

# Large animal induced pluripotent stem cells as pre-clinical models for studying human disease

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## Abstract

The derivation of human embryonic stem cells and subsequently human induced pluripotent stem cells (iPSCs) has energized regenerative medicine research and enabled seemingly limitless applications. Although small animal models, such as mouse models, have played an important role in the progression of the field, typically, they are poor representations of the human disease phenotype. As an alternative, large animal models should be explored as a potentially better approach for clinical translation of cellular therapies. However, only fragmented information regarding the derivation, characterization and clinical usefulness of pluripotent large animal cells is currently available. Here, we briefly review the latest advances regarding the derivation and use of large animal iPSCs.

**Keywords:** induced pluripotent stem cells • large animal iPS • disease modelling • bovine • canine • porcine • primate

## The path to induced pluripotency

The isolation of human embryonic stem cells (ESCs) in 1998 stimulated rapid progression of the field of regenerative medicine research [1]. The ability for ESCs to divide endlessly and to differentiate into all body tissues excited researchers and the public alike. However, ESC research also has been plagued by moral and ethical concerns surrounding the use of human embryos [2]. To bypass past such controversies, scientists have derived human ESC equivalents from adult somatic cells. These novel approaches include fusion protocols involving a combination of a pluripotent donor cell or oocyte with a somatic cell [3–5], methods based on pluripotent cell extracts [6–8], and somatic cell nuclear transfer (SCNT) [9–11]. SCNT has been successful in animal cloning, most notably leading to the birth of Dolly the sheep [12]. Due to its need for large numbers of human oocytes, SCNT has been deemed an unethical and

unsustainable method of reprogramming by many [13]. Ultimately, it was through the seminal work of Takahashi and Yamanaka that a sophisticated yet simple reprogramming method was invented and implemented [14]. By up-regulating OCT4, SOX2, cMYC and KLF4, Yamanaka and colleagues showed that it is possible to ‘reprogram’ mouse and human somatic cells, effectively inducing pluripotency and leading to the derivation of iPSCs [15, 16]. Through reprogramming, researchers have not only avoided the bulk of the moral and ethical problems surrounding pluripotent cell research, but have also opened the door to the possibility of patient-specific regenerative medicine. However, before iPSCs can realize their clinical potential safely, much research is still required. To that end, large animal iPSCs may help usher in a new era of patient-specific cell therapies to the clinic.

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## Discovery of a pan-species pluripotency network

Although Yamanaka's initial approach to reprogram murine cells in 2006 appeared to successfully result in fully pluripotent cells, the iPSCs produced were not germline competent [14]. Subsequently, Yamanaka's group was able to overcome this hurdle and generate murine iPSCs with germ line competency [15]. Around the same time, Jaenisch's group reported viable chimaeras and germline transmission from murine iPSCs, confirming Yamanaka's initial findings and setting the remarkably fast pace that has since defined the field of iPSC research [17]. Following this major milestone, murine iPSCs have been shown to go beyond chimerism and actually give rise to entire progeny through tetraploid complementation, currently the most stringent test of a cell's pluripotency [18–20]. For obvious ethical reasons, this level of stringency is not an assay of human iPSCs or ESCs. Interestingly, the same four Yamanaka factors that were shown to push mouse somatic cells to a fully pluripotent state can also reprogram human somatic cells [16]. A parallel report from Thomson's group showed that while OCT4 and SOX2 were key factors, cMYC and KLF4 could be replaced by the potentially less oncogenic factors, NANOG and LIN28. This and other studies show that the basic pluripotency network is conserved across species, but may not necessarily be regulated in precisely the same way [21].

The discovery of a pan-species, potentially universal pluripotency network that is catalyzed by a common set of factors led researchers to derive iPSCs from monkeys, rats, pigs, sheep, dogs, cows and, most recently, the endangered white rhinoceros, with reprogramming of other mammalian species likely to be underway [22–31]. Although rodents have traditionally been the most common research models for studying human genetic diseases and *in vivo* cell therapies, the derivation of larger animal iPSCs now makes it possible to model autologous cell therapies in animal systems that more closely resemble those of the human body. Several groups have used mouse models to study, ameliorate, and, in some cases, even cure diseases, such as sickle cell anaemia [32], haemophilia [33], diabetes [34], Parkinson's disease [35] and cardiovascular diseases [36]. However, small animal models are limited in their usefulness clinically. For example, while studying heart disease in mice can provide many useful insights, the results are unlikely to be as clinically relevant as those from larger animals (*e.g.*, dogs, pigs and primates), whose lifespan and cardiac physiology are more similar to a human's [37]. See Table 1 for an overview of large animal iPSC derivation.

## Animal iPSCs and disease modelling

The mouse heart beats over twice as fast as the human heart and is therefore under different shear stresses from the human heart and vasculature [37]. Furthermore, the relatively short lifespan of mice (typically <2 years) is often a key limiting factor, and studies have shown that mouse cardiomyocytes are fundamentally different in

design, reducing the relevancy of human heart therapies tested on them [38]. In part to address this need for large animal *in vivo* studies of cardiac disease and injury, we derived canine iPSCs from canine fibroblasts and canine adipose stromal cells (Fig. 1). We then transplanted autologous iPSCs into the *same* animal and followed fate of transplanted iPSCs using positron emission tomography reporter gene imaging and iron oxide labelling by magnetic resonance imaging [30]. As anticipated, transplanting iPSCs in a large animal model was a significant challenge. However, these cells did demonstrate therapeutic potential while shedding light on the specific hurdles of large animal iPSC transplantation, namely the difficulties involved in *in vivo* imaging. Undoubtedly, further studies will be required to further optimize both the imaging protocols and iPSC biology to allow successful translation of pluripotent stem cell based therapies to human patients in the future.

Primates are arguably the best large animal model for comparison with human disease phenotypes. Although both primate ESCs and iPSCs have been previously derived, the use of primates for transplantation experiments remains controversial [39]. In fact, many groups are simply using large animal iPSCs for transplantation in the more traditional mouse model. Zhu *et al.* reported the generation of insulin-producing pancreatic cells from rhesus monkey iPSCs in 2011, yet only tested their efficacy in a diabetic mouse model [40]. Similarly, Zhong *et al.* used genetically modified non-human primate iPSCs in a SCID mouse model to show that suicide genes can be conditionally activated to eliminate pluripotent cells *in vivo*, while leaving the pluripotent state and health of the host unaffected [41]. Although such studies provide clues to how iPSCs might behave following transplantation in humans, transplantation of autologous iPSCs or their derivatives using large animal models would be more insightful by providing better pre-clinical safety data needed to progress towards human clinical trials.

Due to cultural sensitivities, the use of primates and canines as disease research models has generated controversy. The porcine model is less afflicted by the same problems and may prove to be an easier large animal model for pre-clinical iPSC transplantation. Having the appropriate organ size, physiology and lifespan, but with greater ease and availability compared with non-human primates, pigs can serve as a powerful pre-clinical research tool. Porcine iPSCs can be matched to specific pigs, making them essentially 'patient-specific', and can be differentiated into specific lineages. These qualities make it possible to use pigs to test transplantation therapies with iPSCs for safety and efficacy before applying the procedures to human patients. In addition to these favourable characteristics of the porcine model, pigs have a long history of medical and therapeutic use such as their role in providing replacement cardiac valves and insulin [42]. Moreover, in terms of pluripotency, reports of porcine ESCs and iPSCs indicate that they may be more similar to human ESCs and iPSCs than their murine counterparts. Both are stereotypically flat with clearly identifiable nuclei and nucleoli and have a high nucleus-to-cytoplasm ratio, comparable colony morphology, dependence on basic fibroblast growth factor (bFGF), and typically express stage specific embryonic antigen 4 (SSEA-4) on their surface [24, 25, 27, 43, 44]. Unlike pig ESCs, mouse pluripotent stem cells exhibit a more 'rounded up' morphology, require leukaemia inhibitory factor (LIF) to

**Table 1** Large animal iPSC derivation overview

	Date and author	Parental cell source	Feeder layer	Special culture conditions	Differentiation (in vitro)	Differentiation (in vivo)	Markers	Reprogramming factors
Canine iPSCs	2009 Shimada <i>et al.</i>	Canine embryonic fibroblasts	MEFs	6 ng/ml hbFGF, 1000 U/ml hLIF, 1 mM VPA, 0.5 mM PD0325901, 3 mM CHIR99021, 0.25 mM A83-01	AFP, FLK1, Beta III-tubulin positive cells	None	AP, Oct3/4	Retrovirus Canine OKSM
Porcine iPSCs	2009 Lee <i>et al.</i>	Canine fibroblasts, Canine adipose stromal cells	MEFs	5 ng/ml hbFGF, 1000 U/ml hLIF	Embryoid bodies, Endothelial cells	Teratomas	AP, Oct3/4, Sox2, Nanog, TRA-1-60, and SSEA-4	Lentivirus Human OKSM
Porcine iPSCs	2009 Ezashi <i>et al.</i>	Porcine foetal fibroblasts	MEFs	4 ng/ml hbFGF	Embryoid Bodies	Teratomas	AP, SSEA-1, Oct3/4, Nanog, Sox2	Lentivirus Human OKSM
Porcine iPSCs	2009 Esteban <i>et al.</i>	Tibetan miniature pig fibroblast	MEFs	4 ng/ml hbFGF, mLIF, PD0325901, CHIR99021, 39°C	None	Teratomas	AP, Nanog, Sox2, Klf4, Oct3/4, Rex1, Lin28, SSEA4	Retrovirus Mouse and Human OKSM
Sheep iPSCs	2009 Wu <i>et al.</i>	Primary ear fibroblast and primary bone marrow cells	MEFs	DMEM/F12 + 20% KSR + DOX	Embryoid bodies	Teratomas	AP, SSEA3, SSEA4, Tra-1-60, Tra-1-81, Oct3/4, Nanog, Sox2, Rex1 and CDH1.	DOX inducible Lentivirus Human OKSM + Nanog, Lin28
Sheep iPSCs	2010 West <i>et al.</i>	Porcine mesenchymal stem cells	1. MEFs 2. Matrigel	1. DMEM + 20% KSR + 10 ng/ml hbFGF 2. mTeSR1	Embryoid bodies	Chimeric offspring	Nanog, SSEA-4, TRA-1-60	Retrovirus Human OKSM + NANOG, LIN28
Bovine iPSCs	2010 Montserrat <i>et al.</i>	Pig ear fibroblasts	MEFs or Gelatin only	hES/mES media (1:1) 10 ng/ml hbFGF, 1000 U/ml LIF	Embryoid bodies	Teratomas	Nanog, SSEA-4, TRA-1-60	Retrovirus pMXs-OKSM-GFP
Bovine iPSCs	2010 Li <i>et al.</i>	Sheep foetus fibroblast	MEFs	FBS instead of KSR	Embryoid bodies	Teratomas	AP, Oct3/4, Sox2, Nanog, SSEA-4	DOX inducible lentivirus Mouse OKSM
Bovine iPSCs	2011 Sumer <i>et al.</i>	Bovine adult fibroblast	MEFs	10 ng/ml bovine bFGF, 4 ng/ml hLIF, 39°C	Embryoid bodies	Teratomas	AP, Rex1, Oct3/4, Sox2, Nanog, c-Myc, Klf4, SSEA1, SSEA4	Retrovirus OKSM + Nanog

Table 1 Continued

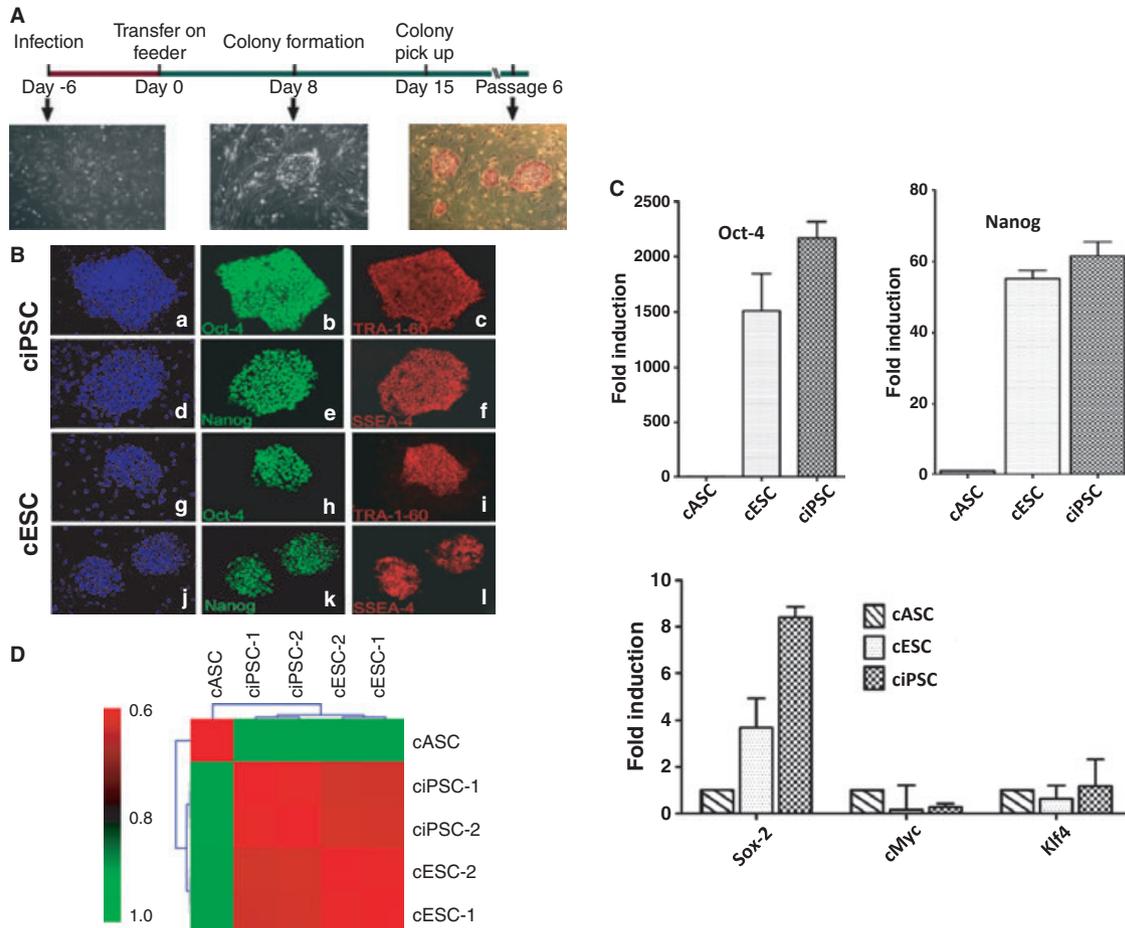
	Date and author	Parental cell source	Feeder layer	Special culture conditions	Differentiation (in vitro)	Differentiation (in vivo)	Markers	Reprogramming factors
Non-human primate iPSCs	2008 Liu <i>et al.</i>	Adult rhesus macaque ear fibroblast	MEFs	KSR medium	Embryoid bodies	Teratomas	AP, Nanog, Oct3/4, Sox2, SSEA4, TRA-1-60, TRA-1-81	Retrovirus Monkey OKSM
	2010 Zhong <i>et al.</i>	Macaca nemestrina oral fibroblasts	MEFs	16 ng/ml hbFGF, 0.5 mM PD0325901, 2 mM VPA, and 10 mM Y-27632	Embryoid bodies Neural precursors, Cardiomyocytes, Hepatocytes	Teratomas	Oct3/4, SSEA3, SSEA4, TRA-1-60, TRA-1-81, Nanog	Retrovirus OKSM
	2011 Deleidi <i>et al.</i>	Cynomolgus macaque skin fibroblasts	MEFs	KSR medium	Neurons	Teratomas	AP, Nanog, Oct3/4, Sox2, Klf4, cMyc, TRA-1-60, TRA-1-81, SSEA4	Retrovirus hOKSM

AP: alkaline phosphatase; bFGF: basic fibroblast growth factor; FBS: foetal bovine serum; KSR: knockout serum replacement; LIF: leukaemia inhibitory factor; MEF: mouse embryonic fibroblasts; VPA: valproic acid; MEF: mouse embryonic fibroblast.

maintain pluripotency, and express SSEA-1 on their surface, an early marker of differentiation on human ESCs and iPSCs [45, 46]. These differences are likely tied to the differing pathways that govern pluripotency and self-renewal between mouse and human ESCs. In the mouse, the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway, regulated by addition of LIF, and bone morphogenetic proteins, found in serum, are the dominant signalling pathways. By comparison, in human the MEK-ERK pathway is activated by bFGF and transforming growth factor beta signalling is more crucial to maintenance of pluripotency [47]. Although no animal model can fully recapitulate the human disease phenotype, greater effort should be made to ensure that the best models are used for basic research to obtain the most relevant and insightful data while limiting animal use.

## Issues with large animal iPSCs

Although human and mouse ESCs and iPSCs are well defined, most other animal models have suffered from a general lack of reliable iPSC and ESC markers. For instance, reports of canine pluripotent cell derivation have shown that the surface marker expression (such as SSEA-1, SSEA-3, SSEA-4, TRA-1-81 and TRA-1-60) varies, despite the common expression of OCT4, SOX2 and NANOG [48–50]. Ungulates, such as pig and cow, also show inconsistencies in surface marker expression [51], and there is a further complication in that porcine and bovine blastocysts show expression of primary pluripotency genes and surface markers, such as OCT4 and SSEA-4, in both the inner cell mass (ICM) and the trophectoderm. Furthermore, as recently reported by Ezashi *et al.*, the same process which results in pig iPSCs also produces the by-product of trophectoderm-like cells. Like iPSCs, these trophectoderm-like cells can grow seemingly limitlessly in iPSC culture conditions, have high expression of telomerase and a subset of pluripotency genes, making them difficult to distinguish from iPSCs following reprogramming [52]. In addition to problems with characterization, multiple groups have shown that continuous passaging of human ESCs and iPSCs frequently results in chromosomal abnormalities, sometimes even within as few as 20 passages. This last finding suggests that long-term culture of large animal iPSCs may result in similar abnormalities, and therefore should be monitored carefully for culture-induced genetic changes [53–55]. In addition, reports also differ on what surface markers porcine iPSCs may express. Although SSEA-1 is clearly associated with pluripotency in murine cells, it has been shown to be an early marker of differentiation in pluripotent human cells. Interestingly, with ungulates such as pigs and cows, SSEA-1 expression varies. In the bovine blastocyst, SSEA-1 and SSEA-4 are expressed on both the inner cell mass, from which ESCs are derived, as well as the trophectoderm cells. Similarly, pig ESCs have been reported as SSEA-1 positive and SSEA-4 negative [24]; however, another group reported contradictory results of SSEA-4 positive and SSEA-1 negative pig iPSCs [56]. The key may lie in the differences in epiblast development, with different groups reprogramming cells towards different points in development, hence requiring different culture conditions and displaying varying marker profiles.



**Fig. 1** Generation of canine induced pluripotent stem cells (ciPSCs). **(A)** Schematic diagram of the generation of ciPSCs. ciPSC colonies can be picked out approximately 12–15 days and are alkaline phosphatase-positive. **(B)** Immunofluorescence staining of pluripotent markers. Similar to cESCs, ciPSCs are positive for pluripotent stem cell markers Oct-4 (*b* and *h*), Tra-1-60 (*c* and *i*), Nanog (*e* and *k*) and SSEA-4 (*f* and *l*), with nuclear staining by DAPI (*a*, *d*, *g* and *j*). **(C)** Quantitative PCR analysis of expression of pluripotent stem cell markers Oct-4, Nanog, Sox-2, c-Myc and Klf-4. Y-axis value represents fold differences (log<sub>2</sub>) in expression of select genes. **(D)** Pearson correlation analysis for gene expression in cASCs, ciPSCs and cESCs using transcripts with SD <0.2 among all samples (17,895 probes,  $P < 1.0E-15$ ). Reprinted with permission from Lee *et al.* [30].

## Conclusions

Despite the rapid progress of the field, iPSCs are difficult to derive from most large animals and there is a general lack of effective reprogramming protocols. Furthermore, more work is needed to develop reliable differentiation protocols capable of becoming different lineages such as neuronal, cardiac, endothelial and hepatic cells. Although no animal study can truly compare with a human study, every effort should be made to ensure that the model system is as close to the human system as possible, particularly when translational medical research is the goal, which is often the case. With this in mind, large animal models should and can play a more significant role in translational research, but they are often overlooked due to difficulty and/or cost. This is especially true with primates

because, although they are arguably the most comparable to humans, they typically require special transport, handling and care. Other large animals such as dogs, sheep and pigs offer many of the same benefits, but are simpler in terms of acquisition and care. However, the various differences in pluripotency networks among species remain a hurdle, making maintenance of large animal iPSCs challenging. Furthermore, it has been our experience that imaging and tracking cells following transplantation is quite difficult in large animals, but since this issue is analogous to humans, it will continue to be an important area to focus on in the future. Following further elucidation of the mechanisms of reprogramming, and improvements in iPSC derivation techniques, new methods to simplify and facilitate characterization of iPSC lines will become possible in the future. New technologies, including non-integrating, xeno-free reprogramming strategies and

genome-wide epigenetic profiling, will make further progress possible in this exciting field.

application; we apologize to those investigators whom we omitted here. This study was supported by the NIH HL089027 and NIH EB009689 (JCW), NIH RC1 HL100490 (MTL) and T32 Training Grant (JRP).

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Due to space limitations, we are unable to include all of the important papers relevant to large animal induced pluripotent stem cell derivation and

## Conflict of interest

The authors confirm that there are no conflicts of interest.

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