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From "ES-like" cells to induced pluripotent stem cells: A historical perspective in domestic animals

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Abstract

Pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) provide great potential as cell sources for gene editing to generate genetically modified animals, as well as in the field of regenerative medicine. Stable, long-term ESCs have been established in laboratory mouse and rat, however, isolation of true pluripotent ESCs in domesticated animals such as pigs and dogs have been less successful. Initially, domesticated animal pluripotent cell lines were referred to as "ES-like" cells due to similar morphological characteristics to mouse ESCs but accompanied by a limited ability to proliferate in vitro in an undifferentiated state. That is, they shared some but not all the characteristics of true ESCs. More recently, advances in reprogramming using exogenous transcription factors, combined with the utilization of small chemical inhibitors of key biochemical pathways, have led to the isolation of induced pluripotent stem cells. In this review, we provide a historical perspective of the isolation of various types of pluripotent stem cells in domesticated animals. In addition, we summarize the latest progress and limitations in the derivation and application of induced pluripotent stem cells.

Keywords

Pluripotent stem cells; Reprogramming; Large animals; Induced pluripotent stem cells

Introduction

Stem cells are characterized by their self-renewal capacity and the potential to differentiate into different cell types, and are generally categorized depending on the range of different cell types they can generate. Stem cells can range from unipotent stem cells, such as muscle progenitor cells [1] to multipotent or pluripotent stem cells. While pluripotent stem cells are able to differentiate into multiple tissues of all three germ layers, multipotent stem cells have more restricted differentiation potential [2]. Pluripotent stem cells includes cell lines isolated from developing embryos such as embryonic stem cells and epiblast stem cells (EpiSCs), or cell lines derived from genital ridges of developing fetuses (embryonic germ cells; EGCs). Embryonic stem cells are isolated from inner cell mass of pre-implantation stage embryo

while epiblast stem cells are derived from post-implantation stage embryos. Multipotent stem cells are isolated from adult tissues and include stem cells such as bone marrow derived mesenchymal stem cell (BM-MSC), and adipose derived mesenchymal stem cells (ADSC). While more restrictive in their ability to differentiate, they are easily collected and expanded allowing autologous transplantation; something that is much more difficult to do with ESCs and EpiSCs. More recently, induced pluripotent stem cells were generated by ectopic expression of various defined transcription factors in somatic cells and these cells were shown to have similar characteristics with embryonic stem cells in morphology, self-renewal and differentiation potency [3]. Their theoretical advantage is that they can be generated from essentially any starting cell type and reintroduced into the donor allowing autologous transplantation without the practical or ethical concerns of ESCs and EpiSCs.

Historical perspectives

Work related to identification of pluripotent stem cells in species other than mice was driven initially by the observations of Bradley et al. that mouse ESCs could contribute to the formation of the whole organism, including the germ line, when injected into the developing blastocysts [4]. This was quickly followed by the demonstration that ESCs could be genetically manipulated in vitro and used to generate germ line chimeras that could transmit the genetic modification to the next generation this allowing the establishment of transgenic mouse lines. [5–8]. This led to the work of Oliver Smithies and Mario Capechhi, showing for the first time that specific genes could be modified by the technique known as homologous recombination [9,10] these work eventually led to the Nobel Prize in Medicine in 2007 [11]. More recently, ESCs have been sub-categorized into “true” ESCs and EpiSCs, and into naïve and primed ESCs, respectively [12–14], with only naive pluripotent being able to contribute to chimeric offspring.

For those of us following the early work, it was obvious that the ES-based approach would have many applications in domestic animals and many of us embarked in a rather frustrating journey attempting to isolate and characterize ES and EG cells from a range of domestic animals [Reviewed by 15–18]. However, while the field has progressed over the last 30 years, we still do not have ESCs in any domestic species that can be considered practical for the generation of transgenic animals.

Domesticated animals such as dogs, pigs and cows are considered excellent models for long-term experiments in regenerative medicines, and biomedical research in general, because of their similarities in physiology with humans compared to the laboratory mouse or rat [19–21]. Furthermore, mice with targeted gene manipulation in most cases failed to reproduce typical human phenotypes [8, 22–24]. As a result, derivation of stem cell lines from large animals such as dogs, pigs, cattle, goats and horses has been of great interests as it benefits both clinical applications to improve human health, and agricultural applications. While there have been many reports of isolated stem cell lines, all of them without exception lack either convincing proof of pluripotency, or require such demanding methods for establishment and maintenance that they are, for all intents and purposes, impractical [Please see the following reviews for details; 15–18].

Another seminal development in the field, the development of somatic cell nuclear transfer (SCNT), by Keith Campbell and Ian Wilmut [25] drastically reduced the need for ESCs as a method to generate transgenic animals. Using SCNT, multiple cell types can, and have been, successfully used for the generation of transgenic and non-transgenic animals [26]. SCNT has been successful in species such as cattle [27], goats [28], pigs [29], cats [30], horses [31] and dogs [32]. While there would be additional benefits derived from a cell line that can remain stable over several rounds of single cell cloning and antibiotic selection (allowing

introduction of multiple genetic manipulations), existing cells lines are adequate for most applications. Thus, the impetus behind the generation of ESCs for the generation of transgenic animals has shifted. The decreased interest of ESCs for gene editing has been accompanied by a vigorous increase in interest in ESCs for application in regenerative medicine. This is an expanding field, and one in which domesticated animals, including dogs and cats, have a critical role to play. This review will focus on advances in the generation of induced pluripotent stem cells and their potential use in regenerative medicine.

Regenerative medicine and stem cells

Since therapeutic approaches based on regenerative medicine generally require large numbers of cells for transplantation, stem cells that can indefinitely self-renew, and have full differentiation potential, hold great promise in regenerative medicine. However, as described previously, attempts at isolating stable pluripotent ESCs in domestic animals have been plagued by difficulties. Additionally there are ethical concerns related to the isolation of ESCs in humans. As a result, others have developed various approaches that overcome ethical concerns and still generate ES or ES-like cell lines. The approaches includes ESCs derived from SCNT [33], reprogramming of cell to pluripotency by co-culture of cells with ESC extracts [34] or oocyte extract [35], cell fusion of differentiated somatic cells with pluripotent cells [36] and overexpression of defined transcription factors to generate induced pluripotent stem cells [3]. This last approach has been successful in developing stable lines in a range of species including humans, mice and rats.

In 2006, Takahashi and Yamanaka demonstrated that mouse embryonic fibroblasts (MEFs) can be reprogrammed into pluripotent state by defined exogenous transcription factors (Oct-4, Klf4, Sox2, and Myc; OKSM) and those cells were termed induced pluripotent stem cells [3]. The isolated iPSCs cells were able to self-renew, were pluripotent as shown by embryoid body formation *in vitro* and teratoma formation *in vivo*, and expressed ESC specific genes. It was also shown that they could also generate germ line chimeras in mice and rats, a key assay demonstrating that the iPSCs and ESCs had almost identical differentiation properties [37, 38]. iPSCs have now been generated in humans [38–40] and other species such as rhesus monkey [41] rat [42], pig [43], dog [44], cattle [45] and horse [46].

While generation of iPSCs in a wide range of species has been successful, there are still many issues to resolve before these cells can be used clinically in a safe and reproducible manner. For instance, transplants derived from iPSCs carry the risk of teratoma formation due to remaining undifferentiated populations of cells as well as the use of oncogenes such as Klf4 and c-Myc [47]. To avoid this risk, iPSCs have been developed using non-integrating vectors, including adenoviruses [48], plasmid vectors [49], small molecules [50], mRNA [51], minicircle DNA [52], episomal vectors [53], recombinant proteins [54] and transposons that integrate into the host genome, but can be subsequently excised [55]. These reprogramming approaches may provide a safe source of cells for future cell-based therapies, but reprogramming efficiency is still low [56]. Moreover, similar to experiences with ESCs, not all species respond similar to the reprogramming process as will be described later.

Of greater concerns for their use in regenerative medicine is their potential immunogenicity as a recent report. Zhao et al. demonstrated that iPSCs were immune-rejected or immunogenic in an autologous transplantation model when those cells were reprogrammed by retrovirus or episomal transfection, respectively [57]. The immune-rejection of iPSCs derived from a donor was an unexpected result as one of the purportedly advantages of iPSCs was autologous transplantation. Zhao et al. further identified nine genes commonly

expressed in the regressed teratomas derived from iPSCs and showed that the overexpression of three genes, Zg6, Hormad1 and Cyp3a11 interfere with teratoma formation when autologous ESCs are transplanted. The authors also confirmed that immune reactions are evoked by T-cell activation after transplantation [57]. However, Okita et al. also commented that Zhao et al. utilized only one line of embryonic stem cells to compare with iPSCs [58], and it has been shown that ESCs have wide range of diversity [59]. Also, undifferentiated iPSCs were used to induce teratoma formation which is not directly relevant to clinical applications [58]. Indeed, a recent study demonstrated that terminally differentiated cells derived from induced pluripotent stem cells do not form a tumor or increase immune responses [60]. In addition, another study also demonstrated that iPSCs derived from MEFs or host bone marrow dendritic cells do not induce immune-rejection in autologous host [61]. Additional researches are needed in multiple lines and species to conclusively demonstrate and develop safe iPSCs that are not immunogenic in an autologous system, and can be used in regenerative medicine. Due to the difficulties in carrying this type of research in human patients, there is a great need for adequate humanized animal models that will allow this type of research to progress at a fast pace.

In terms of differentiation ability, iPSCs have been shown to differentiate into multiple tissues of all three germ layer cells but, as for ESCs, it still is difficult to generate specific cell types with high purity and quality. Thus, before the application of iPSCs considered as safe and efficient, multiple issues have to be resolved. This is one of the reasons why the development of multiple large animal models for the study of iPSCs is very critical as this will allow the identification of both barriers to safe clinical application and methods to overcome those barriers [62].

Induced pluripotent stem cells in pigs

Pigs have been widely used as a model for preclinical studies because of their similarity in size and physiology to humans. Pigs have a longer lifespan than laboratory mice, allowing long-term studies related to disease progression with age. They also have similar organ size and physiology to human making them an appropriate source of organs for xenotransplantation [63, 64]. As a result, there have been many studies focused on the derivation of pig ESCs [65–70]. To date, none of the lines have yet shown either ease of manipulation and maintenance, or required pluripotency criteria such as germ-line transmission in vivo.

Generation of pig iPSC lines, in contrast, has been more successful. The techniques used to generate induced pluripotent stem cells from mouse and human were also suitable in pigs and several groups have reported derivation of pig iPSCs utilizing various cell sources, culture conditions and reprogramming methods (Table 1). Embryonic/Fetal fibroblasts are the most widely used [43, 71–76] while other cell sources such as adult fibroblasts from ear [77–79], bone marrow [77] and mesenchymal stem cells [80] have also been utilized. Both mouse [76, 78, 79] and human [71, 73, 74, 77] transcription factors were able to reprogram the cells into pluripotency. It was also shown that both mouse and human transcription factors could be used to reprogram the same fibroblasts without differences in morphology, or AP expression [77]. Two additional factors, NANOG and LIN28 [72, 80], or NANOG only [75] were also used in combination with OKSM. Most of studies have utilized viral vectors such as retrovirus [71, 73, 78, 79], lentivirus [43, 80] or DOX-inducible lentivirus [75, 77]. Single transfection [72] or repeated transfection methods [76] were also utilized using either episomal or regular plasmids, respectively. Combinations of small molecules inhibitors of mitogen-activated protein kinase (MEK; PD0325901), GSK-3 beta (CHIR99021) [72], FGF receptor (PD173074) [74] or Forskolin alone [73] were able to reprogram and maintain porcine iPSCs in the presence of LIF. The isolated primed iPSCs

resemble human ESCs or mouse EpiSCs in their morphology, gene expression profiling and FGF dependency on maintaining pluripotency [71, 77–79] while naïve-like iPSCs that resemble mouse ESCs in morphology and LIF-dependency were derived using small molecule inhibitors [72–74].

Phenotypically, all of the reported pig iPSC lines express alkaline phosphatase and core pluripotency markers such as OCT4, SOX2 and NANOG [43, 72–76, 79]. Additional markers such as LIN28 [43, 73] and REX1 [74, 76] were also utilized for pluripotency markers. However, pig iPSCs have shown varied expression patterns of the surface markers SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Table 2). SSEA-4 was most commonly expressed, while other surface markers showed variations. This is of concern because it shows the high heterogeneity of the cells being generated by the different induction and maintenance methods. This high heterogeneity makes clinical application difficult, if not impossible.

Embryoid body (in vitro) and teratoma formation (in vivo) or production of a chimeric offspring is now routinely utilized to confirm pluripotency in mouse pluripotent stem cell studies. Pluripotency test by formation of embryoid bodies in vitro and teratoma formation in vivo are also commonly applied to swine while the production of chimeric animals is very limited. In swine, embryoid bodies and teratomas with derivatives of three germ layers (endoderm, mesoderm and ectoderm) have been reported in most studies [43, 72, 77–79]. A recent study reported that teratoma was not formed presumably due to the primed state of derived iPSCs [76]. Chimeric embryo formation was also reported by injecting into parthenogenetic morula, but only shown at the blastocyst stage embryos and efficiency was low (3/29) [73]. Only one study to date has been able to report germ-line transmission via blastocyst injection, but the efficiency was very low (2/34) and the lack of GFP expression (one of the markers utilized) suggests that the cells did not behave as expected for a true naïve pluripotent stem cells [80]. Moreover, one of two chimeric piglets was stillborn and another one died after three days of birth suggesting epigenetic problems during the reprogramming process.

A more disturbing result is the lack of full silencing of the reprogramming factors. Silencing of transduced exogenous factors is a critical marker to distinguish fully reprogrammed cells [81]. In addition, it was shown in a recent study that iPSCs harboring transcriptionally active exogenous transcription factors fail to produce cloned piglets by SCNT, suggesting they are not ideal SCNT donor cells [82]. Only after exogenous transcription factors are silenced through differentiation or treatment of embryos with Scriptaid, can the pig iPSCs produce cloned animals [82]. However, many of the reported porcine iPSCs do not demonstrate complete gene silencing during expansion [71, 73, 74, 78] or showed dependency on DOX induced expression of exogenous factors to stay proliferative and undifferentiated [75, 77]. Two independent studies using different transfection approaches also reported continued transgene expression [72] or transgene genome integration [76] suggesting that maintenance of pig iPSCs, under the culture conditions utilized to date, require exogenous transcription factors.

Though there have been extensive efforts to establish embryonic stem cells from pigs and many reports have demonstrated promising results, truly pluripotent ESCs with a capability of germ-line transmission have not been achieved yet. Pig iPSCs have shown great achievement in isolation of pluripotent cell lines and will serve as an ideal replacement of ESCs as well as a key model to elucidate pluripotency networks.

Induced pluripotent stem cells in dogs

Dogs are excellent models for human diseases as they have similar size and physiology to humans, have breed-associated genetic predispositions to certain disorders, and suffer from many of the same maladies as humans [83]. Many genetic diseases such as Alzheimer's disease, retinal atrophy, muscular dystrophy, cancer, obesity, cardiovascular diseases and diabetes mellitus affect dogs [84, 85]. Thus, establishment of well-defined dog pluripotent stem cells will provide powerful tools not only for the development of therapeutic approaches such as cell transplantation, but also experimental applications for studying human diseases.

The first canine ESC lines were established by using the media conditions used for mouse and human [86–89] but the isolated ESC lines lost pluripotency in long term culture, and failed to form tumors when transplanted into immunodeficient mice. The first dog ESCs capable of teratoma formation was reported in 2008 [90], and the isolation of dog ESC lines required the presence of both LIF and FGF2.

Generation of dog iPSCs can be an alternative for dog ESCs. There have been five reports on the production of dog iPSCs, and three of them used adult skin fibroblasts of various age as the initial cells [91–93]. One study reported the generation of iPSCs from embryonic fibroblasts [44], and another utilized testicular fibroblasts [94]. Both embryonic and skin fibroblasts were able to be reprogrammed into pluripotency by either retro- or lenti-viral transduction of dog [44], human [91, 94] or mouse [93] factors. With the exception of one cell line that required only LIF [91], all other isolated canine iPSCs required both FGF and LIF [44, 91, 93, 94]. The LIF-dependent cell line was reprogrammed using OKSM plus LIN28 and NANOG [92], while the other cell lines utilized only OKSM [44, 91, 93, 94]. Small molecule inhibitors, PD0325901, CHIR99021 and A83-01 were used during the induction of pluripotency [44] or maintenance of established cell lines [93]. In vitro differentiation capacity has been demonstrated by the formation of embryoid bodies [91, 93, 94], but there were few reports of in vivo teratoma formation [91, 93] while germ cell-like tumor was formed when LIF-dependent dog iPSCs were transplanted into SCID mouse [92].

Similar to pig iPSCs studies, positive staining for AP and expression of pluripotency markers OCT4, SOX2 and NANOG was commonly used to identify their pluripotency [44, 91–94]. Cell surface markers expression varies among cell lines. SSEA-4 expression is reported as positive in some lines [91, 92, 94], but not others (Table 2) [93]. In addition, transduced transcription factors still remain positive when examined at different passages [92–94] but may became silent after extended passage (P26) [92].

As for pig iPSCs, current results indicate a high degree of variance in the phenotype and behavior of the isolated iPSCs. An equally disturbing observation is the lack of genetic stability of ESC and iPSC lines as we have reported for dogs [93] and others have reported for mice and humans as will be discussed later in more detail.

Induced pluripotent stem cells in cows

Bovine iPSCs have been generated from fetal fibroblasts [45, 95] and skin fibroblasts [96, 97] by retroviral transduction [45, 96], lentiviral transduction [97] and virus-free poly-promoter vector [95]. Transcription factors cloned from cow [45, 95], human [45, 96] or the combination of human and pig [97] have all been used. iPSCs generated by OKSM (bovine or human) were not stable and could only be passaged 6 times [45]. Only bovine iPSCs reprogrammed using bovine OKSMNL were able to proliferate long term and to show transgene silencing in iPSC-derived SCNT blastocysts [45]. It has also been shown that reprogrammed cells are able to proliferate for longer periods only when NANOG is added to

the OKSM reprogramming factors [96]. However, exogenous transcription factors were still active in parallel to other domestic species [45, 96, 97]. Though there was a report of silencing of exogenous transcription factors from cloned embryos [45], more comprehensive tests in additional lines are needed. In vitro (EBs) and in vivo (teratoma) assays were utilized to test their pluripotency in all studies [45, 95–97]. Both FGF and LIF were used to culture the isolated iPSCs [96, 97] and those cells could be passaged more than 40 passages with normal karyotype [97]. Small molecules, PD0325901 and CHIR99021, with N2/B27 medium were also able to generate LIF dependent bovine iPSCs [95] by using episomal virus free vector. All of the reports demonstrated the positive staining for alkaline phosphatase and the expression of OCT4, SOX2 and NANOG [45, 95–97].

Thus, similar to swine and dogs, bovine iPSCs were successfully generated using the same methodologies used in other species. Isolated bovine iPSCs were able to proliferate long term and differentiate into all three germ layers in vitro and in vivo. However, full silencing of exogenous transcription factors, chimera generation, germ line transmission, and chromosomal instability still need to be further demonstrated.

Genomic instability of pluripotent stem cells

Of the many issues still to be resolved for successful clinical application of iPSCs is their long-term genetic stability in vitro and in vivo. To date, karyotyping protocols and resolution varies a lot, as only traditional G-banding methods have been utilized for karyotyping pluripotent stem cells from domestic species. All of the iPSC lines were reported to be normal in pigs (2N=38), dogs (2N=78) and cows (2N=60) except a report in pigs showing a paracentric inversion of chromosome 16 not present in the parental fibroblasts [43]. Karyotyping with standard banding analysis has the advantage of being low-cost and fast, however, it is limited in resolution especially when chromosomes are relative small as those of dogs and cows. Furthermore, it has been shown by multiple groups that extended passages of human and mouse ESCs and iPSCs frequently induces chromosomal aberrations [98–101]. And those results suggest that it would be important to develop and utilize approaches with higher sensitivity and stringency such as array comparative genomic hybridization (aCGH) to carefully monitor the integrity of the genome during the isolation and maintenance of the cell lines.

It has been suggested that genomic instability in pluripotent stem cells can be caused by various reasons such as pre-existing mutations in parental fibroblasts, viral integration induced DNA damage or replicative stress during in vitro expansion [102]. It has also been demonstrated that there is a higher incidence of mutations such as copy number variations (CNVs) [103] and single nucleotide polymorphisms (SNPs) [104] in the iPSC genome compared to embryonic stem cells. In addition, it has been shown that genomic mutations are caused by expansion in culture in both iPSCs and ESCs [101]. However, Hussein et al. also reported that genomic rearrangements in human iPSCs disappear after extended culture due to selective disadvantages and become homogenous populations [103]. Koh et al. tried to determine the true degree of chromosomal instability in dog iPSCs by utilizing high resolution comparative genomic hybridization (aCGH) combined with fluorescent in situ hybridization (FISH) [93]. And similar phenomenon of gained chromosomal aberrations to those seen in human and mouse pluripotent stem cells were also observed in dog iPSCs. Additionally, high resolution karyotyping revealed that four chromosomes (CFA4, 8, 13 and 16) exhibit copy number increases. Analogous to human iPSC studies, where chromosomes 1, 9 and 12 are gained at high passages (>P40) and human ESCs, where chromosomes 12, 17 and X are prone to karyotypic abnormalities [98, 100, 105]. Collectively, trisomy of chromosome 12 was one of the most common aneuploidy in both human ESCs and iPSCs and the gains are thought to appear upon extended culture during the adaptation period. In

mice, analysis of 127 independent iPSC lines identified a high incidence of chromosome 8 and 11 trisomy and the aberrations were already found from early passages [106]. By syntenic comparison of the affected chromosomes in mouse, human and dog, several regions affected in all three species were identified [93]. However, due to the low amount of available data, especially in large animal studies, we cannot determine whether those chromosomal regions are indicating truly common hotspots of aneuploidies shared by many mammals.

In short, standard cytogenetic techniques such as G-banding analysis have a limitation in their reliability and resolution. For example, domestic dogs have 38 pairs of very small, acrocentric/telocentric chromosomes and it is technically not possible to identify specific chromosome by banding analysis alone [107]. The combined application of array-CGH and FISH provides an analysis tool to overcome those limitations and to evaluate cell lines prior to be used in regenerative medicine.

Conclusions

It has been more than 30 years since the first embryonic stem cell line was isolated in mouse, yet derivation of germ-line competent embryonic stem cells from domestic animals have been not accomplished. Recent advances in cellular reprogramming and availability of iPSCs from different cell sources will provide an opportunity to understand pluripotency networks and to elucidate differences between species. This in turn, is likely to lead to advance in the long-term maintenance of a wide range of stem cell including ESCs. The ability of stem cells to self-renew and differentiate into specific cell types also provide unique opportunities in the field of regenerative medicine as long as issues related to immunogenicity and long term chromosomal stability can be resolved.

List of abbreviations

aCGH	Array comparative genomic hybridization
ADSC	Adipose derived mesenchymal stem cell
BM-MSC	Bone marrow derived mesenchymal stem cell
CNV	Copy number variation
DOX	Doxycycline
EB	Embryoid body
EGC	Embryonic germ cell
EpiSC	Epiblast stem cell
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FISH	Fluorescent in situ hybridization
GFP	Green fluorescence protein
iPSC	Induced pluripotent stem cell
KLF4	Kruppel-like factor 4
KSR	Knockout serum replacer

LIF	Leukemia inhibitory factor
MEF	Mouse embryonic fibroblast
OCT4	Octamer binding transcription factor-4
OKSM	OCT4, KLF4, SOX2 and c-Myc
SCNT	Somatic cell nuclear transfer
SOX2	Sex determining region Y-box 2

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Table 1

Summary of iPSCs generated from domestic animals

Species	Cell sources	Basal media	Media supplements	Feeder layers	In vitro differentiation	In vivo differentiation	Reprogram method	Karyotype / Results	References
Dog	Embryonic fibroblasts	Primate ES medium (ReproCELL, Japan)	bFGF, hLIF, VPA, PD0325901, CHIR99021, A83-01	MEFs	None	None	Retrovirus (Canine OKSM)	None / None	[44]
	Testicular fibroblasts (7 mos)	DMEM/F12, 15% KSR	bFGF, hLIF	MEFs	EB	None	Lentivirus (Human OKSM)	G-banding / Normal	[94]
	Adipose stromal cells, Skin fibroblasts (1 yr)	Knockout DMEM 20% ES qualified FBS	bFGF, hLIF	MEFs	EB	Teratoma	Lentivirus (Human OKSM)	G-banding / Normal	[91]
	Dermal fibroblasts (adult)	Knockout DMEM, 20% KSR	mLIF	MEFs	None	Germ cell-like tumor	Lentivirus (Human OKSMLN)	G-banding / Normal	[92]
	Skin fibroblasts (3 yrs)	DMEM/F12, 20% KSR	bFGF, hLIF, PD0325901, CHIR99021	MEFs	EB	Teratoma	Retrovirus (Mouse OKSM)	Array-CGH and FISH /chromosome gains, CFA4, 8, 13 and 16	[93]
Pig	Primary ear fibroblasts Primary bone marrow (10 wks)	DMEM/F12, 20% KSR	Need continuous DOX	MEFs	EB	Teratoma	DOX-inducible Lentivirus (Human OKSM	G-banding / Normal	[77]
	Embryonic fibroblasts (D37)	DMEM, 10% / 15% FBS	bFGF with 10% FBS or mLIF with 15%FBS	MEFs	None	Teratoma	Retrovirus (Human/Mouse OKSM)	G-banding / Normal	[71]
	Fetal fibroblasts	Knockout DMEM, 20% KSR	bFGF, 4% O_2	MEFs	EB	Teratoma	Lentivirus (Human OKSM)	G-banding / Paracentric inversion, Ch16	[43]
	Mesenchymal stem cells	mTeSR1 (StemCell)		Matrigel	EB	Germline chimera	Lentivirus (Human OKSMLN)	None / None	[80]
	Embryonic fibroblasts	DMEM/F12, 20% KSR	bFGF or LIF, PD0325901, CHIR99021, VPA	MEFs	EB	Teratoma	Episomal plasmids (Human OKSMNL)	None / None	[72]
	Ear fibroblasts (6 mos)	1:1 mix of hES (20% KSR) and mES 20% (FBS) media	bFGF, LIF	MEFs / 0.1% gelatine	EB	Teratoma	Retrovirus (Mouse OKSM)	G-banding / Normal	[78]
	Ear fibroblasts (6 mos)	1:1 mix of hES (20% KSR) and mES (20% FBS) media	bFGF, LIF	MEFs / 0.1% gelatine	EB	Teratoma	Retrovirus (Mouse KSM)	G-banding / Normal	[79]
	Embryonic fibroblasts	Knockout DMEM, 20% KSR	pLIF (conditioned medium), forskolin	Collagen I	EB	Chimeras	Retrovirus (Human OKSM)	G-banding / Normal	[73]
	Fetal fibroblasts	DMEM, 10% FCS	mLIF, N2B27, PD0325901, CHIR, PD173074, Need continuous DOX	MEFs	EB	None	DOX-inducible Lentivirus (Human OKSM)	Normal	[74]
	Fetal fibroblasts	Knockout DMEM, 15% KSR	bFGF, Doxycycline	MEFs	EB	None	DOX-inducible Lentivirus (Human OKSMN)	None	[75]
	Embryonic fibroblasts	DMEM/F10, 15% FBS	bFGF, hSCF	MEFs	EB	None	Transfection (Mouse OKSM)	G-banding / Normal	[76]
Cow	Fetal fibroblasts	DMEM, 20% KSR	bFGF	MEFs	EB	Teratoma	Retrovirus (Bovine OKSM, Bovine OKSMLN, Human OKSM)	G-banding / Normal	[45]

Species	Cell sources	Basal media	Media supplements	Feeder layers	In vitro differentiation	In vivo differentiation	Reprogram method	Karyotype / Results	References
	Skin fibroblasts	MEMα, 20% FBS, ITS	Bovine FGF, hLIF	MEFs	EB	Teratoma	Retrovirus (Human OKSMN)	G-banding / Normal	[96]
	Fetal fibroblasts	1:1 Mix of DMEMF/F12 + N2 and Neurobasal + B27	hLIF, PD0325901, CHIR99021	MEFs	EB	Teratoma	Episomal virus-free poly-promoter vector (Bovine OKSM)	G-banding / Normal	[95]
	Skin fibroblasts (2.5–4 mos)	DMEM, 15% FBS	LIF, bFGF	MEFs	EB	Teratoma	Lentivirus (Human O+ Pig KSM)	G-banding / Normal	[97]

Table 2

Summary of pluripotency-related marker expressions

Species	Stem Cell Markers											References
	AP	OCT4	SOX2	NANOG	LIN28	REX1	SSEA-1	SSEA-3	SSEA-4	TRA-1–60	TRA-1–81	
Dog	+	+										[44]
		+	+	+	+				+			[94]
	+	+	+	+					+	+		[91]
	+	+		+		+			+	+	+	[92]
	+	+	+	+	+	+	+		−			[93]
Pig	+	+	+	+	+	+	−	+	+	+	+	[77]
	+		+	+	+	+			+			[71]
	+	+	+	+	+		+	−	−	−	−	[43]
	+	+	+						+			[80]
	+	+	+	+			+	−	+			[72]
	+			+					+	+		[78]
	+	+	+	+				+	+	+	+	[79]
	+	+	+	+	+		+	+	+	+	+	[73]
	+	+	+	+		+	+					[74]
	−	+	+	+			+	−	+	−	−	[75]
	+	+	+	+		+			+	+	+	[76]
Cow	+	+	+	+			+		+ (Weak)	−	−	[45]
	+	+	+	+		+	+		+			[96]
	+	+	+	+				+	+	+	+	[95]
	+	+		+			+					[97]