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Pluripotent stem cells and livestock genetic engineering

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Abstract The unlimited proliferative ability and capacity to contribute to germline chimeras make pluripotent embryonic stem cells (ESCs) perfect candidates for complex genetic engineering. The utility of ESCs is best exemplified by the numerous genetic models that have been developed in mice, for which such cells are readily available. However, the traditional systems for mouse genetic engineering may not be practical for livestock species, as it requires several generations of mating and selection in order to establish homozygous founders. Nevertheless, the self-renewal and pluripotent characteristics of ESCs could provide advantages for livestock genetic engineering such as ease of genetic manipulation and improved efficiency of cloning by nuclear transplantation. These advantages have resulted in many attempts to isolate livestock ESCs, yet it has been generally concluded that the culture conditions tested so far are not supportive of livestock ESCs self-renewal and proliferation. In contrast, there are numerous reports of derivation of livestock induced pluripotent stem cells (iPSCs), with demonstrated capacity for long term proliferation and in vivo pluripotency, as indicated by teratoma formation assay. However, to what extent these iPSCs represent fully reprogrammed PSCs remains controversial, as most livestock iPSCs depend on continuous expression of reprogramming factors. Moreover, germline chimerism has not been robustly demonstrated, with only one successful report with very low efficiency. Therefore, even 34 years after derivation of mouse ESCs and their extensive use in the generation of genetic models, the livestock genetic engineering field can stand to gain enormously from continued investigations into the derivation and application of ESCs and iPSCs.

Keywords Gene editing · Livestock · Pluripotency · Embryonic stem cells · Transgenic animals

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Introduction

Pluripotent stem cells (PSCs) are characterized by two key features, self-renewal and pluripotency (Kuijk et al. 2011). Self-renewal is the ability to produce new cells with equal characteristics as the original (Kuijk et al. 2011) and pluripotency the ability of a cell to generate all cell types in the adult organism (Wu and Belmonte 2014). These characteristics make PSCs attractive for engineering animal genomes. The ability of PSCs to self-renew basically implies that these cells are immortal in vitro, which provides a great opportunity for genome modification and screening of correctly modified cells, even with the possibility of introducing multiple and complex genetic modifications, such as gene targeting approaches. On the other

hand, the pluripotentiality of PSCs allows them to contribute to formation of embryonic chimeras, and therefore the possibility for contributing to an animal's germline with the respective transmission of the engineered genome to the next generations. Taking advantage of these characteristics, mouse geneticists have made enormous progress in generating transgenic animal models. The approaches used in mice would be less practical for engineering domesticated species, as they require multiple generations and large number of animals (especially if multiple modifications are involved). On the other hand, approaches to reduce the number of generations or the number of animals required to produce founders could be implemented for production of domesticated animals from PSCs. Furthermore, some reports indicate that PSCs can be used as donor nuclei in somatic cell nuclear transfer (SCNT) resulting in higher reprogramming efficiency (Eggan et al. 2001; Kou et al. 2010; Rideout et al. 2000; Wakayama et al. 1999). Because of these potential applications of PSCs for generating genetically modified animals, interest in the derivation of livestock PSCs exists, although success up to date has been limited.

Genetic engineering in domestic animals

Transgenic animals are developed with the goal of changing the characteristics of an animal and its progeny by incorporating new or modified genes to their genomes (Maga 2001). In mammals, introduction of foreign DNA into an organism was first reported in 1980, when recombinant plasmids were microinjected into the pronuclei of fertilized mouse oocytes, and the presence of DNA homologous to the injected plasmid was detected in 2.5 % of the newborns (Gordon et al. 1980). Since that time, many transgenic animals have been developed for several purposes. Farm animals and fish have been genetically modified with the aim to enhance economically important traits (Forabosco et al. 2013). For example, increased growth rate has been attained in pigs (Hammer et al. 1985; Pursel et al. 1997; Vize et al. 1988), Atlantic salmon (Cook et al. 2000), and tilapia (Martínez et al. 1999; Rahman et al. 1998) by modifying growth hormone expression. Cattle (Richt et al. 2007) and goats (Yu et al. 2009a) lacking prion protein or mastitis resistant (Maga et al. 2006; van Berkel et al. 2002; Wall et al. 2005) were produced by knocking out the normal cellular prion protein PrPC or by incorporating the human lysozyme, lactoferrin or lysostaphin genes expressed in the mammary gland. An increase in meat quality was also accomplished in pigs with higher level of PUFAs in their meat (Lai et al. 2006; Saeki et al. 2004). Apart from applications in animal agriculture, genetically modified livestock has been developed for "pharming" (production of recombinant proteins) (Niemann and Kues 2007) and as models for human diseases (Flisikowska et al. 2014; Rogers et al. 2008). Nonetheless these and other successful attempts generating transgenic animals have been reported, production of transgenic animals, in particular livestock, has typically been an inefficient process (Maga 2001). Recent advent of directed meganucleases has opened a new chapter in animal genetic engineering. It is now possible to modify (edit) an animal genome without incorporating new genetic material. This is typically referred to as genome editing and generally involves introducing a small mutation or inducing a small sequence change. Often, these genome edits mimic a naturally occurring gene variant which in principle could be incorporated by standard breeding practices, but whose incorporation into a specific genetic background would take a long time and result extremely costly and inefficient.

Among approaches to create transgenic animals, pronuclear microinjection suffers from serious limitations and low efficiency (Polejaeva and Campbell 2000); retroviral and lentiviral transgenesis, even though efficient, is often associated with insertional mutagenesis and silencing of the transgenes (Reviewed by Nienhuis et al. 2006; Hotta and Ellis 2008). Similarly, generating transgenic animals using SCNT presents limitations for modifying the genome of somatic cells with a finite in vitro proliferation capacity thus it is challenging to introduce complex/multiple genetic modifications before using them for SCNT. The efficiency of generating live animals by SCNT is low, depending among other factors, on the ability of the nuclear donor cell to be fully reprogrammed to an embryonic state (Oback 2008). More recently, the development of programmable nucleases (ZFNs, TALENs and CRISPRs) that can specifically and efficiently create double strand breaks in the DNA, leading to gene disruption by error prone non homologous end joining (NHEJ) or more efficient homologous directed recombination (HDR), has

revolutionized the field of animal transgenesis (Reviewed by Hsu et al. 2014). The high efficiency of these nucleases allows to induce targeted mutations directly in the zygote leading to a high frequency of homozygous gene disruption. Reports of gene-editing applications in livestock include the creation of hornless cattle (Carlson et al. 2012), double muscle sheep and cattle by inactivation of the myostatin gene (Proudfoot et al. 2015), pigs immune to African swine fever virus (Lillico et al. 2013) and resistant to PRRS (Whitworth et al. 2014b). Moreover, high efficiency of homologous recombination by direct injection of CRISPRs and donor plasmid into zygotes has been reported in mice and rats (Remy et al. 2014; Wu et al. 2013; Yang et al. 2013a), which opens the possibility of direct gene editing in livestock species. However, since the changes induced using targeted nucleases by direct injection into a zygote have to happen in a single cell, it is unclear if multiple specific HDR modifications can be efficiently induced before the embryo initiates DNA replication and development. On the other hand, programmable nucleases can be used in PSCs to more effectively create genetic modifications, which could be introduced in sequence if multiple changes to an animal genome are required. For these reasons, PSCs remain as good candidates for genome editing and genetic manipulation.

Types of pluripotent stem cells in rodents and primates

Pluripotent ESCs are obtained from the inner cell mass (ICM) of blastocyst stage embryos and were first derived in 1981 from in vivo mouse embryos (Evans and Kaufman 1981; Martin 1981). Since then many efforts have been made to develop ESCs in many other species, with success in primates (rhesus and human) (Thomson et al. 1995, 1998) several years later. As initially derived, human and mouse ESCs had similar capacity for self-renewal and pluripotency, but differed in culture requirement and other characteristics. While mouse ESCs formed domed colonies, were capable of clonogenic expansion, and depended on leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) signaling; human ESCs established flat colonies, could not be separated as single cells, and were dependent on fibroblast growth factor 2 (FGF2)/Activin signaling. It was the derivation of mouse epiblast stem cells (EpiSCs) that started clarifying the origin of species-specific characteristics. Mouse EpiSCs were derived from post-implantation mouse embryos and showed characteristics resembling human ESCs (Tesar et al. 2007). It is now accepted that human ESCs are the equivalent to mouse EpiSCs. The two different PSCs types are commonly referred to as a naïve (or ground state) and primed state, respectively (Nichols and Smith 2009). Naïve ESCs represent the epiblast of pre-implantation embryos and are characterized by the expression of transcription factors such as Oct4, Sox2, and Nanog (Chambers and Smith 2004; Pera and Tam 2010). Additionally, naïve ESCs maintain both X-chromosomes in an active state in female cells (Wutz and Jaenisch 2000) and efficiently contribute to all three embryonic germ layers (ectoderm, endoderm and mesoderm) when they are injected into host blastocysts (Nagy et al. 1990). Inhibition of mitogen-activated protein kinase (ERK) pathway and glycogen synthase kinase-3beta (GSK3B), accomplished by small molecule inhibitors CHIR99021 and PD0325901 respectively, greatly helps in maintaining mouse ESCs in a naïve pluripotent state (Ying et al. 2003). This culture system, known as '2i' (for two inhibitors) was a key development for the successful isolation of ESCs in rats (previously considered a "difficult species") (Buehr et al. 2008; Li et al. 2008) and mouse strains considered recalcitrant to ESC derivation (Nichols et al. 2009). In contrast to ESCs, post-implantation EpiSCs are the in vitro counterpart of the primed epiblast (Tesar et al. 2007). EpiSCs express core pluripotency factors, Oct4, Sox2, and Nanog, but differ from ESCs in the expression of other transcripts, such as Dax1 (Tesar et al. 2007). Pluripotency in EpiSCs is not maintained by LIF signaling; instead, FGF2 and the Activin A/Nodal cascade are important for maintaining their undifferentiated state (Bao et al. 2009; Li et al. 2009). Even though EpiSCs efficiently form teratomas, they are not competent to contribute to blastocyst chimeras (Tesar et al. 2007). Differential expression of genes associated with the germ cell lineage has also been observed in the ICM versus the post-implantation epiblast (Hayashi 2007). Similar differential expression pattern has been recognized in ESCs and EpiSCs, where genes associated with the germline, including Stella, Piwil2, Stra8 and Dazl are expressed by ESCs cells and significantly decreased or not detected in EpiSCs (Tesar et al. 2007).

Two separate regulatory elements control Oct4 expression, which is downregulated during gastrulation. A distal enhancer drives Oct4 expression in pre-implantation embryos and germ cells. In contrast, a proximal enhancer directs the epiblast-specific expression pattern (Yeom et al. 1996). The distal and proximal enhancers have reciprocal abilities to direct gene expression in ESCs and EpiSCs (Tesar et al. 2007). These differences plus their ability to re-enter embryogenesis at different developmental time points (pre-implantation versus post-implantation, respectively) distinguish ESCs and EpiSCs as existing in two temporally distinct pluripotent states (Fig. 1) (Wu et al. 2015). In fact, the naïve and a primed terminology was introduced to describe an early and late phase of epiblast ontogeny and respective ESCs and EpiSCs derivatives (Nichols and Smith 2009). Compared to naïve cells, primed PSCs exist in a more developmentally advanced state and are poised for rapid and efficient differentiation (Wu et al. 2015). Recently, a novel primed pluripotent cell type has been isolated. Region-selective EpiSCs (rsEpiSCs) are derived from pre- and post-implantation epiblasts under a FGF2 and IWR1 (Wnt canonical pathway inhibitor) based medium (Wu et al. 2015). These newly obtained pluripotent cells colonize the posterior region of post-implantation embryos and have proven efficient generation of intra- and interspecies chimeric embryos. This unique embryo grafting property indicates that rsEpiSCs represent a class of primed-state PSCs with a new spatial identity distinct from conventional EpiSCs (Wu et al. 2015).

Interestingly, the state of pluripotency can be modulated by changing culture conditions and by stimulating/inhibiting different signaling pathways. In this way, human ESCs, originally derived in primed conditions can be programmed to naïve or region selective state by multiple different approaches (Gafni et al. 2013; Takashima et al. 2014; Theunissen et al. 2014; Wu et al. 2015). However, because of ethical consideration, it is still unclear whether human naïve PSCs have the ability to contribute to germline chimeras or not. More recently, non-human primate ESCs derived under primed conditions were induced to naïve state by changing the culture environment, allowing the cells to contribute to chimeric fetuses with contribution to the three germ layers (Chen et al. 2015).

The functional difference between ESCs and EpiSCs in their ability to contribute to different tissues in chimeric animals defines their applications. Naïve state pluripotency has been difficult to achieve in species outside of rodents and primates (Reviewed by Gandolfi 2012), therefore rsEpiSCs are a promising finding which suggests that modulating culture conditions chimera competent primed state cells can be obtained.

Induced pluripotency

Induced pluripotent stem cells (iPSCs) were first derived in 2006 using mouse fibroblasts by overexpression of four key transcription factors: Oct4, Sox2, Klf4, and c-Myc (OSKM) (Takahashi and Yamanaka 2006). These pluripotent cells exhibit morphology and growth properties of ESCs, express ESCs cell marker genes, form teratomas, and after injection into blastocysts can contribute to germline chimeras (Okita et al. 2007; Takahashi and Yamanaka 2006). Human iPSCs were also produced by viral transduction of the same factors to adult fibroblasts (Takahashi et al. 2007). Moreover, a combination of Oct4, Sox2, Nanog and Lin28 was also effective to induce pluripotency in human fibroblasts (Yu et al. 2009b). Reprogramming into PSCs is the result of remodeling the somatic cell transcriptional and epigenetic programs to an ES-like state, including the reactivation of the somatically silenced X-chromosome, demethylation of the Oct4 and Nanog promoter regions, and genome-wide resetting of histone H3 lysine 4 and 27 trimethylation (H3K4me3 and H3K27me3) (Sridharan et al. 2009). Thus, reprogramming is a gradual process that takes several days or weeks and depends on a cascade of self-renewal and pluripotency genes that need to be reactivated (Maherali et al. 2007) and the repression of lineage commitment genes (Sridharan et al. 2009). Furthermore, Stadtfeld and cols. reported that reprogramming mouse iPSCs requires exogenous factor expression for about ten days, at which point the somatic genome becomes poised for conversion into a pluripotent state (Stadtfeld et al. 2008a).

Importantly, reprogramming factors induce epigenetic reprogramming of a somatic genome to an embryonic pluripotent state that reactivates endogenous pluripotency genes (Oct4 and Nanog, Sox2, Klf4,

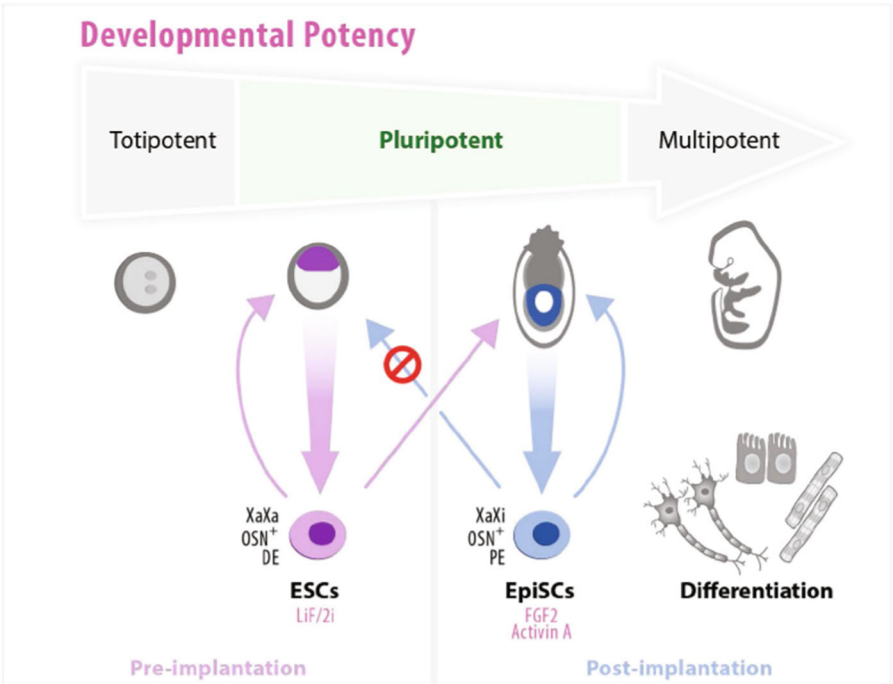

Fig. 1 ESCs and EpiSCs exist in two temporally distinct developmental states. ESCs represent the epiblast of pre-implantation mouse embryos, they can re-enter embryogenesis at any developmental stage, their pluripotency relies on LIF and 2i culture systems, a distal enhancer (DE) drives Oct4 expression, and both X-chromosomes are in an active state in female cells. EpiSCs instead, are derived from post-implantation embryos existing in a more developmentally advanced state. EpiSCs are incapable of re-entering embryogenesis at a pre-implantation stage, their pluripotency relies on FGF2/Activin signaling, a proximal enhancer (PE) drives Oct4 expression, and one of the X-chromosomes in female cells is inactive. Beside these differences, both cell types express core transcription factors OSN (Oct4, Sox2, and Nanog)

etc.). IPSCs become independent of the exogenous reprogramming cocktail and the reactivated endogenous gene expression program ensures maintenance of pluripotency (Wernig et al. 2007). Therefore, a key indication of complete reprogramming is the ability of iPSCs to maintain PSCs characteristics in absence of reprogramming gene expression. Nevertheless, the continuous expression of reprogramming factors in iPSCs does not seem to interfere with self-renewal and pluripotency (West et al. 2010; Zhang et al. 2015). Similar to ESCs, iPSCs can be maintained under different pluripotency states, ranging from naïve to region-selective primed cells. Recently, a new state of induced pluripotency, characterized by the fuzzy appearance of their colonies has been reported (Tonge et al. 2014). The F-class cell state is a Nanog-positive state generated by a sustained and elevated expression of reprogramming factors. F-class cells express many genes at ESC levels; however, they also express transcription factors associated with lineage commitment and when compared to EpiSCs expression profile, F-class cells are transcriptionally distinct (Tonge et al. 2014). This indicates that F-class cells can be considered a pluripotent cell type distinct from ESCs and EpiSCs (Wu and Belmonte 2014).

The availability of well-developed culture conditions for derivation and maintenance of ESCs in primates and rodents has allowed for the extensive development and characterization of methodologies and conditions for induction of pluripotency. The introduction of reprogramming factors to successfully generate iPSCs has been accomplished by several gene delivery systems. Viral vectors are the most common delivery vehicles used, including integrating viral vectors, non-integrating viral vectors and excisable viral vectors. Retroviral and lentiviral vectors integrate into the chromosomes of their targets, a requisite for long-term expression (Zufferey et al. 1998). Retroviral vectors are less efficient than lentiviral vectors; they only transduce cells that divide

shortly after infection (Zufferey et al. 1997) and due to de novo methylation (Jahner et al. 1982) retroviral transgenes get silenced in pluripotent cells (Cherry et al. 2000; Okita et al. 2007). Lentiviral vectors instead, achieve a higher efficiency transducing non-dividing cells (Zufferey et al. 1997) and do not undergo silencing (Seki and Fukuda 2015). Excisable viral vectors have also been used to efficiently generate iPSCs (Soldner et al. 2009). Cre/loxP mediated excision is a sophisticated approach that provides the ability to induce gene ablation in a spatial and temporal manner (Garcia and Mills 2002). Although this system eliminates transgenes and avoids reactivation, the risk of gene disruption is still present (Seki and Fukuda 2015). Adenoviral vectors are considered non-integrating vehicles due to their extremely low integration frequency (Harui et al. 1999), allowing transient, high-level expression of transgenes (Stadtfeld et al. 2008b). They are safer than integrating methods, which are associated with functional gene disruption near the insertion site (Seki and Fukuda 2015) and tumorigenicity risk due to spontaneous reactivation of transgenes (Okita et al. 2007), but their reprogramming efficiency is significantly lower (Stadtfeld et al. 2008b). Non-integrating Sendai virus-based vector systems were developed to improve the efficiency of deriving iPSCs, and to solve the problems associated with integrating vectors (Fusaki et al. 2009). The Sendai virus genome is negative-stranded RNA, which makes these particular RNA vectors highly efficient for gene transfer, expression of foreign genes, and due to the fact they have no DNA phase there is no risk of altering the host genome (Tokusumi et al. 2002). Sendai virus delivered transgenes are gradually depleted with each cell division, can be removed by antibody-mediated negative selection utilizing cell surface markers (Fusaki et al. 2009) or can be removed from the target cells quickly and thoroughly by siRNA (Nishimura et al. 2011). Virus-free reprogramming methods are another approach for iPSCs generation. Many different systems have been shown to successfully reprogram somatic cells into a pluripotent state, including episomes (Okita et al. 2011; Yu et al. 2009b), minicircle vectors (Jia et al. 2010), transposons such as PiggyBac (Kaji et al. 2009; Talluri et al. 2014; Woltjen et al. 2009; Yusa et al. 2009) and Sleeping Beauty (Muenthaisong et al. 2012; Talluri et al. 2014), synthetic mRNAs (Warren et al. 2010), recombinant proteins (Zhou et al. 2009), ESCs specific miRNAs (Anokye-Danso et al. 2011), and small chemical compounds (Hou et al. 2013).

Reprogramming differentiated cells is a slow and sequential process that can be influenced by the cell origin. For example, female somatic cells must overcome more obstacles than male somatic cells to convert to pluripotency (Di et al. 2015), and reprogramming may take longer than in males (Payer et al. 2011). Also, reprogramming efficiency strictly depends on the approach used to deliver the exogenous transcription factors. Each system has its own inconveniences and the ideal, highly efficient, non-integrating and transient delivery method has yet to be developed.

From pluripotent stem cells to genetically engineered offspring

The great advantage of PSCs for generation of genetically modified animals is in their potential for unlimited self-renewal in vitro, and ease of genetic manipulation. However, in order for PSCs to be used for transgenic animal generation, it is important that these cells can contribute to formation of functional gametes for engineered-genome transmission. A standard approach for this has been to create chimeric animals composed of ESCs and a host embryo, in which the ESCs can contribute to the germline (Fig. 2). The early mammalian embryo has a remarkable ability to accommodate alterations in cell numbers. Additional equivalent cells can be introduced into pre-implantation embryos and will readily be incorporated, resulting in chimeric animals (Nichols and Smith 2009). ESCs injected into a pre-implantation embryo can contribute to all three germ layers that give rise to the embryonic and some extraembryonic tissues (Beddington and Robertson 1989) but not to the trophectoderm or primitive endoderm, despite their ability to differentiate into the latter cell type in vitro (Tam and Rossant 2003). In mice germline chimerism can vary greatly between chimeric animals. An approach that has been devised to improve the level of germline contribution from ESCs involves using a host embryo incapable of developing its own germline (Taft et al. 2013). In such case, all germline is contributed by the ESCs resulting in 100 % transgenic offspring after mating and therefore reducing the

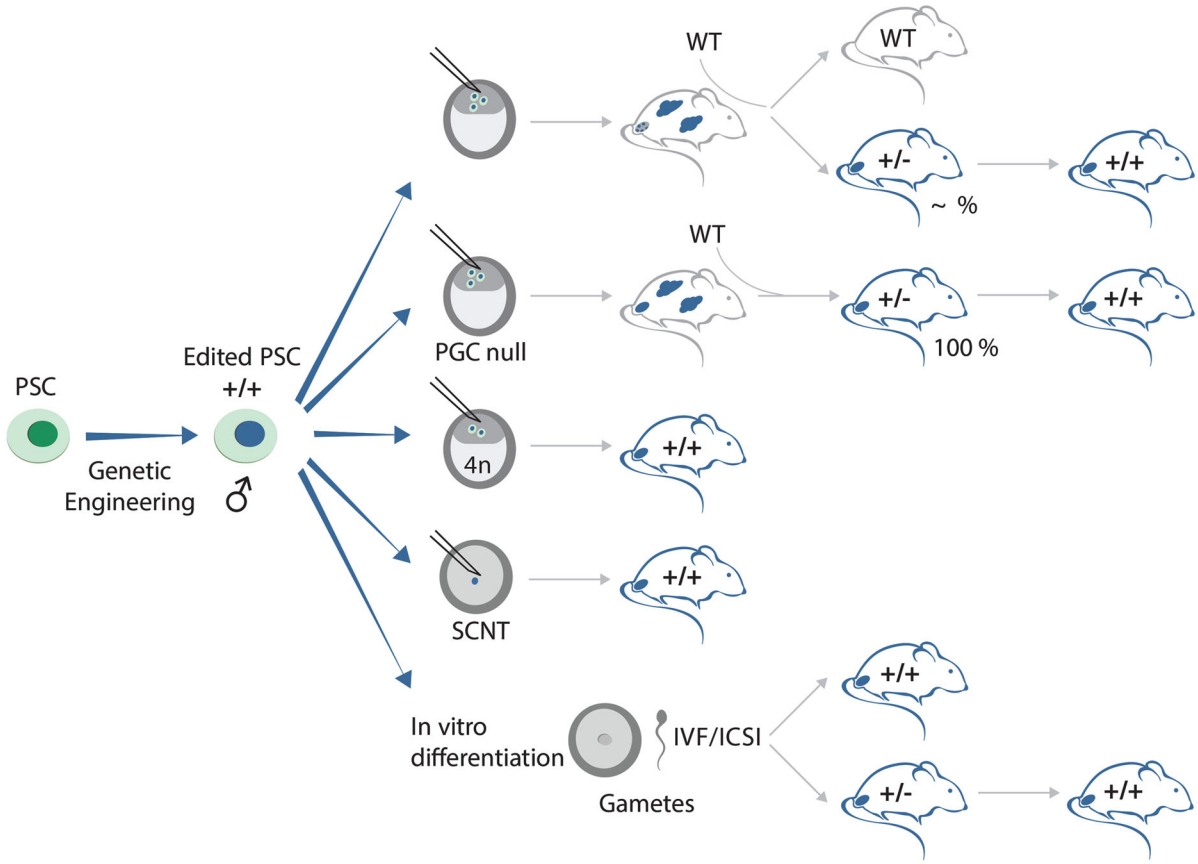

Fig. 2 Approaches for generating transgenic animals from PSCs. Edited PSCs can be used to create transgenic animals by microinjection into a normal host embryo, a host embryo incapable of developing its own germline or a tetraploid host embryo. Respectively, each of these techniques increases the contribution of PSCs to the animal's germline. Edited PSCs can also contribute their nuclei for cloning by nuclear transplantation or potentially be used to generate in vitro functional gametes. The greatest advantage of these methodologies is that homozygous mutants can be produced in just one generation, largely reducing the number of animals and time needed to produce mutant founders

number of animals needed to produce heterozygous founders (Fig. 2) (Taft et al. 2013). The contribution of ESCs to embryonic regions can be maximized by the use of tetraploid host embryos (Fig. 2) (Eakin and Hadjantonakis 2006; Nagy et al. 1993). Tetraploid embryos are not capable of forming viable offspring (Tarkowski et al. 1977), their cells become principally restricted to the extraembryonic membranes with minimal contribution to the proper embryo, resulting in almost completely ESC-derived mice (Nagy et al. 1990). Starting with a homozygous mutant ESC, tetraploid complementation reduces the number of generations required to obtain a homozygous mutant animal to just one; however, it has only been achieved in mice and to a relatively low efficiency (Eggan et al. 2001). Also, ESCs could potentially be used to generate functional gametes or transplantable germ stem cells in vitro and then used to produce animals by ICSI, IVF or germ stem cell transplantation (Fig. 2). Although great progress has been made in developing functional gametes from ESCs in mice (Hayashi et al. 2011, 2012), there is still the requirement for some ex vivo and in vivo culture steps that would have to be resolved before this becomes a practical approach for derivation of ESCs offspring.

ESCs can also contribute to offspring by contributing their nuclei for cloning by nuclear transplantation (Fig. 2) (Wakayama et al. 1999). An advantage of this methodology is that a homozygous mutant animal can be achieved in just one generation. Furthermore, given

the less differentiated state of PSCs compared to somatic cells, the efficiency of the cloning procedure tends to be improved (Eggan et al. 2001; Rideout et al. 2000; Wakayama et al. 1999).

Nowadays, chimera production using genetically modified ESCs is the method of choice for generation of knockout or knockin mice (Polejaeva and Mitalipov 2013). Similarly, rat ESCs and iPSCs are capable of chimera contribution. Interestingly, mouse and rat PSCs can contribute to interspecies chimeras in rat and mouse embryos, respectively (Kobayashi et al. 2010). Only recently, chimeric cynomolgus monkeys have been generated by injecting naïve ESCs into morulas (Chen et al. 2015). Germline contribution in the monkeys is suggested by localization of the ESC derivatives to the fetal gonad (Chen et al. 2015), however, demonstration of successful germline transmission has not yet been shown for this species.

The major applications of PSCs for the generation of transgenic animals are summarized in Fig. 2. Overall by the use of PSCs, the efficiency of each system can be improved and the production of homozygous transgenic founders can be dramatically accelerated.

PSCs in livestock species and applications for generation of transgenic animals

Despite intensive efforts, establishment of robust bona fide ESCs in livestock species has been unsuccessful (Reviewed by Blomberg and Telugu 2012; Gandolfi 2012; Goncalves 2014; Koh and Piedrahita 2014; Kumar et al. 2015; Malaver-Ortega et al. 2012; Nowak-Imialek and Niemann 2012; Park and Telugu 2013; Telugu et al. 2010b). In general, embryo-isolated cells have not demonstrated robust self-renewal and have typically failed to show in vivo pluripotency by teratoma formation assay. This lack of success has been attributed to both extrinsic and intrinsic factors, such as potentially unknown culture requirements or the lack of an equivalent pluripotent state in these species, respectively. Most attempts to derive livestock ESCs have relied on standard mouse (naïve) or human (primed) culture systems. The current new understanding of different pluripotent states in rodents and primates is likely to provide new alternatives for the derivation of livestock ESCs.

On the other hand, reprogramming somatic cells to a pluripotent state has demonstrated some level of success (Goncalves et al. 2014; Kumar et al. 2015; Park and Telugu 2013). Pluripotency has been induced in a number of domestic species including cattle, goats, horses, pigs and sheep (for details see Table 1). Predominantly, these iPSCs have been obtained from embryonic and fetal fibroblasts, and generally using viral vectors to deliver the reprogramming factors. In most cases, pluripotency has been demonstrated by formation of teratomas containing tissue derivatives of the three germ layers. Interestingly, lack of silencing of exogenous transgenes and incomplete reprogramming are a notable feature in most iPSCs from domestic animals. Moreover, continued expression of the reprogramming factors seems to be necessary to maintain pluripotency in iPSCs of farm animals, as shown by differentiation of the cells upon inactivation of the exogenous factors using doxycycline inducible systems (Bao et al. 2011; Hall et al. 2012; Li et al. 2011; Rodriguez et al. 2012). The dependency for continued high expression of the reprogramming factors may indicate that livestock iPSCs could represent F-class type PSCs.

X-chromosome reactivation is an epigenetic hallmark of pluripotent stem cells (Payer et al. 2011). During reprogramming of iPSCs, female cells reactivate the silent X-chromosome that underwent random X-inactivation upon differentiation (Maherali et al. 2007). This is a multiple step process that includes Xist downregulation, H3K27me3 removal, and demethylation of X-linked genes (Di et al. 2015). X-reactivation is a late reprogramming event that occurs at the time of re-expression of telomerase and endogenous pluripotency genes such as Oct4, Sox2 and Nanog and correlates with the time period when cells become independent of the exogenous factor expression (Stadtfeld et al. 2008a). As previously mentioned, in mice the X-chromosome epigenetic make up reflects the pluripotent status in EpiSCs and ESCs; therefore it can be used as an indicator of naïve or primed pluripotency. Interestingly, many of the studies summarized in Table 1 describing derivation of naïve state livestock iPSCs do not evaluate X-chromosome reactivation in female cells. Only three studies (Whitworth et al. 2014a; Zhang et al. 2014, 2015) evaluated H3K27me3 of X-chromosome by immunostaining showing promising results.

Table 1 Characteristics of livestock induced pluripotent stem cells

Species	Cell source	Pluripotent state	Reprograming method	Expresion of RF	Characterization					References
					Markers	Passage	EB	Teratoma	Chimera	
Cattle	Embryonic fibroblasts	Primed	Retrovirus (bovine OSKMLN)	Persistent	AP, Oct4, Sox2, Nanog, SSEA1/4	>16	+	+	NA	Han et al. (2011)
	Embryonic fibroblasts	Naïve	Episomal polypromoter vector (bovine OKSM)	NA	AP, Oct4, Sox2, Klf4, SSEA3/4, TRA-1-60/81	NA	+	+	NA	Huang et al. (2011)
	Skin fibroblasts	Primed	Retrovirus (Human OSKMN)	Persistent	AP, Oct4, Sox2, Klf4, c-Myc, Nanog, SSEA1/4	<12	+	+	NA	Sumer et al. (2011)
	Skin fibroblasts	Primed	Lentivirus (human O + pig SKM)	Persistent Oct4 and Klf4	AP, Oct4, Sox2, Klf4, Nanog, SSEA1	>40	+	+	NA	Cao et al. (2012)
	Fetal fibroblasts	Primed	*PiggyBac* transposon (human OSKM)	Persistent	AP, Oct4, Sox2, Klf4, c-Myc, Nanog, SSEA1/3	>40	NA	+	NA	Talluri et al. (2015)
Goat	Ear fibroblasts	Primed	DOX-inducible lentivirus (human OSKMLN + SV40 large T + hTERT)	Persistent	AP, Oct4, Sox2, Nanog, SSEA1, Tra-1-60/81, Rex1, Cdh1	30	+	+	NA	Ren et al. (2011)
	Ear fetal fibroblasts	Primed	Lentivirus (human OSKM)	Silenced	AP, Oct4, Sox2, c-Myc, Nanog	17	+	+	NA	Song et al. (2013)
	Embryonic fibroblasts	Primed	Lentivirus (human OSKM) + PRMT5	NA	AP, Oct4, c-Myc, SSEA1/4	NA	+	NA	NA	Chu et al. (2015)
	Fetal fibroblasts	Naïve Primed	Lentivirus (bovine OSKMLN) + MIR302/367 Cluster	Persistent, exept Nanog	AP, Oct4, Sox2, Klf4, Nanog	>30	NA	Partial	NA	Sandmaier et al. (2015)
Horse	Fetal fibroblast	Naïve	*PiggyBac* transposon Tet inducible (mouse OSKM)	Persistent	AP, Oct4, Klf4, Nanog, SSEA1/4, TRA-1-60/81	NA	+	+	NA	Nagy et al. (2011)
	Adult fibroblast	Primed	Retrovirus (human OSK)	Persistent	AP, Oct4, Sox2, Nanog, SSEA1/4	>27	+	+	NA	Khodadadi et al. (2012)
	Neonatal and adult fibroblasts	Primed	Retrovirus (mouse OSKM)	Persistent Oct4, partially silenced c-Myc	AP, Oct4, Sox2, Lin28, Nanog, SSEA1/4, TRA-1-60, Rex1	>30	+	+	NA	Breton et al. (2013)
	Adult fibroblast	Naïve[a]	Lentivirus (human OSKM)	Persistent Oct4	AP, Oct4, Nanog, SSEA1/4, TRA-1-60/81, Rex1	>60	+	+	NA	Whitworth et al. (2014a, b)

Table 1 continued

Species	Cell source	Pluripotent state	Reprograming method	Expresion of RF	Characterization					References
					Markers	Passage	EB	Teratoma	Chimera	
Pig	Embryonic fibroblasts	Primed	Retrovirus (human/mouse OSKM)	Persistent	AP, Lin28, Nanog, SSEA4, Rex1	<25	NA	+	NA	Esteban et al. (2009)
	Fetal fibroblasts	Primed	Lentivirus (human OSKM)	Persistent	Oct4, Sox2, Nanog, AP, SSEA1	<20	+	+	NA	Ezashi et al. (2009)
	Ear fibroblasts Bone marrow	Primed	DOX-inducible lentivirus (human OSKM)	Persistent	Oct4, Sox2, Nanog, AP, SSEA3/4, Tra-1-60/81, Rex1, Cdh1	>41	+	+	NA	Wu et al. (2009)
	Mesenchymal cells	Primed	Lentivirus (human OSKMLN)	Persistent	AP, Oct4, Sox2	>50	+	NA	↑Contribution adults ↓Germline transmission	West et al. (2010, 2011)
	Embryonic fibroblasts	Naïve	Episomal vector (human OSKMLN + SV40)	Persistent	AP, Oct4, Sox2, Nanog	30	NA	+	NA	Telugu et al. (2010a, b)
	Embryonic fibroblasts	Naïve	Retrovirus (mouse OSKM)	Down regulated	AP, Oct4, Sox2, Nanog, SSEA1/4, Tra-1-60/81	>20	+	+	Blastocyst	Cheng et al. (2012)
	Adipose tisssue	Primed	Lentivirus (OSKM)	NA	AP, Sox2, Oct4, Klf4, Nanog, SSEA1, Tra-1-60/81	40	+	+	NA	Gu et al. (2012)
	Embryonic mesenchymal stem cells	Naïve	Retrovirus (porcine OK)	Persistent	Oct4, Nanog, SSEA1	>10	Partial	+	NA	Liu et al. (2012a)
	Ear fibroblasts	Primed	Retrovirus (mouse SKM)	Persistent	AP, Oct4, Sox2, Nanog, Lin28, SSEA4, Tra-1-60/81	>30	+	+	NA	Montserrat et al. (2012)
	Fetal fibroblasts	Naïve	DOX-inducible lentivirus (human OSKM)	Persistent	AP, Oct4, Sox2, Klf4, Nanog, Rex1, Stella, Fgf5, Nodal	NA	+	NA	NA	Rodriguez et al. (2012)
	Embryonic fibroblasts	Primed	Retrovirus (mouse OSKM)	NA	AP, Oct4, Sox2, Nanog, Lin28, SSEA4	NA	+	+	NA	Fan et al. (2013a, b)

Table 1 continued

Species	Cell source	Pluripotent state	Reprograming method	Expresion of RF	Characterization					References
					Markers	Passage	EB	Teratoma	Chimera	
	Embryonic fibroblasts	Naïve[a]	Retrovirus (human OSKM)	Persistent	AP, Oct4, Sox2, Klf4, c-Myc, Lin28, Nanog, SSEA1/3/4, Tra-1-60/81, ERas, Stella	>40	+	Negative	Fetus	Fujishiro et al. (2013)
	Fetal fibroblasts	Primed	*Slepping Beauty* transposon (murine OSKM)	NA	AP, Oct4, Sox2, SSEA1, Tra-1-60, Rex1, Esrrb, Dppa5, Utf1	>40	+	+	NA	Kues et al. (2013)
	Ear fibroblasts	Naïve	Lentivirus (human OSKMLN)	NA	AP, Oct4, Sox2, Klf4, Nanog, Rex1, SSEA1	<18	+	+	NA	Kwon et al. (2013)
		Primed			AP, Oct4, ↓Sox2, ↓Klf4, ↓Nanog, SSEA1		Partial	Negative		
	Ear fibroblasts	Primed	Retrovirus (human OSKM)	Persistent	AP, Sox2, Oct4, Nanog, Lin28, SSEA1/4, Tra-1-60/81, Rex1, Cdh1, Dnmt	>25	+	NA	NA	Park et al. (2013)
	Fetal fibroblasts	Primed	*Slepping Beauty* transposon (porcine OSKMN + human L)	Persistent	AP, Oct4, Sox2, Nanog, Rex1, Cdh1, Tdh	>37	Negative	NA	NA	Petkov et al. (2013)
	Embryonic fibroblasts	Naïve	Retrovirus (mouse OSKM + Tbx3, Nr5α2)	Persistent	↑AP, Oct4, Sox2, Nanog, Rex1	>30	+	+	NA	Wang et al. (2013)
		Primed	Retrovirus (mouse OSKM)		AP, Oct4, Sox2, Nanog	NA				
	Adult fibroblasts	Primed	Lentiviral (human OSKMLN)	Down regulated	AP, Oct4, Sox2, Klf4, c-Myc, Lin28, Nanog, Rex1, Cdh1, Dax1, Tdgf1, Tert	22	+	NA	NA	Yang et al. (2013a, b)
	Ear fibroblasts	Primed	Lentivirus (human OSKM)	NA	AP, Oct4, Sox2, Nanog, SSEA1/4, Tra-1-60/81	NA	NA	+	NA	Ao et al. (2014)
	Fetal fibroblasts	Naïve	Retrovirus (mouse OSKM or OSK) + miR-302a, miR-302b, miR200c	Persistent	AP, Oct4, Sox2, Klf4, Nanog, SSEA4, Rex1	NA	+	+	NA	Ma et al. (2014)
	Embryonic fibroblast	Primed	Retrovirus (mouse OSKM + Tbx3, Nr5α2)	Persistent	AP, Oct4, Klf4	<20	NA	+	NA	Xie et al. (2014)

Table 1 continued

Species	Cell source	Pluripotent state	Reprograming method	Expresion of RF	Characterization					References
					Markers	Passage	EB	Teratoma	Chimera	
	Adipose tissue Ear fibroblasts	Naïve[a]	DOX-inducible lentivirus (human OSKM)	Silenced	AP, Oct4, Sox2, Lin28, SSEA3/4, Esrrb, Dppa5, Utf1	>30	+	+	NA	Zhang et al. (2014)
	Adipose tissue derived stem cells	Primed	DOX-inducible lentivirus (human OSKM)	Persistent	↓Oct4, ↓Sox2, SSEA3/4, ↓Lin28, ↓Esrrb, ↓Dnmt3B, ↓Cdh1	>40	+	Negative	NA	Wei et al. (2015)
	Embryonic fibroblasts	Naïve[a]	Retrovirus (porcine OSKM)	Persistent	AP, Oct4, Sox2, SSEA1	>30	+	+	Blastocyst	Zhang et al. (2015)
		Primed			AP, Oct4, Sox2, SSEA4, Tra-1-60/81					
Sheep	Primary ear fibroblasts	Naïve	DOX-inducible lentivirus (human OSKMNL + SV40 large T + hTERT)	Persistent	AP, Oct4, Sox2, Nanog, TRA -1-60/81, Rex1	>30	+	+	NA	Bao et al. (2011)
	Fetal fibroblasts	Primed	DOX Tet-On-inducible lentivirus (mouse OSKM)	Persistent	AP, Sox2, Nanog, SSEA4	<20	+	+	NA	Li et al. (2011)
	Embryonic fibroblasts	Primed	Retrovirus (human OSKM)	Silenced	AP, Oct4, Sox2, Nanog	<20	+	+	Blastocyst	Liu et al. (2012b)
	Embryonic fibroblasts	Primed	Retrovirus (mouse OSKM)	Silenced, except Oct4	AP, Nanog	>25	+	+	↓Contribution live-born lambs	Sartori et al. (2012)
	Fetal fibroblasts	Primed	Retrovirus (mouse OSKM)	Persistent	AP, Oct4, Sox2	NA	+	NA	NA	German et al. (2015)

AP alkaline phosphatase, *EB* embryoid body, *NA* not available, *O* Oct4, *K* Klf4, *S* Sox2, *M* c-Myc, *L* Lin28N, *N* Nanog, *RF* reprogramming factors

[a] Activation of both X chromosomes in female cells is suggested

X-chromosome reactivation is an important feature of naïve state pluripotency and its detection can add valuable information to the epigenetic profile of reprogrammed somatic cells.

Many efforts have been made to generate germline-competent chimeras in farm animals, most of them unsuccessful. Bovine chimeras using pluripotent or genetically modified stem cells have been developed by different methods, but no germline transmission has been reported (Cibelli et al. 1998; Iwasaki et al. 2000; Lim et al. 2011; Saito et al. 2003). Similarly, sheep iPSCs contributed to low-grade chimeras but without demonstration of germline transmission (Sartori et al. 2012). Germline competency of livestock iPSCs has only been demonstrated in pigs (West et al. 2010). By microinjection of iPSCs into blastocyst stage pig embryos West and cols. were able to produce germline-competent chimeric livestock (West et al. 2010). However, from the 29 chimeric animals produced in that study, low rates of germline transmission (4.7 %) were detected; and of the two Oct4 and Nanog transgene positive F1 piglets produced, one was stillborn and its littermate lived for only 3 days (West et al. 2011). Interestingly, these promising attempts to generate livestock chimeric animals have been achieved using iPSCs resembling human ESCs. The low contribution of sheep iPSCs to chimeric lambs (Sartori et al. 2012) and low germline transmission reported by the chimeric sows (West et al. 2011) could be explained by the primed pluripotent state of the iPSCs used. However, the contribution of pig iPSCs to the three germ layers of chimeric animals indicates successful reprogramming and suggests that the rigorous classification of naïve and primed states of pluripotency does not apply to livestock species, and pluripotent states in these animals could be different from the pluripotent states described in mice.

The use of domestic animal iPSCs to produce offspring by nuclear transplantation (NT) has so far being disappointing. While in mice production of cloned animals from iPSCs has similar efficiency to that of ESCs (Kou et al. 2010; Zhou et al. 2010), attempts to clone pigs and sheep from iPSCs have yielded very low efficiencies (Fan et al. 2013b; German et al. 2015; Xie et al. 2014). A large effort to clone from pig iPSCs only proved successful when the iPSC were induced to differentiate before the NT procedure (Fan et al. 2013b). Also, sheep iPSCs demonstrated resistance to reprogramming after NT, resulting in very low embryo production rates (German et al. 2015). It was hypothesized that persistent expression of the exogenous reprogramming factors and the acquisition of chromosomal abnormalities during iPSCs culture may be responsible for the incompatibility of iPSCs with normal development of NT embryos (German et al. 2015). Therefore, development of transgene independent and fully reprogrammed PSCs may be required for realizing the improved cloning efficiency seen when mouse ESCs are used as nuclear donor.

Differences in early embryonic development between rodents and other mammalian species could explain, to some extent, the difficulties generating embryo derived PSCs in domestic animals (Reviewed by Gandolfi et al. 2012). Instead, important advances have been made reprogramming somatic cells from livestock. However, mouse or human culture conditions are broadly used in the derivation of iPSCs from livestock species and their characterization scarcely reports chimeric assays, or X-chromosome reactivation, which is a stringent marker of naïve ground state pluripotency (Payer et al. 2011). The generation of iPSCs is a promising alternative to the elusive ESCs; but there is room for improvement in their derivation and characterization.

Conclusions

ESCs have revolutionized the field of developmental biology and mouse genomics due to their broad applications and because they are perfect candidates for genome editing and genetic manipulation. Despite the efforts, attempts to establish ESC lines have not been successful in large domestic animals, and true chimera-competent ESCs capable of germline transmission had only been validated in the mouse and rat. The derivation of ESCs in these species has allowed their extensive use in the generation of mutant rodents, making the production of transgenic mice models a very strong field. ESCs are a promising tool that could transform the transgenic animal field in livestock by enabling sophisticated genetic modifications (e.g., multiple knockout, knockin or extensive genome editing) to produce biomedical large animal models, and to improve production traits and the ability to resist disease or to produce valuable and high-quality products.

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