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Review Article

Porcine OCT4 reporter system as a tool for monitoring pluripotency states

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ABSTRACT Pluripotent stem cells could self-renew and differentiate into various cells. In particular, porcine pluripotent stem cells are useful for preclinical therapy, transgenic animals, and agricultural usage. These stem cells have naïve and primed pluripotent states. Naïve pluripotent stem cells represented by mouse embryonic stem cells form chimeras after blastocyst injection. Primed pluripotent stem cells represented by mouse epiblast stem cells and human embryonic stem cells. They could not produce chimeras after blastocyst injection. Populations of embryonic stem cells are not homogenous; therefore, reporter systems are used to clarify the status of stem cells and to isolate the cells. For this reason, studies of the *OCT4* reporter system have been conducted for decades. This review will discuss the naïve and primed pluripotent states and recent progress in the development of porcine *OCT4* reporter systems.

Keywords: naïve, oct4, pig, primed, reporter, stem cell

INTRODUCTION

Stem cells could self-renew and differentiate into cells of the three germ layers. Many pluripotent stem cells (PSCs) have been identified. Embryonic stem cells (ESCs) are derived from preimplantation mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981), and embryonic germ cells (EGCs) are derived from *in vitro* cultures of primordial germ cells (PGCs). Mouse epiblast stem cells (EpiSCs) are derived from postimplantation embryos, and induced pluripotent stem cells (iPSCs) are derived from the reprogramming of somatic cells (Matsui et al., 1992; Resnick et al., 1992; Takahashi and Yamanaka, 2006; Tesar et al., 2007).

Various PSCs are classified into two states: naïve and primed, according to the developmental capabilities of PSCs (Nichols and Smith, 2009). Naïve PSCs are represented by mouse ESCs and EGCs. They are developmental ground states similar to early epiblasts of preimplantation embryos. On the other hand, EpiSCs and human ESCs are primed PSCs. They exhibit a more differentiated pluripotency than naïve cells, showing features of late epiblasts in postimplantation embryos. Both states of PSCs in the permissive line can be derived from embryos. However, in nonpermissive lines such as human PSCs, only primed PSCs are derived in the absence of additional treatment such as chemicals and genetic manipulation (Buecker et al., 2010; Hanna et al., 2009; Park et al., 2013).

Embryonic stem cell populations are not homogenous, and thus reporter systems could be used to characterize

the status of stem cells and isolate the cells when needed. Although reporter systems are one of the most necessary tools for studying stem cells and pluripotency, the lack of a reporter system hampers pluripotency research. *OCT4* is one of many pluripotency genes that has been studied as a reporter gene because it is only expressed in pluripotent cells (Jerabek et al., 2014). The transcription factor *OCT4* is an important marker of an undifferentiated status in early mammalian embryonic development and embryonic stem cells. It plays a critical role as a central regulator for maintaining pluripotency and self-renewal. Four conserved regions (CR1, CR2, CR3, and CR4) are located in the 5' upstream regulatory region of various species (Nordhoff et al., 2001). In addition, *OCT4* has a core promoter and two conserved enhancers which are the distal enhancer (DE) and proximal enhancer (PE) (Yeom et al., 1996). The two elements regulated by retinoic acid are located in the PE region. Thus, the loss of occupancy in these elements is called PE1A and PE1B, respectively. DE2A has a similar sequence to PE1A but is located in the DE region (Minucci et al., 1996; Yang et al., 2005). A study of the *Oct4* upstream region in mouse model revealed that the two enhancer were activated differently. The DE region regulates *Oct4* expression in mouse embryonic stem cells, germ cells, and inner cell mass (ICM) cells, whereas the PE region regulates *Oct4* expression in mouse EpiSCs and epiblasts (Yeom et al., 1996).

The *Oct4* upstream regulatory region-derived reporter system has already been developed in various species, including humans, mice, voles, cows, rabbits, goats. It is used to clarify and separate PSCs (Gerrard et al., 2005; Medvedev et al., 2008; Cheng et al., 2011; Li et al., 2011). Many previous studies have shown that luciferase assay is essential for identifying regulatory regions before constructing a reporter system (Yang et al., 2005; Medvedev et al., 2008; Cheng et al., 2011). In pigs, a *OCT4* based GFP reporter systems (Huang et al., 2011; Nowak-Imialek et al., 2011) and a dual reporter system using GFP and RFP were reported previously (Sun et al., 2016). Conserved regions in pig *OCT4* upstream sequence were identified by performing a sequence-based comparative analysis using genome sequences of various mammals. Additionally, a luciferase assay is an essential step in constructing a reporter system, and it has been conducted (Kim et al., 2019).

PSCs exhibit heterogeneity during culture (Tanaka, 2009; Singer et al., 2014; Guedes et al., 2016). Therefore, a dual *OCT4* reporter system could be useful for identifying the states of pluripotency and separating naïve and primed cells in mixed populations of pluripotent cells. Therefore, in this review, the naïve and primed pluripotent states, the porcine-specific *OCT4* reporter system and its application in pig PSC research will be discussed.

NAÏVE AND PRIMED PLURIPOTENT STATES

Human and mouse ESCs differ in many features. Previously, these differences were presumed to be caused by species-specific differences between humans and mice because researchers were not aware of the cause of the difference (Thomson et al., 1998). However, mouse EpiSCs cultured with FGF2 and ActA were similar to human ESCs. PSCs were not classified based on species differences but were divided into two different states according to the pluripotent state and developmental potency: a naïve or primed pluripotent state (Nichols and Smith, 2009; Hanna et al., 2010b). Naïve pluripotent PSCs are derived from early epiblasts in preimplantation blastocysts. Primed pluripotent PSCs are derived from late epiblasts in postimplantation blastocysts. They has a more differentiated pluripotency than naïve cells from the perspective of developmental capacity, gene expression, and epigenetic signatures. Naïve PSCs are characterized by dome-shaped colony morphologies, activation of LIF signaling, and two active X chromosomes in females. However, primed PSCs are defined by flattened colony morphologies and activated FGF signaling pathways. Compared with the primed state, naïve PSCs have developmental and functional ground states that contribute to the formation of blastocyst chimeras and a higher transgenic efficiency (Buecker and Geijsen, 2010; Hanna et al., 2010a).

The ICM produces hypoblasts and pluripotent epiblasts. The epiblast is functionally and molecularly distinct from blastomeres and the early ICM. Epiblasts have a ground state, indicating that they exhibit unlimited proliferation and development potential and the flexibility to differentiate into all embryonic lineages. The epiblast generates the entire fetus, and single epiblast cells that are isolated at this stage and microinjected into another blastocyst contribute to the formation of all 3 germ layer lineages (Gardner and Cockroft, 1998). Preimplantation epiblasts are the developmental ground state, which is also known

as the naïve pluripotent state. Cells that are widely known to present this state include preimplantation epiblasts and mouse ESCs. On the other hand, EpiSCs are the *in vitro* counterpart of primed epiblasts. ESCs are induced to differentiate into EpiSCs by ActA and FGF, but the reverse transition requires transfection with the reprogramming factor *Klf4* (Evans and Kaufman, 1981; Martin, 1981; Guo et al., 2009).

EpiSCs are derived from postimplantation epiblasts under condition with Fgf and ActA and without Lif (Brons et al., 2007; Tesar et al., 2007). These cells express the pluripotency markers, *Oct4*, *Sox2*, and *Nanog*, like naïve ESCs but differ from ESCs in the expression of specification markers such as *Fgf5* and *T* and surface markers such as *SSEA4*, *TRA 1-60*, and *TRA 1-81*. *In vitro*, cultured EpiSCs differentiate into various cell types. However, EpiSCs are not competent to contribute to the formation of blastocyst chimeras (Tesar et al., 2007; Guo et al., 2009) and are developmentally and functionally different from naïve epiblasts and ESCs.

Naïve pluripotent ESCs are immortalized naïve epiblast cells. They have a self-renewal capacity and pluripotency to produce every cell lineage. ESCs also have epigenetic features similar to preimplantation epiblasts, which contain two active X chromosomes in female cells (Heard, 2004). EpiSCs can also be produced from ESCs after culture with ActA and FGF (Guo et al., 2009). This conversion fulfills the criteria for an authentic differentiation process because the reverse transition has not been observed without genetic manipulation. During conversion, one of the X chromosomes is epigenetically silenced in females. EpiSCs express canonical pluripotency factors such as *Fgf5* and *T*. As mentioned above, these cells are converted into naïve PSCs by reprogramming through transfection with *Klf4* (Guo et al., 2009). Reprogrammed stem cells reactivate the inactivated X chromosome, ESC-specific markers, and produce chimeras and germline transmission.

Therefore, naïve pluripotent states are a ground state of pluripotency similar to preimplantation blastocysts. Their embryonic tissue is early epiblasts. They potentially induce blastocyst chimera and teratoma formation. Naïve PSCs express pluripotency factors such as *Oct4*, *Nanog*, *Sox2*, *Klf2* and *Klf4*. Mouse ESCs are representative naïve PSCs. They have a dome-shaped colony morphology and short doubling time. These naïve stem cells

have the ability to form single-cell clones. In the naïve pluripotent state, one of the major pluripotent markers, *OCT4*, is produced by controlling the DE located in the *OCT4* 5' upstream regulatory region. Naïve PSCs exhibit LIF-dependent properties during *in vitro* culture. Mouse ESCs are able to produce whole animals through tetraploid complementation. They express naïve markers such as *Rex1*, *Nr0b1*, and *Fgf4* and the cell surface marker stage-specific embryonic antigen-1 (*SSEA-1*). Naïve PSCs maintain self-renewal in the presence of Lif/stat3 but differentiate in the presence of Fgf/Erk. They have a high clonogenicity. Their XX status is X active because no X chromosome inactivation occurs (Nichols and Smith, 2009).

Comparative aspects of naïve and primed states in animals and humans

Naïve PSCs share many features with the late epiblast in the preimplantation embryo in mice. Recent studies have dealt with the characterization of a similar cell state in other animals including humans. Capturing the exact human equivalent of the mouse naïve PSC is still a difficult goal. However, comparative studies conducted to address this problem have provided a deep understanding of the regulation of pluripotent states in early mammalian development. Since the first report on mouse ESCs (Evans and Kaufman, 1981; Martin, 1981), many studies have been performed to establish PSC from other mammals. However, naïve ESCs have only been validated in mice.

Unlike mouse ESCs, the first established ESCs in pigs and humans were in primed pluripotent states. This pluripotent state is similar to that of postimplantation epiblasts. Post-implantation epiblast cells do not contribute to the formation of blastocyst chimeras (Rossant, 2008), nor do they give rise to ESCs. Their embryonic tissue is an egg cylinder or embryonic disc. Primed PSCs do not contribute to the formation of blastocyst chimeras but enabled teratoma formation after injection into BALB/c nude mice. Because a primed pluripotent state does not produce chimeras, one of the alternative analyses of pluripotency is teratoma formation. Teratomas are tumor containing cells and tissues representative of three germ layers and they occur as germline tumors (Matsui et al., 1992; Resnick et al., 1992). These cancer stem cells are called EC cells. EC cells exhibit a primed pluripotent state, and thus these cells more closely resemble EpiSCs

than ESCs. They express pluripotency factors such as *Oct4*, *Sox2* and *Nanog*. Typically, mouse EpiSCs and porcine and human ESCs are in primed states. They have a flattened colony morphology and long doubling time. Primed PSCs have difficulty forming single-cell clones. In the primed pluripotent state, *OCT4*, one of the major pluripotency markers, is produced by controlling the PE located in the *OCT4* 5' upstream regulatory region. Naïve PSCs exhibit ActA- and FGF-dependent properties during *in vitro* culture. Primed PSCs are unable to produce whole animals through tetraploid complementation. They do not express naïve markers but express specification markers such as *Fgf5* and *T*. They express surface markers such as *SSEA4*, *TRA 1-60* and *TRA 1-81*. Primed PSCs do not respond to Lif/stat3; however, they self-renew in the presence of Fgf/Erk, unlike naïve PSCs, which differentiate in the presence of Fgf/Erk. Primed pluripotent cells have a low clonogenicity. Their XX status in females is one inactive X chromosome due to X chromosome inactivation (Nichols and Smith, 2009).

With the discovery of two pluripotent states, naïve and primed, of mouse PSCs, many studies have tried to establish naïve-state PSCs in nonpermissive species (Buehr et al., 2008; Li et al., 2008). These studies have been conducted to convert primed PSCs into naïve PSCs. The first human naïve PSCs were obtained through exogenous expression of *OCT4* and *KLF4* supplemented with chemicals such as LIF and two inhibitors of GSK and ERK1/2 signaling (Hanna et al., 2010a). However, these cell lines were not maintained without transgene expression. Recent studies have reported that human naïve PSCs were established from primed PSCs by adding several molecules in addition to the aforementioned inhibitors without transgene activation (Gafni et al., 2013; Theunissen et al., 2014). By inhibiting PKC and ROCK signaling, it was successful to derive naïve human ESCs directly from human early embryos (Guo et al., 2016). These cells expressed markers of naïve pluripotency and resembled mouse ESCs in terms of gene expressions and methylation patterns. In FGF2-supplemented media, naïve cells were converted into human primed ESCs. Based on these results, naïve cells exist in early human embryos *in vivo* and modulating signaling pathways is required to maintain naïve PSCs in nonpermissive species.

The homologous recombination efficiency is higher in naïve state PSCs than in primed state PSCs (Buecker et al., 2010). Conversion of the pluripotent state from the primed state to the naïve state has been accomplished by overexpressing exogenous pluripotent genes such as *OCT4* and *KLF4* and inhibiting signaling pathways through treatment with inhibitory molecules In nonpermissive species such as pigs and humans. Naïve PSCs have a shorter doubling time and higher single cell cloning efficiency than primed PSCs according to human studies. Because of these characteristics, the efficiency of homologous recombination in naïve PSCs was higher than counterpart in primed PSCs (Buecker et al., 2010) (Table 1).

Table 1. Comparison between naïve and primed pluripotent states

	Naïve pluripotent state	Primed pluripotent state
Representative cells	Mouse embryonic stem cells	Mouse epiblast stem cells, porcine and human embryonic stem cells
Embryonic tissue	Early epiblasts	Egg cylinder or embryonic disc
Blastocyst chimera and teratoma	May induce blastocyst chimera and teratoma formation	Does not contribute to the formation of blastocyst chimeras, but enables teratoma formation
Pluripotent markers	*Oct4*, *Nanog*, *Sox2*, *Klf2*, and *Klf4*	*Oct4*, *Sox2* and *Nanog*
Representative cellular state	Preimplantation blastocysts	Post-implantation epiblast
Morphology	Dome-shaped colony morphology	Flat colony morphology
Doubling time	Short doubling time	Long doubling time
Single-cell cloning	Single-cell clones are formed	Difficult to obtain single-cell clones
Regulation of *OCT4*	Oct4 is produced by controlling the distal enhancer	Oct4 is produced by controlling the proximal enhancer
Specification markers	Naïve markers such as *Rex1*, *Nr0b1*, and *Fgf4*	Specification markers such as *Fgf5* and *T*
Cell surface markers	Cell surface marker *SSEA-1*	Surface markers such as *SSEA4*, *TRA 1-60* and *TRA 1-81*
Response to Lif/Stat3	Maintains self-renewal through Lif/stat3 signaling	Does not respond to Lif/stat3 signaling
Response to Fgf/Erk	Differentiated through Fgf/Erk signaling	Self-renewal in response to Fgf/Erk signaling
Clonogenicity	High clonogenicity	Low clonogenicity

OCT4 REPORTER SYSTEM FOR MONITORING PLURIPOTENCY

A generic reporter is an indicator of gene expression or cellular phenomena. The reporter measures changes in target genes at various levels. It is divided into two main types: transcription fusion and translational fusion. Transcription fusion reveals changes in transcriptional and posttranscriptional regulatory inputs and events. On the other hand, translational fusion provides information on posttranslational regulatory inputs and events. A reporter system can be measured in cells, tissues, and whole organisms. Therefore, it is a powerful tool for monitoring promoter structure, gene regulation, or signaling pathways (Bamps and Hope, 2008).

Undifferentiated pluripotent cells are characterized by unrestricted proliferation and the ability to differentiate into cells of the 3 germ layers. PSC markers have been identified to verify the pluripotent status. Naïve PSCs express pluripotency factors such as *Oct4*, *Nanog*, *Sox2*, *Klf2* and *Klf4* and markers of the naïve status such as *Rex1*, *Nr0b1*, and *Fgf4*. However, primed PSCs express pluripotency factors such as *Oct4*, *Sox2* and *Nanog* (Nichols and Smith, 2009). Various studies have reported porcine PSCs, and authentic ESCs have been established. However, the characterization of porcine ESCs indicates that the stem cells reported are in a primed state (Choi et al., 2019; Choi and Lee, 2019). Naïve ESCs from pigs have not been reported. An analysis of *OCT4*, one of the key genes showing differences in expression between naïve and primed states in PSCs, is necessary to identify naïve stem cells.

As mentioned earlier, *OCT4* is a pluripotency marker and reporter candidate gene (Jerabek et al., 2014). It is an important marker of undifferentiated status in early mammalian embryonic development and ESCs. In mouse embryonic development, *Oct4* is expressed in oocytes before fertilization, and all cells during cleavage. *Oct4* is expressed in all cells of the epiblast, but downregulated in trophoblasts. In addition, it is temporarily expressed in hypoblasts, but not in all extraembryonic cells after implantation (Palmieri et al., 1994). According to knockout studies on *Oct4*, it is not necessary for the formation of blastocysts but required for the ICM. Epiblasts and hypoblasts are not formed without *Oct4* (Nichols et al., 1998).

OCT4 and *SOX2*, which are required for embryonic development and PSCs, work closely and play a role in the negative feedback (Rizzino and Wuebben, 2016). *CDX2* suppresses *OCT4* and *SOX2* expression in trophectoderm (TE) during the embryonic development (Wu and Schöler, 2014). In mice, *Oct4* and *Cdx2* are specifically expressed in the ICM and TE, respectively. However, in TE cells from humans and pigs, *OCT4* and *CDX2* are coexpressed for a relatively long time in TE cells compared to mice (Liu et al., 2015). Based on these results, the expression and mechanism of transcription factors binding to the *OCT4* regulatory region may differ between species.

APPLICATION OF AN OCT4 REPORTER SYSTEM

The stem cells in culture are not all in the same state (Tanaka, 2009). An *OCT4* reporter system can be used for pluripotent research through the role of identifying, distinguishing, and separating the pluripotent cells in heterogeneous population (Gerrard et al., 2005; Medvedev et al., 2008; Cheng et al., 2011; Li et al., 2011). Using the human *OCT4* DE GFP reporter system, human naïve PSCs were identified. The mouse *Oct4* reporter system is well developed and has been widely applied in pluripotency studies (Choi et al., 2016). The hOCT4-ΔPE-GFP reporter system was problematic in human cells. Its DE has weak activity in primed human PSCs, while it was used to distinguish naïve from primed cells (Theunissen et al., 2014). Although research on porcine PSCs is important for human therapeutic research (Hall, 2008; Choi and Lee, 2019; Choi et al., 2020), naïve PSCs and authentic iPSCs have not been reported. One of the reasons for the limited information is that fewer studies have been conducted to test useful tools for studying species-specific pluripotency including reporter systems compared to other species. So, many researchers have attempted to develop a porcine-specific reporter system.

Mouse Oct4 and human *OCT4*-based reporter systems were applied in porcine pluripotent cells. But the limitation is that they are not the porcine-specific reporter systems (Nowak-Imialek et al., 2011). A porcine *OCT4*-eGFP reporter system was introduced in porcine embryonic fibroblasts and operated after SCNT and reprogramming. However, there was no distinction between DE and PE (Huang et al., 2011). A porcine *OCT4* enhancer-based dual reporter system that worked in mouse PSCs has been
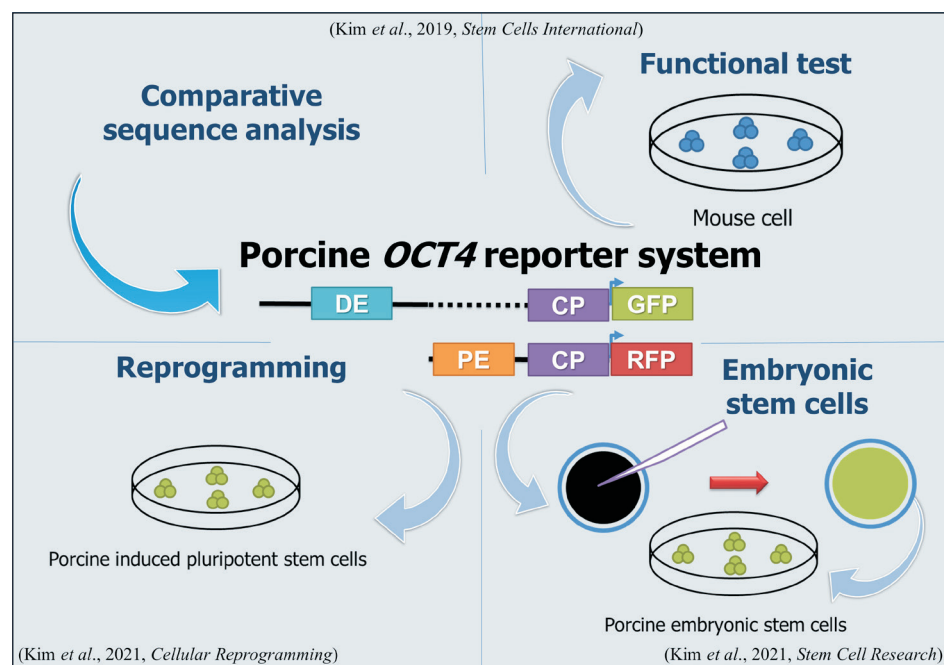
Fig. 1. Scheme of construction and application of the porcine OCT4 reporter system.

reported (Sun et al., 2016). But a luciferase assay did not conducted, and function of a reporter system in porcine pluripotent cells was not tested. In 2019, the results of an analysis of the *OCT4* upstream region showed that the sequence and function of DE and PE of porcine *OCT4* were similar to those of other mammals. However, a substantial difference was observed in the nucleotide sequence of the *Oct4* upstream region between species when the Oct4 upstream region-based reporter systems constructed from one species were inserted into another species. For instance, porcine PE-based vectors did not function properly in mouse PSCs (Kim, et al., 2019). Therefore, a porcine-specific *OCT4* reporter system is essential for the functional evaluation of porcine-derived pluripotent cells. Functional tests of the porcine-specific *OCT4* reporter system in porcine ESCs and iPSCs were conducted in 2021 (Kim et al., 2021a; Kim et al., 2021b). Porcine *OCT4* upstream region-derived dual reporter systems serve as live naïve/primed pluripotency indicators for porcine-iPSCs establishment. Research examining the activity and function of the pig *OCT4* enhancer in the porcine early embryonic development stage and porcine authentic ESCs is underway.

Overall, a reporter system is needed to identify species-specific pluripotency. A sequence analysis was conducted to confirm the possibility of species-specific pluripotency, and luciferase assays were conducted for a enhancer

analysis (Kim et al., 2019). In addition, the function of the reporter was tested in porcine-origin pluripotent cells (Kim et al., 2021a; Kim et al., 2021b) (Fig. 1). Research using the reporter system will become more diverse. These applied studies will promote research on stem cells and mechanisms of pluripotency in pigs and will also help in applying these stem cells.

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