG*. The endogenous cat primers did not cross-react with non-cat samples (Fig. 2A). These two genes were expressed only in the snow leopard iPS cells and not in sLF. Further, RT-PCR also confirmed that all the human transgenes were transcribed at P4, however the pluripotency-related *OCT4, SOX2* and *NANOG* transgenes were silenced at P14. These results were confirmed by subsequent analysis at P36. In contrast, the transgenes associated with proliferation (*cMYC* and *KLF4*) were still detectable at P14 and P36 (Fig. 2B).

3.4. Karyotype

Chromosomal analysis showed that 55/60 (92%) of the snow leopard iPS cells tested displayed a euploid male karyotype of 38, XY with 18 matched pairs of autosomes (Fig. 3).

3.5. Teratoma formation

At 10 wk after subcutaneous injection of snow leopard iPS cells at P18 into the thigh muscle of a male SCID mouse, a solid tumor (approximately 8 mm) was observed. Histologic examination showed that the tumor was a fully differentiated teratoma containing cells and tissues that were representative of the three primary germ layers: keratinocytes (ectoderm), cartilage (mesoderm), and secretory epithelium (endoderm) as confirmed by a veterinary pathologist (Fig 4 A, B, C).

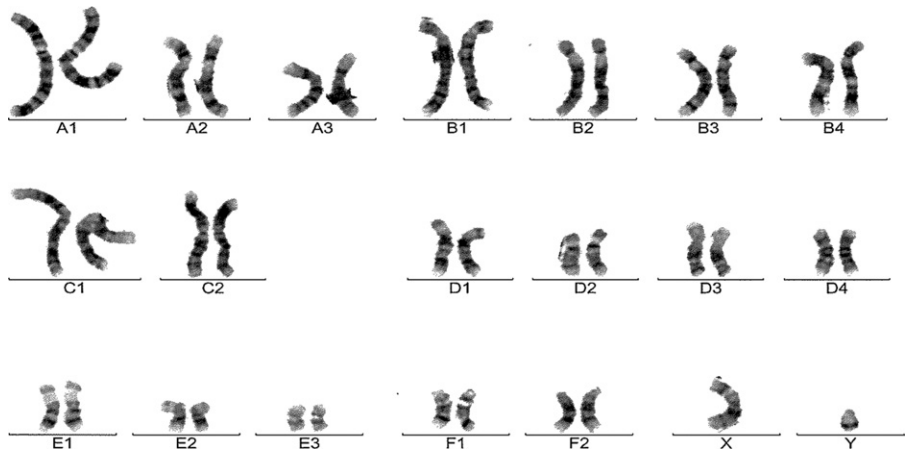


Fig. 3. Chromosome counts on snow leopard iPS cells at P14. Karyotype showing a total of 19 pairs of chromosomes in snow leopard iPS cells.

Genomic PCR analysis demonstrated the presence of all five transgenes in snow leopard iPS cells at P18 and in teratoma tissue, but not in mouse cells (Fig. 4D).

4. Discussion

Since the initial generations of murine iPS cells [11], there have been numerous attempts to derive iPS cells from a range of other species. However with the exception of rodents, primates and rabbit complete reprogramming of somatic cells has not been reported. To date the lack of silencing of inserted transgenes has been a hallmark of iPS cells in large animals, including dog, sheep, monkey, horse, pig and cattle [18,20-23,29-32].

Preliminary experiments examining the generation and maintenance of snow leopard (*Panthera uncia*) iPS

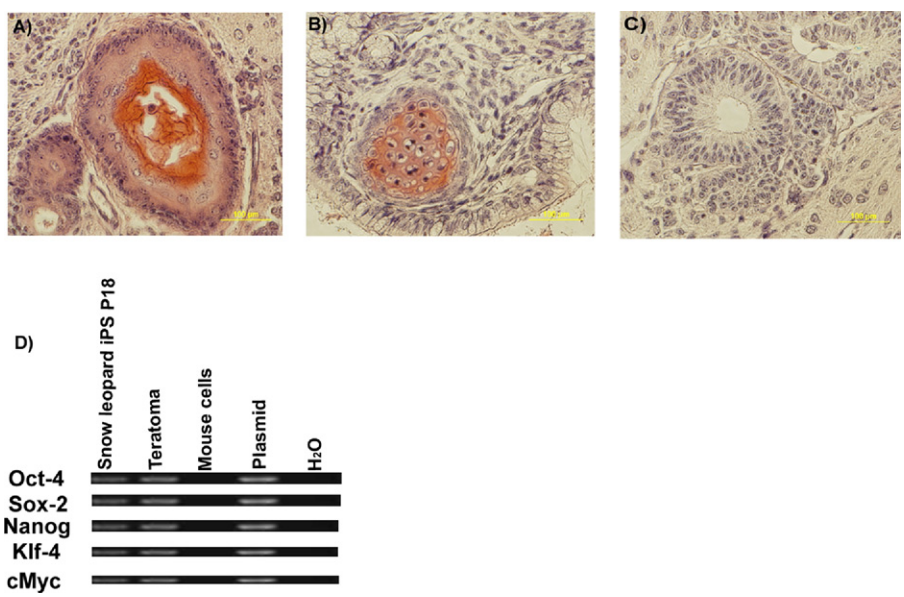


Fig. 4. Cross-sections of a snow leopard iPS cells teratoma developed in an SCID mouse and genomic PCR on snow leopard iPS cells and teratoma. Cross-section of a teratoma obtained at 10 wk after injection of snow leopard iPS cells at P18 in an SCID mouse, stained with hematoxylin and eosin with tissues representative of the three primary germ layers (A) Keratinocytes (ectoderm), (B) Cartilage (mesoderm) and (C) Secretory Epithelium (endoderm). D, Genomic PCR confirming the presence of human transgenes pMX-*OCT4*, pMx-*SOX2*, pMX-*NANOG*, pMX-*KLF4*, pMX-*cMYC*, in snow leopard iPS cells at P18, plasmid and teratoma tissue but not in mES (mouse cells).

cells with the four Yamanaka factors resulted in generation of putative iPS cell colonies. However these colonies could not be maintained past four passages, suggesting for snow leopard that additional pluripotency associated factors may be required for robust generation of iPS cells. We report the successful derivation of iPS cells from ear fibroblasts of the snow leopard transfected with five human factors *OCT4, SOX2, KLF4, cMYC* and *NANOG*. We believe the high transduction efficiency (96%) for this study, measured using a pMx-GFP construct, was important in achieving a successful outcome.

Early morphologic changes in transfected snow leopard cells comparable with those seen in mouse iPS cells were detected, surprisingly, as early as D3 post-infection, which enabled early detection of colonies and expansion on feeder cells from D5 after transfection. These colonies were routinely passaged, by enzyme dissociation (0.5% trypsin-EDTA) without losing their pluripotent characteristics. The cells were positive for AP and showed positive immunofluorescent staining for OCT4, NANOG, and SSEA-4 at P14. RT-PCR demonstrated expression of endogenous *OCT4* and *NANOG* using cat specific primers [28] that were shown not to amplify transcripts from the human transgenes. Expression of all five inserted human transgenes genes was observed at P4, but by P14, transcripts from the inserted *OCT4*, *SOX2* and *NANOG* transgenes were silenced while cat specific transcripts of *OCT4* and *NANOG* continued to be expressed, suggesting reprogramming of the endogenous pluripotency associated genes. However the transgenes for *cMYC* and *KLF4*, which are associated with proliferation, a second key attribute of stem cells, continued to be expressed. We interpreted this to be incomplete reprogramming of the proliferation capability by the snow leopard iPS cells. Incomplete transgene silencing has also been described in human and rat iPS cells and especially in large animal iPS cells [18,20-23,29-32].

In humans, the addition of Nanog as a reprogramming factor to the four Yamanaka factors was shown to alter the growth and proliferation characteristics of resulting iPS cells (hiPS) [27]. In cattle we have also shown that the addition of Nanog was essential for generation of stable iPS cell line [23].

The effect of sustained expression of the c-Myc transgene in snow leopard iPS may have influenced tumor formation. However the multilineage differentiation potential of these cells, that was clearly demonstrated histologically by the presence of representative tissues from the three primary germ layers in the resulting teratoma, is an essential demonstration of the pluripotent potential of the snow leopard iPS cells. Genomic PCR for the trangenes confirmed that the teratoma tissue was derived from snow leopard iPS cells. Moreover, snow leopard iPS cells maintained a stable karyotype of 38 chromosomes at P14.

In summary, we have described a method for transducing ear fibroblasts from the snow leopard into iPS cells and characterized their pluripotency. Of particular importance was the observation that the three key exogenous pluripotency transgenes (OCT4, SOX2, NANOG) were silenced at later passages. In conclusion, we believe this is the first report on the derivation of iPS cells from both a felid and as well as an endangered species. This is also the first report on the induction of pluripotency in a large animal with concomitant silencing of the pluripotency-associated transgenes. The iPS cell technology has the potential to impact on conservation of endangered species at a number of levels. It can provide insights into pluripotency and development in species where embryos are difficult to access. Furthermore, iPS cells generated from the endangered species can be easily expanded for banking of genetic material or used as a reprogrammed donor cell to improve NT outcomes. They may also create opportunities to prevent extinction in a wide range of threatened animals in the future. For example, it may eventually be possible to differentiate cell lines with proven pluripotency *in vitro* to produce gametes or use these cell lines *in vivo* in conjunction with tetraploid complementation to produce whole animals. This report has relevance to understanding pluripotency in big cats and also has application in domestic cats, which are companion animals and are unique biomedical models to study genetic diseases (e.g. arthritis and diabetes).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.theriogenology.2011.09.022.

Acknowledgments

We thank Sally Padey, (Director) and Hannalie Vander Merwe, (Officer in charge) of Mogo Zoo in New South Wales (Australia) for their generous support of this project by providing tissue samples. We also thank to all the staff at Mogo Zoo, veterinarians Dr. Mary Atkinson and Dr. Peter Atkinson and veterinary pathologist Dr. Mark Williamson (Gribbles Pathology,

Australia). Rajneesh Verma acknowledges a Ph.D. scholarship awarded by Professor Bryan Williams and the Monash Institute of Medical Research, Melbourne, Australia. The authors thank Jun Liu for his guidance in tissue culture throughout the project. This research was also supported by the Victorian Government's Operational Infrastructure Support Program.

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Suppl Table 1
Specific domestic cat primer sequences reported previously [28]. These primers were used to detect the expression of endogenous genes in snow leopard iPS cells at P4 and P14.

Oligo's name	Primer sequences
GAPDH(F)	5′GCAAAGTGGACATTGTCGCC3′
GAPDH (R)	5′CCTTCTCCATGGTGGTGAAG3′
OCT4 (F)	5′GGAGTCCCAGGACATCAAAG3′
OCT4 (R)	5′GCCTGCACAAGTGTCTCTGC3′
NANOG (F)	5′AAGCCACAGTGTGATACAGC3′
NANOG (R)	5′AGCCAAAGCTACGGAATCCTC3′

Suppl Table 2
Primer sequences of human transgenes reported previously [27]. These primers were used to detect the expression of transgenes in snow leopard iPS cells at P4, P14 and P36.

Oligo's name	Primer sequences	Annealing temperature (°C)
OCT4 (F)	CTAGTTAATTAAGAATCCCAGTG	47
OCT4 (R)	CACTAGCCCCACTCCAACCT	47
SOX2 (F)	CTAGTTAATTAAGGATCCCAGG	47
SOX2 (R)	TGTTGTGCATCTTGGGGTTCT	47
cMYC (F)	CTAGTTAATTAAGGATCCCAGTG	47
cMYC (R)	CAGCAGCTCGAATTTCTTCC	47
KLF4 (F)	ACAAAGAGTTCCCATCTCAAGGTG	48
KLF4 (R)	TCCAAGCTAGCTTGCCAAACCTACAGG	48
NANOG (F)	TCAATGATAGATTTCAGAGACAG	47
NANOG (R)	GGGTAGGTAGGTGCTGAGGC	47