Using, Assisted Reproductive Technologies For The Conservation of Endangered Wild Cats

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Declaration

This thesis contains no materials which has been accepted for the award for any other degree or diploma at any university and to the best of my knowledge and belief contains no material that was previously or written by another person, except where due reference is made in the text of the thesis.

The thesis includes two original papers (Chapter 4 and 5) that were published in peer-reviewed journals.

Rajneesh Verma

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This PhD is dedicated to my "Kids"

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Abstract

The advent of induced pluripotency has opened alternate avenues to produce ES-like cells from somatic cells, that provide a unique tool to elucidate both pluripotency and lineage assignment. To investigate whether this technology could be applied to endangered species, where the limited availability of gametes makes production of and research on embryonic stem cells difficult, we first investigated the appropriate species transcription factors to use for induction of pluripotency in wild cats. Octamer binding transcription factor 4 (Oct4), also known as POU5F1, is a protein critically involved in stem cell renewal and pluripotency and a key factor involved in reprograming. To identify the suitability of using either mouse or human Oct4 constructs, which pre-existed in the laboratory to derive induced Pluripotent Stem Cells (iPSC) from endangered cats, the coding sequence of domestic cat Oct4 was compared with that from other known species. Cat Oct4 protein has 94.44% and 83.33% sequences similarity compared with human and mouse Oct4, respectively. (Publication 1-paper submitted). In addition, the coding sequence for Oct4 were successfully amplified and cloned from fibroblasts of the three wild cats and compared in silico. Bioinformatics analysis revealed that although the sequences of coding regions vary slightly between snow leopard, Bengal tiger and jaguar, the predicted theoretical protein consist of 360 amino acids and is identical between these species. By contrast to the exons, introns and 5' region close to the exon1 are less conserved in the three species. A phylogenetic tree was constructed using the neighbour-joining method based on the alignment of the coding sequences of Oct4 gene. As Oct4 is usually expressed in oocytes/early embryos, which are difficult to obtain from endangered species, the verification of the mRNA sequence of the gene posed obvious challenges. In conclusion, this study is the first to describe the molecular cloning and bioinformatics analysis of snow leopard, Bengal tiger and jaguar Oct4 gene (Publication 1-submitted). Information on the specific sequences of Oct4 in these endangered big cats will

be useful to understand and manipulate pluripotency in these species. This has an exciting potential to contribute to real-life species preservation, particularly for endangered felids. Therefore we determined to use human sequences for induction of pluripotency in snow leopard (*Panthera uncia*) fibroblasts by retroviral transfection with Moloney-based retroviral vectors (pMXs) encoding four human 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). We hypothesized the addition of *NANOG*, another transcription factor critically linked to Oct4 mediated pluripotency, to the transfection cocktail would enhance generation and maintenance of stable iPSC colonies, which formed as early as Day3 (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. Reverse Transcriptase PCR (RT-PCR) also confirmed that endogenous *OCT4* and *NANOG* were expressed by snow leopard iPSC from P4. All five human transgenes were transcribed at P4, but *OCT4*, *SOX2* and *NANOG* transgenes were silenced as early as P14; signifying reprogramming of the endogenous pluripotent genes had occurred. When injected into severe combined immuno-deficient (SCID) mice, snow leopard iPSC formed teratomas containing tissues representative of the three germ layers. In conclusion, this was the first report on derivation of iPSC from an endangered felid and the first report on induced pluripotency in felid species and it demonstrated the addition of NANOG to the reprogramming cocktail was essential for derivation of stable iPSC lines in this felid (Publication 2- published).

Next, to examine whether the importance of Nanog for generation of iPSC was restricted to snow leopard or more generally applicable to wild felids globally, we studied 3 geographically diverse and evolutionarily divergent felids from Asia (Bengal tiger, *Panthera*

tigris), Africa (serval, *Leptailurus serval*), and the Americas (jaguar, *Panthera onca*). Dermal fibroblasts were transduced with genes encoding the human transcription factors *OCT4, SOX2, KLF4,* and *cMYC* with or without *NANOG*. Both four- and five-factor induction resulted in colony formation at day 3 in all three species tested; however, we were not able to maintain colonies that were generated without *NANOG* beyond passage (P) 7. Five-factor induced pluripotent stem cell (iPSC) colonies from wild cats were expanded in vitro on feeder layers and were positive for alkaline phosphatase and protein expression of OCT-4, NANOG, and stage-specific embryonic antigen-4 at P4 and P14. Reverse-transcription polymerase chain reaction confirmed that all five human transgenes were transcribed at P4; however, *OCT4, SOX2*, and *NANOG* transgenes were silenced by P14. Endogenous *OCT4* and *NANOG* transcripts were detected at P4 and P14 in all cell lines confirming successful reprogramming. At P14, the iPSC from all three species remained euploid and differentiated in vivo and in vitro into derivatives of the three germ layers. Our study demonstrated unequivocally that iPSC from the three felids examined all required the addition of Nanog to the reprogramming cocktail to ensure derivation of stable iPSC lines. Interestingly iPSC from all three species silenced the reprogramming transgenes (*OCT4*, *SOX2* and *NANOG),* but not the transgenes implicated in proliferation (*KLF4* and *cMYC)* (Publication 3- published).

This thesis investigated induced pluripotency in endangered felids and reports successes in effectively inducing pluripotency in four endangered wild cats from across the globe and importantly identifies Nanog as an essential factor in the reprogramming cocktail. Efficient production of iPSC from endangered felids creates a unique opportunity to preserve these species using these iPSC in future gamete production, nuclear transfer and embryo complementation.

Chapter 1

1.1. Introduction and Literature Review

One of every four animal species on the planet is threatened by extinction. Historically, strategies for preserving biodiversity have focused on saving habitat and, by default, species in these native environments (*in situ*) (Ben-Nun *et al.* 2011b). However, the magnitude of the crisis is so dire owing to ecosystem loss, fragmentation and climate change that more direct, species-targeted action is necessary.

One support strategy is creating and managing 'insurance' populations *ex situ* for hundreds of mammals, birds, reptiles, amphibians and fish. This preserves genetic integrity, allows basic research to be conducted and is a source of animals for reintroduction programs. *Ex situ* species propagation approaches are complex and demand expertise and resources, including specialized animal space and facilities, and secure reintroduction and release sites. Even then, achieving reproduction by natural means is challenging. Animals can be paired but not breed because of unrealised sexual partner preferences, and it is expensive and complicated to transfer wild or captive animals between locations to make ideal genetic matches to retain maximal heterozygosity. As a result, assisted reproductive technologies have been explored for helping to manage successful breeding outcomes in *ex situ* wildlife populations.

Much progress has been made during the last decade in the development of assisted reproductive techniques, not only in the economically-driven fields of human infertility and domestic animal breeding, but also in species conservation. Endangered felids are often difficult to breed both in captivity and under natural conditions. One of the most important reasons for infertility or sub-fertility in this group is inbreeding depression caused by decreased genetic diversity, due to genetic bottlenecks as a consequence of geographical isolation and population contraction. Because of this there has been increasing interest in maintaining genetic diversity for the conservation of wild felids and preservation of valuable cat breeds. However, for a successful assisted reproduction programme, a basic understanding of the reproductive biology (ovarian cyclicity, time of ovulation, optimal time for mating, pregnancy etc.) of the animals is necessary.

Assisted reproductive technology (ART), comprised of techniques such as artificial insemination (AI), Intra-Cytoplasmic Sperm Injection (ICSI), In-vitro fertilization (IVF),

Nuclear transfer (NT), has been promoted over the past 25 years as a potential means to conserve and manage threatened wildlife populations. But the application of ART is limited due to the inaccessibility of gametes (Verma *et al* 2012).

Assisted reproductive technology tools have many advantages. Artificial insemination eliminates the need to pair particular animals, which can result in antagonistic behaviours and injuries. Embryo transfer allows multiple embryos of a valuable female to gestate to term in a surrogate dam. Using chilled or frozen gametes and embryos allows genetic material to be cheaply moved long distances or stored for future use. Many wildlife offspring from giant pandas to bottlenose dolphins and koalas have been produced by artificial insemination with fresh or thawed sperm (Oliveira *et al.* 2008). Progeny that resulted from the artificial insemination of whooping cranes and black-footed ferrets have been used to reintroduce these species back to nature Wildt *et al.* 1986; Loi *et al.* 2001). By contrast, embryo transfer after *in vitro* fertilization or intra-cytoplasmic sperm injection has resulted in only a few milestone births in wildlife species. Notable and repeatable successes have occurred in a few felids (cats) using *in vitro* fertilization and embryo transfer of thawed embryos. Similarly, offspring have been produced using somatic cell nuclear transfer (SCNT, or 'cloning')-derived embryos in two small wild cat species (sand cat and African wild cat), with embryos gestated in domestic cat surrogates (Gomez *et al.* 2004).

But even in the face of this modest progress, not one wildlife species has been managed with the help of embryo-based technologies. This is because there is little species-specific information. Requirements for the creation and culture of embryos differ greatly among species: requirements for cow embryos are substantially different from those for cheetah or scimitar-horned oryx, for instance. Embryo transfer requires finding a surrogate and learning how to synchronize the newly created embryo with the recipient's uterus. And finally there is always the most important question: what does one do with the endangered embryo, especially as transferring embryos between species has been rarely successful. It would not be logistically or economically practical to maintain large numbers of generic elephants or tigers, for instance, as surrogate populations for embryo transfer. Clearly, it is easier to generate an embryo from a wild species than to find, hormonally prepare or care for suitable surrogate dams.

Pluripotent stem cells have the capacity to differentiate *in vitro* into all the cell types in the body, including gametes, while retaining the capacity for indefinite self-renewal. The best

example are Embryonic Stem Cells (ESC) that (1) can contribute to the formation of embryos for e.g. chimeras, (2) can be differentiated to form gametes in-vitro and (3) can be used as donor cells for nuclear transfer. ESC have traditionally been derived from embryos, which are destroyed in the process, raising moral, ethical and logistical concerns for the derivation of stem cell lines in endangered species. For species in which embryos are particularly difficult to obtain, or those, which are endangered, this approach has not been particularly useful and feasible, and so far no true ESC have been reported in any domestic and endangered species (Malaver-Ortega *et al.* 2012; Verma *et al* 2012).

Despite this reality check on what is possible today, it is essential to consider what might be promising for future intensive management of endangered species, especially with the rapid advancement in embryo and molecular technologies. So it is natural to consider how induced pluripotent stem cells (iPSC) generated by reprogramming adult or differentiated somatic cells back to a primordial stem cell-like state using defined transcription factors can be used to benefit endangered species conservation.

In a seminal study, Japanese scientists (Takahashi & Yamanaka 2006) used viral transduction of mouse fibroblasts to screen a combination of 24 candidate genes with putative roles in pluripotency and remarkably found that four previously known transcription factors (Oct3/4, Sox2, Klf4 and cMyc) could reprogram mouse embryonic fibroblasts (MEFs) and tail tip fibroblasts into ES like cells, which were almost indistinguishable from mouse ESC in terms of pluripotency called Induced Pluripotent Stem Cells (iPSC).

iPSC have now been isolated from rodents (mouse and rats) (Takahashi *et al.* 2007b; Honda *et al.* 2010), primates (human and monkeys) (Liu *et al.* 2008; Park *et al.* 2008), livestock (pigs, horse, cattle and sheep) (Ezashi *et al.* 2009a; Nagy *et al.* 2011b; Sumer *et al.* 2011c; Liu *et al.* 2012a) and endangered species (Ben-Nun *et al.* 2011b; Verma *et al.* 2012b). The use of iPSC technology to provide a source of pluripotent cells for felid conservation was considered to be likely a more successful approach than isolating ESC from endangered felids embryos because it is a non-invasive technique, which only requires somatic cells to revert them back to embryonic stem-like cells. In mice, iPSC are similar to embryonic stem cells (ESC) and can form chimeric embryos when injected into blastocysts (Liu *et al.* 2011a). A new chapter in the applications of stem cell science opened with this discovery, offering the possible option to produce cells from skin to ESC regardless of age and gender of donor.

Whereas reprogramming has provoked enormous interest for human regenerative medicine, future benefits might extend to helping conserve faunal biodiversity.

The major obstacle in applying assisted reproductive technologies to wildlife is the extreme diversity in reproductive mechanisms and strategies among species, even those that re taxonomically related. That iPSC apparently can be produced relatively easily across species is therefore potentially important for advances in assisted reproductive technologies.

During the last few years a set of reprograming factors for inducing pluripotency, including Oct4, Sox2, Klf4, c-Myc and Nanog, have been extensively studied due to the rising importance of iPSC particularly in regenerative medicine (Wu & Hochedlinger 2011) and the livestock industry (Sumer *et al.* 2011a). Among these factors, Oct4 is essential in the reprogramming process for human (Wu & Hochedlinger 2011), mouse (Li *et al.* 2010) and ruminants (Malaver-Ortega *et al.* 2012) fibroblasts into iPSC.

The first cloning of Oct4 and its expression in adult human tissues was reported in 1992 (Takeda *et al.* 1992). More recently orthologs of the Oct4 gene have been cloned and characterized in cattle, buffalo, goat, cat and rabbit (Van Eijk *et al.* 1999, Shi *et al.* 2008, Yu *et al.* 2009, Cheng *et al.* 2011, Singh *et al.* 2011). These studies showed high sequence identities in Oct4 between orthologous genes at the nucleotide and amino acid level as well as similarities in its genomic organization, gene localization and regulatory regions. Oct4 contains POU and Homeobox protein domains which both have a high degree of amino acid sequence conservation between species. A number of Oct4 pseudogenes are present in the human genome and alternative splicing, as well as the use of alternative translation initiation codons, results in multiple isoforms of this gene in human.

In this study, I discovered the exact sequence of Oct4, the most important stem cell gene, in Bengal tiger, snow leopard and jaguar, by using bioinformatics analysis to examine similarities and/or dissimilarities between them compared with the human Oct4 gene. Like the similarities seen between human and mouse Oct4, I found the endangered felid Oct4 was 94% similar to human compared with 83% to mouse. I then generated the first iPSC from snow leopard ear fibroblasts by forced expression of human reprogramming factors and later from other geographically separated wild felids such as Bengal tiger (Asia), serval (Africa) and jaguar (Americas) (Verma *et al* 2012; Verma *et al.* 2013).

1.2. Literature Review

Conservation biology is the scientific study of the nature and status of Earth's biodiversity with the aim of protecting species, their habitats, and ecosystems from excessive rates of extinction and the erosion of biotic interactions. The aim of animal conservation is to maintain biodiversity because removal of single species can affect the functioning of global ecosystems (Margules & Pressey 2000). Habitat preservation is one of the best ways to conserve biodiversity (Wildt *et al.* 1986; Loi *et al.* 2001). Small population propagation is also part of a multi-disciplinary approach to conservation, including genetic and ecological characterizations and other strategies (Comizzoli *et al.* 2000).

In situ conservation is on-site conservation or the conservation of genetic resources in natural populations of plant or animal species, such as forest genetic resources in natural populations of tree species. It is the process of protecting an endangered plant or animal species in its natural habitat, either by protecting or cleaning up the habitat itself, or by defending the species from predators and illegal human trade (Bainbridge & Jabbour 1998a). One benefit of *in situ* conservation is that it maintains recovering populations in the surrounding where they have developed their distinctive properties. Another is that this strategy helps ensure the ongoing processes of evolution and adaptation within their environments. As a last resort, *ex situ* conservation may be used on some or all of the population, when *in situ* conservation is too difficult, or impossible.

Ex situ conservation means literally, "off-site conservation". It is the process of protecting an endangered species of plant or animal outside of its natural habitat; for example, by removing part of the population from a threatened habitat and placing it in a new location, which may be a wild area or within the care of humans. While *ex situ* conservation comprises some of the oldest and best known conservation methods, it also involves newer, sometimes controversial laboratory methods (Pope 2000). The best method of maximizing a species chance of survival (when *ex situ* methods are required) is by relocating part of the population to a less threatened location. It is extremely difficult to mimic the environment of the original colony location given the large number of variables defining the original colony (microclimate, soils, symbiotic species, absence of severe predation, etc.) It is also technically challenging to uproot (in the case of plants) or trap (in the case of animals) the required organisms without undue harm (Holt *et al.* 2004).

Endangered animal species are preserved using similar techniques. The genetic information needed in the future to reproduce endangered animal species can be preserved in gene banks, which consist of cryogenic facilities used to store living cell lines, sperm, eggs, or embryos (Oliveira *et al.* 2008).

In situ and *ex situ* conservation programs for some endangered mammalian species can benefit from modern reproductive biotechnologies or assisted reproductive techniques (ART) including artificial insemination (AI), embryo transfer (ET), *in vitro* fertilization (IVF), gamete/embryo micromanipulation, semen/embryo sexing and genome resource banking (GRB). With more knowledge emerging on the basic biology of reproduction, cloning or somatic cell nuclear transfer (SCNT) have been suggested as a potentially integral part of wildlife conservation programs (Gomez *et al.* 2003). To date, however, natural breeding coupled with traditional ART has been the preferred method for increasing endangered animal populations, due to the poor efficiency of SCNT (Gomez *et al.* 2000). With future progress in the field of cloning, this technology will also become more useful for saving species at risk of extinction. Application of modern biotechnologies or ART to mammalian species threatened with extinction will allow more offspring to be obtained from selected parents to ensure genetic diversity and may reduce the interval between generations. Therefore, this review will analyse the use and current status of ART for endangered mammalian species in the context of the broad variability in, and sparse knowledge of reproductive physiology between species (Pope CE 2006).

1.2.1. Strategies for application of reproductive biotechnologies in endangered species

Within the past few decades, a powerful new approach has emerged for conservation of threatened wildlife species, through *in situ* and *ex situ* conservation programs. Hanks (2001) in his review on conservation strategies suggested that zoo-based captive breeding programs should also be regarded as a supplement rather than an alternative to *in situ* conservation activities. Also captive breeding programs should essentially be guided by rational priorities for *ex situ* conservation, ideally focusing on threatened species or groups with which zoos already have space and husbandry experience (Gomez *et al.* 2008).

One of the major problems with the implementation of *in situ* and *ex situ* conservation programs is the lack of availability of the biological material which is required for a better

understanding of reproductive patterns as well to maximize reproductive efficiency. This constraint arises from the strict procedures adopted for restraining or anaesthetizing free-living animals for collection of biological/reproductive samples. However, this has been partially resolved by the development of non-invasive methods of hormonal monitoring the assessment of hormonal profiles from voided urine and faeces. There have been a number of examples of the application of non-invasive endocrine monitoring techniques for *in situ* or zoo-based studies. The first non-invasive measurement of testosterone in the urine of free-ranging African elephants around the time of behavioural musth, characterized by unpredictable, dominant, and excitable behaviour, was reported by (Poole *et al.* 1984). Other examples include Creel *et al.* (1992) who conditioned dwarf mongooses to urinate on a rubber pad during the course of scent marking. This approach provided hundreds of urine samples, which were analysable for hormonal metabolites, allowing an elegant examination of behavioural and endocrine mechanisms of reproductive suppression in this species. Monfort *et al.* (1990) monitored ovarian function and pregnancy in the Eld's deer by evaluating urinary steroid metabolite excretion and Wasser (1995) analysed faecal hormonal metabolites from baboons to understand how dominance is related to conception and reproduction. This technique was also use to provide new evidence suggesting that many female cheetahs inexplicably experienced periods of anoestrus unrelated to season. Ostner and Heistermann (2003) have characterized female reproductive status during breeding season and gestation in wild red-fronted lemurs through analysing immunoreactive 5α-pregnane-3βol-20-one and total estrogens in faeces. Czekala *et al.* (1994) and Robbins and Czekala (1997) measured gonadal steroids in protected mountain gorillas, including quantitating urinary glucocorticoid excretion to assess social and environmental impacts on animal well being. Faecal progesterone hormone monitoring has also revealed major differences in reproductive patterns, estrous cycles and seasonality among rhinoceros species (Schwarzenberger *et al.* 1998; Brown *et al.* 2001; Roth *et al.* 2001; Garnier *et al.* 2002). Non-invasive endocrine monitoring has also proved to be an essential tool for assisting in better understating of basic reproductive biology of elephants (Brown 2000). In a review, Pukazhenthi and Wildt (2004) refers to unpublished observations of Monfort and Brown on the possibility of detecting the ovulatory luteinising hormone (LH) surge in urine of giant panda and killer whale using radioimmunoassay or enzyme immunoassay techniques. Also there is a study claiming accurate profiles of gonadal steroid metabolites by analysing the faeces of giant panda (Kersey *et al.* 2003). These studies have highlighted the possibilities for

non-invasive, remote monitoring of reproductive status in a number of endangered mammalian species. Further development of techniques that allows the instantaneous assessment of the endocrine status of animals living in nature would offer exciting opportunities to interrelate their physiology, especially that of reproduction, with their natural environment. This information would also help to apply available ART like AI and ET more efficiently for *in situ* or zoo-based conservation of endangered species.

Other developments have taken place in the collection of biological material, like semen from aggressive males, by the use of internal artificial vaginas or vaginal condoms (Bainbridge & Jabbour 1998b). Semen can also be collected from the epididymides following the death of an animal or when males are killed during the rut period. Additionally, post-coital sperm recovery has been used successfully in marmoset monkeys (Morrell & Hodges 1998) and rhinoceros (Wildt & Roth 1997). For embryo recovery, non-surgical or less invasive methods like transcervical embryo collection have been applied in the oryx, bongo, eland, greater kudu (Schiewe *et al.* 1991) and laparoscopic embryo collection has been used in an endangered swine breed, the silver fox (Jalkanen & Lindeberg 1998) and the bear (*Ursus americanus*) (Boone *et al.* 1999).

1.2.2. Background information on Wild Felids

1.2.2.1. Snow Leopard

The snow leopard (*Uncia uncia*) is a moderately large cat native to the mountain ranges of Central Asia. They live between 3,000 and 5,500 metres (9,800 and 18,000 ft) above sea level in the rocky mountain ranges of Central Asia. However, their secretive nature means that their exact numbers are unknown, although it has been estimated that between 3,500 and 7,000 snow leopards exist in the wild and between 600 and 700 in zoos worldwide (Kleihman & Garman 1978).

1.2.2.2. Bengal Tiger

The Bengal tiger, or Royal Bengal tiger (*Panthera tigris tigris*, previously *Panthera tigris bengalensis*), is a subspecies of tiger, found in India, Bangladesh, Nepal and Bhutan. The Bengal tiger is the most numerous of the tiger subspecies. According to WWF, there are about 2,100 Royal Bengal tigers in the wild today, including 1,411 in India, 200 in Bangladesh, 150 in Nepal and 100 in Bhutan (Hemmer 1968; Pope 2000)

1.2.2.3. Jaguar

The jaguar (*Panthera onca*) is a big cat, a feline in the *Panthera* genus, and is the only *Panthera* species found in the Americas. The jaguar is the third-largest feline after the tiger and the lion, and the largest and most powerful feline in the Western Hemisphere. The jaguar's present range extends from Mexico across much of Central America and south to Paraguay and northern Argentina. Apart from a known and possibly breeding population in Arizona (southeast of Tucson), the cat has largely been extirpated from the United States since the early 1900s (Hemmer 1968)

The jaguar is a near threatened species and its numbers are declining. Threats include habitat loss and fragmentation. While international trade in jaguars or their parts is prohibited, the cat is still regularly killed by humans, particularly in conflicts with ranchers and farmers in South America.

1.2.2.4. Serval

The serval is a medium-sized cat, measuring 59 to 92 centimetres (23 to 36 in) in head-body length, with a relatively short, 20 to 38 centimetres (7.9 to 15 in) tail, and a shoulder height of about 54 to 66 centimetres (21 to 26 in). The serval is native to Africa, where it is widely distributed south of the Sahara. It was once also found in Morocco, Tunisia, and Algeria, but may have been extirpated from Algeria and only remains in Tunisia because of a reintroduction programme. Its main habitat is the savanna, although melanistic individuals occur in mountainous areas at elevations up to 3,000 metres (9,800 ft). The serval needs watercourses within its territory, so it does not live in semi-deserts or dry steppes. Servals also avoid dense equatorial jungles, although they may be found along forest fringes. They have dwindled in numbers due to human populations taking over their habitat and also hunting for their pelts. The serval is sometimes preyed upon by leopards and other large cats (Sassa *et al.* 2010)

1.2.3. Reproduction in Wild Felids

Despite variations within the Felidae family in phenotype, genotype and physiology, all species face a similar challenge (i.e.), each is threatened or endangered with extinction somewhere in its natural range. For this reason, establishing reservoir populations in captivity is considered useful as a hedge, or insurance policy, while at the same time offering research opportunities that would not be available in nature. However, creating self-sustaining

populations of wild felids *ex situ* has been challenging. Although, some species can be paired readily, but breeding inexplicably does not occur (e.g., cheetah) (Wildt & Roth 1997). Others exhibit extreme male–female aggression upon introduction, which if not managed carefully can result in the death of one of the pair. However, when pairs are successfully established, then breeding can be prolific (e.g., clouded leopard (*Neofelis nebulosa*) and fishing cat (*Prionailurus viverrinus*)). It is interesting that, although most consider the domestic cat (*Felis catus*) to be fertile (sometimes excessively so), this species actually presents many reproductive challenges under highly intensive, captive management situations. This is particularly relevant for those domestic cats that are used as models to study certain human genetic diseases ranging from metabolic disorders like muco-polysaccharidosis (Driscoll *et al.* 2002) and porphyria to chromosomal abnormalities like Klinefelter's syndrome . These cat colonies are often comprised of small, inbred populations of heterozygous individuals whose disease conditions interfere with successful breeding (Mitsouras *et al.* 2011). Therefore, assisted reproductive technologies, including artificial insemination (AI), in vitro fertilization (IVF) and embryo transfer (ET) in conjunction with embryo and sperm cryopreservation, potentially offer practical solutions for overcoming breeding difficulties encountered with either wild felids or domestic cats managed as human disease models (Gomez *et al.* 2000; Pope CE 2006; Gomez *et al.* 2008). Although both AI and IVF/ET have been successful in selected felid species or populations, the overall incidence of success remains low (<20%) (Pope 2000). Successful assisted reproduction can be elusive because there are so many interacting variables that must be understood and controlled before offspring can be consistently produced. One of the most challenging hurdles is ensuring a reliable and normal ovarian response for either ovulation (AI) or oocyte aspiration (IVF) (Gomez *et al.* 2003).

1.2.3.1. AI (Artificial Insemination)

Although intra-vaginal AI has been successful in the tiger and domestic cat, very high concentrations (10^7–10^8) of sperm have been required to achieve pregnancy (Pope 2000). Early studies identified anesthesia and induced ovulation are the factors that potentially interferes with both sperm transport and ovulation in the domestic cat, probably through mechanisms that slow uterine tract contractility and somehow inhibit follicular release of the ovum. These findings have necessitated the development of post-ovulatory, intrauterine AI procedures (Ketz-Riley *et al.* 2003), which have resulted in living offspring in the intensively

managed laboratory cat, including cat models for studying human diseases as well as eight wild felid species. Using fresh spermatozoa, AI has been reasonably successful in certain species such as the domestic cat and cheetah, sufficiently so to contribute to genetic management. However, breeding success has been far less in other species for reasons that remain mostly unknown (Wildt & Roth 1997; Ketz-Riley *et al.* 2003). Some rudimentary studies have explored the impact of female age, demonstrating in the cheetah (for example) that nulliparous females older than 7 years are poor AI candidates. This is particularly interesting given that cheetahs commonly live to 12– 15 years of age in zoos (Bosman *et al.* 2007). Thus, reproductive fitness may decline at relatively early ages in cats indicating that, for assisted breeding to be most effective, it should be directed at younger females rather than serving as a last ditch tool for producing pregnancies in individuals at or near reproductive senescence. Interestingly, ovarian response to exogenous gonadotropins does not appear altered by age, with older individuals responding with normal follicle and ovulation numbers post-eCG/hCG treatment. Poor pregnancy rates are, therefore, attributed to alterations in uterine function in aging females as is commonly seen in domestic cats, although diminished oocyte quality also could be playing an important role as is seen in mice and humans (Mitsouras *et al.* 2011) (Figure 1).

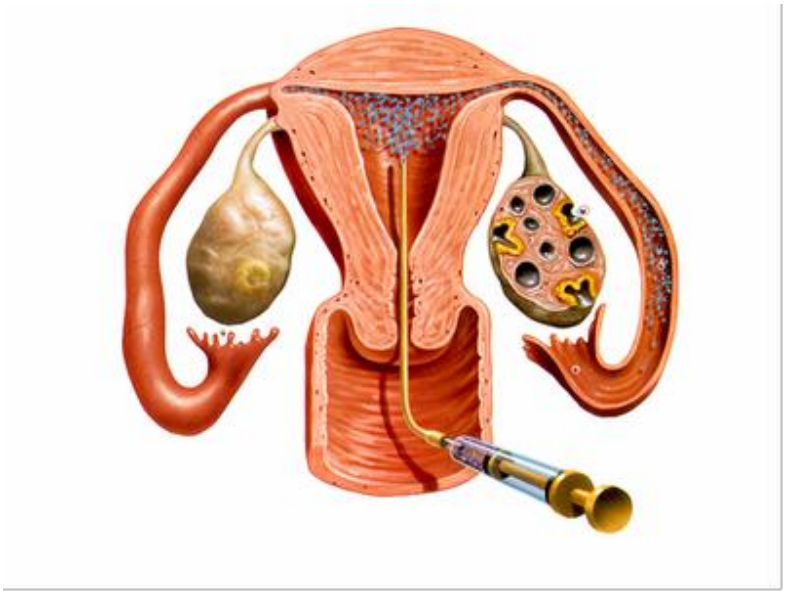

Figure 1: *Artificial Insemination, a diagrammatic representation of the intra-uterine approach* (Howard *et al.* 1992).

1.2.3.2. IVF (In-vitro Fertilization)

In vitro fertilization has been also tried in various wildlife species but with sporadic success.

The major studies involving IVF are in puma (Miller *et al.* 1990), tiger (Donoghue *et al.* 1990), cheetah (Donoghue *et al.* 1990), Indian desert cat (Pope *et al.* 1993), African wild cat (Pope 2000), jaguar, ocelot, tigrina. Live births have been only reported in tiger and Indian desert cat using IVF. Several IVF protocols also have been developed in the domestic cat and adapted to multiple wild felid species. In most cases, 'successes' with the production of living offspring after ET have been highly sporadic. Because of this inconsistency, IVF/ET is not being used as a genetic management tool for the dependable production of offspring in any felid species. A prerequisite to IVF, of course, involves treatment with exogenous gonadotropins prior to oocyte aspiration either by laparoscopy or laparotomy (Wildt & Roth 1997). As might be expected from the results of AI study, ovarian response to gonadotropin stimulation for oocyte recovery has been as variable as the phenotypes of the species investigated. Although there are differences between species, in general it has been possible to recover viable, in vivo matured oocytes that fertilize in vitro when exposed to either fresh or thawed spermatozoa (Wildt & Roth 1997). It should not be surprising that the highest IVF success occurs in the domestic cat, no doubt because this species has been studied most intensively. ET after IVF has been successful in the laboratory cat, in cats used as models for mucopolysaccharidosis in humans and in four wild felid species: the tiger (Panthera tigris), African wild cat (Felis sylvestris lybica), Indian desert cat (Felis sylvestris ornata), and caracal (Caracal caracal). When at least five embryos are transferred per recipient, pregnancy success has ranged from 0 to 50% (Figure 2).

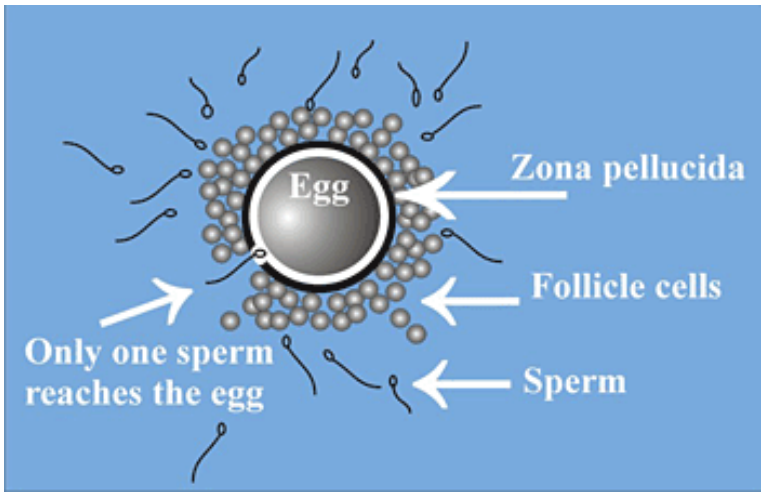

Figure 2: *In-vitro fertilization of egg with sperm. (Diagramatic representation)*

1.2.3.3. Intra-Cytoplasmic Sperm Injection (ICSI)

The development of ICSI in humans has lead to its application in non-human primate species, a number of which are endangered. Similarly, improvements in ICSI techniques in some domesticated animals could be useful for conservation programs of endangered species belonging to the same families. It should be noted that for the fertilization of feline and murine oocytes, ICSI has been less effective than sub-zonal insemination, raising the possibility that the successful application of ICSI, like most of the other reproductive biotechnologies, is also species-specific. Despite this, there is a preference for applying ICSI to wildlife conservation, rather than Sub Zonal sperm Injection (SUZI), because there is a total absence of polyspermy with ICSI (Pope 2000).

The different procedures used for sperm insertion (ICSI and SUZI) will have an important role to play in future conservation efforts, particularly for endangered species in which males might develop a higher proportion of abnormal sperm and no other method except ICSI or SUZI would be available for successful IVF. This is particularly important for some feline species, such as the clouded leopard and cheetah, where a high level of abnormalities have been detected in the spermatozoa of animals in captive populations (Wildt *et al.* 1986).

Micromanipulation of embryos by treatments such as zona drilling or partial zona dissection could raise their chances of implantation. Drilling holes in the zona pellucida aims to facilitate earlier hatching of embryos from the zona pellucida when it has been hardened by ovarian stimulation and/or embryo culture. Results obtained by Loskutoff et al. (1999) indicate that partial zona dissection improves the hatching frequencies of bovine blastocysts produced in vitro and co-culture conditions can affect survival after thawing. Hence, it is probable that the embryos of endangered wildlife species will also benefit from micro-manipulated hatching techniques (Figure 3).

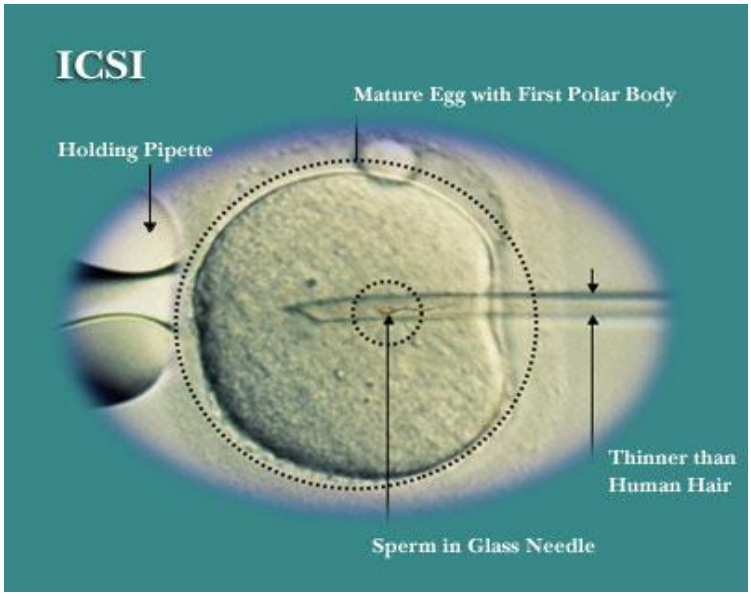

Figure 3: *Intra-Cytoplasmic Sperm Injection (ICSI)* (Suemori *et al.* 2001)

1.2.3.4. Somatic Cell Nuclear Transfer (SCNT)

Somatic cell nuclear transfer (SCNT) represents an alternative for the production of genetically identical animals and offers the possibility of preventing the extinction of wild species. However, owing to the limited availability of oocytes from wild animals, the cloning of endangered species usually requires the use of donor oocytes from a related domestic species. Interspecies SCNT consists of the construction of a cloned embryo using a donor cell and recipient oocyte from a different species, but from the same genus; whereas intergeneric SCNT consists of the construction of a cloned embryo in which the donor nucleus and recipient cytoplast differ both in species and in genus. Several studies have demonstrated that it is possible to produce embryos from endangered species by interspecies or inter-generic SCNT (Gomez *et al.* 2008), however, few live cloned wild mammals have been produced and these animals were derived from embryos reconstructed with donor oocytes of the same genus. Viable offspring from inter-generic SCNT have not been produced in any mammalian species, although pregnancies have been established with inter-generic cloned embryos after transfer into sheep or domestic cat recipients (Gomez *et al.* 2000).The successful development of interspecies and inter-generic cloned embryos is dependent on a variety of factors, which are similar to those reported for intra-species SCNT including source of oocyte cytoplasts, cell cycle synchronisation and genotype of the donor cells. Moreover, increasing evidence shows that aberrant epigenetic alterations that arise during SCNT may be associated with perinatal and neonatal losses and the production of abnormal offspring (Figure 4).

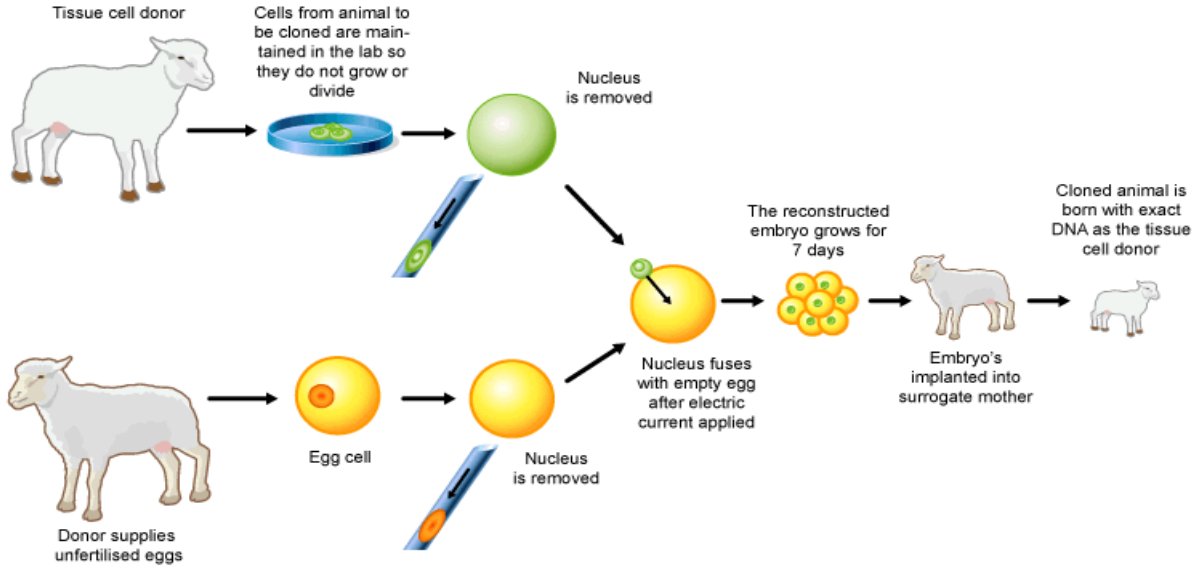

Figure 4: *Somatic Cell Nuclear Transfer (SCNT)* (Wilmut *et al.* 2002)

1.2.4. Stem Cells and Potency

A stem cell is a cell that is capable of self-renewal while maintaining the ability to differentiate into other distinct cell types. There are several levels of potency which describes a stem cell's differentiation potential. The zygote and blastomeres of the two and four cell stage embryos in mice are totipotent, that is, they are capable of differentiating into any cell of the three germ layers including the germline as well as extra-embryonic tissues and so, alone can form an entire organism. On the other hand, cells that are pluripotent are capable of differentiating into any cell of the three germ layers but cannot alone form a whole organism due to their inability to contribute to the extra-embryonic tissues. Multipotent cells are normally only capable of differentiating into cells of one lineage. Adult stem cells such as haematopoietic stem cells (HSCs) are multipotent and can usually differentiate only into cells of the haematopoietic lineage, while unipotent cells, such as spermatogonial stem cells are precursor cells that can only differentiate into one cell type, spermatozoa. Unipotent cells are distinct from nullipotent cells, which describe terminally differentiated cells such as lymphocytes that have a very limited self-renewal capacity and cannot differentiate into any other cell type. Given that pluripotent cells have been used to generate established cell lines with unlimited proliferation and also offer the greatest differentiation potential, they have garnered much interest. Different types of pluripotent stem cells have been derived from the embryo at various stages in mice which include: embryonal carcinoma cells, ESC, embryonic

germ (EG) cells, multipotent germline stem cells and epiblast stem cells but not in any endangered species, due to the inaccessibility of embryos (Figure 5).

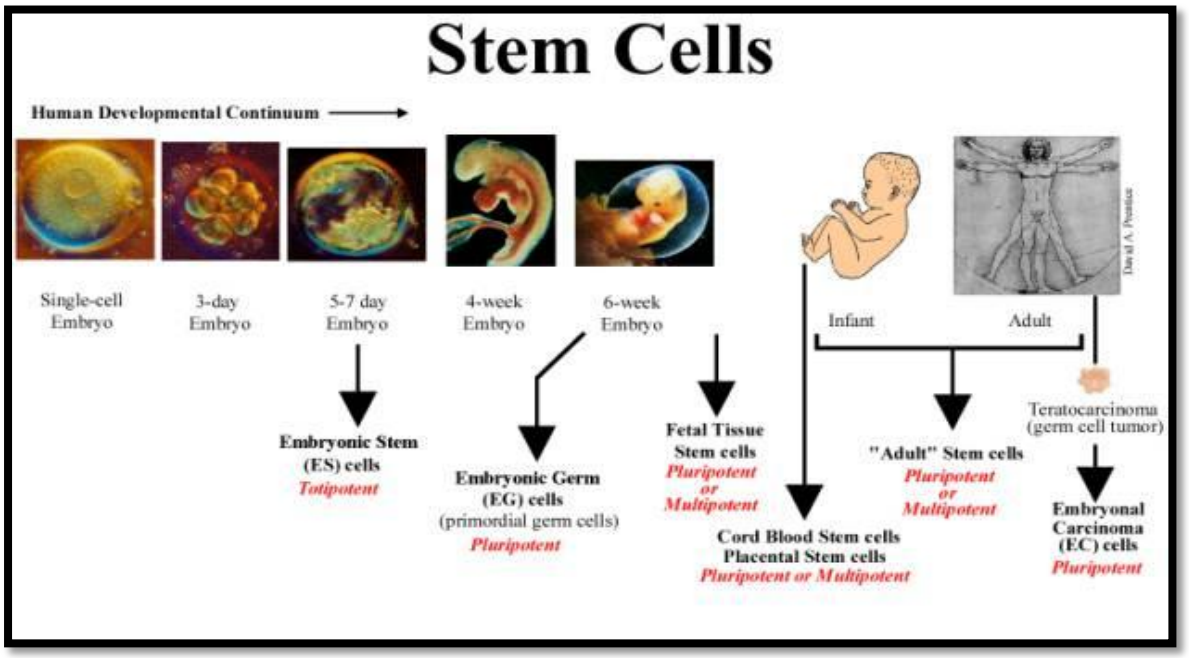

Figure 5: *Different types of Stem cells* (Arrigoni *et al.* 2009b)

1.2.4.1. Reprogramming

When pluripotent cells such as ESC differentiate into more specialised cells, they lose their pluripotency. Differentiation is due to the systematic silencing and activation of certain genes. The mechanisms underlying such silencing and activation are not well characterised but are thought to be due mainly to epigenetic modifications (Pralong *et al.* 2006). Epigenetic modifications refer to the alterations in gene expression patterns without changing the DNA sequence. Such epigenetic modifications include DNA methylation, histone acetylation and methylation and chromatin remodelling, which act by regulating gene specific transcriptional activation or repression (Agarwal *et al.* 2012). The epigenetic status of ESC is distinct from that of differentiated somatic cells. It is accepted that once genes are silenced, they become more difficult to reactivate due to the persistence of stable repressive nucleoprotein complexes (Levine *et al.* 2012). This central dogma implies that once cells become differentiated they can no longer revert back to their pluripotent characteristics. Remarkably, the developmental stage of cells can be reversed. The reversion of somatic nuclei, which have a restricted differentiation potential, back to a pluripotent or even totipotent state is known as reprogramming (Liu *et al.* 2011b). The process of reprogramming can be achieved through

several methods including nuclear transfer, cell fusion, and treatment with cell extracts and induced pluripotency (Figure 6) (Tat *et al.* 2010).

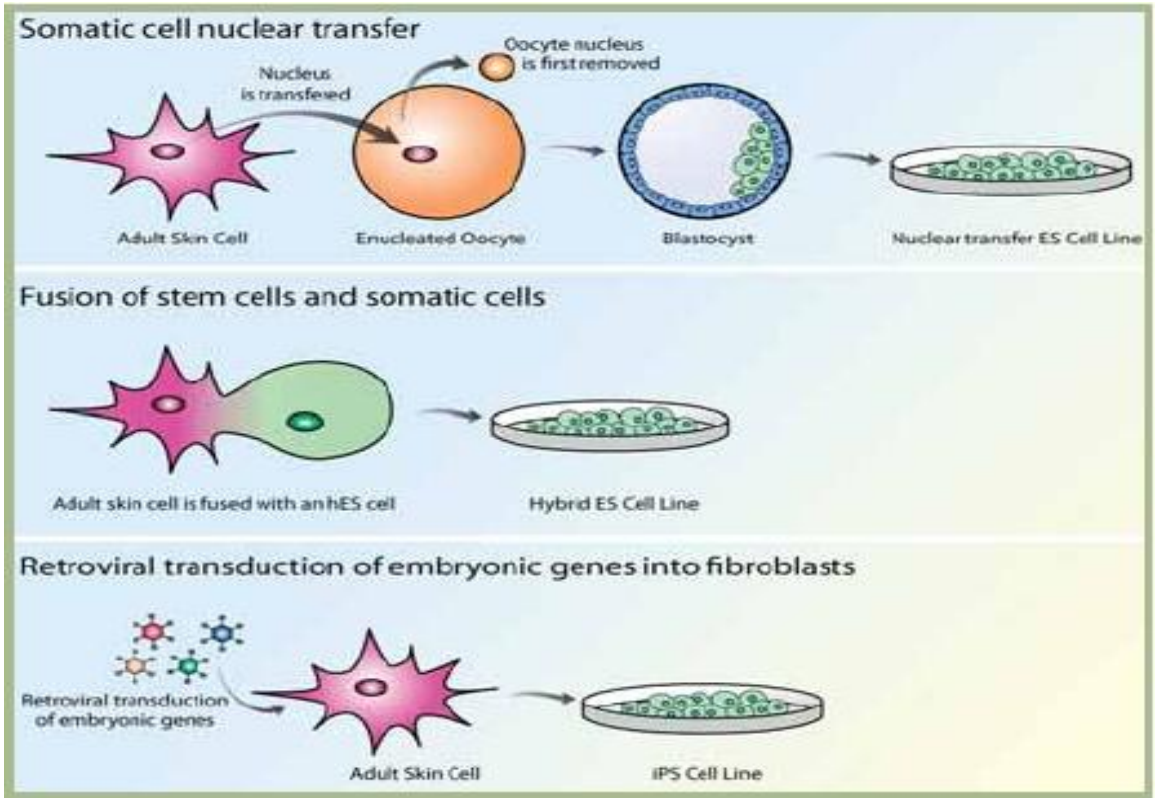

Figure 6: *Different types of reprogramming applications* (Tat *et al.* 2010)

1.2.5. Generation of Induced Pluripotent Stem Cells (iPSC)

Pluripotent cells, such as ESC have the ability to form all cell types of the body. iPSC have resulted from an exciting new discovery that four stem cell genes when introduced into a somatic cell can reprogram the adult cell to behave like an ESC. iPSC can be created from cells that do not have pluripotent qualities, such as mouse and human skin cells. Typically to create an iPSC, retroviruses are used to insert the stem cell genes into the somatic cell. Within weeks small colonies of iPSC begin to appear. They can be maintained using conditions developed for ESC for the species. In recent studies it has been shown that the stem cell genes do not have to be inserted into the somatic cells to form iPSC and the tissue source of donor can significantly affect the reprogramming outcomes, which makes iPSC technology very promising as a source of stem cells (Tat *et al.* 2010; Heffernan *et al.* 2013).

For large animals, iPSC have been reported for pigs (Okita *et al.* 2007a), , horse (Khodadadi *et al.* 2012), bovine (Sumer *et al.* 2011b), , rhino (Ben-Nun *et al.* 2011a), sheep (Liu *et al.* 2012b) snow leopard (Verma *et al* 2012). However there are a number of groups

internationally, vying to develop methods to generate and characterize iPSC from a number of mammalian species.

Takahashi *et al.* (2007a; 2007c) initially demonstrated the derivation of iPSC through retroviral delivery and constitutive expression of four key transcription factors Oct4, Sox2, Myc and Klf4. The pluripotent state of the resultant colony-forming iPSC was confirmed by gene/surface antigen expression, epigenetic remodelling, contribution to embryonic lineages following blastocyst injection resulting in live born chimeras and commitment to all three germ layers in teratomas generated in SCID mice (Hamilton *et al.* 2009). Importantly, i*n vitro* directed differentiation of iPSC yield cells capable of treating mouse models of humanized disease. Genome wide analysis of promoter binding and expression shows strong overlap between iPSC and ESC. Of the ESC promoter regions co-bound by 3 or 4 of the key iPSC factors, 64% of the corresponding loci are similarly bound in fully reprogrammed iPSC (Okita *et al.* 2007b). In loci bound by 1 or 2 factors, the similarity between ESC and iPSC increases to 87%. It is noteworthy that very few promoters co-bound by 3 or 4 factors in ESC are not bound by any key factor in iPSC.

Originally demonstrated in mouse, iPSC have been generated in rat, rhesus monkey and a range of human cells including keratinocytes, dermal fibroblasts and $CD34^+$ peripheral blood. However, low efficiency of conversion from somatic to iPSC continues to hamper this method. Initially, only 0.02% of human fibroblasts converted to a pluripotency phenotype (Takahashi *et al.* 2007a; Takahashi *et al.* 2007c). Many cells become partially reprogrammed that both form colonies and express pluripotency markers such as SSEA1, but are incapable of reactivating pluripotency genes or the silent X chromosome as observed in fully reprogrammed cells. Experiments using homogenous populations of cells harbouring inducible transgenes of each key reprogramming factor at identical loci confirm that differential integration sites account for little of the low efficiency observed in iPSC technology (Figure 7) (Park *et al.* 2008).

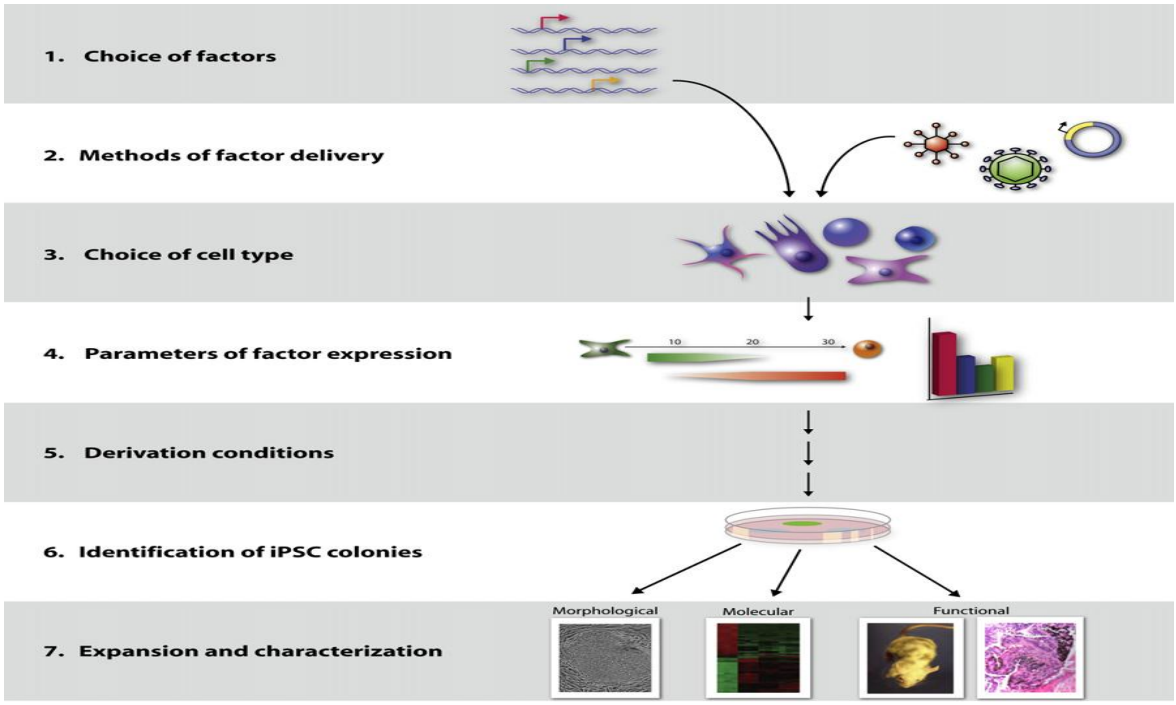

Figure 7: *Different parameters for generating and validating iPSC in any species* (Esteban *et al.* 2009).

1.2.5.1. Description of Transcription Factors:

a) Oct4

Oct4 (octamer-binding transcription factor 4, also known as Oct 3 and Pou5f1) was first described as a protein present in unfertilized oocytes, ESC and primordial germ cells (Sumer *et al.* 2010b). Its expression is essential for the development of the inner cell mass (ICM) in vivo, the derivation of ESC and the maintenance of a pluripotent state. The precise levels of Oct-3/4 govern three distinct fates of ESC. Within a narrow window of expression, ESC retains an undifferentiated, pluripotent state. A less than twofold increase in expression causes differentiation into primitive endoderm and mesoderm, whereas repression of Oct-3/4 induces loss of pluripotency and differentiation into trophectoderm (Sumer *et al.* 2010a).

Yu *et al.* (2009) determined the nearly complete Coding Sequence (CDS) of *cPOU5F1* in the domestic cat ES-like cells, including 249 base pairs (bp) of the 3'-UTR. The sequence was deposited into the GenBank database under accession number EU366914. They performed a sequence alignment of the putative amino acid sequences of *cPOU5F1* (272 amino acids based on a CDS of 819 bp) and its human (GenBank accession no. NM_002701) and mouse

(GenBank accession no. NM_013633) orthologs. The sequence identities of *cPOU5F1* and human and mouse *POU5F1* were 92 and 82% at the nucleotide level and 94 and 83% at the amino acid level, respectively. We identified a POU-specific domain and a POU homeodomain in *cPOU5F1*, which were linked by a non-conserved linker (Yu et al 2009) There was a high degree of conservation between the 60 amino acid-POU homeodomains of the cat and human (98%) and the cat and mouse paired with the human and mouse, respectively, and a corresponding insertion of 30 bp. The sequence identity of the cat and human homeodomains was 80%, and that of the cat and mouse was 66%. Yu et al (2009) demonstrated that *POU5F1* is strongly conserved among the cat, human and mouse at the nucleotide and amino acid sequence levels (Figure 8). In addition, we identified a POU-specific domain and POU homeodomain in *cPOU5F1*, which are also present in *POU5F1* homologues from several other mammals.

Figure 8: *Comparison of the predicted amino acid sequences of cPOU5F1 and its human and mouse orthologs* (Yu *et al.* 2009).

b) Sox2

SRY (sex-determining region Y)-box 2, known as Sox2, is a transcription factor involved in the self-renewal of ESC (Lowry *et al.* 2008). It has an important role in maintaining ES-cell pluripotency and heterodimerizes in a complex with Oct4. In human ESC, SOX2 has 1279 binding sites and OCT4 has 623 binding sites. A total of 404 of these are overlapping and 87% (353) of these sites also overlap with NANOG (1687 binding sites) targets. In addition to ESC, Sox2 is also expressed in the extra-embryonic ectoderm, trophoblast stem (TS) cells and the developing central nervous system (neural stem cells). In these cell lineages, Sox2

expression is restricted to cells with stem cell characteristics supporting their self renewal capability and is no longer expressed in cells with a more restricted developmental potential. Interestingly, forced expression of Oct4 can compensate for loss of Sox2 in ESC and, in direct reprogramming, Sox2 can be replaced by Sox1, Sox3 and, to a lesser extent, Sox15 or Sox18 (Tat *et al.* 2010).

c) c-Myc

c-Myc is a pleiotropic transcription factor that has been linked to several cellular functions, including cell-cycle regulation, proliferation, growth, differentiation and metabolism (Martinez-Fernandez *et al.*). This factor tends to be highly expressed in the majority of rapidly proliferating cells and is generally low or absent during quiescence. c-Myc also functions during both self renewal and the differentiation of stem and progenitor cells, particularly in interactions between stem cells and the local microenvironment. A large number of binding sites have been reported throughout the genome and c-Myc appears to be involved in recruiting chromatin-remodeling activities to promoters (Wu *et al.* 2009). The role of c-Myc in reprogramming is not clear yet. It is dispensable for the generation of iPSC in mouse and human but the efficiency of reprogramming decreases dramatically. It can also be replaced by other family members, such as n-Myc and l-Myc, to reprogram somatic cells to an ESC-like status.

d) Klf4

Kruppel-like factor 4 (Klf4) is a transcription factor expressed in a variety of tissues, including the epithelium of the intestine, kidney and the skin (Nagy *et al.* 2011b). Depending on the target gene and interaction partner, Klf4 can both activate and repress transcription and a growing body of evidence suggests that Klf4 can function both as an oncoprotein and tumour suppressor. Constitutive expression of Klf4 suppresses cell proliferation by blocking G1–S progression of the cell cycle. In human colorectal carcinoma, KLF4 appears to be down-regulated, with evidence of hyper-methylation and loss of heterozygosity. Recently, it has been demonstrated that the forced over-expression of Klf4 in ESC inhibits differentiation in erythroid progenitors, suggesting a role for this factor in ESC function. Its exact role in the reprogramming process is also not fully understood and it can be replaced with other Klf family members (Klf2 and Klf5) or the unrelated factors Nanog and Lin28 (Aasen *et al.* 2008).

e) Nanog

Nanog was first described as a factor that was involved in maintaining ESC self-renewal and pluripotency (Dixon *et al.* 2010). Smith and colleagues termed the factor Nanog, after the mythological Celtic land of the ever young, 'Tir nan Og'. Nanog is thought to operate in concert with other crucial 'stemness' factors, such as Oct4 and Sox2, to establish the identity of ESC. Nanog expression is found in the interior cells of the compacted morula and the ICM of the blastocyst. On implantation, Nanog expression is detected only in the epiblast and is eventually restricted to primordial germ cells. It is of interest that it was reported initially that Nanog-deficient ESC completely lose their self-renewal capability, differentiating into extra-embryonic cell lineages (Chambers *et al.* 2003).

1.2.5.2. Delivery of reprogramming factors via retroviral Vectors

Retroviral vectors are widely used gene transfer systems for both clinical gene therapy and basic research because their biology is well understood and they have high transduction efficiency. Retroviral vectors can either be replication competent or replication-defective. Replication-competent viral vectors contain the essential genes for virion synthesis, and continue to propagate themselves once infection occurs (Hamilton *et al.* 2009). Because the viral genome for these vectors is large, the cloning capacity for any genes is limited. Consequently, these vectors have not been used to generate iPSC. Conversely, replication-defective vectors are the most common choice in studies because the viruses are deleted in the coding regions for the genes necessary for additional rounds of virion replication and packaging (Gonzalez *et al.* 2009). Viruses generated from replication-defective vectors can infect their target cells and deliver their viral payload, but avoid triggering the lytic pathway, which would result in cell lysis and death. Replication defective viral vectors can usually hold inserts of up to 10 kb, dependent on the types of vectors. The major drawback of the retrovirus-mediated gene delivery approach is the requirement for cells to be actively dividing for transduction to occur. As a result, slowly dividing or non-dividing cells such as neurons are very resistant to infection and transduction by retroviruses. Stable integration of retroviral DNA into the host genome leads to persistent expression of transgenes, which may lead to insertional mutagenesis and result in cancers. The vector used to produce iPSC is a Moloney murine leukaemia virus (MMLV)-based retrovirus vector, and the transgenes were driven by the 5'MMLV long terminal repeat (LTR) promoter, which usually is silenced in ESC and embryonic carcinoma (EC) cells. Indeed, reprogramming factors were silenced by

methylation in their generated iPSC. However, the MMLV LTR promoter often became reactivated and drove c-Myc expression in differentiated cells derived from iPSC subsequently causing tumor formation in iPSC-derived chimeric mice (Yusa *et al.* 2009). Because of this phenomenon, the Yamanaka group omited c-Myc from reprogramming factors, and unexpectedly obtained fully functional iPSC derived from human and mouse fibroblasts by retroviral delivery. Although they achieved reprogramming of human and mouse somatic cells with three transcription factors (devoid of c-Myc), and the mice derived from the iPSC were tumor free, the reprogramming efficiency was much lower than the combined four-factor process, indicating the impracticality of this approach. To improve efficiency, several reprogramming methods have been developed including usage of different cell types, and combination of the four defined reprogramming factors with other factors or small molecules (Lyssiotis *et al.* 2009).

Additionally, the Daley group successfully isolated iPSC from primary skin fibroblasts by retroviral transduction of the defined four factors with hTERT and SV40 large T antigen via a murine stem cell virus (MSCV) based retroviral vector, despite the low efficiency of this approach (Park *et al.* 2008). The MMLV LTR promoter is susceptible to silencing in ESC, due to harbouring several silencers such as the negative control region (NCR), direct repeat (DR) enhancer, CpG rich promoter, and primer binding sites (PBS). In contrast, the MSCV LTR promoter deleted or mutated some of these silencers, and thus is more potent and able to drive expression of transgenes in ESC (Geijsen *et al.* 2004). However, in this study, the reprogramming factors driven by MSCV LTR were still silenced in generated human iPSC, suggesting that the silencing may due to somatic cell reprogramming. Nevertheless, the MSCV-based retroviral vectors may be more effective than MMLV-based vectors in generating iPSC, although it needs side-by-side comparison studies to compare their reprogramming efficiency (Stadtfeld *et al.* 2008).

In the recent study of bovine iPSC, the VSVG pseudotyped retroviral delivery by using GP2-293 packaging cells was identified as the most efficient method of transgene delivery to bovine adult fibroblasts (Sumer *et al.* 2011b). The reprogramming efficiency of the retrovirus-mediated gene delivery approach is partially dependent on somatic cell types. Aasen et al. 2008 used human keratinocytes as a reprogrammed target cell and had a 100-fold higher reprogramming efficiency than that achieved in human fibroblasts by retroviral delivery of the four defined factors. Two factors (Oct4 and Klf4 or c-Myc) can convert mouse

adult neural stem cells into pluripotent stem cells because of high levels of endogenous Sox2 and c-Myc (Aasen *et al.* 2008). Human fibroblasts can be reprogrammed into iPSC through two reprogramming factors and small molecules, such as Oct4 and Klf4 plus BIX-01294 and Bayk8644 or Oct4 and Sox2 plus VPA. Similarly, (Giorgetti et al. 2009) generated iPSC from human cord blood stem cells via retroviral delivery of only two reprogramming factors (Oct4 and Sox2). Interestingly, it was shown that Oct4 generates pluripotent stem cells from adult mouse neural stem cells and that the one-factor iPSC are similar to embryonic stem cells *in vitro* and *in vivo* (Giorgetti et al. 2009) These approaches will make iPSC safer and practical to use.

Significantly, the Jaenisch group reported the generation of iPSC from sickle cell anaemia by transduction of retroviruses expressing Oct4, Sox2 and Klf4 factors, and lentiviruses expressing an excisable c-Myc gene. They corrected the sickle haemoglobin allele in these anaemia-specific iPSC by gene specific targeting and transplanted hematopoietic cells derived from these corrected iPSC into mice with sickle cell anaemia (Shenoy *et al.* 2012). Their data demonstrated that all systemic parameters of sickle cell anemia were improved in these animals. In addition, (Remenyi *et al.* 2003) it was shown that injection of iPSC-derived endothelial/endothelial progenitor cells into liver corrected the hemophilia a phenotype in mice. The therapeutic potential has been demonstrated for treatment of Parkinson's and heart diseases (Park *et al.* 2008). The neural precursor cells derived from iPSC were shown to improve symptoms of rats with Parkinson's disease, upon injection into the adult brain. It was shown that intra-myocardial delivery of iPSC yielded progenies that properly engrafted without disrupting cyto-architecture in immune-competent recipients. In contrast to parental non-reparative fibroblasts, iPSC treatment restored post-ischemic contractile performance, ventricular wall thickness, and electric stability, while achieving in situ regeneration of cardiac, smooth muscle, and endothelial tissue (Wernig *et al.* 2008). Together, these studies demonstrated that iPSC were inherently powerful potential tools for therapeutic application and studying treatment and mechanism of diseases (Luo *et al.* 2011).

1.2.5.3. Role of Four Key Transcription Factors in Conversion to iPSC

The role of the key reprogramming factors in ESC biology highlights their importance in iPSC conversion. Indeed, functional redundancy could not be achieved through substitution of any iPSC factor with alternative factors, even when c-Myc and Klf4 tumor-related factors were replaced with other oncogenes (Takahashi *et al.* 2007a; Takahashi *et al.* 2007c). Sox2

maintains pluripotency in ESC directly through gene expression, and also indirectly through maintenance of Oct4 expression levels. Half of the hundreds of Oct4 promoter and enhancer transcriptional targets incorporate proximal Sox2 recognition sequences, where they coordinate activation and repression of a number of genes (Remenyi *et al.* 2003). Unsurprisingly, transcriptionally-inactive genes co-bound by Oct4/Sox2 in ESC are enriched for lineage specification, and many are also co-occupied by Klf4. Over-expression of Klf4 in ESC suppresses expression of differentiation genes and maintains pluripotency in ESC in the absence of LIF (Ema *et al.* 2008).

In conversion to a pluripotent iPSC, emerging evidence suggests a sequential and biphasic sequence of events. Interestingly, Myc appears most critical for the initial stages of reprogramming, upregulating expression of genes implicated in metabolism, translational control, RNA splicing, cell cycle and energy production. In addition, Myc may facilitate binding of other factors to their transcriptional targets, all of which may highlight why efficiency is compromised when Myc is removed from the reprogramming cocktail and why reprogramming in these cells is delayed. In the majority of cells, rapid down-regulation of lineage-specific genes (Thy1 in the case of fibroblasts) occurs within days of infection. However, only a small subset of these cells (~3-4%) progress to alkaline phosphatase expressing cells, evident 3-4 days post-infection. A direct link between Myc and AP expression has not been established, but may induce global histone acetylation allowing Oct3/4 and Sox2 to bind targets later in the reprogramming cascade (Heffernan *et al.* 2012).

The expression of stage-specific embryonic antigen-1 (SSEA1) in approximately 4% of cells at day 9 post-infection, the appearance of small colonies and resetting of histone methylation profile characteristic of ESC denotes an intermediate stage of reprogramming. The majority of these cells remain partially reprogrammed, never converting to a fully reprogrammed state; despite successfully down-regulating lineage-specific genes, they appear incapable of up-regulating genes associated with pluripotency. Promoter binding profiles are less conserved between partially reprogrammed cells and ESC, with genes lacking ES-like binding being more often targets of Nanog.

The transition from a partially reprogrammed to fully reprogrammed cell (day 16 post-infection) is distinguished by promoter de-methylation and expression of endogenous Oct4 and Nanog loci (confirmed by knock-in reporter gene expression). This observation is supported by increased efficiency of reprogramming in cells treated with *de novo* methylation

agents or specific DNA demethylase-1 inhibitors. Reactivation of the somatically silenced X chromosome in female cells also distinguishes partial and full reprogrammed cells. These cells adopt and maintain characteristics of ESC outlined above (Sumer *et al.* 2011b).

1.2.5.4. What are the differences between iPSC and ESC?

ESC are isolated from early embryos and were discovered over a decade ago in humans. They are pluripotent therefore they are a valuable resource for fundamental biology, to understand how an embryo develops into all the cells of the body. In addition the hope is that these cells can be grown into the specific type of cells required, such as cardiac cells, kidney cells, or neural cells for therapeutic benefit to people with diseases and spinal cord injuries (Pal *et al.* 2012). However, the concern for cell therapy is that as the ESC are not identical to the patient they will be rejected by the patient's immune system unless they are matched, in a manner similar to that used for organ transplant patients (Sumer *et al.* 2010b).

iPSC on the other hand can be isolated from somatic cells obtained from an adult individual. In other words they can be generated from the patient's adult cells to produce cells, which are 'matched' or autologous to the patient (Pralong *et al.* 2006). Autologous pluripotent cells are also regarded as the tool most likely to provide the breakthrough for understanding conditions such as type 1 diabetes, heart disease, multiple sclerosis and Parkinson's disease (Arrigoni *et al.* 2009a). As iPSC can be generated from adult cells taken from patients with the specific diseases mentioned above and reprogrammed to produce pluripotent cells harbouring the disease, they will provide a valuable resource for research and drug discovery.

In summary both iPSC and ESC are pluripotent, however, iPSC provide a novel approach to generating a pluripotent cell by reprogramming an adult cell. This opens a range of opportunities to study and potentially treat a number of presently incurable diseases. It also allows the generation of pluripotent cells, which are genetically matched to an individual (Smithers *et al.* 2003). While iPSC are being hailed as a scientific breakthrough, and may be a preferable alternative to working with ESC, there are several hurdles that must be overcome. Currently, the technique used to create iPSC can only convert between 1 in 5000 to 1 in 10,000 somatic cells to every iPSC, therefore the cells are difficult to generate and rare (Hu *et al.* 2010). Scientists are therefore working on ways to improve the efficiency and methods to generate iPSC before they can hope to use iPSC as a tool to combat disease (Martinez-Fernandez *et al.*).

iPSC and ESC have only been isolated from rodents (mouse and rats) and primates (human and monkeys). To date true pluripotent cells, either ESC or iPSC, have not been isolated from any large animal.

1.2.5.5. iPSC in Endangered Species

Cultured fibroblasts senesce and can eventually be depleted, thereby limiting availability. By contrast, the iPSC option (with appropriate culture conditions) could provide a self-renewing, inexhaustible resource of material from wildlife species (Figure 2) (Ben-Nun *et al.* 2011b). Frozen stocks could thus, be sampled in a sustainable way, regenerating cells as needed.

Furthermore, frozen fibroblasts from banks are used for SCNT studies, which to date have yielded poor results. iPSC may offer advantages here as well, trumping previously touted SCNT methods(Wildt & Roth 1997). Using pluripotent blastomeres as donor cells appears to increase cloning efficiency in mice because only minimal nuclear reprogramming is required. The iPSC from wild mammals most probably will resemble cells of the inner cell mass or ESC (Figure 9).

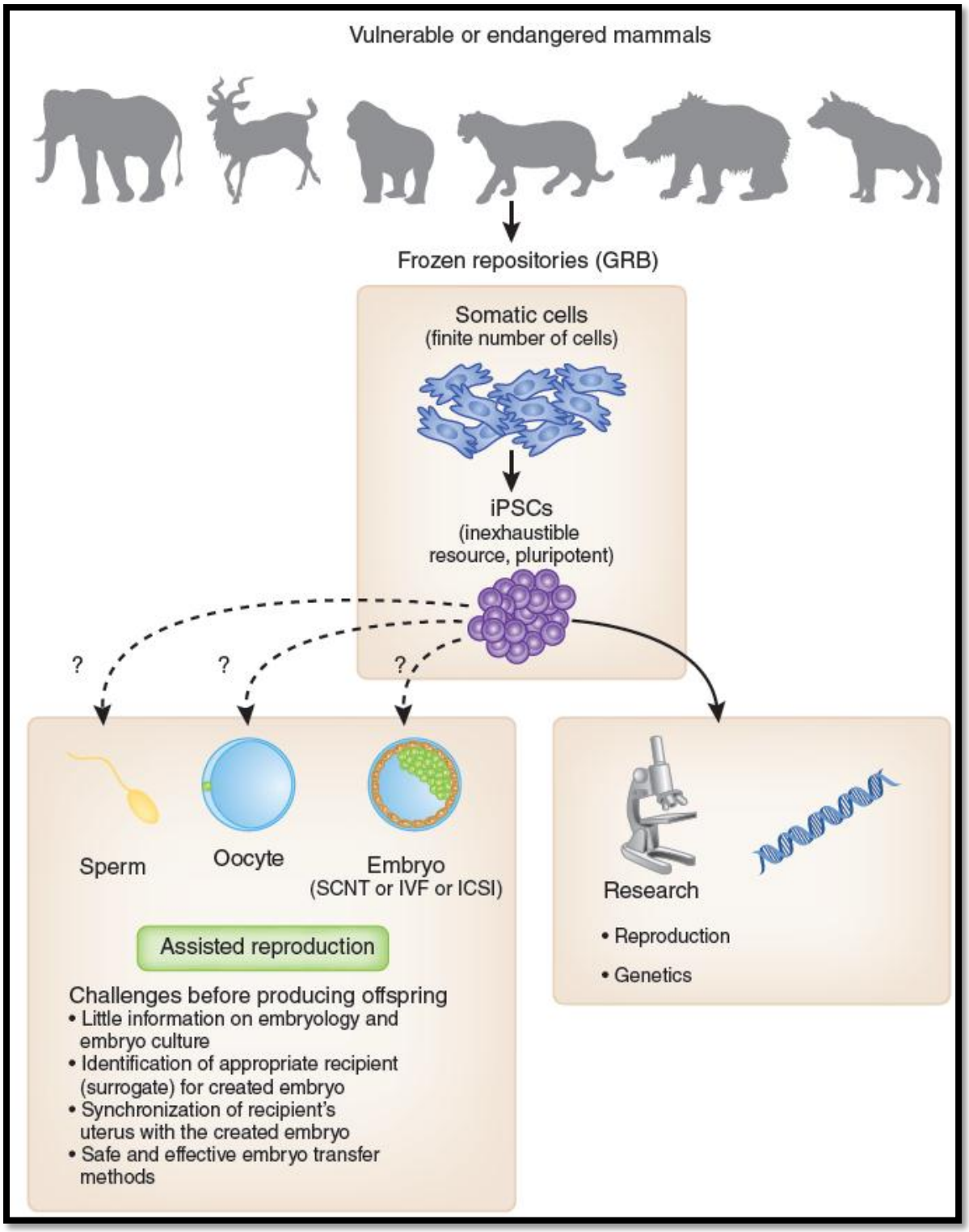

Figure 9: *Inclusion of iPSC from threatened or endangered species in genome resource banks (GRBs)* (Selvaraj *et al.* 2011).

1.2.5.6. Future potential challenges in the use of iPSC for the conservation of endangered species

If the many challenges associated with advanced embryo culture, finding the appropriate surrogate mother and synchronising the embryo to the uterus can be overcome, then iPSC may well improve application of SCNT for producing viable offspring (Holt *et al.* 2004).

Generating sperm from iPSC derived from frozen somatic cell samples from long-dead animals would provide a way to infuse much needed genetic diversity using already proven AI methods. An analogous approach using iPSC-derived oocytes could provide an endless resource for fundamental investigations into IVF, ICSI and SCNT (Holt *et al.* 2004).

There is also potential for these cells as a scalable resource of rare genetic material. Making iPSC for diverse species and populations available to every interested institution would accelerate research progress on analysing phylo-geographic structure, delineating subspecies, tracing paternities, evaluating gene flow and assessing genetic variation- information critical for decision–making in managing both *ex situ* and *in situ* wildlife populations (Pope 2000).

Therefore, wildlife–related studies will need to focus on fidelity measures for the reprogramming process to ensure the production of legitimate and 'fit' iPSC. As these are rare species and some methods for generating the cells rely on viral vectors, the process also has to be proven indisputably safe for offspring (Geijsen *et al.* 2004).

1.3. Hypothesis

Endangered species natural breeding and assisted reproductionhas been a limitation for their survival and expansion. No true stem cells has yet been reported which brings the possibility of producing individuals through assisted reproduction. I hypothesize that using iPSC technology for wild cats can create alternatives to the generation of ESC, as it doesn't require reproductive cells. Further, that additional reprogramming factors may be required for successful reprogramming in these species.

1.4. Specific Aims

The aims of this study are:

1. to sequence full-length Oct-4 gene for Snow leopard, Bengal tiger, Jaguar and Snow leopard iPSC and examine the homology between these three geographically

separated species and also to determine whether any similarity or dissimilarity exists between the human Oct4 gene sequence and wild cat Oct4.

2. to induce pluripotency in adult snow leopard fibroblasts by forced expression of 4 or 5 transcription factors (Oct-4, Sox-2, Klf-4, cMyc and Nanog).
3. to determine if the combination of factors above will work the same way in globally diverged species such as Bengal tiger (Asia), Jaguar (Americas) and Serval fibroblasts (Africa).

Chapter 2

2.1. Materials and Methods

2.1.1. 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 Institutional Animal Experimentation Ethics Committees.

2.1.2. Collection of tissues of ear from snow leopard, tiger, jaguar and serval.

Tissues samples were collected from the ear pinnae (Figure 10) of wild cats (Figure 11, Figure 12, Figure 13), which died of natural causes or were euthanized due to health-related problems identified by a zoo veterinarian. All samples were kindly donated by Mogo Zoo, NSW (Australia) (www.mogozoo.com.au).

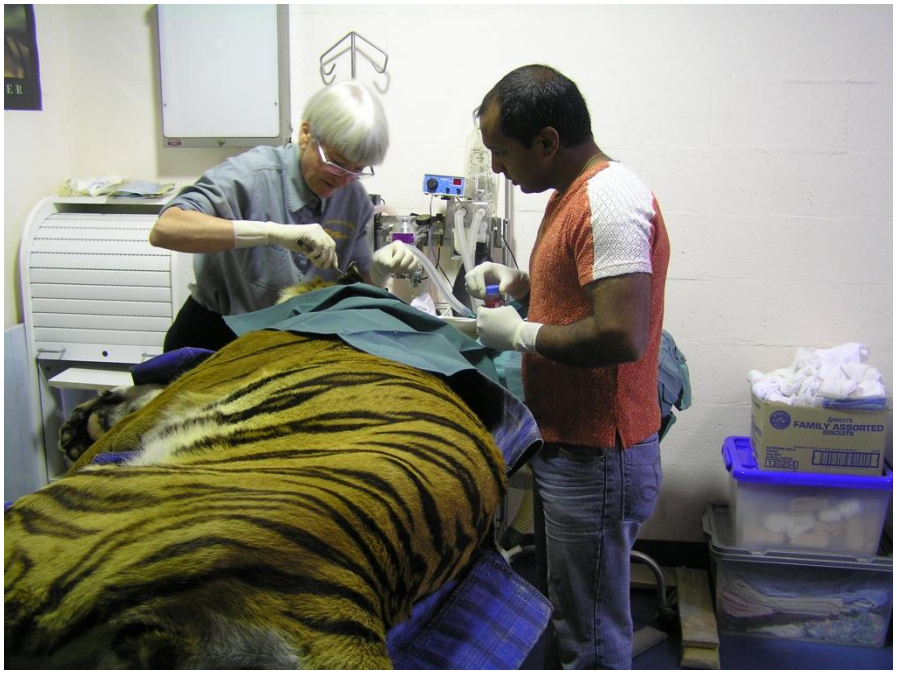

Figure 10: *Collection of ear tissue from a euthanized tiger along with the zoo veterinarian*

Figure 11: *Snow Leopard named "Mangal" used in this project*

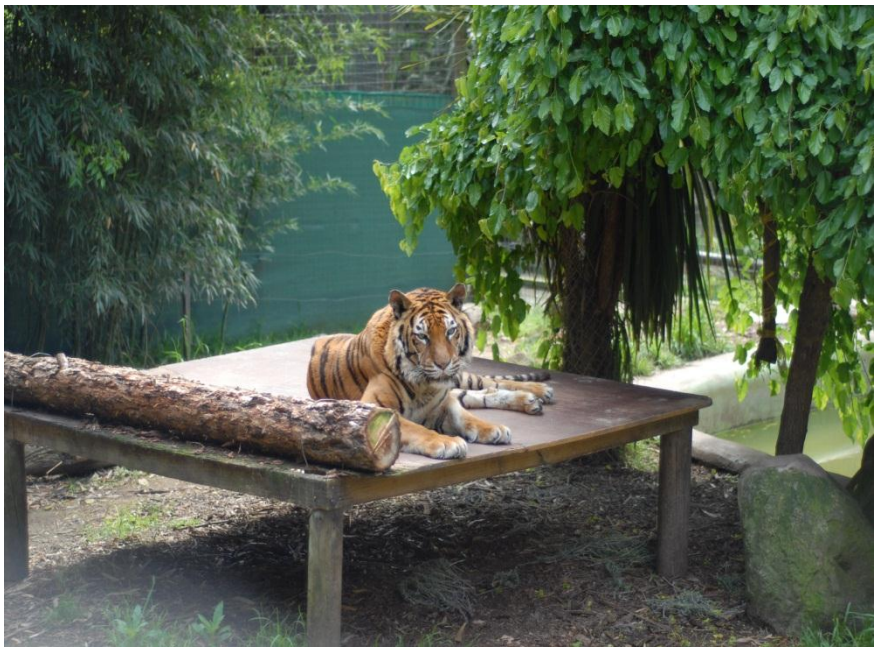

Figure 12: *Bengal Tiger named "Rocco" used in the project*

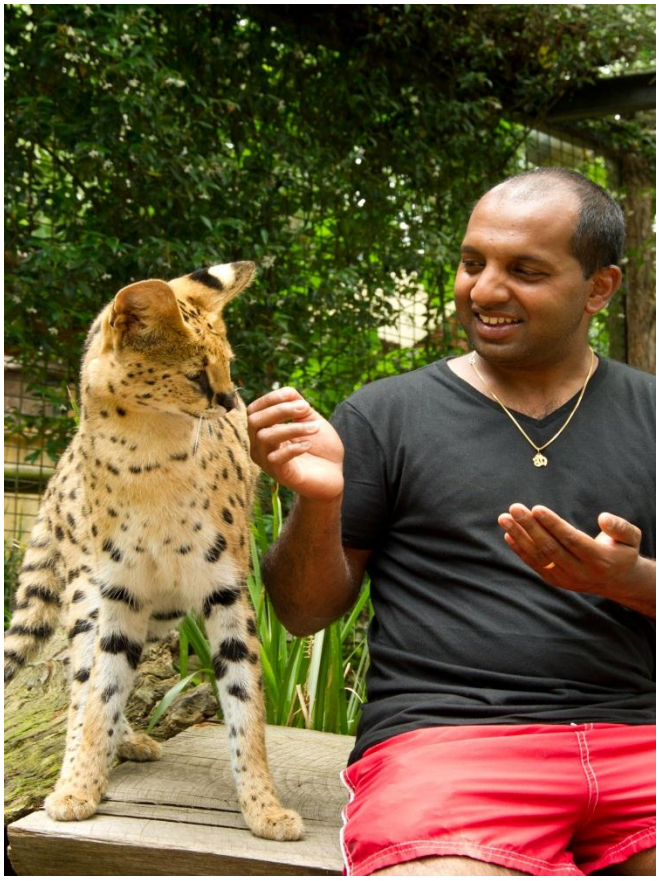

Figure 13: *Serval named "Gunda" used in this project*

Figure 14: *Jaguar named "Jelly" used in this project*

2.1.3. Tissue Culture

All reagents were sourced from Gibco-Invitrogen, Australia unless otherwise stated. All media were filter sterilised using 0.22μm filters (Chemicon-Millipore). Cells were cultured in tissue culture coated flasks or plates (BD Falcon^{TM}, BD Biosciences, Australia) at 37°C in a humidified incubator containing 5% CO_2 /95% air.

2.1.3.1. Isolation of ear fibroblasts from ear tissues of wild cats

Somatic cells were derived from the tissue samples using standard isolation and culture techniques. A small sample of ear tissue (~5mm^2) was minced using sterile surgical instruments and plated into 6 well-dishes with Fibroblast Plating (FP) medium, containing Dulbecco's modified Eagle's medium, high glucose with penicillin/streptomycin (Invitrogen) and 10%(v/v) fetal bovine serum (JRH), The tissue was cultured in a 38.5°C incubator in an atmosphere of 6%CO_2 in air for 7 days. Fibroblast outgrowths from the tissue explants were then transferred to two T175cm^2 flasks for expansion.

2.1.3.2. Derivation and maintenance of mouse embryonic fibroblasts (mEFs)

Pre-pubertal (4-6 weeks old) female mice Quakenbush (QS) were superovulated with intra-peritoneal injections of 5 IU of pregnant mare serum gonatrophin (PMSG, Folligon) and 5 IU of human chorionic gonadotrophin (hCG, Chorulon) 24 hours apart followed by mating with male mice (QS). Female mice were checked for seminal plugs the day after mating and separated. At 13.5 dpc (days post coitum), pregnant females were humanely killed by carbon dioxide gas (> 70% in air). Fetuses were collected and washed in Dulbecco's phosphate buffered saline with calcium and magnesium (PBS+) supplemented with 1x penicillin – streptomycin and 1x amphotericin B. mEFs were derived from foetuses according to Conner (2000). Briefly, fetuses were decapitated, internal organs removed and the remaining carcass minced using scalpels. TrypLE (stable trypsin-like enzyme) was added and the embryonic tissue was further dissociated by homogenising several times through a 3ml syringe (Terumo) fitted with a 15mm gauge needle. The homogenised cell mixture was collected into a 15ml tube and shaken for 1min at 80-90 rpm on a vortex mixer (Vtron, VM 2000, Bartelt Instruments). mEF medium consisting of high glucose Dulbecco's minimum essential media (DMEM), 10% Fetal bovine serum (FBS from JRH Biosciences, Australia), 1 x non-essential amino acids, 1 x GlutaMAX and 1 x penicillin-streptomycin was added to inactivate TrypLE. The cell mixture was then centrifuged at 400g for 5 mins (Eppendorf centrifuge 5702). The

supernatant was aspirated and the cell pellet re-suspended in 1 ml of mEF media supplemented with 1 x amphotericin B. the cells were counted using a haemocytometer (Brand, Germany) and plated at a density of 6x 10^4 cells/cm^2. Primary cells were cultured in mEF media supplemented with 1x amphotericin B, however subsequent mEF cultures did not contain amphotericin B.

mEFs were split 1:3 or 1:4 once confluent. Passaging was done by first washing mEFs with PBS+ and then incubating at 37°C with TrypLE until cells visibly lifted off the flask/plate. Visual confirmation was done by observing cells under the microscope (Nikon Eclipse TE 2000U). TrypLE was inactivated by the addition of mEF media at 2 times the volume of trypLE and the entire cell suspension transferred to a 15ml tube. This was centrifuged for 5 mins at 400g, the supernatant aspirated and the cell pellet re-suspended in the appropriate amount of mEF media and seeded on plates/flasks before returning to the incubator.

2.1.3.3. Generation of inactivated mEFs as feeder cells

Passage 3 or 4 QS mEFs were inactivated by treating cells with mitomycin-C (MMC) at 10µg/ml for 2.5-3 hours. After MMC treatment, mEFs were washed twice with PBS+ and trypsinised as per the mEF passaging protocol. Inactivated mEFs were counted using a haemocytometer and either cryopreserved for future use or used immediately by plating onto 0.1% gelatinised plated/flasks at a density of 1.25x10^4 cells/cm^2.

2.1.4. Induced Pluripotency

2.1.4.1. Maintenance of packaging cell lines

Platinum A packaging (Plat A) cells derived from 293T cells (Cell Biolabs, USA) were used for generating retroviruses for wild cats cell lines. Plat A cells were cryopreserved, thawed, , cultured and passaged as per mEFs. However, they were cultured with 10µg/ml Blasticidin S Hydrochloride (Funakoshi Corporation, Japan) and 1µg/ml Puromycin (Sigma-Aldrich).

2.1.4.2. Transfection and Transduction

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). Amphotropic viral particle production by Plat-A packaging cells (Jomar Biosciences, Kensington, SA, Australia) was performed as described previously. Nine µg of each vector described above were co-transfected to Plat-A cells using Fugene 6 (Roche, Dee

Why, NSW, Australia) according to manufacturer's instruction. Virus-containing supernatants were collected 48 and 72 hours post-transfection and filtered through a 0.45 µm pore-size filter and supplemented with 8 µg/ml of polybrene (Sigma-Aldrich, St. Louis, MO, USA). Felids fibroblasts were plated 24 hours prior to infection at a density of 4×10^4 cells/cm^2. Equal parts of the four or five transcription factors-containing retroviral supernatant were added to the plated felids fibroblasts. Two rounds of infection were performed 24 hours apart. The culture medium was changed to mouse ESC medium at day 5 post-infection. The iPS colonies were picked based on ESC-like morphology at day 3. Felids fibroblast cells were infected with GFP-containing retroviral supernatant in parallel, to monitor the transduction efficiency (Figure 14).

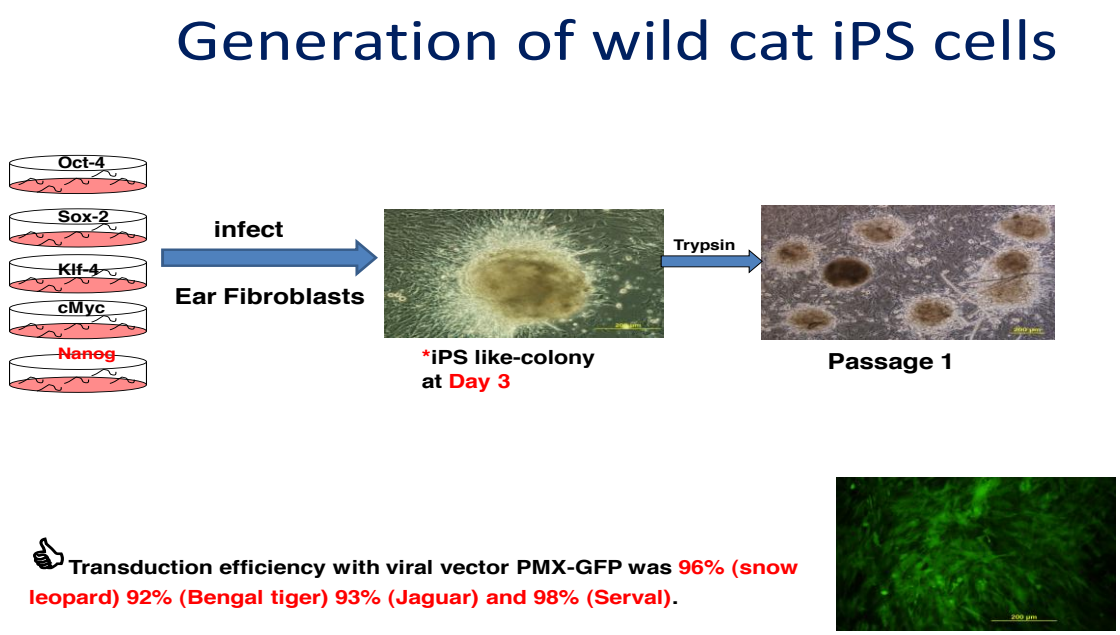

Figure 15: *Generation of iPSC from wild cats' ear fibroblasts using viral method*

2.1.4.3. Maintenance of wild cats induced pluripotent stem cells (iPSC)

Three days after transduction, pMXs-GFP infected cells were analysed by flow cytometry to determine transduction efficiencies. Five days after transduction, five factor infected cells were passaged onto mytomycin C treated mEFs previously plated in a 60mm /100mm tissue culture dish coated with 0.1%gelatin. The wild cats iPSC were cultured in medium consisting of DMEM supplemented with 0.1mmol/litre 2-beta-mercaptoethanol (βMe), 1M Non-essential amino acid (NEAA), 2mM Glutamax, 20% (v/v) Hyclone serum, Pen/strep and 10^3

units/ml murine LIF (ESGRO; Invitrogen) in a 6% CO_2 incubator at 38.5°C. Medium was changed daily and iPSC were passaged manually every 3-4 days onto fresh feeder layers.

2.1.5. Cryopreservation and thawing of cell lines

All cells were cryopreserved as follows: the volume of the prepared cell suspension was adjusted using the appropriate medium and an equal volume of freezing medium which consisted of 20% dimethyl sulphoxide (DMSO) in FBS (filter sterilised) was slowly added to the cells. Cell stocks were prepared in 1.5ml cryovials (Nunc, Denmark) and slowly frozen in a cryo freezing container "Mr Frosty: (Nalgene) containing isopropanol (BDH) in a -80°C freezer. For long-term storage, vials were transferred to liquid nitrogen tanks.

All cells were thawed by heating cryovials in a 37°C water bath until the ice had melt. The cell suspension was then transferred to a 15ml tube and mEF media added drop-wise (10ml of media per thawed vial of cells). This cell suspension was then centrifuged at 400g for 5 mins and the cell pellet re-suspended in the appropriate media and cultured accordingly. 24 hours after thawing, a medium change was performed for cultured cells to remove residual DMSO and dead cells.

2.1.6. Flow Cytometry

Flow cytometry was performed at the Monash Health and Research Precinct Flow cytometry facility using either the MoFlo® BTA analyser (for analysis), DakoCytomation systems, Fort Collins Colorado, USA. Data was analysed using the Summit software version 4.3. Viable cells were identified and gated using a forward scatter/side scatter (FSC/SSC) dot plot and then analysed with the appropriate channels.

2.1.7. Microscopy

Felid iPSC were examined and images were captured by phase-contrast and bright-field microscopy using a Nikon Elite 2000 microscope or an Olympus IX71 microscope. Image of immuno-stained iPSC were taken by confocal microscopy at the micro-imaging facility of Monash Institute of Medical Research.

2.1.8. Immunostaining

Wild cats iPSC were cultured in a 0.1% gelatinised 4 well glass chamber slides (BD Falcon) for 3-4 days prior to fixation. For fixation, cells were washed 2 times with PBS+ and then incubated at room temperature with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10mins.

PFA was aspirated and the cells washed 2 times with PBS+. Fixed cells were stored at 4°C in PBS+.

2.1.9. Alkaline Phosphatase

Fixed cells were stained using the Alkaline Phosphatase detection kit (Chemicon-Millipore) as per manufacturer's instructions. Briefly, fixed cells were covered with staining solution (Napthol/Fast red violet/water) in the dark at room temperature for a maximum of 15 mins. The stain was then washed twice and covered with PBS+. Red-pink staining indicative of positive staining was examined under bright field using an inverted microscope (1x71 Olympus, Melville, NY). Photographs were taken using a DP70 camera (Olympus, Melville, NY) attached to the microscope.

2.1.10. Antibody staining

For antibody staining, 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 Oct-4 and Nanog were blocked with PBS pH7.5 supplemented with 1% (w/v) BSA, 5%(v/v) goat serum, and 0.1%(v/v) Tween and those tested for SSEA-1 were blocked with 1% (w/v) BSA, 5%(v/v) goat serum in PBS pH7.5 for 60 min at RT. Mouse anti-human Oct-4 IgG (SC-5279, Santa Cruz, Heidelberg, Germany), mouse anti-mouse SSEA-1 IgM (SC- 4305, Chemicon-Millipore) and rabbit polyclonal to Nanog IgG (Abcam, ab-80892) primary antibodies were used at 1:100 dilution in PBS containing 5%(v/v) goat serum and then incubated overnight at 4°C. The next day, the cells were washed three times with PBS and a secondary antibody was added in PBS containing 5%(v/v) goat serum for one hour at RT (Oct-4 and Nanog: anti-IgG Alexa 488 1:500, SSEA-1: anti-IgM Alexa 594 1:500). Sections were subsequently washed three times with PBS, mounted in Vectashield and then examined using an Olympus 1X71 Fluorescent microscope initially under phase contrast optics and then under ultra violet light UV2A- 360-370 (excitation filters) 420-460 (emission filters). Fluorescence in the green range was also assessed using U-MIGA2- 540-550 (excitation filters) 575-625 (emission filters) and photographic records of the cells were obtained.

2.1.11. Molecular Biology

2.1.11.1. RT-PCR

Samples used for RNA extraction were snap frozen using dry ice and stored at -80°C. RNA extraction was performed using RNeasy kit (Qiagen) according to manufacturer's

instructions. Briefly, cells were homogenised in lysis buffer and 70% ethanol added to promote selective binding of RNA to the RNAeasy membrane. Contaminants were eliminated by successive washed through the RNeasy membrane column. RNA was eluted by the addition of RNase free water and stored at -20°C. The amount and purity of extracted RNA was measured by absorbance at 260/280nM on a Nanodrop ND 1000 Spectrophotometer (Nanodrop^{TM}, ThermoScientific).

2µg of RNA was used for subsequent cDNA generation. Total RNA was first treated with RQ1 DNase at 37°C for 30mins to remove contaminating genomic DNA. DNase treatment was stopped by the addition of stop buffer and incubated at 65°C for 10mins. To further exclude the possibility of genomic DNA contamination, control reactions without reverse transcriptase enzyme (RT-) were conducted in parallel. Next, random primers, RNase out and MQ water were added to the reverse transcriptase (RT) reaction and incubated at 65°C for 5mins. The RT reaction was cooled on ice for 5mins and then, Superscript buffer, 0,1M dithioreitol (DTT), 10mM dNTP mix and Superscript III reverse transcriptase added. This RT reaction was incubated at 50°C for 1 hr and the enzyme subsequently inactivated by incubating the mixture at 70°C for 15mins. cDNA were stored at -20°C and 1-2µl were used for subsequent PCR reactions.

I 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 (Tat *et al.* 2010). Reactions were carried out for endogenous genes under the following conditions: 95°C for 10min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, with final extension at 72°C for 10 min (Oct-4 and GAPDH) or 95°C for 10 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with final extension at 72C° for 10 min (for NANOG) (Table 1).

For exogenous gene expression, the house keeping gene and human transgenes (Table 2) reaction was carried out under the following conditions: 95°C for 5min, 35 cycles of 94°C for 30 sec, 58°C (β-actin), 47°C (Oct-4), 47°C (Nanog), 47°C (Sox-2), 48°C (Klf-4) and 47°C (cMyc) for 30 sec and 72°C for 1 min, with final extension at 72°C for 5min (Sumer *et al.* 2011b).

The PCR products were run on a 2% agarose gel at 80-100V for 2 hours. Gels were visualised using the Bio-Rad Universal hood II GelDoc system (BioRad Laboratories Inc.) and images taken using the Quantity One version 4.6.3 software.

Table 1: *Domestic cat primer sequences used to detect the expression of endogenous genes in felid iPSC at P4 and P14* (Yu *et al.* 2009).

Oligo's Name	Primer Sequences
GAPDH(F)	5'GCAAAGTGGACATTGTCGCC3'
GAPDH (R)	5'CCTTCTCCATGGTGGTGAAG3'
OCT-4 (F)	5'GGAGTCCCAGGACATCAAAG3'
OCT-4 (R)	5'GCCTGCACAAGTGTCTCTGC3'
NANOG(F)	5'AAGCCACAGTGTGATACAGC3'
NANOG (R)	5'AGCCAAAGCTACGGAATCCTC3'

Table 2: *Primer sequences of human transgenes14 used to detect the expression of transgene in felid iPSC at P4 and P14* (Liu *et al.* 2008).

Oligo's Name	Primer Sequences	Annealing Temperature (°C)
OCT-4 (F)	CTAGTTAATTAAGAATCCCAGTG	47 °C
OCT-4 (R)	CACTAGCCCCACTCCAACCT	47 °C
SOX-2 (F)	CTAGTTAATTAAGGATCCCAGG	47 °C
SOX-2 (R)	TGTTGTGCATCTTGGGGTTCT	47 °C
cMYC (F)	CTAGTTAATTAAGGATCCCAGTG	47 °C
cMYC (R)	CAGCAGCTCGAATTTCTTCC	47 °C
KLF-4 (F)	ACAAAGAGTTCCCATCTCAAGGTG	48 °C
KLF-4 (R)	TCCAAGCTAGCTTGCCAAACCTACAGG	48 °C

NANOG (F)	TCAATGATAGATTTCAGAGACAG	47 °C
NANOG (R)	GGGTAGGTAGGTGCTGAGGC	47 °C

2.1.12. Cytogenetic analysis

2.1.12.1. Karyotyping

Cells of interest were grown to 70-80% confluency in a single well of a 6-well plate and then sent to the Department of Cytogenetics, Monash Medical Centre for karyotype analysis.

2.1.12.2. Chromosome counts

Cells of interest were grown to 70-80% confluency in a 100mm dish. The day prior to chromosome counting, 30µl of 10mM BrdU (5-Bromo-2-Deocyuridine) was added for every 1ml of culture medium and incubated at 37°C. After 14 hours, the BrDU containing medium was aspirated and fresh culture medium was added. To this, 10µl of KaryoMax® Colcemid solution (10µg.ml) was added for every 1ml of culture medium and cells incubated at 37°C for 4 hours. After this time, cells were passaged as per the normal protocol and re-suspended in 50µl of medium. To this cell suspension, 10ml of 75mM Potassium Chloride solution (Sigma-Aldrich) was added slowly and then incubated at 37°C for 15mins. Next, 2ml of freshly made fixative (3 parts methanol, Sigma-Aldrich to 1 part Galcial acetic acid, BDH) was added and mixed by gentle inversion. The cell suspension was centrifuged at 400d for 10mins and supernatant poured out. A plastic Pasteur pipette was used to re-suspend the cells in any remaining liquid and then 8ml of fixative was added, followed by gentle inversion. The cell suspension was centrifuged at 400g for 10mins and again the supernatant poured out. The re-suspension of cells, addition of 8ml fixative and centrifugation was repeated 3 times. Upon the final centrifugation step, the supernatant was poured out and the cell pellet re-suspended in approximately 1ml of fixative (this was dependent upon cell pellet size). 2-3 drops of the cell suspension were dispended onto the surface of a clean microscope glass slide (SuperFrost®, Thermo Scientific) held at a slight angle. These glass slides were allowed to dry at room temperature for at least 30mins (maximum 2hours). Slides were then stained for 5-10mins with Leishman's staining solution, which consists of 15% Leishman's Stain stock solution (stock solution is made by dissolving 2g of Leishman's stain, Sigma-Aldrich in 1L of methanol) in Gurrs Gemsa buffer, pH6.8. Leishman's stain was washed off slides under

running tap water and then dried using either a hair dryer or left to air-dry overnight. A few drops of Histomount (National Diagnostics) were dispensed onto the dried slides and a glass coverslip mounted. Chromosome spreads were visualised using the 100x oil immersion lens of a DNA upright fluorescence microscope (Leica Microsystems).

2.1.13. Embryoid body formation

10^6 cells were transferred to a 10cm Petri dish (non-tissue culture coated) containing 10ml of mEF media and returned to the incubator. After 4 days in culture, cells were split 1:4 by the addition of mEF media and transferred by pipetting to new Petri dishes. 7-10 days after initial plating, embryoid bodies (EBs) were either individually picked using a 200µl pipette or transferred to a single well of a 24 well plate previously coated with 0.1% gelatin or snap frozen at -80°C in a 1.5ml eppendorf tube for RNA extraction.

2.1.14. Teratoma formation

Between 2.5x10^6cells were collected using the standard passaging protocol and the cell pellet re-suspended in 100µl of the appropriate media. Cells were then injected into the hind leg of severe combined immunodeficient (SCID) mouse using a gauge needle. Between 3-6 weeks, mice were monitored for teratoma formation. Once teratomas visibly reached 10mm in diameter they were excised and fixed in Histochoice (Amresco). Histological processes were performed by the Monash Institute of Medical Research Histology Facility. Slides were stained with either haematoxylin or eosin (H&E) or Masson's trichrome.

2.1.15. Cell culture of Wild cats Fibroblasts and nucleic acid purification

Wild cats fibroblasts were plated in 6cm organ culture dishes. The FP medium containing DMEM (high glucose, Invitrogen) supplemented with 20% (v/v) Hyclone serum, 0.5% penicillin/streptomycin. The medium was changed every day and were cultured in 6% CO_2 at 38.5°C. Nucleic acid purification was done by using DNeasy Blood & Tissue Kit (QIAGEN Inc.) according to the manufacture instruction.

2.1.16. PCR, cloning and sequencing of Oct4 gene

To isolate the Oct4 coding sequence, the following oligonucleotide primers were designed by using primer-BLAST (Ye *et al.* 2012) based on the published domestic cat (*Felis catus*) genome sequence. Two fragments about 1760 and 1500 bp in length were successfully amplified for Exon1 and Exon2-5, respectively. Amplification reactions for both fragments

were done by using the following constituents: in a final volume of 50 µl 1× high fidelity PCR buffer containing 1 unit of Platinum *Taq* DNA polymerase (Invitrogen, USA), 0.2 mM each of dNTPs (Bioline, USA), 2 mM $MgSO_4$, 0.3 µM each of primers and 100 ng of purified DNA isolated from tiger, leopard and jaguar cultured cells. Amplification was performed in a thermal cycler My-Cycler (BioRad, USA) with the following program; after an initial denaturation step at 95 °C for 2 min, 35 cycles were programmed as follows: 94 °C for 30s, 55 °C (exon1) 58.5 °C (exon2-5) for 30s, 72 °C for 90s and final extension at 72 °C for 15 min. Three microliters of the PCR products were mixed with a ligation reaction including 50 ng pGEMt-Easy vector (Promega), 3 units T4 DNA ligase (Promega) in 1× T4 DNA ligase buffer, incubated at room temperature for 16 hours, then transformed into DH5α *E. coli* competent cells (Invitrogen, USA) and plated on LB-ampicillin-XGal plates. White colonies were selected for plasmid DNA extraction using a Miniprep kit from Qiagen Inc. (USA) and sequenced with T7 (5'- taa tac gac tca cta tag gg-3-) and SP6 (5'- tat tta ggt gac act ata g-3') universal primers using Applied Biosystems 3130xl Genetic Analyzer.

2.1.17. Bioinformatics analysis

To determine intron/exon boundaries, the tiger, leopard and jaguar Oct4 gene sequences were aligned to the cat genomic sequence using CLC Main workbench software (Version 5.5). Multiple sequence alignments were conducted in CLC Main Workbench program, and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei 1987). The accession numbers of Oct4 sequences used for alignment and for the phylogenetic tree are listed in Table 3. The molecular weight and isoelectric point (pI) were calculated by Compute pI/Mw (http://us.expasy.org/tools/pi_tool.html). The domain structure of the putative protein was analysed on the SMART (http://smart.embl.de/) server.

Table 3: *Pairwise comparison and list of the various Oct4 orthologs coding sequences used in the construction of the phylogenetic tree to analyze the evolution of Bengal tiger, snow leopard and jaguar Oct4.*

		1	2	3	4	5	6	7	8	9	10
Felis=NM_001173441	1		99.82	99.91	99.91	93.44	92.61	91.97	91.87	52.54	80.98
Jaguar Oct4	2	0.00		99.72	99.72	93.26	92.43	91.78	91.69	52.63	80.89
Tiger Oct4	3	0.00	0.00		99.82	93.35	92.71	92.06	91.97	52.54	80.98
Leopard Oct4	4	0.00	0.00	0.00		93.35	92.52	91.87	91.78	52.45	80.89
Canis=XM_538830	5	0.07	0.07	0.07	0.07		90.86	89.01	88.92	52.17	80.06
Sus=NM_001113060	6	0.08	0.08	0.08	0.08	0.10		90.21	90.12	52.45	81.44
Homo(Variant1)=NM_002701	7	0.08	0.09	0.08	0.09	0.12	0.10		98.15	52.35	81.90
Macaca=NM_001114955	8	0.09	0.09	0.08	0.09	0.12	0.11	0.02		52.63	82.73
Mus=NM_001252452	9	0.75	0.75	0.75	0.75	0.76	0.75	0.76	0.75		59.40
Rattus=NM_001009178	10	0.22	0.22	0.22	0.22	0.23	0.21	0.21	0.20	0.58	

Upper comparison
Percent identity
Upper comparison gradient
min max
Lower comparison
Distance
Lower comparison gradient
min max

Chapter 3

Cloning and sequencing Oct4 gene from endangered wild cats to determine whether human or mouse transcription factors can be used for the generation of iPSC. (Paper1 submitted)

Octamer binding transcription factor 4 (Oct4), also known as POU5F1, is a protein critically involved in stem cell renewal and pluripotency and a key-reprogramming factor involved in reprograming somatic cells back to a pluripotent state. To identify the suitability of either mouse or human Oct4 to derive induced Pluripotent Stem Cells (iPSC) from endangered cats, such as Bengal tiger, snow leopard and jaguar, the coding sequence of domestic cat Oct4 was compared with that from other known species. Cat Oct4 protein has 94.44% and 83.33% sequences similarities compared to human and mouse Oct4, respectively. The coding sequence for Oct4 was successfully amplified and cloned from fibroblasts of the three species and compared in-silico. In conclusion, this study is the first to describe the molecular cloning and bioinformatics analysis of Oct4 gene. Information on the specific sequences of Oct4 in these endangered big cats was useful to understand and manipulate pluripotency in these species. This has an exciting potential to contribute to real-life species preservation, particularly for endangered felids.

Shortened Title

CLONING AND BIOINFORMATIC ANALYSIS OF WILD CATS OCT4 GENE

CLONING, CHARACTERIZATION AND BIOINFORMATICS ANALYSIS OF OCT4, THE MOST IMPORTANT PLURIPOTENCY GENE, IN ENDANGERED WILD CATS; BENGAL TIGER, SNOW LEOPARD AND JAGUAR

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ABSTRACT

Octamer binding transcription factor 4 (Oct4), also known as POU5F1, is a protein critically involved in stem cell renewal and pluripotency and a key-reprogramming factor involved in reprograming somatic cells back to a pluripotent state. To identify the suitability of either mouse

or human Oct4 to derive induced Pluripotent Stem Cells (iPSC) from endangered cats, the coding sequence of domestic cat Oct4 was compared with that from other known species. Cat Oct4 protein has 94.44% and 83.33% sequences similarities compared to human and mouse Oct4, respectively. The coding sequence for Oct4 was successfully amplified and cloned from fibroblasts of the three species and compared in-silico. Bioinformatic analysis revealed that although the sequences of coding regions vary slightly between leopard, tiger and jaguar, the predicted theoretical protein consist of 360 amino acids and is identical between these species. By contrast to the exons, introns and 5' region close to the exon1 are less conserved in three species. A phylogenetic tree was built using the neighbor-joining method based on the alignment of the coding sequences of Oct4 gene. As Oct4 is usually expressed in oocytes/early embryos, which are difficult to obtain from endangered species, the verification of the mRNA sequence of the gene posed obvious challenges. In conclusion, this study is the first to describe the molecular cloning and bioinformatics analysis of tiger, leopard and jaguar Oct4 gene. Information on the specific sequences of Oct4 in these endangered big cats will be useful to understand and manipulate pluripotency in these species. This has an exciting potential to contribute to real-life species preservation, particularly for endangered felids.

Keywords: Tiger, Jaguar, Leopard, Cloning, Oct4.

INTRODUCTION

It is generally agreed that induced pluripotent stem cells (iPSCs) is a useful new agent in developing strategies for preventing the extinction of some critically endangered species (1, 2, 3). During last few years a set of reprograming factors for inducing pluripotency, including Oct4,

Sox2, Klf4, c-Myc and Nanog, have been extensively studied due to the rising importance of iPSCs particularly in regenerative medicine (4) and the livestock industry (5). Among these factors, Oct4 is essential in the reprogramming process for human (4), mouse (6) and ruminants (7) fibroblasts into iPSCs.

The first cloning of Oct4 and its expression in adult human tissues was reported in 1992 (8). More recently orthologs of Oct4 gene have been cloned and characterized in the cattle, buffalo, goat, cat and rabbit (9, 10, 11, 12, 13). These studies showed high sequence identities of Oct4 between orthologous genes at the nucleotide and amino acid level as well as similarities in its genomic organization, gene localization and regulatory regions. Oct4 contains POU and Homeobox protein domains which both have a high degree of amino acid sequence conservation between species. A number of Oct4 pseudogenes are present in the human genome and alternative splicing, as well as the use of alternative translation initiation codons, results in multiple isoforms of this gene in human.

We recently reported the generation of wild cats iPSCs by forced expression of human pluripotency factors in Bengal tiger, snow leopard, serval and jaguar fibroblast cells (3, 14). However, due to the lack of genome information on these species the exact sequence of Oct4 gene is not available yet. Here we report the first sequencing and bioinformatics analysis of the Oct4 gene in tiger (India), leopard (central Asia) and jaguar (south America) to know if there is any difference in Oct4 sequence between the three geographically separated species. And also to see any similarity or dissimilarity between the human Oct4 gene which has been used in previous studies to make wild cat iPSCs (3, 14). Further, to study the evolutionary relationship between the wild cat Oct4 gene and those of other selected species, a phylogenetic tree was constructed based on the alignment of coding sequences.

MATERIAL AND METHODS

Cell culture and nucleic acid purification

Wild cats fibroblasts were plated in 6cm organ culture dishes. The FP medium containing DMEM (high glucose, Invitrogen) supplemented with 20% (v/v) Hyclone serum, 0.5% penicillin/streptomycin,. The medium was changed every day and were cultured in 6% CO_2 at 38.5°C. Nucleic acid purification was done by using DNeasy Blood & Tissue Kit (QIAGEN Inc.) according to the manufacture instruction.

PCR, cloning and sequencing of Oct4 gene

To isolate the Oct4 coding sequence, the following oligonucleotide primers were designed by using primer-BLAST (15) based on the published domestic cat (*Felis catus*) genome sequence (Table 1). Two fragments about 1760 and 1500 bp in length were successfully amplified for Exon1 and Exon2-5, respectively (Figure 1). Amplification reactions for both fragments were done by using the following constituents: in a final volume of 50 µl 1× high fidelity PCR buffer containing 1 unit of Platinum *Taq* DNA polymerase (Invitrogen, USA), 0.2 mM each of dNTPs (Bioline, USA), 2 mM $MgSO_4$, 0.3 µM each of primers and 100 ng of purified DNA isolated from tiger, leopard and jaguar cultured cells. Amplification was performed in a thermal cycler My-Cycler (BioRad, USA) with the following program; after an initial denaturation step at 95 °C for 2 min, 35 cycles were programmed as follows: 94 °C for 30s, 55 °C (exon1) 58.5 °C (exon2-5) for 30s, 72 °C for 90s and final extension at 72 °C for 15 min. Three microliters of the PCR products were mixed with a ligation reaction including 50 ng pGEMt-Easy vector (Promega), 3 units T4 DNA ligase (Promega) in 1× T4 DNA ligase buffer, incubated at room temperature for 16 hours, then transformed into DH5α *E. coli* competent cells (Invitrogen, USA) and plated on

LB-ampicillin-XGal plates. White colonies were selected for plasmid DNA extraction using a Miniprep kit from Qiagen Inc. (USA) and sequenced with T7 (5'- taa tac gac tca cta tag gg-3-) and SP6 (5'- tat tta ggt gac act ata g-3') universal primers using Applied Biosystems 3130xl Genetic Analyzer.

Bioinformatics analysis

To determine intron/exon boundaries, the tiger, leopard and jaguar Oct4 gene sequences were aligned to the cat genomic sequence using CLC Main workbench software (Version 5.5). Multiple sequence alignments were conducted in CLC Main Workbench program, and a phylogenetic tree was constructed using the neighbor-joining method (16). The accession numbers of Oct4 sequences used for alignment and for the phylogenetic tree are listed in Table 2. The molecular weight and isoelectric point (pI) were calculated by Compute pI/Mw (http://us.expasy.org/tools/pi_tool.html). The domain structure of the putative protein was analyzed on the SMART (http://smart.embl.de/) server.

RESULTS AND DISCUSSION

Cloning and sequence analysis of Oct4 gene

Two fragments of 1760 and 1500 bp were obtained and cloned from the extracted genomic DNA of the tiger, leopard and jaguar. The amplified fragments comprised a 5' region before exon1, exons 1 to 5 as well as some part of intronic regions (Figure 1). A pairwise comparison of aligned open reading frame sequences (Table 3) showed that introns were less conserved in all three species and identified a total number of 22 single nucleotide polymorphisms in intronic regions of Oct4 gene between the three species. Comparing the 5' regions before exon1 in tiger, leopard and jaguar, a 13 bp deletion ranging from 204-192 before start codon was identified in

the jaguar. This region may be part of either 5' untranslated region or 5' proximal promoter region, however, further studies are required to demonstrate if this deletion could affect the expression of Oct4 in jaguar (shown in figure 2).

Oct4 is the most important regulatory gene in cell reprograming and its endogenous expression isdeterminant for pluripotency (17, 18). Induced pluripotent stem cells (iPSCs) have the potential to provide the basis for a new strategy to conserve highly endangered species by the reintroduction of genetic material into breeding populations, especially those *ex situ* populations in which inbreeding has resulted in extremely low levels of genetic diversity (1, 3).

Analysis of the translated amino acid sequence of felid Oct4

Based on the predicted open reading frame, the deduced Oct4 protein is a 360 amino acid single polypeptide with a predicted molecular mass of 38.59 kDa and an estimated isoelectric point of 5.85. Although this study identified small differences in the genomic sequences of the tiger, leopard and jaguar Oct4 gene, the predicted translated proteins are identical with cat Oct4 protein. The 360 amino acid protein is common with the Oct4 orthologs in human, dog, cattle, pig, and monkey (GenBank accession numbers are listed in Table 2), however, (11) reported a putative 272 amino acid protein based on a sequenced open reading frame of 819 bp of cat Oct4 which does not correlate with the current information on cat Oct4 available in NCBI Whereas with our findings 360 amino acid length was standard in all the three wild cats used in this study.

One POU domain at the position 138 to 212 (E-value: 3.599e-48) and one HOX domain at the position 230 to 292 (E-value: 1.343e-18) were predicted by the SMART site (http://smart.embl.de/). POU proteins are eukaryotic transcription factors containing a bipartite DNA binding domain (19), and the homeobox domain is a DNA-binding factor that is involved in the transcriptional regulation of key developmental processes (20, 21).

Phylogenetic analysis of Oct4 gene

The results of alignment analysis showed that the coding sequence of the Oct4 gene from the tiger, leopard and jaguar have 52.45%-99.82% identity to the Oct4 gene of other selected species (Table 2). As shown in Figure 3, felid Oct4 genes were located at the base of the tree, considered ancestors at the molecular level, and close to the *Canis* which belongs to Carnivores. The Oct4 genes of rodents were clustered together and clearly separated from Primates (Human and Monkey), with high bootstrap probability which agrees with the known fact that they are belong to Euarchontoglires (22). These results are in agreement with the mammalian phylogenetic tree reported by (22).

CONCLUSION

In conclusion, we report the first sequence of Oct4 gene in tiger, leopard and jaguar by using various bioinformatics tools to analyze the gene and predicted protein sequences and their phylogenetic relationships. Based on our results although there were some differences between genomic DNA sequences of Oct4 gene of wild cats, but the amino acid sequence of the putative protein was identical.

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Figure 1. Location of designed primers used to amplify coding sequence of Oct4 gene

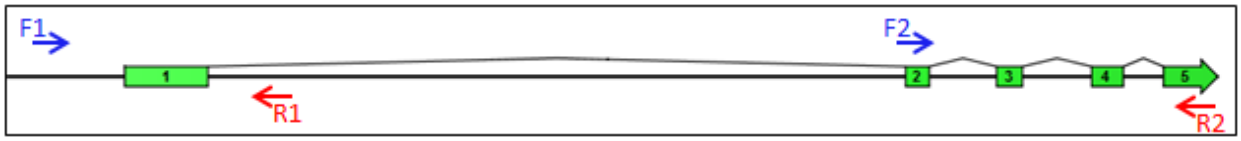


Table 1. Primer pairs used to amplify the Oct4 gene in Tiger, Leopard and Jaguar

Oct4 genomic region	Primer	Oligonucleotide sequences (5' to 3')	Product size
Exon1	F1	CCGTTGGAGATGGGGTGAAA	1760
	R1	ACCCTACATTTGGGCGATGG	
Exons 2-5	F2	GCAGTCCCAGGACATCAAAG	1500
	R2	TCAGTTTGAATGCATGGGAGAGC	

Table 2. Pairwise comparison and list of the various Oct4 orthologs coding sequences used in the construction of the phylogenetic tree to analyze the evolution of Tiger, Leopard and Jaguar Oct4.

		1	2	3	4	5	6	7	8	9	10
Felis=NM_001173441	1		99.82	99.91	99.91	93.44	92.61	91.97	91.87	52.54	80.98
Jaguar Oct4	2	0.00		99.72	99.72	93.26	92.43	91.78	91.69	52.63	80.89
Tiger Oct4	3	0.00	0.00		99.82	93.35	92.71	92.06	91.97	52.54	80.98
Leopard Oct4	4	0.00	0.00	0.00		93.35	92.52	91.87	91.78	52.45	80.89
Canis=XM_538830	5	0.07	0.07	0.07	0.07		90.86	89.01	88.92	52.17	80.06
Sus=NM_001113060	6	0.08	0.08	0.08	0.08	0.10		90.21	90.12	52.45	81.44
Homo(Variant1)=NM_002701	7	0.08	0.09	0.08	0.09	0.12	0.10		98.15	52.35	81.90
Macaca=NM_001114955	8	0.09	0.09	0.08	0.09	0.12	0.11	0.02		52.63	82.73
Mus=NM_001252452	9	0.75	0.75	0.75	0.75	0.76	0.75	0.76	0.75		59.40
Rattus=NM_001009178	10	0.22	0.22	0.22	0.22	0.23	0.21	0.21	0.20	0.58	

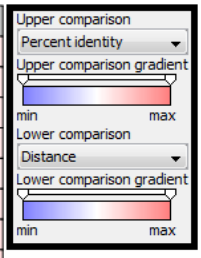


Figure 2. The 13 bp deletion close to exon1 of Jaguar Oct4 gene.

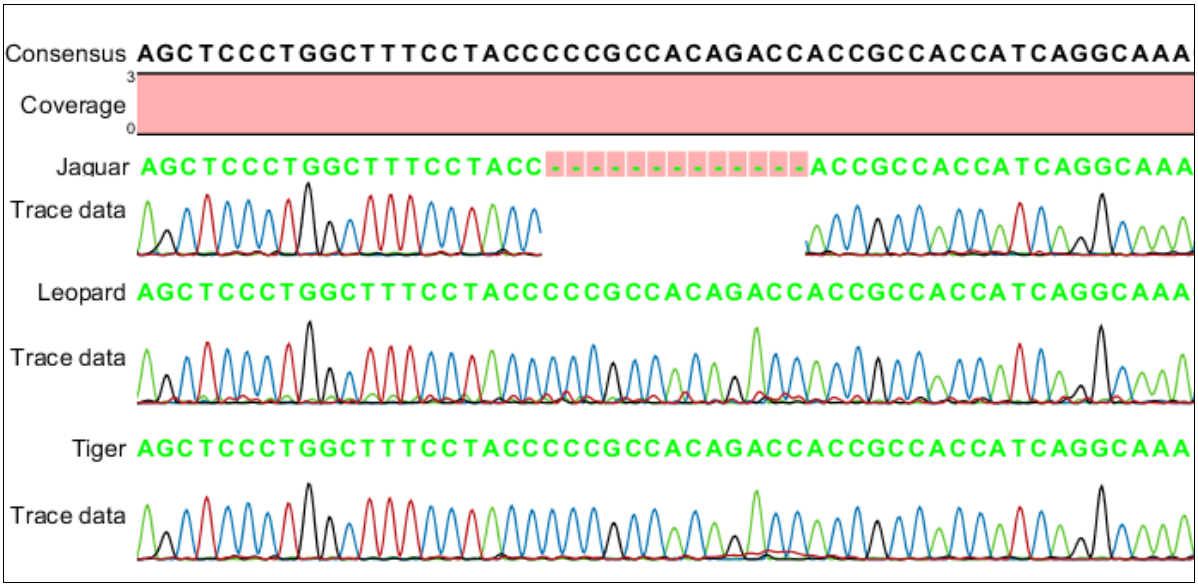
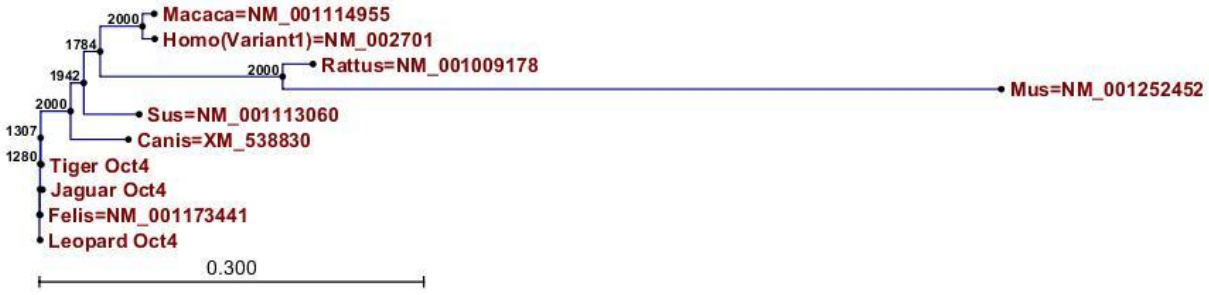


Figure 3. Phylogenetic tree of the Oct4 gene showing the ancestoral relationship between the four felids and other known mammalian species. The scale bar allows conversion of branch length in the dendrogram to genetic distance between clades (0.3_30% genetic distance). Bootstrap-support values are based on 2000 replicates and are indicated on the nodes.



Chapter 4

Generation of First endangered Snow leopard iPSC by using five human factors (Paper 2 published)

While stem cell biology and assisted reproduction technologies can prove invaluable in understanding development in endangered animals and providing conservation alternatives, the critical limitation to date has been the scarcity of gametes and embryos. The seminal discovery by Shinya Yamanaka's team that it was possible to revert first mouse and then human somatic cells back to an embryonic stem cell equivalent, by insertion of 4 pluripotency associated genes, opened up potential opportunities for other species. However, despite numerous publications on generation of induced pluripotent stem iPSC, the work is restricted to rodents and primates, with pig being the only large animal species from which the generation of iPSC has been reported to date.

I aimed to generate iPSC from an endangered big cat, the snow leopard (*Panthera uncia*) and in initial studies observed that insertion of the 4 "Yamanaka" factors alone was insufficient to generate an iPSC. I hypothesized that additional stem cell factors were required and added Nanog as an additional reprogramming factor (5 factors) to the reprogramming mix. Following this I observed the appearance of stem cell-like colonies, which could be maintained in culture and expressed markers of pluripotency, including the formation of teratomas, when injected in SCID mice.

So in summary I believe this research would be of great interest as it provides the first insight into the need for additional reprogramming factors for generating iPSC from endangered snow leopard.

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

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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.

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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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

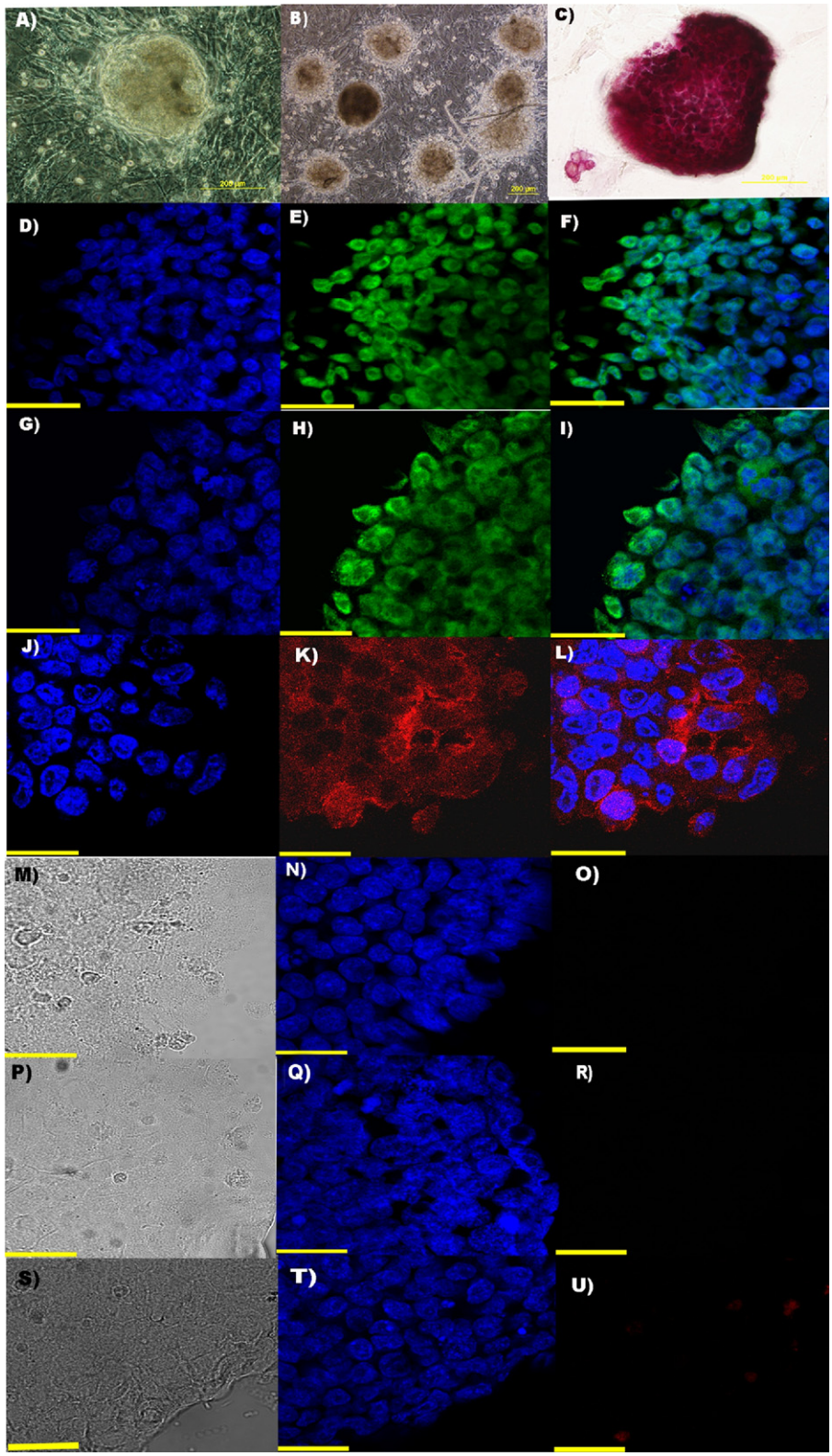
Mitotically inactivated mEF were plated in six-cm organ culture dishes. The FP medium was replaced with mES cell medium containing DMEM (high glucose, Invitrogen) supplemented with 0.1 mM 2-beta mercaptoethanol (β-ME), 1M non-essential amino acids (NEAA), 2 mM GlutaMAX, 20% (v/v) HyClone serum, 0.5% 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)
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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).

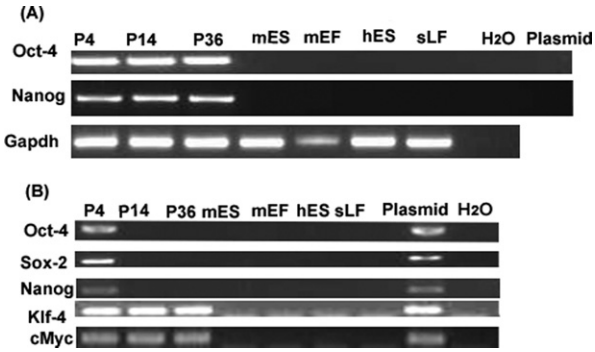

Fig. 2. RT-PCR of cat (Specific) endogenous genes and human transgenes. (A) Gene expression of endogenous cat *OCT4* (286 bp), *NANOG* (246 bp) and *GAPDH* (271 bp) and (B) human transgenes expression of pMX-*OCT4*, pMX-*SOX2*, pMX-*NANOG*, pMX-*KLF4* and pMX-*cMYC* on snow leopard iPS cells at P4, P14, 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).

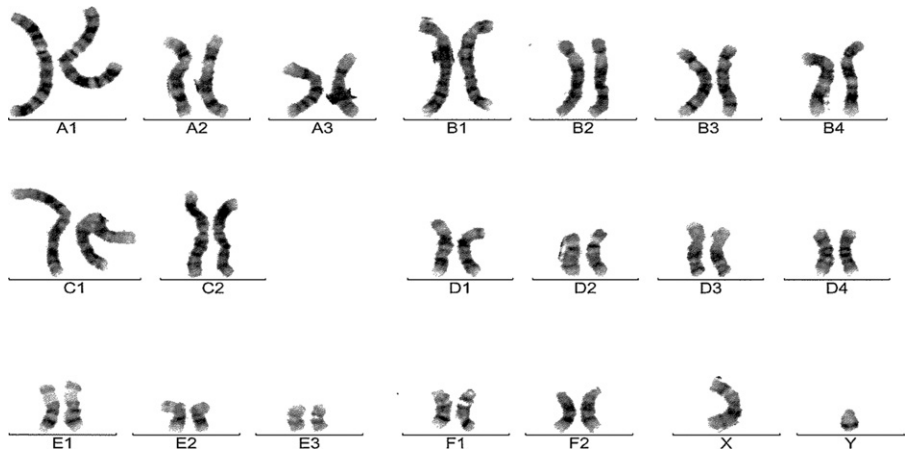

Fig. 3. Chromosome counts on snow leopard iPS cells at P14. Karyotype showing a total of 19 pairs of chromosomes in snow leopard iPS cells.

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

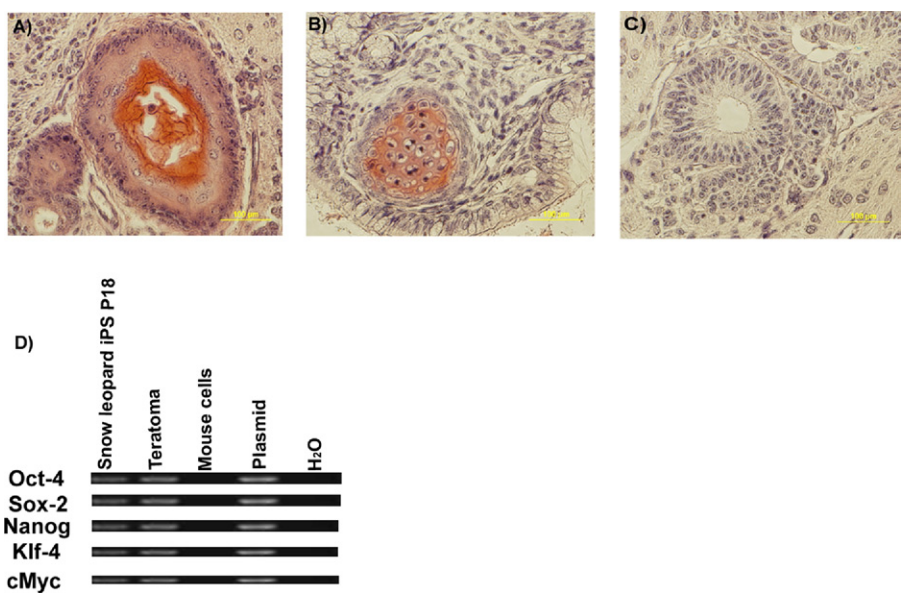

Fig. 4. Cross-sections of a snow leopard iPS cells teratoma developed in an SCID mouse and genomic PCR on snow leopard iPS cells and teratoma. Cross-section of a teratoma obtained at 10 wk after injection of snow leopard iPS cells at P18 in an SCID mouse, stained with hematoxylin and eosin with tissues 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.

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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

Chapter 5

Confirming "Nanog" as the essential additional factor required for generating iPSC from geographically diverse Wild felids. (Paper 3 published)

Generation of induced pluripotent stem cells (iPSC) from endangered species raises fascinating possibilities for species conservation. However numerous challenges hinder their use for assisted reproduction (nuclear transfer, embryo complementation) in endangered species with a primary challenge being the limited availability of compatible recipient eggs and surrogate dams for embryos generated. In Felidae, endangered and non-endangered species can interbreed to produce hybrids (*e.g.* tigon, jaglion, Savannah cat), demonstrating embryo-uterine and mitochondrial compatibility for embryo surrogacy.

iPSC with unlimited proliferation have been generated in rodents and primates, but in domestic species continued proliferation is dependent on expression of exogenous transgenes reflecting species differences in pluripotency, a factor potentially restricting isolation of embryonic stem cells (ESC) from these species.

Nanog plays a critical role inducing and maintaining pluripotency in some domestic species. Therefore this chapter aimed to determine if "Nanog" was required to derive felid iPSC, and examine the induction of pluripotency in three geographically separated and evolutionarily diverse felid species. The Bengal tiger (*Panthera tigris*; Asia) and jaguar (*Panthera onca;* Americas), from the *Panthera* lineage, have been separated for >2My[4] and diverged from the modern radiation of felids ~11Ma, and the serval (*Leptailurus* serval; Africa), from the caracal lineage, diverged ~8.5Ma[5].

I describe an effective method for inducing pluripotency in cells from endangered wild cats from around the globe and confirm Nanog as an essential factor in the reprogramming milieu for these species. Felids are suitable models for studying diseases including HIV but lack of germ-line competent ESC has limited the ability for genome modification in the cat. The efficient production of iPSC from felids therefore creates a unique opportunity for species preservation through assisted reproduction and provides an alternative for elusive cat ESC for biomedical research.

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BRIEF REPORT

Nanog Is an Essential Factor for Induction of Pluripotency in Somatic Cells from Endangered Felids

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Abstract

Nanog has an important role in pluripotency induction in bovines and snow leopards. To examine whether it was required for wild felids globally, we examined the induction of pluripotency in felids from Asia (Bengal tiger, *Panthera tigris*), Africa (serval, *Leptailurus serval*), and the Americas (jaguar, *Panthera onca*). Dermal fibroblasts were transduced with genes encoding the human transcription factors *OCT4*, *SOX2*, *KLF4*, and *cMYC* with or without *NANOG*. Both four- and five-factor induction resulted in colony formation at day 3 in all three species tested; however, we were not able to maintain colonies that were generated without *NANOG* beyond passage (P) 7. Five-factor induced pluripotent stem cell (iPSC) colonies from wild cats were expanded *in vitro* on feeder layers and were positive for alkaline phosphatase and protein expression of OCT-4, NANOG, and stage-specific embryonic antigen-4 at P4 and P14. Reverse-transcription polymerase chain reaction confirmed that all five human transgenes were transcribed at P4; however, *OCT4*, *SOX2*, and *NANOG* transgenes were silenced by P14. Endogenous *OCT4* and *NANOG* transcripts were detected at P4 and P14 in all cell lines confirming successful reprogramming. At P14, the iPSCs from all three species remained euploid and differentiated *in vivo* and *in vitro* into derivatives of the three germ layers. This study describes an effective method for inducing pluripotency in three endangered wild cats from across the globe and confirms Nanog as an essential factor in the reprogramming event. Efficient production of iPSC from endangered felids creates a unique opportunity for species preservation through gamete production, nuclear transfer, embryo complementation, and future novel technologies.

Key words: conservation; induced pluripotent stem cells; Nanog; wild cats

Introduction

Induced pluripotency is a new approach to produce embryonic stem-like cells from somatic cells[1] and provides a unique approach to understand both pluripotency and lineage assignment. Generation of induced pluripotent stem cells (iPSCs) from endangered species[2,3] raises fascinating possibilities for species conservation by augmenting the available technologies, such as nuclear transfer and embryo complementation, for assisted reproduction. However, some challenges hinder this application, with the primary challenge being limited availability of compatible recipient eggs and surrogate dams for embryos generated,[4] though recent advances in the field hold promise.[5,6] In the Felidae, endangered and nonendangered species can interbreed to produce viable hybrids (e.g., tigon, jaglion, Savannah cat), demonstrating embryo-uterine and mitochondrial compatibility for embryo surrogacy.[7] This circumvents the potential barriers in selected endangered felids for using iPSCs to assist reproduction.

iPSCs with properties that are equivalent to embryonic stem cells (ESCs) have been generated in rodents, primates, and some domestic species.[8–11] In most domestic species, however, maintenance of iPSCs is dependent on continued expression of exogenous transgenes[12] reflecting species differences in pluripotency, a factor potentially also restricting isolation of ESCs from these species.

We have recently identified the critical role of Nanog in pluripotency induction and maintenance in some large animal species.[3,11] To determine whether Nanog was an absolute

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requirement for robust derivation of iPSCs from felids globally, we examined the induction of pluripotency in fibroblast cultures from three geographically separated and evolutionarily diverse felid species:[13] the Asian Bengal tiger (*Panthera tigris*), the African serval (*Leptailurus serval*), and the South American jaguar (*Panthera onca*).

Materials and Methods

Animal handling and experimentation was conducted in accordance with the code of practice of the Australian National Health and Medical Research Council (NHMRC; 2004) and was approved by Monash University Animal Experimentation Ethics Committee.

Fibroblasts were isolated from tissues taken from the ear pinnae of Bengal tiger, jaguar, and serval, which died of natural causes or were euthanized due to health-related problems. The tissue ($\sim 5\,mm^2$) was minced using sterile surgical instruments and plated in six-well dishes with fibroblast plating (FP) medium. The FP medium was composed of Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with penicillin/streptomycin and 10% (v/v) fetal bovine serum (all from Invitrogen, Mulgrave, Australia) The tissue was cultured in a 6% CO_2 incubator at 38.5°C for 7 days.[10]

Mouse embryonic fibroblasts (MEFs) were isolated from fetuses collected from Quackenbush (QS) mice on day 13.5 post-coitum and used as a feeder layer for the iPSCs as previously described.[3] QS-MEFs were cultured in MEF medium which consisted of DMEM (high glucose) supplemented with 10% (v/v) fetal bovine serum, 2 mM Glutamax, penicillin/streptomycin, and 1 M nonessential amino acids (Invitrogen). MEFs were treated with 10 μg/mL mitomycin C (Invitrogen) for 3 h to arrest mitosis.[14]

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). Amphotropic viral particle production by Plat-A packaging cells (Jomar Biosciences, Kensington, Australia) was performed as described previously.[3] Nine micrograms of each vector was co-transfected to Plat-A cells using Fugene 6 (Roche, Dee Why, 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 8 μg/mL of polybrene (Sigma-Aldrich, St. Louis, MO). Felids fibroblasts were plated 24 h prior to infection at a density of 4×10^4 cells/cm^2. Equal parts of the retroviral supernatant containing four or five transcription factors were added to the plated felid fibroblasts. Two rounds of infection were performed 24 h apart. The culture medium was changed to mouse ESC medium at day 5 post-infection. The iPSC colonies were picked based on ESC-like morphology at day 3. Felids fibroblast cells were infected with GFP-containing retroviral supernatant in parallel, to monitor the transduction efficiency.

The felid iPSCs were cultured in medium consisting of DMEM supplemented with 0.1 mmol/L 2-beta-mercaptoethanol, 1 M nonessential amino acid, 2 mM Glutamax, 20% (v/v) Hyclone serum, penicillin/streptomycin, and 10^3 U/mL murine leukemia inhibitory factor (LIF) (ESGRO; Invitrogen) in a 6% CO_2 incubator at 38.5°C. Medium was changed daily, and iPSCs were passaged manually every 3–4 days onto fresh feeder layers.

Putative iPSC colonies were tested for alkaline phosphatase (AP) using a diagnostic AP substrate kit according to the manufacturer's specification (SK-5300, Vector Laboratories, Inc., Burlingame, CA). For immunocytochemistry, colonies were fixed in 40 mg/mL paraformaldehyde for 15 min at room temperature and washed three times with phosphate-buffered saline (PBS, pH 7.5). Cells examined for OCT-4 and NANOG were blocked with PBS (pH 7.5) supplemented with 10 mg/mL bovine serum albumin (BSA), 5% (v/v) goat serum, and 0.1% (v/v) Tween. Those tested for stage-specific embryonic antigen (SSEA)–4 were blocked with 10 mg/mL BSA, 5% (v/v) goat serum in PBS pH 7.5 for 60 min at room temperature. Mouse anti-human OCT-4 immunoglobulin G (IgG; SC-5279, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-mouse SSEA-4 IgG (MC 813-70, Millipore, Melbourne, Australia) and rabbit polyclonal IgG to NANOG IgG (ab-80892, Abcam, Waterloo, 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. Cell preparations were then washed three times with PBS and a secondary antibody was added in PBS containing 5% (v/v) goat serum for 1 h at room temperature (OCT-4: anti-IgG Alexa 488 1:500; NANOG: anti-IgG Alexa 488 1:500; SSEA-4: anti-IgG Alexa 594 1:500). In addition, control cell lines with the primary antibodies excluded were used as negative controls. At the end of the secondary antibody reaction all cell preparations were washed three times with PBS, mounted in Vectashield + 4′,6-diamidino-2-phenylindole (DAPI; Abacus) and cover-slipped.

Total RNA was extracted from felid iPSCs at passage (P) 4 and P14, and also from human ESC, mouse ESC, and tiger, jaguar, and serval ear fibroblasts using an RNA-easy kit (Qiagen, Doncastor, VIC, Australia) according to the manufacturer's instructions. We used *GAPDH* expression as an internal control for the amount of template in each reaction. Polymerase chain reaction (PCR) was carried out using Go Taq DNA polymerase (Sigma). Primers used in this study[15] are listed in Supplementary Tables S1 and S2.

To test for embryoid bodies (EBs) formation, felid iPSCs at P14 were typsinized and 20×10^3 cells were plated in low-attachment plastic six-well dishes. iPSC medium was used without LIF and was changed every second day till day 10.[16] EBs were collected after 10 days for RNA extraction and preparation of cDNAs for reverse-transcription (RT)–PCR. The primers used for detecting three germ layers were Nestin (endoderm), CD 31 (mesoderm), and *FoxA2* (ectoderm).[17]

Felid iPSCs at P14 were harvested using 5 mg/mL trypsin-EDTA (Invitrogen). Two million cells were injected subcutaneously into the thigh muscle of 5- to 7-week-old severe combined immunodeficient (SCID) male mice.[17] Ten weeks after injection the mice were euthanized and the resulting tumors were harvested and fixed in 40 mg/mL paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

At least 60 metaphase spreads were counted for tiger, jaguar, and serval iPSCs (TiPSCs, JiPSCs, and SiPSCs, respectively) at P14 for karyotype analysis.[3]

Results and Discussion

Moloney-based retroviral vectors (pMXs) encoding the human transcription factors *OCT4*, *SOX2*, *KLF4*, and *cMYC*

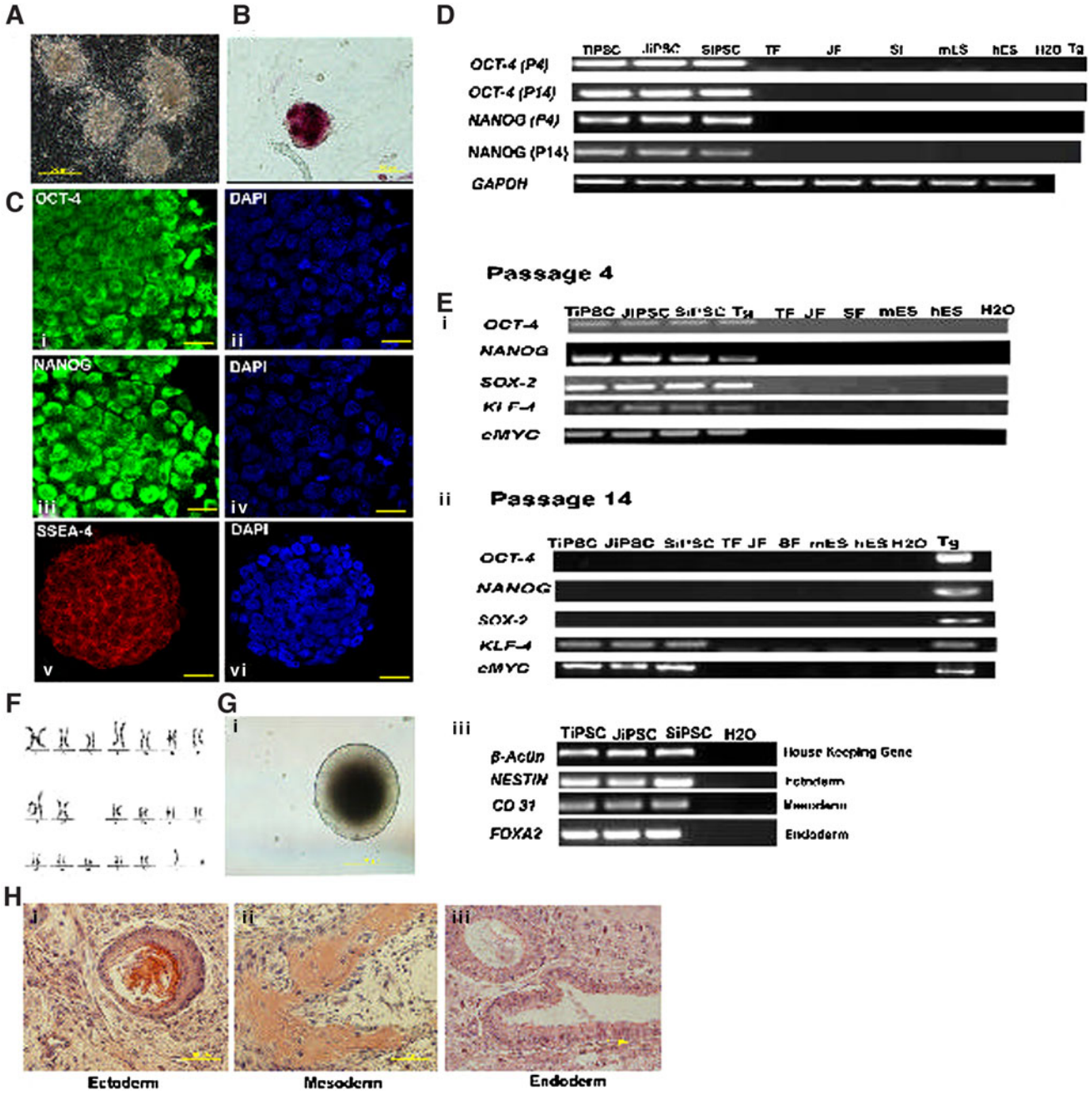

FIG. 1. Characteristics of Bengal tiger induced pluripotent stem cells (TiPSCs). **(A)** Morphology of TiPSCs at P1. **(B)** Alkaline phosphatase (AP) staining of a putative TiPSC colony at P14. **(C)** Confocal images of immunofluorescence staining of TiPSC at P14. OCT-4: **(i)** green fluorescence, **(ii)** 4′,6-diamidino-2-phenylindole (DAPI); NANOG: **(iii)** green fluorescence, **(iv)** DAPI; stage-specific embryonic antigen (SSEA)-4: **(v)** red fluorescence, **(vi)** DAPI. The scale bar for all confocal images represents 25 μm. **(D)** Reverse-transcription polymerase chain reaction (RT-PCR) gene expression analysis of endogenous cat *OCT4* (286 bp), *NANOG* (246 bp), and *GAPDH* (271 bp) at P4 and P14 in Bengal tiger, jaguar, and serval iPSCs (TiPSCs, JiPSCs, and SiPSCs, respectively) and fibroblasts (TFs, JFs, and SFs), mouse and human embryonic stem cells (mESCs and hESCs), transgene (Tg), and water (H_2O). **(E, F)** Expression of human transgenes pMX-*OCT-4*, pMX-*NANOG*, pMX-*SOX-2*, pMX-*KLF-4*, and pMX-*cMYC* in TiPSCs, JiPSCs, SiPSCs, Tg, TFs, JFs, SFs, mESCs, hESCs, and H_2O at P4 and P14. **(G)** Karyotype of TiPSCs at P14 showing a euploid count of 18 autosomal and one XY pair of chromosomes. **(H)** TiPSCs differentiated *in vitro* to form embryoid bodies. **(I)** Confirmation of differentiation of embryoid bodies into the three germ layers by specific gene expression by RT-PCR (housekeeping gene: β-actin; ectoderm: *NESTIN*; mesoderm: *CD31*; and endoderm: *FOXA2*) on TiPSCs, JiPSCs, SiPSCs, and H_2O. **(J)** Cross-section of a teratoma obtained at 10 weeks after injection of TiPSCs at P14 in a severe combined immunodeficient (SCID) mouse, stained with hematoxylin and eosin with tissues representative of the three primary germ layers: **(i)** ectoderm: keratinizing stratified squamous epithelium; **(ii)** mesoderm: bone surrounded by connective tissue; **(iii)** endoderm: ciliated epithelium.

with or without *NANOG* were used to infect dermal fibroblasts from each species. Infection efficiency of the retrovirus was ascertained using pMX-GFP transgene expression and averaged 91.3%±1.2%, 92.3%±1.2%, and 98.3%±0.6% in tiger, jaguar, and serval, respectively, from three repeated experiments (Supplementary Table S3). Both four- and five-factor induction initiated colony formation at day 3; however, colony formation and programming efficiency were reduced in four-factor inductions (SupplementaryTable S3) and we were not able to maintain colonies that were generated without *NANOG* beyond seven passages (P7).

Five-factor–induced iPSC colonies from tiger, jaguar, and serval were expanded *in vitro* (Figs. 1A and 2A, A′) and were positive for AP (Figs. 1B and 2B, B′) and protein expression of OCT-4, NANOG, and SSEA-4 at P14 (Figs. 1C and 2C, C′). Endogenous *OCT-4* and *NANOG* were detected by RT-PCR at P4 and P14 (Fig. 1D) indicating reprogramming and reactivation of the endogenous pluripotency genes. RT-PCR confirmed that all five transgenes were transcribed at P4 (Fig. 1E); however, *OCT-4*, *SOX-2*, and *NANOG* transgenes were silenced by P14 (Fig. 1F). Karyotypes of the iPSC lines (Figs. 1G and 2D, D′) were normal (18XX or 18XY) in all three species. Under *in vitro* differentiation conditions, embryoid bodies developed from all iPSC lines (Figs. 1H and 2E, E′) and randomly differentiated into ectoderm, endoderm, and mesoderm as detected by RT-PCR of specific marker genes (Fig. 1I). Further, when injected into SCID mice, P14 iPSCs from all three felid species formed teratomas containing tissues from the three germ layers (Figs. 1J and 2F, F′).

In conclusion, we describe an effective method for inducing pluripotency in cells from large endangered felids and confirm Nanog as an essential factor in the reprogramming milieu for these species. Felids are also suitable models for studying diseases, including HIV, but lack of germ-line–competent ESCs has limited the ability for genome modification in the cat.[7] The efficient production of iPSCs from felids therefore creates a novel opportunity for species preservation through assisted reproduction and provides an alternative for elusive cat ESCs for biomedical research.

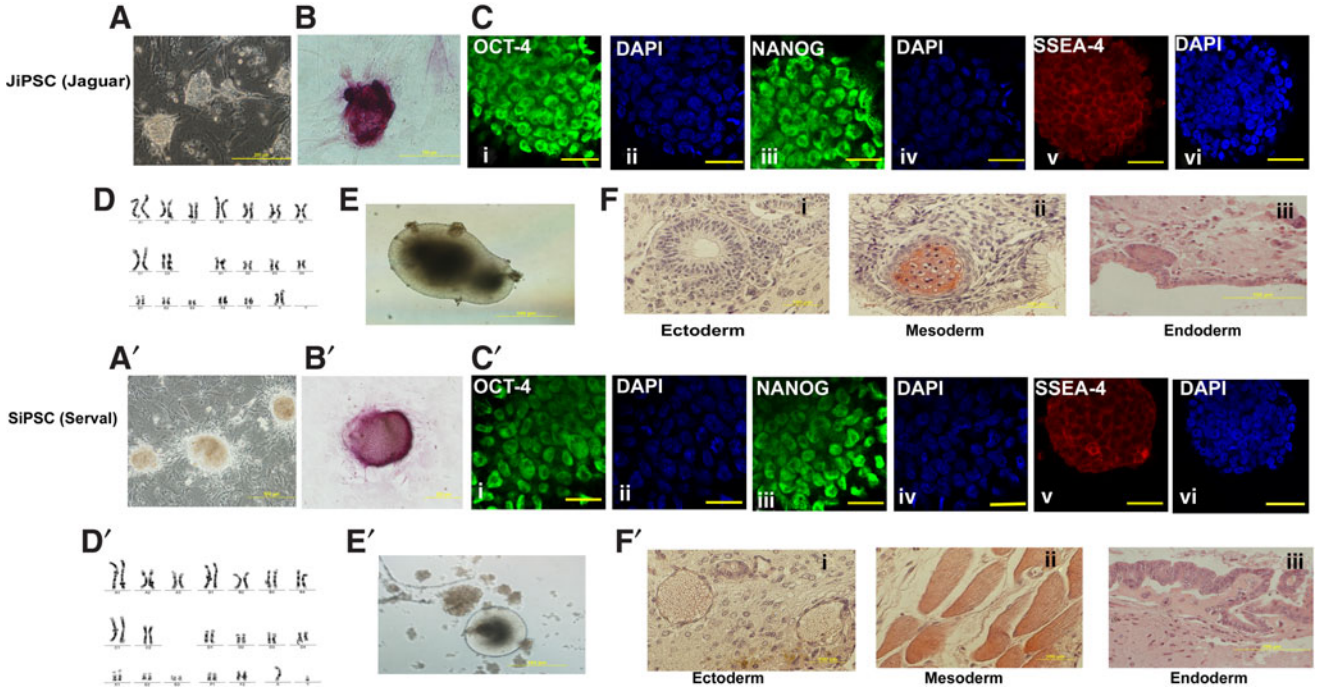

FIG. 2. Detailed characterization of JiPSCs and SiPSCs at P14. **(A–F)** Morphology and characterization of JiPSCs. **(A)** Morphology of JiPSCs at P1 on feeder layers. **(B)** AP staining of a putative JiPSC colony at P14. **(C)** Confocal images of immunofluorescence staining of JiPSCs at P14. OCT-4: **(i)** green fluorescence, **(ii)** DAPI; NANOG: **(iii)** green fluorescence, **(iv)** DAPI; SSEA-4: **(v)** red fluorescence, **(vi)** DAPI. The scale bar for all confocal images represents 25 μm. **(D)** Karyotype of JiPSCs at P14 showing a euploid count of 18 autosomal and one XX pair of chromosomes. **(E)** JiPSC differentiated in vitro to form embryoid bodies. **(F)** Cross-section of a teratoma obtained at 9 weeks after injection of JiPSCs at P14 in a SCID mouse, stained with hematoxylin and eosin with tissues representative of the three primary germ layers: **(i)** ectoderm: nervous tissue; **(ii)** mesoderm: bone surrounded by connective tissue; **(iii)** endoderm: cuboidal epithelium. **(A′–F′)** Morphology and characterization of SiPSCs. **(A′)** Morphology of SiPSC at P1 on feeder layers. **(B′)** AP staining of a putative SiPSC colony at P14. **(C′)** Confocal images of immunofluorescence staining of SiPSCs at P14: OCT-4: **(i)** green fluorescence, **(ii)** DAPI; NANOG: **(iii)** green fluorescence, **(iv)** DAPI; SSEA-4: **(v)** red fluorescence, **(vi)** DAPI. The scale bar for all confocal images represents 25 μm. **(D′)** Karyotype of SiPSCs at P14 showing a euploid count of 18 autosomal and one XY pair of chromosomes; **(E′)** SiPSCs differentiated in vitro to form embryoid bodies. **(F′)** Cross-section of a teratoma obtained at 9 weeks after injection of SiPSCs at P14 in a SCID mouse, stained with hematoxylin and eosin with tissues representative of the three primary germ layers: **(i)** ectoderm: nervous tissue with blood cells (vascular differentiation); **(ii)** mesoderm: muscle; **(iii)** endoderm: columnar epithelium.

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Author Disclosure Statement

All authors declare that no competing financial interests exist.

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Chapter 6

Using stem cells to study and preserve bio-diversity in endangered Big Cats (Publication 4- Book Chapter)

6.1. Introduction

One of every four animal species on the planet is threatened by extinction. Historically, strategies for preserving biodiversity have focused on saving habitat and, by default, species in these native environments (*in situ*) (Ben-Nun *et al.* 2011b).

One support strategy is managing 'assurance' populations *ex situ* for hundreds of mammals, birds, reptiles, amphibians and fish. This preserves genetic integrity, allows basic research to be conducted and is a source of animals for re-introduction programs. *Ex situ* species propagation approaches are complex and demand expertise and resources, including specialized animal space and facilities. Even then, achieving reproduction is challenging. Animals can and often do have preferences for sexual partners, and it is expensive and complicated to transfer wild animals between locations to make ideal genetic matches to retain maximal heterozygosity. As a result, assisted reproductive technologies (ART) have been explored for helping manage *ex situ* wildlife populations.

Much progress has been made during the last decade in the development of assisted reproductive techniques, not only in the economically driven fields of human infertility and domestic animal breeding, but also in the area of animal conservation. Endangered felids are often difficult to breed either in captivity or even under natural conditions. One of the most important reasons for infertility or sub-fertility in this group is decreased genetic diversity caused by inbreeding, due to genetic bottlenecks as a consequence of geographical isolation and population contraction. Because of this there has been increasing interest in maintaining genetic diversity for the conservation of wild felids and preservation of valuable cat breeds. ART comprising of techniques such as artificial insemination (AI), Intra-Cytoplasmic Sperm Injection (ICSI), In-vitro fertilization (IVF) and Somatic Cell Nuclear transfer (SCNT), has been promoted over the past two decades as a potential means to conserve and manage threatened wildlife populations. But one of the major problems with the implementation of *in situ* and *ex situ* conservation programs is the lack of availability of suitable biological material, which is required for a better understanding of reproductive patterns as well to maximize reproductive efficiency. This constraint arises from the strict procedures adopted

for restraining or anaesthetizing free-living animals for collection of biological/reproductive samples due to inherent risks. (Verma *et al.* 2012b)

6.2.SCNT, an alternative to ART

SCNT also called cloning represents an alternative for the production of animals genetically (chromosomally) identical to the donor somatic cell, albeit with mitochondria being contributed by the recipient oocyte, and offers the possibility of preventing the extinction of wild species. However, owing to the limited availability of oocytes from wild animals, the cloning of endangered species requires the use of donor oocytes from a related domestic species. Interspecies SCNT consists of the reconstruction of a cloned embryo by use of a donor somatic cell and a recipient oocyte from a different species, but from the same genus; whereas inter-generic SCNT consists of the reconstruction of a cloned embryo in which the donor nucleus and recipient cytoplast differ both in species and in genus, with oocyte specific mitochondria in both cases. Several studies have demonstrated that it is possible to produce embryos from endangered species by interspecies or inter-generic SCNT (Gomez *et al.* 2008); however; few live cloned wild mammals have resulted and these animals were derived from embryos reconstructed with donor oocytes of the same genus. Viable offspring from inter-generic SCNT have not been produced in any mammalian species, although pregnancies have been established with inter-generic cloned embryos after transfer into sheep or domestic cat recipient occytes (Gomez *et al.* 2000).The successful development of interspecies and inter-generic cloned embryos is dependent on a variety of factors, which are similar to those reported for intra-species SCNT including source of oocyte cytoplasts, cell cycle synchronisation, mitochondrial compatibility and genotype of the donor cells. Moreover, increasing evidence shows that aberrant epigenetic alterations that arise during SCNT may be associated with perinatal and neonatal losses and the production of abnormal offspring (Wu *et al.* 2010).

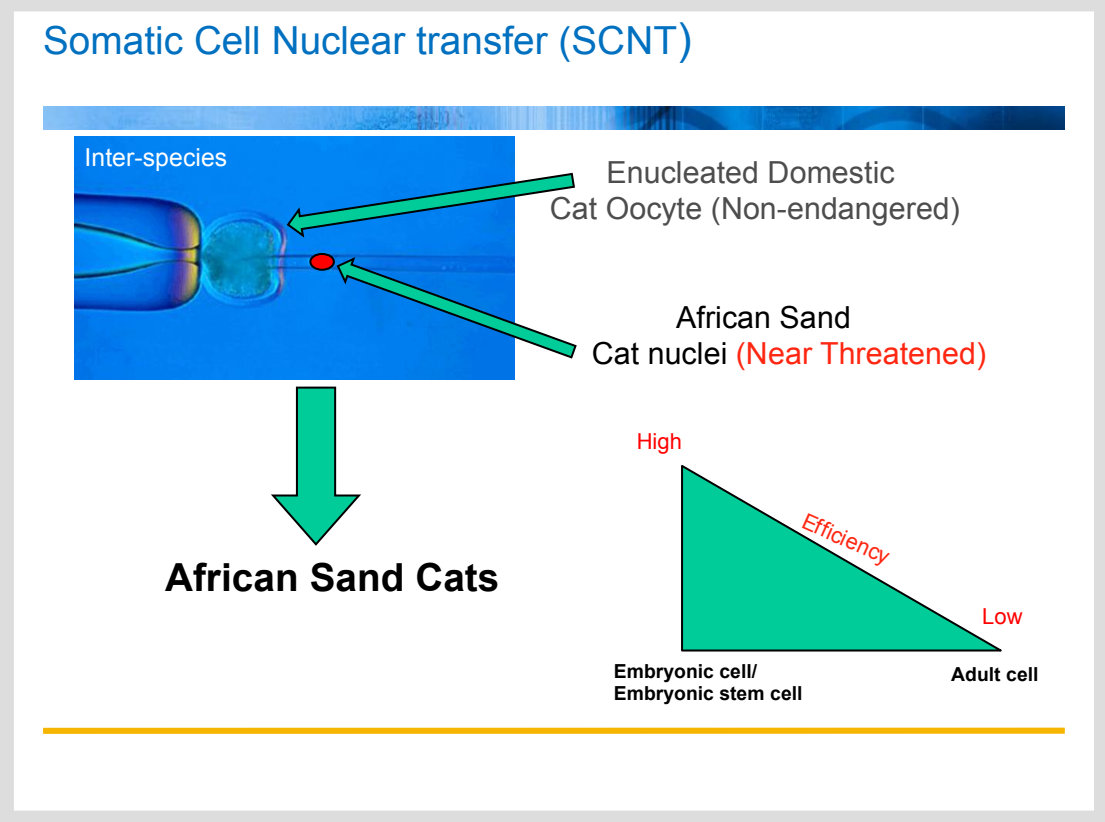

Figure 16: *Inter-species cloning and efficiency parameter*

6.3. Pluripotent Stem Cells

Pluripotent stem cells differentiate into all the cell types in the body including gametes in vivo, while retaining the capacity for indefinite self-renewal in-vitro. The best known example of pluripotent cells are Embryonic Stem Cells (ESC) and the potential application of these cells are that they (1) can contribute to embryos for e.g. chimeras (2) can be differentiated to gametes in-vitro (3), and can be used as a donor cell for nuclear transfer. ESC are typically derived from Inner Cell Mass (ICM) of blastocysts, which are destroyed in the process, raising ethical and logistical concerns for the derivation of stem cell lines from endangered species. Therefore for species in which embryos are particularly difficult to obtain or those, which are endangered, this approach was not particularly useful and feasible. In addition, to date no true ESC has been reported for any species for other than rodents (Malaver-Ortega *et al.* 2012; Verma *et al.* 2012).

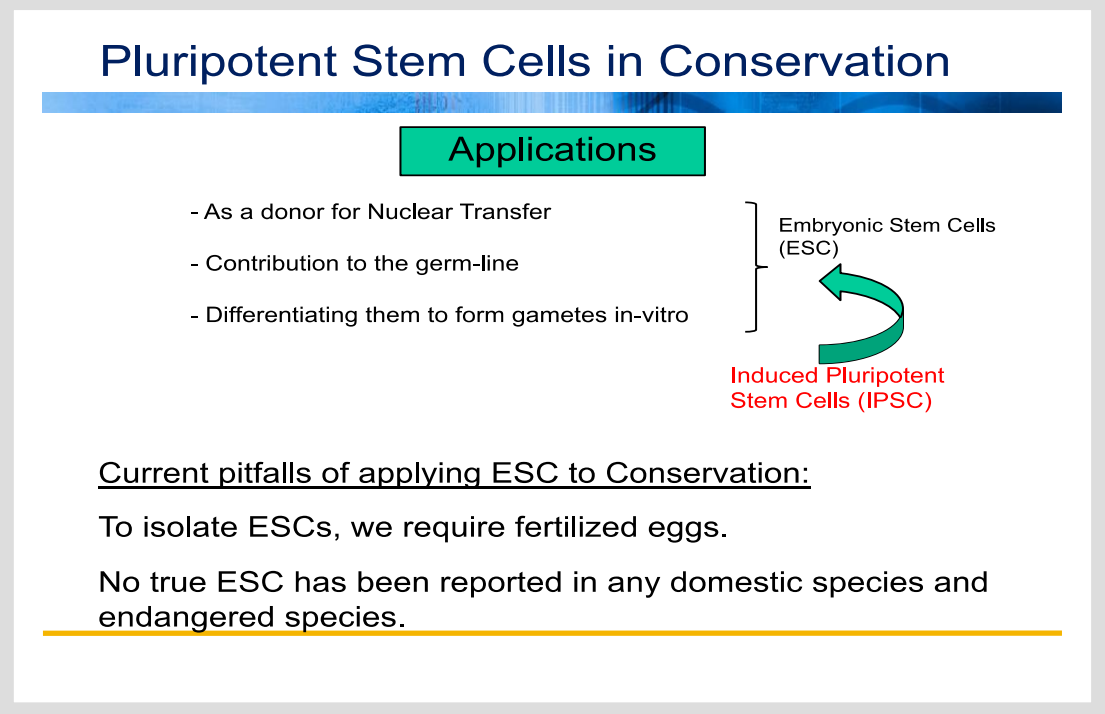

Figure 17: *Pluripotent Stem Cells and its applications in Conservation*

6.4. Induced Pluripotent Stem Cells (iPSC)

Despite this reality check on what is possible today, it is essential to consider what might be promising for future intensive management of endangered species, especially as embryo and molecular technologies advance. So it is imperative to consider the potential benefits offered by iPSC generated by reprogramming adult or differentiated somatic cells to a pluripotent stem cell-like state using defined transcription factors.

In a seminal study, Japanese scientists (Takahashi & Yamanaka 2006) used viral transduction of mouse fibroblasts to screen a combination of 24 candidate genes with putative roles in pluripotency and remarkably found that four previously known transcription factors (Oct3/4, Sox2, Klf4 and cMyc) could reprogram mouse embryonic fibroblasts (MEFs) and tail tip fibroblasts into ES-like cells, which were almost indistinguishable from mouse ESC in terms of pluripotency and coined the term Induced Pluripotent Stem Cells (iPSC).

iPSC have now been isolated from rodents (mouse and rats) (Takahashi *et al.* 2007b; Honda *et al.* 2010), primates (human and monkeys) (Liu *et al.* 2008; Park *et al.* 2008), livestock (pigs, sheep, horse and cattle) (Ezashi *et al.* 2009b; Nagy *et al.* 2011a; Sumer *et al.* 2011b; Khodadadi *et al.* 2012; Liu *et al.* 2012b) and endangered species (Ben-Nun *et al.* 2011b;

Verma *et al.* 2012). The use of iPSC technology to provide a source of pluripotent cells for use in felid conservation was considered likely to be a more successful approach than isolating ESC from endangered felids embryos because it is non-invasiveand only requires somatic cells. In mice, iPSC are similar to embryonic stem cells (ESC) and can form chimeric embryos when injected into blastocysts. This opened a new direction in the use of iPSC technology by offering the possibility of converting cells from skin to ESC regardless of age and gender of donor, and to use them for ART in various forms.

6.5.Snow leopard

The snow leopard (*Uncia uncia*) is a moderately large cat native to the mountain ranges of Central Asia. They live between 3,000 and 5,500 metres (9,800 and 18,000 ft) above sea level in the rocky mountain ranges of Central Asia. However, their secretive nature means that their exact numbers are unknown, although it has been estimated that between 3,500 and 7,000 snow leopards exist in the wild and between 600 and 700 in zoos worldwide (Kleihman & Garman 1978).

6.5.1. First Snow leopard iPSC

Before attempting to produce iPSC from snow leopard we came across a number of unknowns as listed below.

LIMITATION 1: Choice of Factors; Mouse /human,

LIMITATION 2: Cell Type, no access to any other

LIMITATION 3: Derivation Conditions; medium

LIMITATION 4: No specific stem cell markers of wild cats for *in vitro* analysis. No knowledge on differentiation

Figure 18: *Limitations for producing snow leopard iPSC*

Later, we reported the successful derivation of iPSC 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.

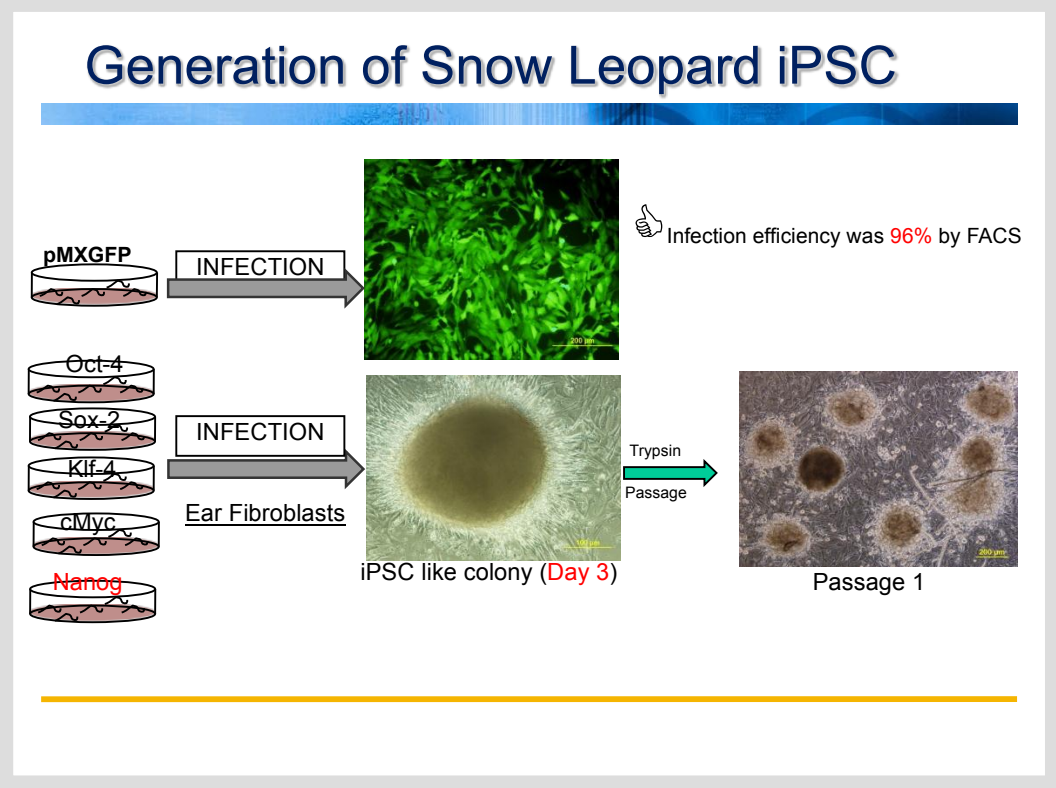

Figure 19: *Experimental diagram to show the procedure for producing snow leopard iPSC*

Of particular importance was the observation that the three key exogenous pluripotency transgenes (OCT4, SOX2, NANOG) were silenced at later passages. Interestingly, we observed a similar requirement of transcription factors for induction of pluripotency and an identical pattern of subsequent silencing of transgenes for three globally diverse endangered felids in a subsequent study (Verma *et al.* 2013). During the course of our study we observed that snow leopard iPSC at P14, when tagged with mCherry reporter and injected into the peri-vitelline space of mouse morulae, did not compromise development of the mouse embryos. We next examined whether these iPSC were able to contribute to developing mouse embryos by incorporation into the ICM. We propose this as a novel *in vivo* assay to assess the embryo contribution ability of iPSC and pluripotent cells from exotic species where species-specific testing is impossible because of the often extremely limited access to gametes, especially

oocytes and embryos.

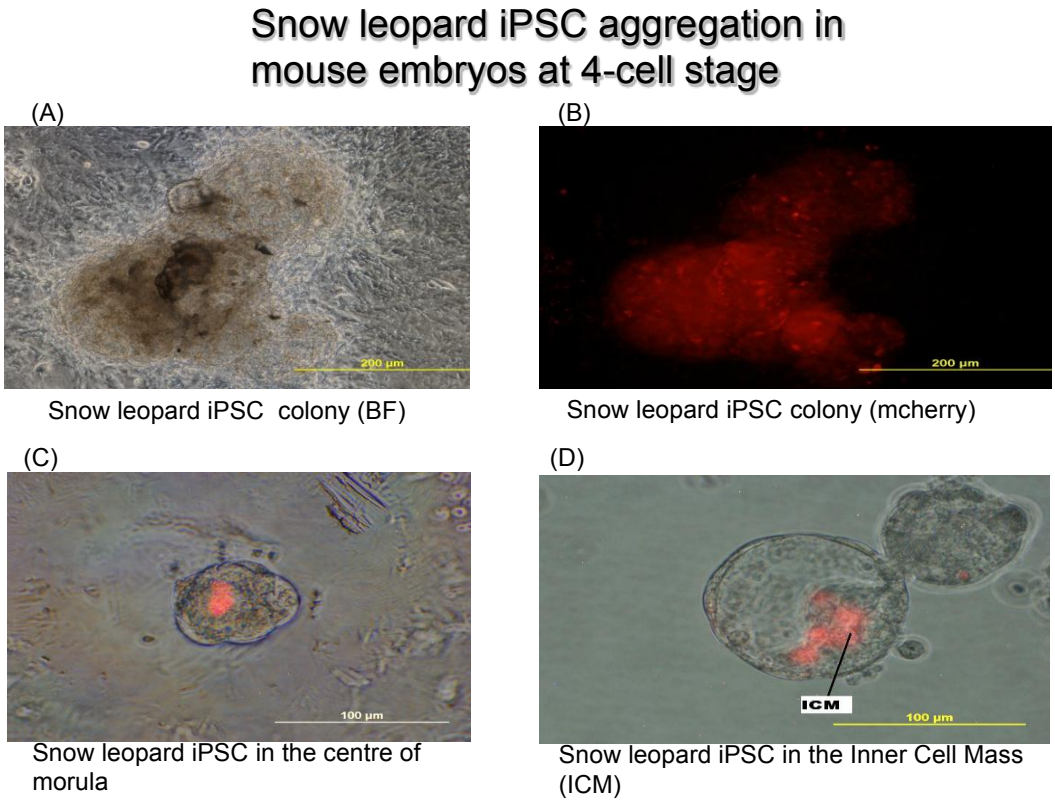

Figure 20: *Potential in-vivo assay for assessing snow leopard iPSC.*

Snow leopard iPSC tagged with mCherry reporter construct and aggregated with mouse embryos at the 4-cell stage (A) Snow leopard iPSC colony (Bright field), (B) Snow leopard iPSC colony expressing mCherry reporter, (C) Snow leopard iPSC localized into a morula and (D) Snow leopard iPSC seen to appear or localize in the ICM of hatching mouse blastocyst.

In conclusion, I believe this is the first report on the derivation of iPSC 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 iPSC 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, iPSC 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. HIV, arthritis and diabetes; (Verma *et al.* 2013).

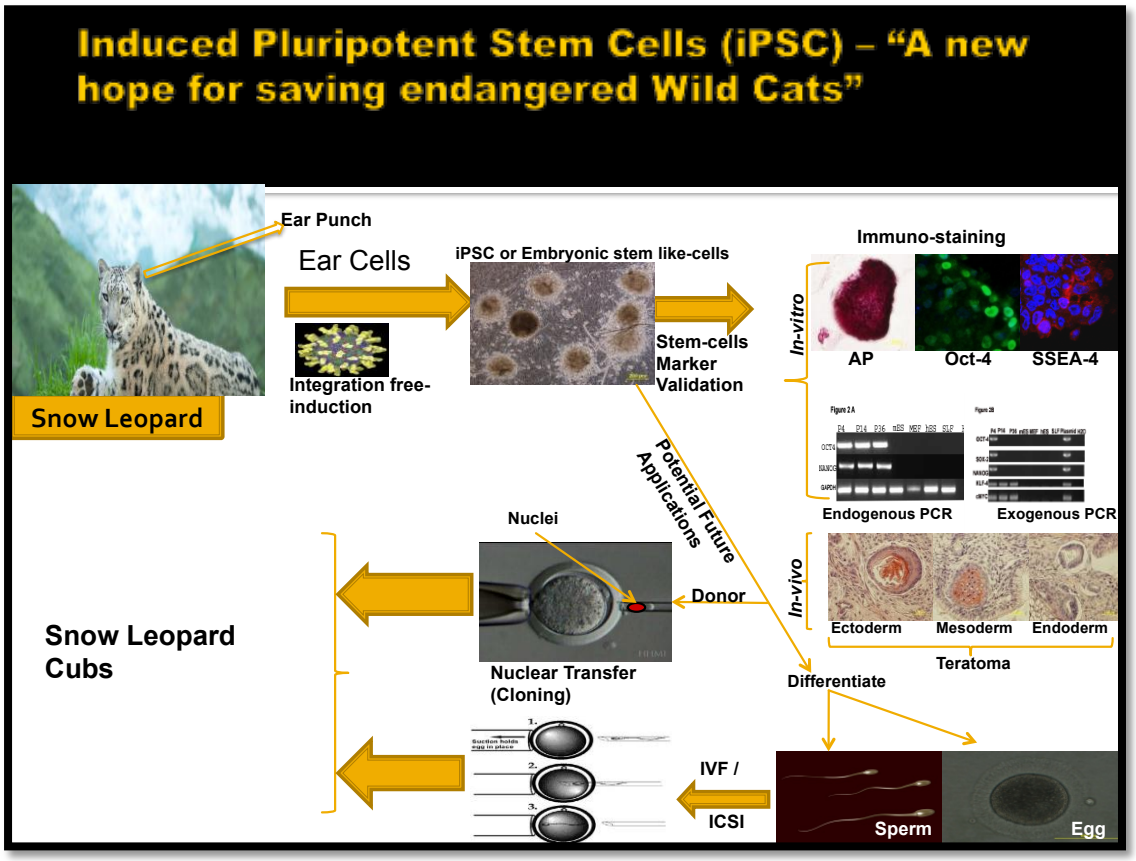

Figure 21: *Experimental layout of generating snow leopard iPSC and future applications.*

6.6. Future of iPSC in endangered species

If the many challenges associated with advanced embryo culture, finding the appropriate surrogate mother and synchronising the embryo to the uterus can be overcome, then pluripotent cells may well improve application of SCNT for producing viable offspring (Holt *et al.* 2004).

Generating sperm from iPSC derived from frozen somatic cell samples from long-dead animals would provide a way to infuse much needed genetic diversity using already proven AI methods. An analogous approach using pluripotent cell-derived oocytes could provide an endless resource for fundamental investigations into IVF, ICSI and SCNT (Holt *et al.* 2004).

There is also potential for these cells as a scalable resource of rare genetic material. Making iPSC for diverse species and populations available to every interested institution would accelerate research progress on analysing phylo-geographic structure, delineating subspecies, tracing paternities, evaluating gene flow and assessing genetic variation- information critical for decision–making in managing both *ex situ* and *in situ* wildlife populations (Pope 2000).

Therefore, wildlife–related studies will need to focus on fidelity measures for the reprogramming process to ensure the production of legitimate and 'fit' iPSC. As these are rare species and some methods for generating the cells reply on viral vectors, the process also has to be proven indisputably safe for offspring (Verma *et al.* 2013).

Chapter 7

7.1. Discussion and conclusion

Since the initial generations of murine iPSC, there have been numerous attempts to derive iPSC from a range of other species and as well as endangered species such as the rhinocerus and mandrill (Wildt & Roth 1997). However with the exception of rodents, primates and the pig, robust induction and reprogramming of somatic cells has not been reported, and to date the lack of silencing of inserted transgenes has been a hallmark of iPSC in the pig.

Nanog has an important role in pluripotency induction in bovines and snow leopards. To examine whether it was required for wild felids globally, we examined the induction of pluripotency in felids from Asia (Bengal tiger, *Panthera tigris*), Africa (serval, *Leptailurus serval*), and the Americas (jaguar, *Panthera onca*). Preliminary experiments examining the generation of wild cats iPSC with the 4 Yamanaka factors resulted in generation of putative iPSC colonies, however the colonies could not be maintained past 4-7 passages (12-21 days in culture), suggesting for wild cats that additional pluripotency associated factors may be required for robust generation of iPSC. This observation has recently been reported for pigs (West *et al.* 2010). In ear fibroblasts, when transfected with 5 human factors Oct-4, Sox-2, Klf-4, cMyc and Nanog (O, S, K, M, N), early morphological changes comparable with those seen in mouse iPSC were detected. These changes were observed, surprisingly, as early as day 2 post-infection, which enabled early detection of colonies and passage on to feeder layers. These colonies were subsequently routinely passaged enzymatically (0.5% Trypsin-EDTA) without losing their pluripotent characteristics, which is atypical for iPSC from most mammals with the exception of rodents. The cells were positive for alkaline phosphatase at P4, P14 and P36 and showed immunofluorescent staining for Oct-4, Nanog and SSEA-1 at P4 and P14. RT-PCR demonstrated high levels of expression of endogenous Oct-4 and Nanog using cat specific primers (Yu *et al.* 2009) that were shown not to amplify transcripts from the human transgenes. Expressions of all five inserted transgenes were observed at P4, suggesting limited reprogramming had occurred. By P14, transcripts from the inserted Oct-4, Sox-2 and Nanog were silenced but endogenous transcripts of Oct-4 and Nanog continued to be expressed, suggesting reprogramming of the pluripotency associated genes. However the transgenes for cMyc and Klf-4, which are associated with proliferation, a second key attribute of stem cells, were still being expressed.

This may suggest incomplete reprogramming of the proliferation capability by the wild cats iPSC. However, the reason for continued expression of the two exogenous transgenes in our cell line is unclear. Incomplete transgene silencing has also been described in human, rat and especially in pig iPSC lines (West *et al.* 2010).

In human cells, the addition of Nanog as a reprogramming factor to the 4 Yamanaka factors was shown to alter the growth and proliferation characteristics of resulting iPSC and in addition changed their dependence from bFGF to LIF. Interestingly LIF dependent HiPSC in this study formed colonies reminiscent of mouse ESC and spontaneously activated the inserted transgenes (Liu *et al.* 2011b).

Multilineage differentiation potential of iPSC of wild cats was clearly demonstrated histologically by the presence of representative tissues from the three primary germ layers in the teratoma that resulted from intramuscular injection of iPSC into the thigh of a SCID mice. Under *in vitro* differentiation conditions, embryoid bodies developed from all iPSC lines and randomly differentiated into ectoderm, endoderm and mesoderm as detected by RT-PCR of specific marker genes. This was an essential demonstration of the pluripotent potential of the wild cats iPSC produced.

In this study, the Oct4 gene was sequenced in Bengal tiger, snow leopard and jaguar and pairwise comparison of sequences showed that introns were less conserved in comparison to the exons. The 13 bp deletion ranging from 204-192 before start codon that was identified in the jaguar may be part of either 5' untranslated region or 5' proximal promoter region, however, further studies are required to demonstrate if this deletion could affect the expression of Oct4 in jaguar.

Bioinformatics analysis of the predicted Oct4 open reading frame showed that the deduced Oct4 protein is a 360 amino acid single polypeptide with a predicted molecular mass of 38.59 kDa and an estimated isoelectric point of 5.85. Although this study identified small differences in the genomic sequences of the tiger, leopard and jaguar Oct4 gene, the predicted translated proteins are identical with cat Oct4 protein. The 360 amino acid protein is common with the Oct4 orthologs in human, dog, cattle, pig, and monkey. Yu *et al.* (2009), however, reported a putative 272 amino acid protein based on a sequenced open reading frame of 819 bp in cat Oct4 which does not correlate with the current information on cat Oct4 available in NCBI. In contrast, our findings show that the 360 amino acid length was standard in all the three wild cats used in this study.

The results of alignment analysis showed that the coding sequence of the Oct4 gene from the tiger, leopard and jaguar have 52.45%-99.82% identity to the Oct4 gene of other selected species. Based on the constructed phylogenetic tree, felid Oct4 genes were located close to the *Canis* family, which belongs to the carnivore phylogeny. The Oct4 genes of rodents were clustered together and clearly separated from primates (Human and Monkey), with high bootstrap probability, which agrees with the known fact that they are, belong to Euarchontoglires (Margulies *et al.* 2007). These results are in agreement with the mammalian phylogenetic tree reported by (Margulies *et al.* 2007).

This thesis and the associated publications report the first derivation of iPSC from felids as well as from an endangered species. This is also the first report on the induction of pluripotency in a large mammal with concomitant silencing of the pluripotency-associated transgenes.

The study describes an effective method for inducing pluripotency in cells from large endangered felids and confirms Nanog as an essential factor in the reprogramming milieu for these species. As well as the important conservation implications, felids are also suitable models for studying diseases including HIV but lack of germ-line competent ESC has limited the ability for genome modification in the cat. The efficient production of iPSC from felids therefore creates a novel opportunity for species preservation through assisted reproduction and provides an alternative for elusive cat ESC for biomedical research. We propose this as a novel *in vivo* assay to assess the embryo contribution ability of iPSC and pluripotent cells from exotic species where species-specific testing is difficult because of the often extremely limited access to gametes, especially oocytes and embryos.

We report the first sequence of Oct4 gene in Bengal tiger, snow leopard and jaguar by using various bioinformatics tools to analyse the gene and predicted protein sequences and their phylogenetic relationships. Based on our results, although there were some differences between genomic DNA sequences of Oct4 gene of wild cats, the amino acid sequence of the putative protein was identical.

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