The quest for pluripotency: a comparative analysis across mammalian species

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Abstract

Pluripotency is the developmental potential of a cell to give rise to all the cells in the three embryonic germ layers, including germline cells. Pluripotent stem cells (PSCs) can be embryonic, germ cell or somatic cell in origin and can adopt alternative states of pluripotency: naïve or primed. Although several reports have described the differentiation of PSCs to extra-embryonic lineages, such as primitive endoderm and trophoctoderm, this is still debated among scientists in the field. In this review, we integrate the recent findings on pluripotency among mammals, alternative states of pluripotency, signalling pathways associated with maintaining pluripotency and the nature of PSCs derived from various mammals. PSCs from humans and mouse have been the most extensively studied. In other mammalian species, more research is required for understanding the optimum *in vitro* conditions required for either achieving pluripotency or preservation of distinct pluripotent states. A comparative high-throughput analysis of PSCs of genes expressed in naïve or primed states of humans, nonhuman primates (NHP) and rodents, based on publicly available datasets revealed the probable prominence of seven signalling pathways common among these species, irrespective of the states of pluripotency. We conclude by highlighting some of the unresolved questions and future directions of research on pluripotency in mammals.

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Introduction

The zygote is a single cell, endowed with the amazing potency to develop into an entire organism that harbours extreme complexity (reviewed in [Leung & Zernicka-Goetz 2015](#)). The most important aspect is the cell fate decisions during its development to form an embryo. In terms of development, pluripotency is a transitory state exhibited by specialised cells in the blastocyst, for a very short period of time, possessing the ability to differentiate into all the cells within the three embryonic germ layers, including the germline cells ([Adjaye et al. 2005](#); reviewed in [Weinberger et al. 2016](#)). PSCs can be derived from the pre-implantation (embryonic stem cells (ESCs)), post-implantation embryos (epiblast stem cells (EpiSCs)), primordial germ cells (PGCs) (embryonic germ cells (EGCs)) or somatic cells (somatic cell nuclear transfer (SCNT)-derived ESCs, induced PSCs (iPSCs)). Embryonic germ cells (EGCs) share similar properties with ESCs; however, they sometimes exhibit imprint erasure (reviewed in [Leitch & Smith 2013](#)).

Under *in vitro* conditions, pluripotent stem cells (PSCs) can beget all the cell types of embryonic and extra-embryonic lineages (reviewed in [Beddington &](#)

[Robertson 1989](#), [Xu et al. 2002](#), [Pera et al. 2004](#), [Das et al. 2007](#), [Niakan et al. 2010](#), [Sudheer et al. 2012](#), [Leitch & Smith 2013](#), [Morgani et al. 2013](#), [Roberts et al. 2014](#), [Kojima et al. 2017](#)). The potency of PSCs in giving rise to cells of the extra-embryonic lineage was first reported in mice, this was based on the presence of colonisation pattern of ESCs in trophoctoderm (TE) of chimeras ([Beddington & Robertson 1989](#)). Later on, the competency of hPSCs to differentiate into extra-embryonic cells, such as trophoblast and primitive endoderm when exposed to BMP4 was described ([Xu et al. 2002](#), [Pera et al. 2004](#), [Das et al. 2007](#), [Sudheer et al. 2012](#), [Horii et al. 2016](#), [Kojima et al. 2017](#)). In spite of several publications from different groups on the differentiation ability of human PSCs to extra-embryonic lineages, these protocols and concept are still debated among scientists in the field ([Roberts et al. 2014](#)). Our understanding of pluripotency is on a surge, as several coordinated molecular events underlying pluripotency and differentiation are being discovered, such as the role of signalling pathways, transcription factors, epigenetic factors (DNA and histone modification) and non-coding RNAs ([Davidson et al. 2002](#), [Babaie et al. 2007](#), [Brons](#)

et al. 2007, *Tesar et al.* 2007, *Thomson et al.* 2011, *Sudheer et al.* 2012, *Guo et al.* 2017; reviewed in *Li et al.* 2017).

It has been more than three decades since the description of the derivation of mouse PSCs and now, we are capable of reprogramming somatic cells into induced pluripotent stem cells (iPSCs) (*Evans & Kaufman* 1981, *Thomson et al.* 1998, *Takahashi & Yamanaka* 2006, 2007, *Yu et al.* 2007). The nature and state of PSCs may change with respect to the donor organism, the developmental stage, the cell of origin (embryonic, germ or somatic) and the culture conditions (*Kerr et al.* 2006, *Dejosez & Zwaka* 2012, *Martello & Smith*

2014; reviewed in *Weinberger et al.* 2016). Different cell culture conditions can influence the state of the derived PSCs, which can also lead to failure in capturing the developmental state, from which the cells were originally derived.

According to the specific developmental stage of the early embryo or the *in vitro* culture conditions, PSCs can exist in at least two distinct states – naïve (ground) or primed, which differ in both morphology and molecular characteristics (*Fig. 1* and *Tables 1, 2*) (*Greber et al.* 2007b, *Ying et al.* 2008, *Nichols & Smith* 2009, *Sudheer et al.* 2016). Naïve pluripotency is the earliest state of pluripotency (ground state), closely resembling

Figure 1 Embryonic pluripotency in mouse and humans.

Table 1 Comparative features of naïve and primed states of pluripotency.

	Features	
	Naïve state of pluripotency	Primed state of pluripotency
Donor cells	ICM of pre-implantation blastocyst	Epiblast cells of post-implantation blastocyst
Gene expression profiling	<i>Oct4</i> , <i>Nanog</i> , <i>Sox2</i> , <i>Klf4</i> , <i>Esrrb</i> , <i>Tbx3</i> , <i>Tfcp2l1</i> , <i>Gbx2</i>	<i>Oct4</i> , <i>Sox2</i> , <i>Fgf5</i> , <i>Pou3f1</i> , <i>Meis1</i> , <i>Sox11</i>
Chimera-forming ability	Yes	No
Cellular respiration profile	Oxidative phosphorylation, glycolysis	Glycolysis
X-chromosome status	XaXa	XaXi
Global DNA methylation	Hypomethylation	Hypermethylation
Response to 2i	Self-renewal	Differentiation
Response to LIF/Stat3	Self-renewal	None
Response to Fgf/Erk	Differentiation	Self-renewal
OCT4 enhancer utilisation	Distal	Proximal

embryonic epiblast in the pre-implantation blastocyst, in which the cells possess an unbiased ability to form all the embryonic lineages, including germline cells and are capable of giving rise to germline chimeras. On the other hand, primed pluripotency, closely resembling *in vivo* immediate post-implantation epiblast, is a later state of pluripotency, in which the cells are primed towards lineages ectoderm, mesoderm and endoderm (the three germ layers) and lack germline chimera-forming capability. The two states differ in various characteristics, including gene expression, cellular respiration or metabolism, X-chromosome status, DNA methylation, OCT4 enhancer utilisation and germline chimera formation capability (Fig. 1 and Tables 1, 2) (Ying *et al.* 2008, Nichols & Smith 2009; reviewed in Sudheer *et al.* 2016, Weinberger *et al.* 2016). Soon after the discovery of the two states, scientists showed that PSCs are interchangeable between the primed and naïve states, by using specific culture conditions and over-expression of the pluripotency factors via transgene expression (Babaie *et al.* 2007, Bao *et al.* 2009, Guo *et al.* 2009, Hanna *et al.* 2009).

In this review, we discuss the nature of PSCs, the states of pluripotency and the comparative analysis between PSCs of humans, NHP and rodents. In this regard, we performed a comparative analysis of three transcriptome datasets, one from each species (Human (GSE75868); Theunissen *et al.* 2016), crab-eating macaque (*Macaca fascicularis* (GSE69708); Chen *et al.* 2015) and mouse (GSE99494) (Bao *et al.* 2018), to identify common signalling pathways, irrespective of species differences. To obtain comparable numbers of expressed genes in human and mouse (Affymetrix data), detection *P* value <0.001 was used as gene expression threshold. For next-generation sequencing (NGS) data from the monkey, a gene expression threshold of FPKM > 1 was considered. The analysis revealed both similarities and differences in terms of gene expression and led to the identification of seven signalling pathways presumably active in PSCs of all three species. Although these pathways are known to have important roles in maintaining pluripotency, this analysis provides clues to the evolutionary conservation of their roles as gatekeepers of pluripotency and thus warrants attention.

Pluripotency and its states

During development, the totipotent zygote gradually loses its flexibility and transforms into an embryo. The polarisation in a developing embryo begins from the eight-cell stage blastomeres (in case of mouse), continues in the morula stage and ends in the first differentiation and lineage segregation, of the inner cell mass (ICM) and trophectoderm (TE) in the blastocyst stage (Kelly 1977; reviewed in Nichols & Smith 2012; reviewed in De Paepe *et al.* 2014, Wu & Schöler 2016). Expression of pluripotency-associated genes – *Oct4* and *Nanog* – in the ICM continues only up to somitogenesis *in vivo* and in contrast, pluripotency can be maintained indefinitely *in vitro*, thus enabling the manipulation and directed differentiation of PSCs into specific cell lineages found in a developing embryo (Brink *et al.* 2008, Osorno *et al.* 2012; reviewed in Smith 2017).

The discovery of self-renewal and the multipotent nature of embryonic carcinoma cells (ECCs) paved the way for a new era of stem cell research (Solter *et al.* 1970, Stevens 1970). Malignant teratomas contain ECCs, which show overlapping features with PSCs, but are not bona fide PSCs – they are the stem cell component of teratocarcinomas, which are derived from germ cell tumours (GCTs) (Andrews *et al.* 2005, Greber *et al.* 2007a, Jung *et al.* 2010). Earlier studies on ECCs led to the successful isolation and culture of mouse ESCs (mESCs) (Evans & Kaufman 1981).

ESCs are generally derived from blastocyst and efficient derivation of clonal embryonic stem (ES) cell lines can only be from mid- and late blastocyst stages (Boroviak *et al.* 2014). However, ESCs have also been successfully derived from various pre-implantation stages and from single blastomeres (Delhaise *et al.* 1996, Tesar 2005, Chung *et al.* 2006, Wakayama *et al.* 2007), resulting in ESCs with similar characteristics (reviewed in Nichols & Smith 2012). Other than the PSCs derived from embryos, they can also be derived from somatic cells through somatic cell nuclear transfer (SCNT-derived ESCs) or artificial reprogramming (induced pluripotent stem cells – iPSCs) (Takahashi & Yamanaka 2006, Tachibana *et al.* 2013). The ECCs derived from PGCs are similar to ESCs and are considered as naïve PSCs, but bona fide ECCs

Table 2 Details of pluripotent stem cells obtained from different mammals and their responses towards various culture conditions.

PSCs	Culturing conditions*	State of pluripotency shown by cells in response to different signalling pathways					Transgenes expressed	Status of X-chromosome	Potential of chimera formation	References
		JAK/STAT (exogenous LIF)	BMP	MEK/ERK	TGFβ/activin A	FGF				
Human	2i, LIF	Naïve	Naïve	Naïve (inhibition)	Primed (long term)		OCT4, KLF4 or KLF4, KLF2	Pre-inactivation		Hanna <i>et al.</i> (2010)
	2i, LIF, Forskolin	Naïve	Naïve (LIF dependent)	Naïve (inhibition)	Primed		OCT4, SOX2, KLF2 ± MYC, LIN28	Pre-inactivation	Yes (in mouse host embryos)	Gafni <i>et al.</i> (2015)
	2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, TGFβ	Naïve	Naïve (LIF dependent)	Naïve (inhibition)	Primed					
	2i, LIF, PKCi, MEF	Naïve		Naïve (inhibition)			NANOG, KLF2	Pre-inactivation		Takahima <i>et al.</i> (2015)
	2i, LIF, SAHA, HDACi, FGFRi, Activin A, FGF2	Naïve		Naïve (inhibition)	Primed		OCT4, KLF4, KLF2	Pre-inactivation	Yes	Ware <i>et al.</i> (2014)
	2i, LIF, JNKi, p38i, TGFβ, bFGF, MEF	Naïve	Naïve (LIF dependent)	Naïve (inhibition)	Primed		NANOG, KLF2			Warrier <i>et al.</i> (2017)
	2i, LIF, tankyrase inhibitor	Naïve	Naïve	Naïve (inhibition)	Naïve			Pre-inactivation		
	2i, BRAFi, ROCKi, SRCi, LIF, Activin A, MEF (FGF2, JNKi)	Naïve	Naïve	Naïve (inhibition)	Primed	Naïve	OCT4, SOX2, KLF4	Pre-inactivation	Yes (in Mouse host embryos)	Zimmerlin <i>et al.</i> (2016)
	FGF2, TGFβ, 2i, BMPi, LIF, MEF	Naïve		Naïve (inhibition)	Primed		DOX-inducible KLF2, NANOG	Pre-inactivation	Yes (in Mouse host embryos)	Theunissen <i>et al.</i> (2014)
	FGF2, TGFβ or FGF2, Activin A or FGF2, FBS or FGF2, MEF (Primed conditions)	Primed	Primed (LIF independent)	Naïve (inhibition)	Primed	Primed		Inactivated	No	Theunissen <i>et al.</i> (2016)
Rhesus Monkey	2i LIF, bFGF or 2i, LIF, bFGF, JNKi, p38i	Naïve		Naïve (inhibition)		Naïve	OCT4, KLF4	Pre-inactivation		Fang <i>et al.</i> (2014)
	Activin A, bFGF, GSK3i, IWR1, ROCKi, MEF		Primed		Naïve	Naïve	OCT3/4, SOX2, KLF4, L-MYC, LIN28			Zhang <i>et al.</i> (2017)
	bFGF, MEF or BMP4, Activin A, IWR1 (Primed conditions)	Primed	Primed	Primed	Primed	Primed		Inactivated		
	2i, LIF, p38i, JNKi, PKCi, FGF2, TGFβ, Vit-C	Naïve		Naïve (inhibition)				Pre-inactivation	Yes	Chen <i>et al.</i> (2015)
Cynomolgus Monkey	2i, LIF, Forskolin, MEF	Naïve		Naïve (inhibition)			KLF2, NANOG			Honda <i>et al.</i> (2017)
	bFGF, FBS, MEF	Primed	Primed	Primed	Primed	Primed		Inactivated		

Mouse	2i, LIF	naïve	Naïve (inhibition)	Naïve	naïve	<i>Klf4</i>	Pre-inactivation	Yes	Guo <i>et al.</i> (2009)
	LIF, FBS	Naïve	Naïve (inhibition)	Naïve	Naïve	<i>Oct4, Sox2, Klf4, c-Myc</i>	Pre-inactivation	Yes	Hanna <i>et al.</i> (2010)
	3i, LIF	Naïve	Naïve (inhibition)	Naïve	Naïve		Pre-inactivation	Yes	Ying <i>et al.</i> (2008)
	SRCi, GSK-3β	Naïve	Naïve (inhibition)	Naïve	Naïve		Pre-inactivation	Yes	Shimizu <i>et al.</i> (2012)
	LIF, BMP4	Naïve	Naïve (inhibition)	Naïve	Naïve		Pre-inactivation	Yes	Onishi <i>et al.</i> (2014)
	2i, LIF, PKCi	Naïve	Naïve (inhibition)	Naïve		<i>Oct4, Sox2, Klf4, c-Myc</i>			Dutta <i>et al.</i> (2011)
	Activin A, BMP4, CH, LIF	ASCs*	ASCs (inhibition)	ASCs		<i>Oct4</i>		Yes	Bao <i>et al.</i> (2018)
	FGF2, Activin A or FGF2, TGFβ	Primed	Primed	Primed	Primed		Inactivated		
Rat	2i, LIF, MEF	Naïve	Naïve (inhibition)	Naïve	Naïve			Yes	Chen <i>et al.</i> (2013)
	LIF, MEKi, PKCi	Naïve	Naïve (inhibition)	Naïve	Naïve	<i>Oct4, Sox2, Klf4, c-Myc</i>		Yes	Rajendran <i>et al.</i> (2013)
	2i, LIF	Naïve	Naïve (inhibition)	Naïve	Naïve	<i>Oct4, Sox2, Klf4, c-Myc</i>			Sherstyuk <i>et al.</i> (2017)
	2i/3i, LIF	Naïve	Naïve (inhibition)	Naïve (inhibition)	Naïve (inhibition)	<i>Oct4, Sox2, Nanog</i>	Pre-inactivation	Yes	Buehr <i>et al.</i> (2008)
	3i, LIF	Naïve	Naïve (inhibition)	Naïve (inhibition)	Naïve (inhibition)	<i>Oct3/4, Klf4, Sox2</i>		Yes	Hamanaka <i>et al.</i> (2011)
	FGF2, Activin A or FGF2, TGFβ	Primed	Primed	Primed	Primed		Inactivated		
Chimpanzee	FGF2, IGF, ROCKi, MEF					<i>OCT3/4, SOX2, KLF4, L-MYC, NANOG</i>			Romero <i>et al.</i> (2015)
Common marmoset	LIF, MEF					<i>OCT3/4, SOX2, KLF4, c-MYC, NANOG, LIN28</i>			Tomioka <i>et al.</i> (2010)
	bFGF, ROCKi, MEF					<i>OCT4, SOX2, KLF4</i>			Wu <i>et al.</i> (2010)

Some cells have been left blank due to unavailable/unclear information on those specific details.

*Growth factors, inhibitors and small molecules which affects the behaviour of the cells. *Advanced pluripotent stem cells (ASCs) derived from EpiSCs. 2i, 2 inhibitors (MEK inhibitor (PD03259010) GSK3 inhibitor (CHIR99021)); ALK5i, TGFβ pathway inhibitor; bFGF, basic fibroblast growth factor; BMPi, bone morphogenetic protein inhibitor; DOX, doxycycline; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; FGFRi, fibroblast growth factor receptor inhibitor; GSK3i, glycogen synthase kinase 3 inhibitor; HDACi, histone deacetylase inhibitor; IGF, insulin-like growth factor; IWR1, WNT pathway inhibitor; JNKi, Jun-like kinase inhibitor; LIF, leukaemia inhibitory factor; MEF, mouse embryonic fibroblasts; p38i, p38 inhibitor; PKCi, protein kinase C inhibitor; ROCKi, RHO-associated protein kinase 1 inhibitor; SAHA, suberoylanilidehydroxamic acid; SRCi, MEK1/2 inhibitor; TGFβ, transforming growth factor-β.

having naïve pluripotency have been derived only from rat and mouse till now (Matsui *et al.* 1992, Resnick *et al.* 1992, Shambloott *et al.* 1998, Mueller *et al.* 1999, Leitch *et al.* 2010; reviewed in Nikolic *et al.* 2016).

Mouse PSCs of embryonic origin can be derived from pre-implantation blastocyst (E3.5 (3.5-day post coitum)), termed ESCs and post-implantation blastocyst (E5.5–E7.5), termed EpiSCs (Evans & Kaufman 1981, Martin 1981, Thomson *et al.* 1998, Brons *et al.* 2007, Tesar *et al.* 2007). Pre-implantation ESCs are in a naïve state of pluripotency, possessing germline chimera-forming ability and the post-implantation EpiSCs are in a primed state of pluripotency, and are more restricted, not being able to form germline chimeras (Fig. 1 and Tables 1, 2) (Ying *et al.* 2008, Nichols & Smith 2009; reviewed Sudheer *et al.* 2016, in Weinberger *et al.* 2016). Naïve PSCs tend to grow as dome-shaped colonies and can be passaged as single cells, thus facilitating clonal propagation. Furthermore, they can give rise to germline chimeras, when introduced into pre-implantation blastocysts (Nichols & Smith 2009). In contrast, primed PSCs (mEpiSCs and hESC) form flat colonies but also give rise to germline chimeras (Fig. 1 and Tables 1, 2) (Thomson *et al.* 1998, Brons *et al.* 2007, Tesar *et al.* 2007). The common characteristics shared between naïve (mESC) and primed PSCs (mEpiSCs and hESC) include the properties of pluripotency, teratoma formation and embryoid body formation. Numerous pluripotency-associated genes are expressed in common in these cells, but there are also differences in the expression profiles of several genes such as Klf4, Esrrb, Tbx3, Tfcp2l1, Gbx2, Fgf5, Pou3f1, Meis1 and Sox11 (Table 1) (Nichols & Smith 2009).

In earlier days, mESCs were considered to be similar to the ICM of the early blastocyst, but more recent studies have shown that ESCs are more similar to the epiblast of a pre-implantation blastocyst than the ICM of an early blastocyst (Boroviak *et al.* 2014). Mouse ESCs can be maintained on feeder layer using a medium containing leukaemia inhibitory factor (LIF), supplemented with serum (LifS condition), which is the 'conventional' culture condition for the derivation of mESCs (Evans & Kaufman 1981, Smith *et al.* 1988, Williams *et al.* 1988, Niwa *et al.* 1998, 2009). However, the efficiency of mESC derivation in this condition is higher for permissive strains, such as 129, but not for non-permissive strains (Kawase *et al.* 1994, Suzuki *et al.* 1999). Depending on the efficiency of mESC derivation in LifS condition, mouse strains have been classified as permissive or non-permissive. Mouse ESCs can be maintained under feeder-free conditions in the presence of LIF and two small molecules, that inhibit GSK3 β (CHIR99021) and ERK/MAPK signalling (PD0325901) (2i/LIF condition or popularly known as the 2i Media) (Ying *et al.* 2008). The 2i/LIF condition promotes naïve pluripotency and makes the mESC derivation from

non-permissive strains also highly efficient (Ying *et al.* 2008, Nichols *et al.* 2009). LIF, a cytokine, which activates Stat3, induces mitochondrial respiration and the activity of several transcription factors (Oct4, Nanog, Sox2) that have a decisive role in the gene regulatory network of naïve pluripotency (Kawase *et al.* 1994, Suzuki *et al.* 1999, Ying *et al.* 2008, Nichols *et al.* 2009, Carbognin *et al.* 2016).

In place of 2i/LIF, which supports naïve pluripotency, when mESCs are continuously exposed to bFGF/ACTIVIN A, they develop to a primed state of pluripotency, which is similar to mEpiSCs derived from post-implantation embryo (Thomson *et al.* 1998, Brons *et al.* 2007, Tesar *et al.* 2007). Mouse EpiSCs can be converted into a naïve state by maintaining them in a 2i/LIF condition, along with over-expression of Klf4 (Guo *et al.* 2009). Hence, the states of pluripotency, naïve and primed, are inter-convertible, based on the growth conditions/factors used, in both human and mouse (Table 2, Thomson *et al.* 1998, Brons *et al.* 2007, Tesar *et al.* 2007, Guo *et al.* 2009, Gafni *et al.* 2015).

Pluripotency in mammals other than primates and rodents

So far, other than human and mouse, many studies have concentrated on the derivation of PSCs from mammals such as mink, pig, bovine, rabbit, cat, dog, sheep, goat and horse (Sukoyan *et al.* 1993, Li *et al.* 2003, Telugu *et al.* 2010; reviewed in Ezashi *et al.* 2016, Ogorevc *et al.* 2016, Osteil *et al.* 2016). In most of these cases, ESCs have either not been tested for or do not satisfy all the criteria for pluripotency (Fig. 1, Tables 1, 2 and Supplementary data 1, see section on supplementary data given at the end of this article). Therefore, the characteristics of the cells derived from these mammals are not completely defined. In contrast to ESCs, iPSCs derived from these animals have been shown to satisfy many of the criteria for pluripotency, except the germline chimera formation ability (Supplementary Table 1).

Mink (a semi aquatic mammal) ESCs and iPSCs have been derived from different developmental stages, such as morula, blastocyst (Sukoyan *et al.* 1993) and embryonic fibroblasts (Menzorov *et al.* 2015) respectively. However, the characterisation of mink PSCs was only based on methods such as X chromosome status, glucose-6-phosphate dehydrogenase (G6PD) activity, embryoid bodies (EB), teratoma formations and RNA-seq analysis. The mink PSCs were shown to express pluripotency-associated markers, along with lineage-specific markers, such as Otx2 and Gata6, which hint towards a primed state, but these studies lack clarity. Despite this progress, there is a need for a more elaborate characterisation of mink ESCs with respect to the signalling pathways required to isolate and maintain them in culture and their ability to form germline chimeras.

ESCs and iPSCs generated from rabbit depend on FGF2 and show features of primed pluripotency (Tables 1, 2 and Supplementary data 1) (Schmaltz-Panneau *et al.* 2014). When exposed to LIF, they show naïve-like characteristics (Tables 1, 2 and Supplementary data 1) (Honsho *et al.* 2015, Osteil *et al.* 2016, Taponnier *et al.* 2017). Rabbit PSCs (rbPSCs) do not depend on LIF, neither LIF withdrawal nor JAK inhibition leads to differentiation in the presence of feeders (Taponnier *et al.* 2017). But, RbESCs can be converted to a naïve-like state, using LIF, along with forskolin and CHIR990021 (Tables 1, 2 and Supplementary data 1) (Honsho *et al.* 2015), but failed to form germline chimeras, the golden standard indicative of naïve pluripotency. However, there is still a lack of clear understanding of the signalling pathways that support naïve and primed states of pluripotency of rbPSCs and this could be addressed with elaborate experiments under defined culture conditions, without feeders.

iPSCs derived from porcine/pigs resemble mEpiSCs and hESCs in terms of morphology and expression of pluripotency-associated markers and are likely dependent on FGF2/ACTIVIN/NODAL signalling. When porcine embryonic fibroblasts were reprogrammed to iPSCs in the presence of 2i/LIF, they resembled naïve mESCs in colony morphology and LIF dependence (Tables 1, 2 and Supplementary data 1) (Telugu *et al.* 2010). In the presence of three kinase inhibitors (GSK-3 β , MAPK, MEK), along with LIF, porcine ESCs and iPSCs exhibit naïve-like properties (Tables 1, 2 and Supplementary data 1) (Li *et al.* 2003, Telugu *et al.* 2010, Petkov *et al.* 2016).

Bovine ESCs and iPSCs are also LIF dependent and have limited proliferation capacity. They acquire naïve-like characteristics, when grown in 2i/LIF (Cong *et al.* 2014, Kawaguchi *et al.* 2015, Talluri *et al.* 2015) and in the presence of FGF2/IWR1 (an inhibitor of canonical Wnt signalling pathway), bovine ESCs acquire a stable primed state of pluripotency (Tables 1, 2 and Supplementary data 1) (Bogliotti *et al.* 2018).

In case of goat, ESCs are LIF dependent in nature and in the presence of FGF2, they show primed state-like characteristics (Tables 1, 2 and Supplementary data 1). Sheep iPSCs attain a naïve-like state, with an exposure of 2i/LIF and BMP4 (Behboodi *et al.* 2011, Sartori *et al.* 2012).

Similar to sheep and goat, horse-iPSCs show an FGF2-dependent primed state of pluripotency (Tables 1, 2 and Supplementary data 1) (Nagy *et al.* 2011). Horse-derived ESCs differentiate into cells representative of the three germ layers, but failed to form teratomas (Li *et al.* 2006, Guest & Allen 2007).

ESCs of canine and feline are in a primed state, with morphologies similar to EpiSCs of the mouse (Tables 1, 2 and Supplementary data 1). However, feline ESCs have limited self-renewal potential and lack teratoma formation capability (Gómez *et al.* 2010). The self-

renewal property of canine PSCs can be improved by using LIF and FGF2 (Vaags *et al.* 2009, Koh *et al.* 2013).

Among the discussed mammals, germline chimera formation ability, 'the golden standard' of naïve pluripotency was not tested in most of these cases. A detailed understanding of the regulation of states of pluripotency in these mammalian ESCs and their culture conditions require further investigation and standardisation. It will be interesting to investigate if the dependence of naïve state on LIF and primed state on FGF2 are conserved among all mammals. An intriguing question that still needs to be addressed is, 'if a spectrum of pluripotent states exists, representing different developmental stages and if so, what is the exact signalling environment and differentiation potential of each of these states and their evolutionary conservation among mammals'.

Pluripotency in primates

Totipotency of human zygote persists until E3.5 (6- to 8-cell stage) and at E4, the first lineage segregation to TE and the ICM occurs at E4 stage (reviewed in De Paepe *et al.* 2014). Under conventional culture condition (FGF2 and ACTIVIN/TGF β), without any manipulations, hESCs exhibit primed state of pluripotency and are very similar to mEpiSCs. The states of pluripotency are interchangeable between the primed and naïve states, using suitable culture conditions, over-expression of the pluripotency factors by transgene expression (Table 2 and Supplementary data 1) (Bao *et al.* 2009, Guo *et al.* 2009, Hanna *et al.* 2009, Hanna *et al.* 2010, Xu *et al.* 2010) and modification of epigenetic factors (histone decetylase (HDAC) inhibition) (Guo *et al.* 2017). Naïve hESCs can be directly derived from individual cells of the ICM and maintained in t2iLGÖY medium (LIF, PD0325901, CHIR99021, GÖ6983 along with ROCK inhibitor (Y-27632) and ascorbic acid) (Guo *et al.* 2016). The molecular signature of human-naïve PSCs includes expression of pluripotency-associated markers, over-expression of members in the SINE-VNTR-Alu (SVA) family of transposable elements and epigenetic factors (X-chromosome status and DNA methylation) and developmental potential (interspecies chimera formation) (Theunissen *et al.* 2016). However, the generation of interspecies chimeras by injection of naïve human cells into early mouse embryos was too sporadic for definitive assessment of functional contribution, as naïve human cells rarely contributed to interspecies chimeras (Theunissen *et al.* 2014, 2016, Ware *et al.* 2014). Hence, till now, including interspecies chimera formation as a part of the molecular assessment of naïve human pluripotency has not been widely accepted.

Like humans, iPSCs have been derived from NHPs, such as chimpanzee, bonobo, Gorilla and monkeys, by over-expression of the transcription factors—OCT3/4, SOX2, KLF4, C-MYC and LIN28 (Table 2, Liu *et al.* 2008,

Chan *et al.* 2010, Tomioka *et al.* 2010, Wu *et al.* 2010, 2012, Ben-Nun *et al.* 2011, Deleidi *et al.* 2011, Okamoto & Takahashi 2011, Hong *et al.* 2014, Wunderlich *et al.* 2014). In most of these studies, after successful reprogramming, the endogenous transcription factors were upregulated and the transgenes were downregulated (Liu *et al.* 2008, Chan *et al.* 2010, Tomioka *et al.* 2010, Wu *et al.* 2010, Ben-Nun *et al.* 2011).

Pluripotent cells of NHP share a lot of characteristics in common (Marchetto *et al.* 2014, Romero *et al.* 2015), but transcriptome analysis revealed significant variation among closely related primate species (Wunderlich *et al.* 2014). RNA sequencing and DNA methylation analysis of iPSCs of the closest relatives of human proved that their PSCs share a high degree of similarity between these species than their somatic counterparts (Romero *et al.* 2015). The differences in the regulation of long interspersed element-1 (*LINE-1*) transposons and the expression of *REX1*, between humans and NHPs highlight some of the evolutionary changes that might have occurred during the differential shaping of the genomes of humans and NHPs (Marchetto *et al.* 2014).

Unlike mouse naïve PSCs, the state of the naïve PSCs obtained from NHP was bFGF-dependent, in addition to 2i/LIF (Fang *et al.* 2014). The need for suppression of MAPK signalling in primate naïve PSCs is an intriguing question that arises, based on the role of other pathways, downstream of FGF signalling, such as PI3K/AKT and PLC γ (Lanner & Rossant 2010). Human-naïve PSCs are dependent on TGF- β signalling (Theunissen *et al.* 2014, Gafni *et al.* 2015), whereas monkey-naïve PSCs have been observed to be independent of the same (Fang *et al.* 2014). In case of rhesus monkey naïve PSCs, the inhibition of protein kinase C (PKC) and ROCK-induced differentiation (Fang *et al.* 2014); however, in human naïve PSCs, inhibition of PKC is essential for the maintenance of pluripotency (Chan *et al.* 2013, Gafni *et al.* 2015). Despite species-specific differences, it is not clear, if LIF dependence of the naïve state and the FGF-dependence of the primed state, among PSCs of primates is conserved. This requires an enhanced understanding of signalling pathways and gene regulatory networks and comparative studies, involving synchronisation of culture conditions in PSCs from each species.

Pluripotency in rodents

Like mESCs, rat ESCs (rESCs) are capable of forming germline chimeras, but they express lineage-specific markers when cultured in 2i/LIF (Buehr *et al.* 2008, Li *et al.* 2008). In mESCs, the presence of inhibitors of MEK (MEKi) and GSK3 (CHIR99021) suppress the expression of lineage determining factors such as *Cdx2* and *T* (Smith *et al.* 1988; reviewed in Ye *et al.* 2014, Huang *et al.* 2015, Weinberger *et al.* 2016). In rat ESCs, the overinhibition of Gsk3 leads to the activation of differentiation-associated genes, such as *Cdx2* and *T*

and reduced inhibition of Gsk3 promotes clonogenicity and self-renewal, thus highlighting the importance of attaining an optimal level Gsk3 inhibition (Chen *et al.* 2013). ESCs derived from rodents can be maintained in a naïve state of pluripotency, when cultured in 2i/LIF (Meek *et al.* 2013) and show differentiation in the presence of FGF4 or FGF2 and ACTIVIN A/TGF β enhancers (Tesar *et al.* 2007).

Pluripotency in rodents vs primates

Human and mouse embryos show morphological similarities during the development of pre-implantation blastocyst. They show considerable differences in their developmental stages (occurrence of zygotic genome activation (ZGA)), transcriptome and state of pluripotency (*in vitro* condition). The first embryonic lineages in the mouse are formed between E3 and E3.5, but in human, it happens at the E4 stage of development. The newly formed ICM in mouse and human pre-implantation blastocyst can act as an origin of ESCs of both species (reviewed in De Paepe *et al.* 2014, Wu & Schöler 2016, Brink *et al.* 2008). In addition to ESCs, trophoblast stem cells (TS) and extra-embryonic endoderm stem cells (XEN) have been derived from mouse blastocysts (Chung *et al.* 2006, Yamanaka *et al.* 2006) and more recently, TS cells from human blastocysts (Okoe *et al.* 2018).

In rodents, pluripotency and self-renewal of ESCs are strictly dependent on JAK/STAT3 signalling pathways (Fig. 2). BMP4, a possible molecule, which inhibits MEK/ERK activity and co-operates with LIF, contributes to self-renewal in mESCs (Ying *et al.* 2003, Qi *et al.* 2004, Schnerch *et al.* 2010). The features of standard primate ESCs are entirely distinct from rodent ESCs and pluripotency is entirely dependent on FGF2 and ACTIVIN A signalling pathways (reviewed in Schnerch *et al.* 2010, Warrier *et al.* 2017). Activation of the MEK/ERK signalling pathway in rodent PSCs results in the differentiation. In contrast, in hESCs, FGF2 maintains a crosstalk between MEK/ERK and ACTIVIN/NODAL signalling pathways to enable pluripotency (Vallier *et al.* 2005, Li *et al.* 2007). IGF promotes the activation of PI3K/AKT signalling pathway, which is also crucial for stem cell self-renewal and pluripotency. The activity of MEK/ERK and PI3K/AKT pathways increases the levels of GSK3 within cytoplasm, which has a negative effect on β -catenin, resulting in the inhibition of WNT signalling pathway and maintaining the primed state of pluripotency in primates (reviewed in Huang *et al.* 2015).

Like PSCs, pluripotency properties of early embryonic stages among these mammals have also been explored by different groups. These studies concentrated on the role of signalling pathways (Boroviak *et al.* 2015) and transcription factors (Nakamura *et al.* 2016) involved in the maintenance of pluripotency in different developmental stages of primates and rodents. Along with different signalling pathways and transcription

Figure 2 Signalling pathways regulating pluripotent states of PSCs.

factors, several transposable elements (TEs) are involved in maintaining the molecular harmony of pluripotency. The members of LTR and SRV families show an elevated expression level during different developmental stages of mammalian embryos (Goke *et al.* 2015; reviewed in Hutchins & Pei 2015, Gerdes *et al.* 2016). More studies, concentrating on gene regulatory networks and signalling pathways involved in establishing and

maintaining pluripotency and its distinct states-naïve and primed in primates and their comparison to other mammalian species would help in increasing our meagre understanding of the general principles of pluripotency conserved in evolution.

Comparative analysis of pluripotency among primates and mouse

We performed a comparative analysis of datasets from three species namely human (GSE75868; Theunissen *et al.* 2016), crab-eating macaque (*Macaca fascicularis*, GSE69708; Chen *et al.* 2015) and mouse (GSE99494; Bao *et al.* 2018), to identify common represented signalling pathways, irrespective of their differences. The studies concentrate on both naïve and primed states of pluripotency and satisfy the criteria, such as the source of donor cells, gene expression profiling, germline chimera-forming ability, cellular respiration profile, X-chromosome status, global DNA methylation, OCT4 enhancer utilisation, and so forth (Tables 1, 2 and Supplementary data 2). The dataset for the analysis was selected mainly on the basis of the platform used for sequencing, state of pluripotency (Table 2 and Supplementary data 2) and the origin of the cells. Bao *et al.* (2018) successfully derived advanced PSCs (ASCs) from EpiSCs, using Activin A, BMP4, CH and LIF. These cells are hyper-methylated with high levels of potency and can contribute to both embryonic and extra-embryonic mesoderm in germline chimeras.

Our comparison unveiled both overlapping and distinctly regulated genes, between naïve and primed states of the three species (Fig. 3 and Supplementary Tables 3, 4, 5, 6, 7, 8, 9, 10). Here we have not discussed specific genes in detail, as this requires experimental validation. The most prominent observation from our

Figure 3 Comparative analysis of pluripotent states in primates (human, monkey) and rodents (mouse). (A) Naïve state of human versus monkey, (B) primed state of human versus monkey, (C) naïve state of mouse versus primate (human and monkey) and (D) primed state of mouse versus primate (human and monkey).

analysis is the common regulation of 12 signalling pathways in common between these species, irrespective of naïve or primed state of pluripotency (Supplementary Tables 6, 7, 8 and 9): Neurotrophin, thyroid hormone, FoxO, phosphatidylinositol, p53, AMPK, Hedgehog, Hippo, sphingolipid, mTOR, HIF-1 signalling pathway and insulin signalling pathway. This implies a probable conservation of these pathways during the course of evolution, in maintaining pluripotency and demand experimental validation.

Neurotrophin signalling pathway has a role in maintaining pluripotency and it also maintains a cross-talk with other signalling pathways involved in pluripotency (Pyle *et al.* 2006). Insulin signalling pathway and insulin-like growth factors (IGF) are also important for upholding pluripotency (Wang *et al.* 2007, Dalton 2013). mTOR pathway is very essential for the proliferation and survival of the pluripotent cells (Warrier *et al.* 2017). p53 is highly expressed in mouse ESCs in the cytoplasm under basal conditions (Aladjem *et al.* 1998, Solozobova *et al.* 2009) and functions to maintain genome stability in ESCs by inducing apoptosis upon DNA damage and differentiation (He *et al.* 2016). p53 reduces reprogramming efficiency of somatic cells to pluripotent stem cells and its inhibition enhances reprogramming efficiency (reviewed in Lin & Lin 2017).

With respect to differences, the most prominent, included MAPK signalling, WNT signalling and VEGF signalling, which were found significantly over-represented (P and q value <0.05) in the genes expressed in common in primates and mouse in the primed state, but not in the naïve state (Supplementary Tables 4 and 5). The differences in MAPK/ERK signalling between naïve and primed state are obvious as reflected by the inhibition of ERK signalling to induce the naïve state (Ying *et al.* 2008). The role of WNT signalling is controversial, because non-neural differentiation is induced upon WNT stimulation and residual neural differentiation is eliminated by WNT in the context of ERK and FGFR inhibition (Ying *et al.* 2008). Our finding of significant over-representation of WNT signalling in the primed state, compared to the naïve state could mainly be influenced by the culture conditions used for the naïve state in human which lacks the GSK3 inhibitor (4i) (Theunissen *et al.* 2016). Thus, while Ying *et al.* (2008) and Theunissen *et al.* (2016) induced the naïve state with and without the help of WNT induction, the advantages of WNT induction might be debatable.

Oxidative phosphorylation pathway ($P=0.02$, $q=0.08$) was significantly over-represented in the naïve state, but not in the primed state, in the set, common between primate and mouse. This is in line with expectations concerning a shift of metabolism from oxidative phosphorylation to glycolysis in the maintenance of pluripotency (Prigione & Adjaye 2010, Prigione *et al.* 2010, Carbognin *et al.* 2016, Weinberger *et al.* 2016).

In general, maintenance of pluripotency is achieved in a balanced state of self-renewal, differentiation, proliferation and cell survival, dependent on a well-orchestrated regulation of gene expression and a network of various signalling pathways (reviewed in Sui *et al.* 2013). The future challenges include standardisation of culture conditions that absolutely support naïve or primed state of pluripotency in mammals, other than human and mouse. Additionally, detailed investigation of pluripotency and the mechanisms for directed lineage specification *in vitro*, and a comprehensive analysis of gastrulation in each mammal will facilitate their comparison. For all the mammalian PSCs derived to date, other than germline chimera formation ability, that has been defined as the 'golden standard of naïve pluripotency', an additional test for their integration to tetraploid embryos, the most stringent test for pluripotency, would prove their potential, although this does not test for the ability to form extra-embryonic lineages (Wernig *et al.* 2007, Jaenisch & Young 2008, Kang *et al.* 2009).

This is the era, where a spectrum of pluripotent states is being discovered. Investigation of a panel of EpiSC lines from mouse post-implantation embryos at embryonic day E5.5–E7.5 showed heterogeneity in the gene expression patterns and differentiation potential (Bernemann *et al.* 2011). Two distinct cell populations of mEpiSCs exist, which represent either early or late epiblast, the former possessing the ability to readily contribute to chimeras (Han *et al.* 2010). In contrast to the early-stage EpiSCs, the late-stage EpiSCs cannot be converted to naïve state with 2i medium, but require CK1alpha inhibition for the same (Illich *et al.* 2016). As a clear understanding is still lacking, it will be interesting to find out more about the states of pluripotency, and a question remains, if pluripotent states, other than naïve and primed states, with varied differentiation potencies, representing different developmental stages exist. The existence of such states is beginning to be unveiled (Smith 2017, Bao *et al.* 2018). Another intriguing question that needs addressing is, do all mammalian PSCs rely on similar signalling environment to maintain their states of pluripotency and therefore preservation of evolutionary conservation.

Methods

Bioinformatic analysis

Datasets for human (GSE75868; Theunissen *et al.* 2016), crab-eating macaque (*Macaca fascicularis*, GSE69708; Chen *et al.* 2015) and mouse (GSE99494; Bao *et al.* 2018) were downloaded from NCBI GEO. Expressed genes were determined using a threshold for FPKM (fragments per kilobase of exon model per million reads mapped) >0.1 for the NGS data of monkey. For the NGS pileup and read data of human and mouse a threshold of $t=10$ was used. Expressed genes

in ENSEMBL gene notation were mapped to gene symbols via the ENSEMBL Biomart version 92 annotations. Interspecies comparisons were directly based on these symbols. Venn diagram analyses and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway over-representation analyses were performed in the R/Bioconductor (Gentleman *et al.* 2004) environment using the package Venndiagram (Chen & Boutros 2011) and the test method phyper for the hypergeometric distribution. KEGG pathway data (Kanehisa *et al.* 2017) was downloaded from KEGG in March 2018 corresponding to version Release 85.1.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0083>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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