

# Porcine OCT4 Reporter System can Monitor Species-Specific Pluripotency During Somatic Cell Reprogramming

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## Research

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# Abstract

**Background:** The present study examined the activity and function of pig *OCT4* enhancer in porcine reprogramming cells. Dual fluorescent protein reporter systems controlled by the upstream regulatory region of *OCT4*, which is one of the master regulators for pluripotency, are widely used in studies of the mechanism of pluripotency. We analyzed how this reporter system functions in FGF- or LIF-dependent reprogrammed porcine pluripotent stem cells using the previously established porcine-specific reporter system.

**Results:** Porcine embryonic fibroblasts were coinfecting with the p*OCT4*- $\Delta$ PE-eGFP (DE-GFP) and p*OCT4*- $\Delta$ DE-DsRed2 (PE-RFP) vectors, and GFP and RFP expression was verified during a DOX-dependent reprogramming process. We demonstrated that the porcine *OCT4* distal enhancer and proximal enhancer were activated in different expression patterns simultaneously as the changes in the expression of pluripotent marker genes during the establishment of porcine-induced pluripotent stem cells (iPSCs).

**Conclusions:** Porcine *OCT4* upstream region-derived dual fluorescent protein reporter systems serve as live naïve/primed pluripotency indicators for porcine induced pluripotent cell establishment. This work demonstrates the applicability of the porcine *OCT4* upstream region-derived dual fluorescence reporter system, which may be applied to investigations of species-specific pluripotency in porcine-origin cells. These reporter systems may be useful tools for studies of porcine-specific pluripotency, early embryo development and embryonic stem cells.

## Background

Characteristics of pluripotent stem cells are proliferation and differentiation, which may be useful tools for therapeutic research, such as regenerative medicine. Pluripotent stem cells (PSCs) have two different states of pluripotency. Naïve and primed PSCs are related to the activation of LIF or FGF signaling, respectively. The mouse is a well-researched model for the pluripotent states naïve and primed, which have a clear distinction. The reference cells of pluripotent states are present according to pluripotent level, such as mouse embryonic stem cells (naïve), mouse epiblast stem cells and embryonic carcinoma cells (primed) [1–4]. Various research was also reported about porcine pluripotent stem cells [5–7]. A recent remarkable achievement was made in pigs, which is the establishment of authentic embryonic stem cells [8]. However, naïve pluripotent stem cells of pig were not reported [8, 9]. One key difference between naïve and primed states in pluripotent stem cells is *OCT4*.

The pluripotent marker *OCT4* is one of many pluripotency-related genes that was studied as a reporter gene because it is only expressed in pluripotent cells [10]. The transcription factor *OCT4* is an important marker of undifferentiated status in early mammalian embryonic development and embryonic stem cells. It plays a critical role as a central regulator in maintaining pluripotency and self-renewal. *OCT4* contains a core promoter and two conserved enhancers, the distal enhancer (DE) and proximal enhancer (PE) [11–13]. Enhancers have multiple cognate binding sites where various transcription factors may be attached

to regulate the expression of genes. Some sites are close to the promoter, and other sites are far away. The former sites are called proximal enhancers (PE), and the latter sites are called distal enhancers (DE) [14]. These two enhancers are used to produce *OCT4* in various pluripotent cells and work simultaneously or sequentially. Because different factors exist depending on the pluripotent state, *OCT4* has two enhancers to form *OCT4* in different environments, and it is configured to operate in two different environments. One study of a mouse *Oct4* upstream region model revealed that the two enhancer regions were activated differently. The DE is a key element of the *Oct4* gene in naïve pluripotent cells, such as mouse embryonic stem cells, germ cells, and inner cell mass [11, 15], and the *Oct4* PE is specifically primed in pluripotent cells, such as mouse epiblast and embryonic carcinoma [11, 13].

Pluripotent stem cells have heterogeneity during culture [16–18]. Therefore, an *OCT4* upstream-based dual reporter system may be used to classify the states of pluripotency in mixed populations of pluripotent cells and separate more naïve cells and more primed cells in these mixed populations. Although it is one of the most necessary parts of studying stem cells and pluripotency, the function of a porcine-specific *OCT4* reporter system in porcine origin cells was not reported. Therefore, it was necessary to compare and confirm the activity of the *OCT4* enhancers with the acquisition of pluripotency in the reprogramming process. Many studies were performed using the species-specific reporter system in other species for the introduction into cells of the species. The mouse *Oct4* reporter system is well-developed and well-applied for pluripotency [12, 19]. The h*OCT4*- $\Delta$ PE-GFP reporter system had problems in humans, such as slight activity in primed human pluripotent stem cells, but it could be used to distinguish naïve from primed cells [20]. Although research of porcine pluripotent stem cells is important for human therapeutic research [21], naïve pluripotent stem cells and authentic induced pluripotent stem cells have not been reported [8, 9, 22–25]. One of the reasons is few research on useful tools for studying species-specific pluripotency, including porcine *OCT4* upstream-based reporter system compared to other species. Many researchers have tried to develop a porcine-specific reporter system because of its importance.

An m*Oct4* and h*OCT4*-based reporter were introduced in porcine pluripotent cells, but it is not a porcine-specific reporter system [26]. A porcine *OCT4* upstream region-based eGFP reporter system was introduced in porcine embryonic fibroblasts and worked after SCNT and reprogramming, but activities of the distal enhancer and proximal enhancer were not separated [27]. A porcine *OCT4* enhancer-based dual reporter system worked in mouse pluripotent stem cells, but luciferase assay and analysis of porcine pluripotent cells with a reporter system was not performed [28]. Our previous study analyzed the *OCT4* upstream regions. The results showed that the sequence and function of distal and proximal enhancers of porcine *OCT4* were similar with other mammals. However, a large difference was found in the *Oct4* upstream region nucleotide sequence between species, and the differences in expression occurred when the *Oct4* upstream region-based reporter systems constructed from one species were inserted into another species. Porcine proximal enhancer-based vectors did not work properly in mouse pluripotent stem cells [29]. Therefore, a porcine-specific *OCT4* reporter system, which is important for porcine pluripotency research, required functional evaluation in porcine origin pluripotent cells. We needed to establish a reference by checking the activity and difference of the porcine *OCT4* enhancers according to

the state of pluripotency during the process of reprogramming. Therefore, we performed functional tests of the porcine *OCT4* reporter system in porcine-induced pluripotent stem cells in this study.

## Methods

### Porcine OCT4 upstream region-derived dual reporter system

Porcine-specific OCT4 upstream region-derived dual reporter systems were produced as described previously [29]. The following two vectors were used primarily in this study: p*OCT4*- $\Delta$ PE-eGFP vector (DE-GFP) containing Conserved regions (CR) 1 and 4, and a p*OCT4*- $\Delta$ DE-DsRed2 vector (PE-RFP) containing CR1, 2, and 3. For analysis of the expression pattern of distal and proximal enhancers using two previously constructed reporter system, an identical fluorescent (eGFP) vector was used. The p*OCT4*- $\Delta$ PE-eGFP vector (DE-GFP) and p*OCT4*- $\Delta$ DE-eGFP vector (PE-GFP), which contained CR1, 2, and 3 in the eGFP vector [24], were transfected in porcine embryonic fibroblasts cultured in LIF- or FGF-reprogramming conditions. All vectors were linearized by *Asel* and *Hind* III before use for removing the CMV promoter and for cell transfection.

## Cell culture, transfection and porcine embryonic fibroblast reprogramming

Porcine embryonic fibroblast cells (PEFs) with inducible systems containing human Oct4, Sox2, Klf4, and Myc were prepared in a previous experiment following the protocols described by Choi et al. (2016)[7]. PEFs were cultured in feeder-free conditions on gelatin-coated culture dishes. Each reporter construct was transfected into cells using Fugene HD reagent (Promega) according to the manufacturer's protocol. After 24 hours of culture, selective media containing 300  $\mu$ g/mL G418 (Sigma) were added, and the cells were cultured for another 7 days. G418-resistant cell colonies were selected and cultured after subculture. The reprogramming media contained DMEM (Welgene) supplemented with 15% KSR, 2 mM glutamax (Gibco), 0.1 mM  $\beta$ -mercaptoethanol (Gibco), 1  $\times$  MEM nonessential amino acids (Gibco), 1  $\times$  antibiotic/antimycotic (Gibco), 2 ng/ml doxycycline (dox), and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, MN, USA) or 1000 unit/ml leukemia inhibitory factor (LIF; Millipore, MA, USA) (Fig. 1). Media were changed daily, and all cells were cultured under humidified conditions with 5% CO<sub>2</sub> at 37 °C.

## Immunocytochemistry

Immunocytochemistry was performed to evaluate the expression of genes related to pluripotency. Before staining, all cell samples were preincubated for 10 minutes at 4 °C and fixed with 4% paraformaldehyde for 30 minutes. After washing twice with Dulbecco's phosphate-buffered saline (DPBS; Welgene), samples were treated for 1 hour with 10% goat serum in DPBS to prevent nonspecific binding. Serum-treated cells were incubated overnight at 4 °C with the primary antibodies. The following primary antibodies were used: OCT4 (1:200; Santa Cruz Biotechnology, CA), eGFP (1:200; Invitrogen) and DsRed2 (1:200;

Invitrogen). When using antibodies for intracellular proteins, fixed cells were treated for 5 minutes with 0.2% Triton X-100 (Sigma-Aldrich) before serum blocking. After incubating with the primary antibody, the cells were treated for 3 hours at room temperature with Alexa Fluor-conjugated secondary antibodies 488 and 594. Nuclei were stained with Hoechst 33342 (Molecular Probes). Images of the stained cells were captured using an inverted microscope.

## **Total genomic DNA isolation and PCR**

To check transfection, total genomic DNA was extracted from porcine embryonic fibroblasts using the G-spin Total DNA Extraction Kit (iNtRON Biotechnology, Korea). Genomic DNA samples were amplified using 10 pmol of species-specific primers (Table 1) and 2 × PCR master mix solution (iNtRON Biotechnology). PCR was performed in a thermocycler under the following conditions: 95 °C for 5 minutes; followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; with a final extension at 72 °C for 7 min. Amplified PCR products were electrophoresed in 1% (w/v) agarose gels and stained with RedSafe Nucleic Acid Staining Solution (an alternative to ethidium bromide; iNtRON Biotechnology).

Table 1  
Primer sequences used in the gene expression analysis.

mRNA	Primer sequences (5'-3')
GAPDH	F : TGCTCCTCCCCGTTTCGAC R : ATGCGGCCAAATCCGTTTC
E-CADHERIN	F : ATTCTGGGAGGCATCCTTGC R : GTTGTCCCGGGTGTTCATCTT
SALL4	F : TACCAGAGCCGAAGTCCAGA R : ATCTCAGTGCGGCTGTTCTC
CRIPTO	F : TCCCAGTTTGTACCATCCAC R : TAGAAGGAAGGAGGGCAAGC
NANOG	F : CATCTGCTGAGACCCTCGAC R : GGGTCTGCGAGAACACAGTT
DAX1	F : GGTACCAGGCCAGATTTGCT R : CAGCTCCTGTACTTGGGTGG
KLF2	F : TTCGGTATCTTTGACGACCCG R : GGCTTGGCCTCTAGTAGCTC
OCT4A	F : CTTGGAGAGCCCTGGTTTTACT R : GCCAGGTCCGAGGATCAAC
SOX2	F : CGGCGGTGGCAACTCTAC R : TCGGGACCACACCATGAAAG
GFP	F : AAGGGCATCGACTTCAAGG R : TGCTTGTCGGCCATGATATAG
RFP	F : TGAAGGGCGAGACCCACAA R : TGGACTTGA ACTCCACCAGGTA

## Analysis of mRNA expression by quantitative real-time PCR

To verify the gene expression levels in pluripotent cells, we performed quantitative real-time PCR (qPCR). Total mRNA from individual samples was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed using a High Capacity RNA-to-cDNA (cDNA Reverse Transcription) Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's

instructions. Briefly, cDNA synthesis was performed at 37.5 °C for 60 minutes at a final volume of 20 µL, and samples were subsequently incubated at 95 °C for 5 minutes to inactivate the reverse transcription reaction. Synthesized cDNA samples were stored at – 80 °C until subsequent use. qPCR was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems). A DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific, Rockford, IL, USA) was used for real-time quantification of the PCR products. For amplification, 0.1 µM of each primer listed in Tables 1 and 0.5 µL of cDNA was added to a 10-µL reaction mixture. The reactions were performed under the following conditions: 1 cycle at 95 °C for 10 minutes; 40 cycles at 95 °C for 15 seconds; and 60 °C (or 64 °C for *OCT4A*, *SOX2*) 15 seconds at the annealing temperature. The dissociation curve was analyzed to confirm the specificity of the PCR product. GAPDH was used as a control gene to determine the relative quantity. The expression ratio relative to the PEF cells was calculated using the  $\Delta\Delta C_t$  method.

## Immunofluorescence analysis using flow cytometry

For flow cytometry analysis, D15 reprogrammed porcine embryonic fibroblasts at a concentration of 1 million cells/mL were preincubated for 10 minutes at 4 °C, and fixed with 4% paraformaldehyde for 30 minutes. The fixed cells were sorted with a BD FACSAria II and subjected to FACS analysis using the following antibodies: GFP (1:500, Invitrogen), RFP (1:500, Invitrogen), Alexa Fluor 488 dye (1:2000, BD Biosciences), and Alexa Fluor 647 dye (1:2000, BD Biosciences). Prior to the experiment, the compensation was performed using a negative control and each single dyeing cell. Data analysis was performed using FlowJo software (Tree Star, Inc.).

## Results

### Reprogramming of pig embryonic fibroblasts carrying porcine OCT4 upstream region-based dual reporter system in FGF2- or LIF-containing media

First, we investigated whether the porcine *OCT4* upstream-based dual reporter system worked in the porcine-origin pluripotent stem cells. For this experiment, porcine embryonic fibroblasts introduced with the porcine *OCT4* upstream region-based dual reporter system were essential. The porcine embryonic fibroblast cells with inducible systems of *OCT4*, *SOX2*, *KLF4* and *MYC* used in this study was generated in a manner reported previously [7]. Transfection of porcine *OCT4* upstream-based dual reporter systems and selection were performed. Naïve pluripotent cells exhibit activation of LIF signaling, but primed pluripotent cells exhibit activation of the FGF signaling pathway [3, 4]. Therefore, the experiment consisted of two groups: FGF-dependent reprogramming condition and LIF-dependent reprogramming condition. These experimental groups referred to previous reports of several researchers who studied porcine-induced pluripotent stem cells in FGF [30] or LIF [5] or both conditions [31]. The cells were cultured in a medium supplemented with LIF, Dox or bFGF, Dox (Fig. 1A). Transfection of the *OCT4* dual reporter system was checked using PCR. The results confirmed that DE-GFP and PE-RFP vectors were introduced well (Fig. 1B). In FGF- and LIF-dependent conditions, cell numbers increased and morphology changed during Dox reprogramming. This reprogramming process showed that cell proliferation was faster and greater under FGF-dependent conditions than LIF-dependent conditions (Fig. 1C). *OCT4* is a

pluripotency marker that was not expressed on day 0, but it was expressed on day 15 using immunostaining in FGF- and LIF-dependent conditions. The ICC results showed that the PEFs expressed *OCT4* after reprogramming, which shows that it became iPSCs (Fig. 1D). Therefore, our results indicate that porcine embryonic fibroblasts with a porcine *OCT4* upstream region-based reporter system were well established and reprogrammed in Dox-containing reprogramming media.

## Expression of pluripotent marker genes in LIF- or FGF-dependent DOX reprogramming media culture conditions

LIF signaling was closely related to naïve-type pluripotent stem cells, but FGF signaling was related closely to primed-type pluripotent stem cells. Therefore, we investigated which signaling more affected the reprogramming process in porcine pluripotent stem cells. To minimize variables, the FGF-dependent and LIF-dependent reprogramming conditions were the same except for supplementation with FGF or LIF. In both reprogramming conditions, the reprogramming process was confirmed by identifying changes in the expression levels of pluripotent genes. Porcine embryonic fibroblasts with *OCT4*, *SOX2*, *KLF4*, and *MYC* were cultured in reprogramming culture media supplemented with FGF or LIF, and qPCR was performed in samples collected after culturing for 0, 3, 6, 9, 12 and 15 days (Fig. 2). Analysis of the qPCR results showed that pluripotent-related markers increased during the reprogramming process in different expression patterns.

*E-CADHERIN*, also as known as *CDH1*, is an early-phase reprogramming and naïve marker [32], and *DAX1* and *KLF2* are late-phase reprogramming markers [33]. These genes were expressed similarly in LIF- and FGF-dependent reprogramming conditions. Notably, *DAX1*, also as known as *NROB1*, is a naïve pluripotency marker in mouse [1], It is also expressed in porcine early embryo and D10 epiblasts [34]. *DAX1* expression correlated with the activity of porcine *OCT4* distal and proximal enhancers in the FGF-dependent condition (Figs. 2 and 3A). *KLF2* is a conserved naïve marker in mouse and human [35], but it is not a naïve marker in pig, in which it exhibits low expression compared to mouse and human [22, 34]. *E-CADHERIN* also has low expression levels in porcine preiPSCs compared to human and mouse pluripotent stem cells [22]. Notably, we found two genes with distinctly different expression under FGF- or LIF-dependent conditions, *CRIPTO* and *SALL4*. *CRIPTO* is expressed in porcine early embryo development [34], and it was highly activated in LIF-dependent conditions. *CRIPTO* is important for stem cell maintenance. Stem cells with insufficient *CRIPTO* become small, which reduces stem cell stability, such as increasing *CDX*, and promote differentiation [36]. *SALL4* had a high level of expression in the FGF-dependent condition, and it is an intermediate-phase reprogramming marker [37] that is and important for stem cell maintenance. *OCT4* may be expressed without *SALL4*, but if the expression of *SALL4* increases, the expression of *OCT4* increases [38]. The quantitative PCR results showed that the expression of *OCT4* in the FGF-dependent condition was higher than the LIF-dependent condition. These results show that *SALL4* is attached to the *OCT4* promoter region. *SALL4* blocks *CDX* to prevent differentiation, and without it, stem cell proliferation decreases. In our results, the cell proliferation rate in the FGF-dependent condition was higher than the LIF-dependent condition (Fig. 1C and 4A). *SALL4* is an intermediate-phase

reprogramming marker [37], and it was only expressed in the FGF-dependent reprogramming condition. *CRIPTO* is expressed in porcine early embryo development [34], and it was only expressed in the LIF-dependent reprogramming condition. *OCT4* was highly expressed during the early reprogramming stage in both conditions. *SOX2* was expressed during the late reprogramming stage only in the LIF-dependent reprogramming culture condition. We observed that *OCT4* was expressed in both conditions, but its expression level in the FGF-dependent condition was higher than the LIF-dependent condition. During D12-15, *NANOG*, which is a conserved naïve marker in mouse and human [35], was not expressed continuously in the LIF-dependent reprogramming condition. *SOX2*, which is core pluripotency marker in mouse and human [35] and is expressed in pig early embryo and D12-primed embryonic discs, was expressed in the LIF-dependent condition. Some pluripotent marker genes were not expressed in LIF (*SALL4*, *NANOG*) or FGF (*CRIPTO*, *SOX2*) conditions (Fig. 2 and Table 2), which means that both reprogramming conditions are incomplete for the successful reprogramming of porcine embryonic fibroblasts, and porcine preiPSCs in both reprogramming conditions have different pluripotent networks.

Table 2

Pluripotent-related marker gene expression in porcine embryo and induced pluripotent stem cells.

	Porcine embryo development			Porcine-induced pluripotent stem cells	
	Early embryo	Epiblast	Embryonic disc	FGF-dependent	LIF-dependent
OCT4	+	+	+	+(higher than LIF)	+
SOX2	+	-	+	-	+
NANOG	+	+(weak)	+	+	+(not detected on D15)
DAX1	+	+	-	+	+
SALL4	+			+	-
E-CADHERIN	+			+	+
KLF2	-	-	+	+	+
CRIPTO	+	+	+	-	+
Expression of mRNA related to pluripotency, such as <i>OCT4</i> , <i>SOX2</i> , <i>NANOG</i> , <i>DAX1</i> , <i>KLF2</i> , <i>CRIPTO</i> [34], <i>SALL4</i> [48] and <i>E-CADHERIN</i> [54] in porcine embryo development and induced pluripotent stem cells.					

These results suggest that *DAX1* in porcine pluripotent cells is related to *OCT4* enhancer expression in the FGF condition, and *SALL4* and *CRIPTO* expression are related to FGF and LIF signaling, respectively. Naïve pluripotent markers, such as *E-CADHERIN*, *KLF2* and *DAX1*, in mouse and human were not applicable in porcine preiPSCs. *DAX1* expression in porcine epiblasts [34] is related to FGF signaling and had similar expression patterns with activation levels of porcine *OCT4* distal and proximal enhancers.

Expression of *OCT4* was higher in the FGF-dependent condition than the LIF-dependent condition. *NANOG*, which is expressed in porcine-primed embryonic disc [34], and it was expressed after reprogramming in the FGF-dependent condition. It is one of the important factors for maintaining pluripotency and self-renewal in mouse and human embryonic stem cells, and it was also important for the reprogramming of induced pluripotent stem cells [39]. *CRIPTO*, which is related to LIF, and *SOX2*, which is expressed in porcine early embryo [34], were expressed in the LIF-dependent condition. Therefore, LIF signaling is also needed for successful porcine reprogramming. *SOX2* is another important factor for pluripotent stem cells [40] (Fig. 2 and Table 2). However, our results showed that porcine-induced pluripotent stem cells have primed state and FGF-signaling preference, but the pig is thought as a nonpermissive species [7].

## Distal and proximal enhancer activity patterns in LIF- or FGF-dependent DOX reprogramming media culture condition

To measure the activity of the two enhancers by the expression level of mRNA, it was necessary to confirm that the mRNA of GFP correlated with the transfection of the amount of DNA introduced in the analysis of mRNA. With the y-axis on a log scale, the quality control results were almost straight in a dose-dependent manner (data not shown). Comparison of before and after reprogramming, GFP and RFP expression on day 15 was higher than day 0 in the FGF- and LIF-dependent conditions. Activity of distal enhancers in LIF-dependent reprogramming continued to increase as the reprogramming progressed. However, the activity of the distal enhancer in FGF-dependent reprogramming increased until day 6 and was accompanied with increased *OCT4*, *SOX2* and *DAX1*. On day 12, the distal enhancer was activated to the highest level in the FGF-dependent condition. In the LIF-dependent condition, proximal enhancer was highly activated on day 9, and it was the highest on day 12 in the FGF-dependent condition. the proximal enhancer expression level declined on day 15 in both conditions. The activation pattern of the proximal enhancer was similar to the *DAX1* expression pattern in the FGF-dependent condition (Figs. 2 and 3A). However, the two conditions were similar to embryonic carcinoma cells than embryonic stem cells in mouse. In a previous luciferase analysis experiment in mouse, the porcine *OCT4* distal enhancer was highly activated in the naïve state on E14, and the proximal enhancer was highly activated in the primed state on P19 [29]. However, the proximal enhancer in the porcine embryonic fibroblast reprogramming process was more activated than the distal enhancer in both reprogramming culture conditions (Fig. 3B).

We confirmed the function of our reporter system at the protein level. A large number of porcine fibroblasts went through reprogramming and expressed at least one fluorescence. Porcine iPSCs are in a heterogenous state and contain several intermediate unstable cell types because of spontaneous reprogramming in FGF- or LIF-dependent culture conditions. Therefore, various fluorescence expression patterns in each cell colony were detected. This result demonstrated that the culture population in a FGF- or LIF-dependent reprogramming medium was heterogenous, and the different subcell types were classified using our dual-fluorescence reporter. Our reporter system in porcine embryonic fibroblast results indicated that porcine *OCT4* upstream region-based reporter system worked during porcine

reprogramming at the protein level (Fig. 4A). According to the FACS analysis, the fluorescent-positive rates in FGF-dependent and LIF-dependent reprogramming conditions were 93.1% and 97.7%, respectively. A high percentage of cells were double positive, 90.1% in FGF and 96.1% in the LIF-dependent condition. These results suggest that pigs do not use only one enhancer in pluripotent states, but two enhancers at the same time, and there is only a difference in degree (Fig. 4B).

## Discussion

### Porcine OCT4 upstream region-based reporter system could work similar to human reporter analysis during porcine reprogramming

The reprogramming process and epigenetic reversion were checked in mouse using an *Oct4* reporter system [41–43]. Transgenic mice were generated that had a mouse *Oct4* upstream region-based dual reporter system to check the control of enhancers in embryonic and germ cell development. The dual reporter system worked and distinguished mouse naïve and primed stem cells [19, 44]. The mouse *Oct4* reporter system worked very well by cell-type and developmental stage because the activation of mouse *Oct4* enhancers were distinguished clearly by pluripotent states in mouse pluripotent cells. However, researchers attempted to establish naïve-state human pluripotent stem cells using a h*OCT4* upstream region-based reporter system. Some results indicated that human *OCT4* enhancers did not express and clearly silence genes, similar to mouse *Oct4* enhancers, in naïve or primed state pluripotent stem cells [20, 45]. This experiment identified that porcine *OCT4* enhancers acted differently in naïve and primed pluripotency states in mouse pluripotent stem cells and functioned in porcine pluripotent stem cells. Our results found that porcine-induced pluripotent stem cells in FGF- or LIF-dependent conditions were similar to the primed pluripotent state. When porcine *OCT4* enhancers acted in porcine pluripotent stem cells, the distal and proximal enhancers worked similar to a human *OCT4*-derived reporter system rather than a mouse reporter system. Porcine *OCT4* expression was similar to human *OCT4* expression rather than mouse *Oct4* expression [46]. Our results indicated that porcine *OCT4* enhancers and other reporter systems also worked similar to human, not mouse systems.

The characteristics of mouse *Oct4*, human *OCT4* and porcine *OCT4* exhibited many differences, such as low homology of sequences, but various features of porcine *OCT4* and its enhancers were more similar to human *OCT4* than the mouse *Oct4*. Nucleotide sequences from the porcine *OCT4* upstream region were more similar to the human *OCT4* upstream region rather than counterpart of mouse *Oct4*. In all conserved regions and a 3.2-kb upstream region, total nucleotide, including distal and proximal enhancers, homology between human and pig is higher than between mouse and pig [29]. Enhancers are key contributors to gene expression patterns via various mechanisms [14]. Research of *Oct4* in mouse indicated that distal enhancers activated *Oct4* expression in mouse embryonic stem cell, morula, ICM and primordial germ cells, and proximal enhancers inhibited the expression of *Oct4* in the epiblast [11]. However, the *OCT4* gene was expressed in the ICM and trophectoderm, and it was epiblast-specific in human [47] and pig [48]. These results indicated that the expression of distal and proximal enhancers in mouse *Oct4* was clearly distinct during embryo development, but not in human and pig.

Our previous research found that putative transcription factor binding sites exist in porcine *OCT4* upstream regions. One of the 4 conserved regions is conserved region 4 (CR4), which is farthest from the core promoter and has distal enhancer site 2A and a binding domain for Oct4/Sox2 [29]. *OCT4* and *SOX2* are required in embryo development and pluripotent stem cells, and these genes were controlled in embryo and stem cells. These genes work together closely, and they have a negative feedback loop balance [49]. During embryo development, *CDX2* in TE inhibits *OCT4* and *SOX2* expression by affecting the *OCT4* upstream CR4 region [50]. *Oct4* and *Cdx2* were specifically expressed in ICM and TE, respectively, in mouse. *Oct4* was excluded from TE, and *Cdx2* was excluded from ICM. However, *OCT4* and *CDX2* are coexpressed for a relatively long time in TE cells in human and pig [48, 51]. These results indicated that the expression and mechanism of transcription binding factors in the *OCT4* distal enhancer region may be different between species. This research supports our results that porcine enhancers and our porcine-based reporter system were more similar to human rather than mouse.

### **Applications of porcine *OCT4* upstream region-based reporter system could help investigation of porcine-specific pluripotency**

Pluripotency, naïve and primed markers vary from species to species. For example, naïve markers in mouse and human are not applicable in pig [34], and various human-specific pluripotent markers were reported [35]. Our results also indicated that naïve and primed markers in mouse and human were not applicable in porcine embryo and pluripotent stem cells. Despite the importance of porcine-specific markers for naïve and primed conditions, this research remains an ongoing endeavor. Under these circumstances, a porcine reporter system would help identify new porcine-specific pluripotent state markers. Our *OCT4* reporter system would help distinguish between naïve and primed pluripotent states and identify porcine-specific markers in porcine embryo and pluripotent stem cells. In the LIF-dependent condition, the activity of a distal enhancer was slightly higher than in the FGF-dependent condition. However, the expression level of distal and proximal enhancers in both conditions differed in naïve-state mouse embryonic stem cells, and it was rather similar to the primed-state mouse embryonic carcinoma (Fig. 3B). This difference means that the porcine-induced pluripotent stem cells in both conditions were closer to a primed pluripotent state than naïve. Our reporter system was applied in porcine pluripotent cells, a distinction between the naïve and primed states may be performed in the current situation where the porcine-specific naïve and primed pluripotent markers were not identified. The porcine *OCT4* upstream region has various transcription factor binding sites, and these transcription factors may relate to species-specific pluripotency [29]. Our reporter system will also help find the naïve and primed markers after separating the cells in naïve or primed states.

During the reprogramming process, pluripotent marker genes with unique expression patterns were found. *DAX1* affected *OCT4* via *LRH-1* mediated activation. The *LRH-1* (*NR5A2*) binding site in the *OCT4* promoter region was confirmed using chromatin immunoprecipitation. *DAX1* is related to *OCT4* expression [52], and its expression was similar to the expression pattern of the enhancers of porcine *OCT4* enhancers. Therefore, the activities of *OCT4* enhancers affect its promoter region, and our results indicated that *DAX1* was a representative *OCT4* enhancer in the FGF condition. Two genes (*SALL4* and

*CRIPTO*) thought to be related to FGF and LIF signaling were also found, and these genes may be candidates for the marker genes identified in porcine-induced pluripotent stem cells that were defined as primed. *SALL4* affects *OCT4* expression via binding its distal enhancer DE2A in CR4, which is one of the 4 *OCT4* upstream conserved regions [38]. Considering that the basic fibroblast growth factor signaling pathway maintains the stemness of primed pluripotent stem cells [53], and *SALL4* was highly expressed in FGF-dependent reprogramming conditions in pig, the distal enhancer of porcine *OCT4* upstream region played an important role. Although the expression was lower than the proximal enhancer in primed state porcine pluripotent stem cells, unlike the mouse *Oct4* distal enhancer, which is specifically activated in naïve state pluripotent stem cells. Further verification of the *OCT4* enhancers is required to analyze the unique mechanism of porcine enhancers and establish naïve state pluripotent stem cells in porcine-specific enhancer studies and research on reporter systems.

## Conclusions

In summary, we introduce a porcine-specific *OCT4* reporter system that was constructed previously into cells of porcine embryonic fibroblasts and analyzed GFP and RFP expression during the reprogramming process. This reporter system may be used for the identification of naïve or primed pluripotent status of porcine-induced pluripotent stem cells when porcine-specific pluripotent state markers were not identified. Based on the results of these experiments, the reporter system functioned properly, and this system showed that the porcine iPSCs created by this experiment was close to a primed state, not a naïve state. Porcine iPSCs proliferated well in the FGF-dependent condition and showed a preference for FGF signaling. However, both FGF and LIF signaling are needed. Our results indicated that porcine enhancers and the reporter system were more similar with humans rather than mouse. The mechanisms and functions of distal and proximal enhancers of porcine *OCT4* were different from the mouse. Studies of porcine *OCT4* enhancers and porcine-based reporter system will be helpful to understand porcine-specific pluripotency and to establish naïve pluripotent stem cells in pig.

## Abbreviations

<b>FGF</b>	<b>Fibroblast growth factor</b>
LIF	Leukemia inhibitory factor
DE	Distal enhancer
PE	Proximal enhancer
PSC	Pluripotent stem cell
GFP	Green fluorescent protein
RFP	Red fluorescent protein
CR	Conserved region
CMV	Cytomegalovirus
PEF	Porcine embryonic fibroblast
PCR	Polymerase chain reaction
FACS	Fluorescence-activated cell sorting

## Declarations

### Ethics approval and consent to participate

The authors assert that all procedures in this work complied with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. The Institutional Animal Care and Use Committee, Seoul National University approved the care and experimental use of pigs (SNU-181024-8).

### Consent for publication

Not applicable.

### Availability of data and material

All data generated or analyzed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

S.-H. Kim, K.-H. Choi and C.-K. Lee designed research; S.-H. Kim, K.-H. Choi and M. Lee performed research; S.-H. Kim, K.-H. Choi, M. Lee and D.-K. Lee analyzed data; S.-H. Kim and C.-K. Lee wrote the paper; C.-K. Lee finally approved the manuscript.

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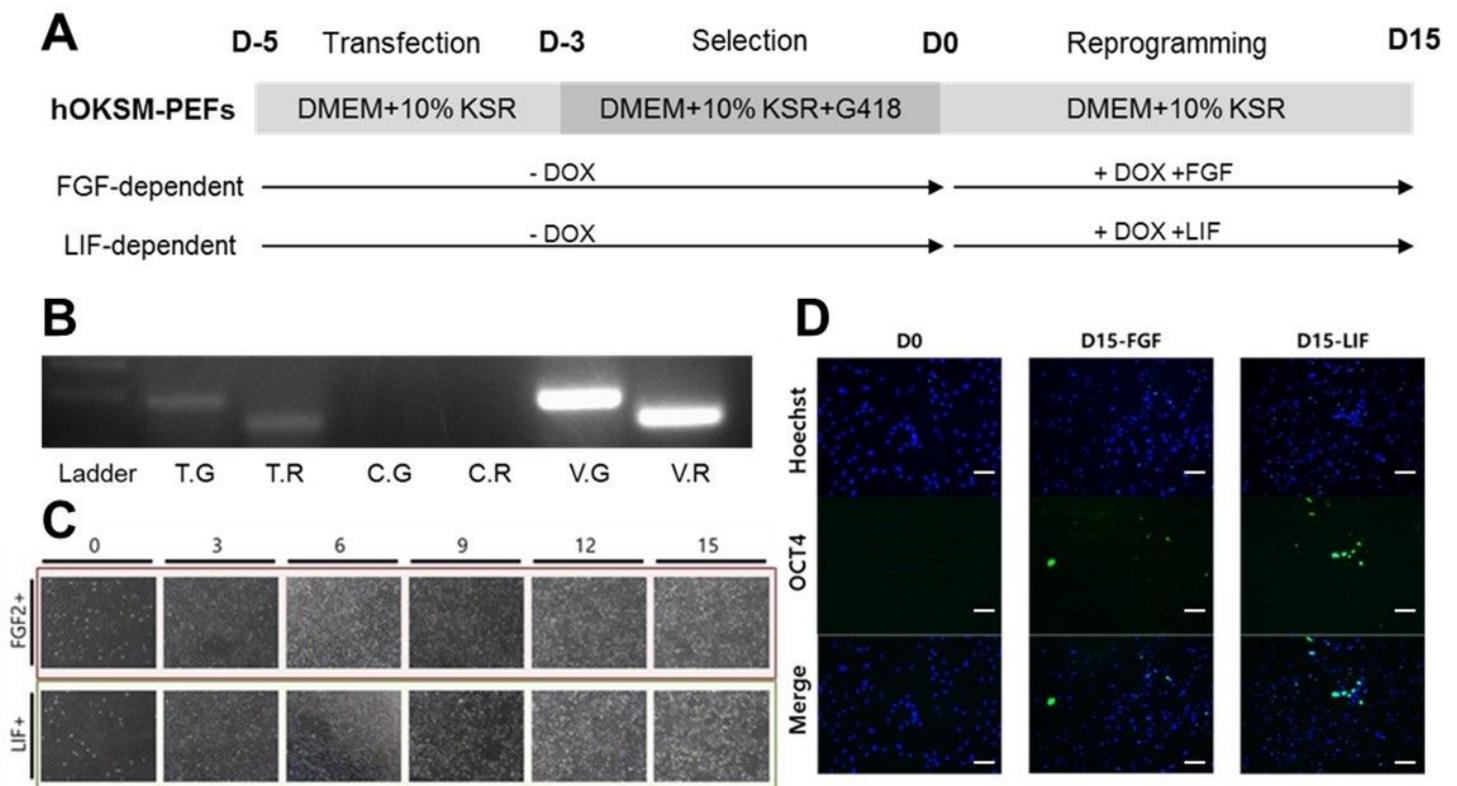
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55. **To whom it may concern.**
56. The paper "Porcine *OCT4* reporter system can monitor species-specific pluripotency during somatic cell reprogramming" by Chang-Kyu Lee was edited by Elsevier Language Editing Services.
57. regards  
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58. **Elsevier Webshop Support.**

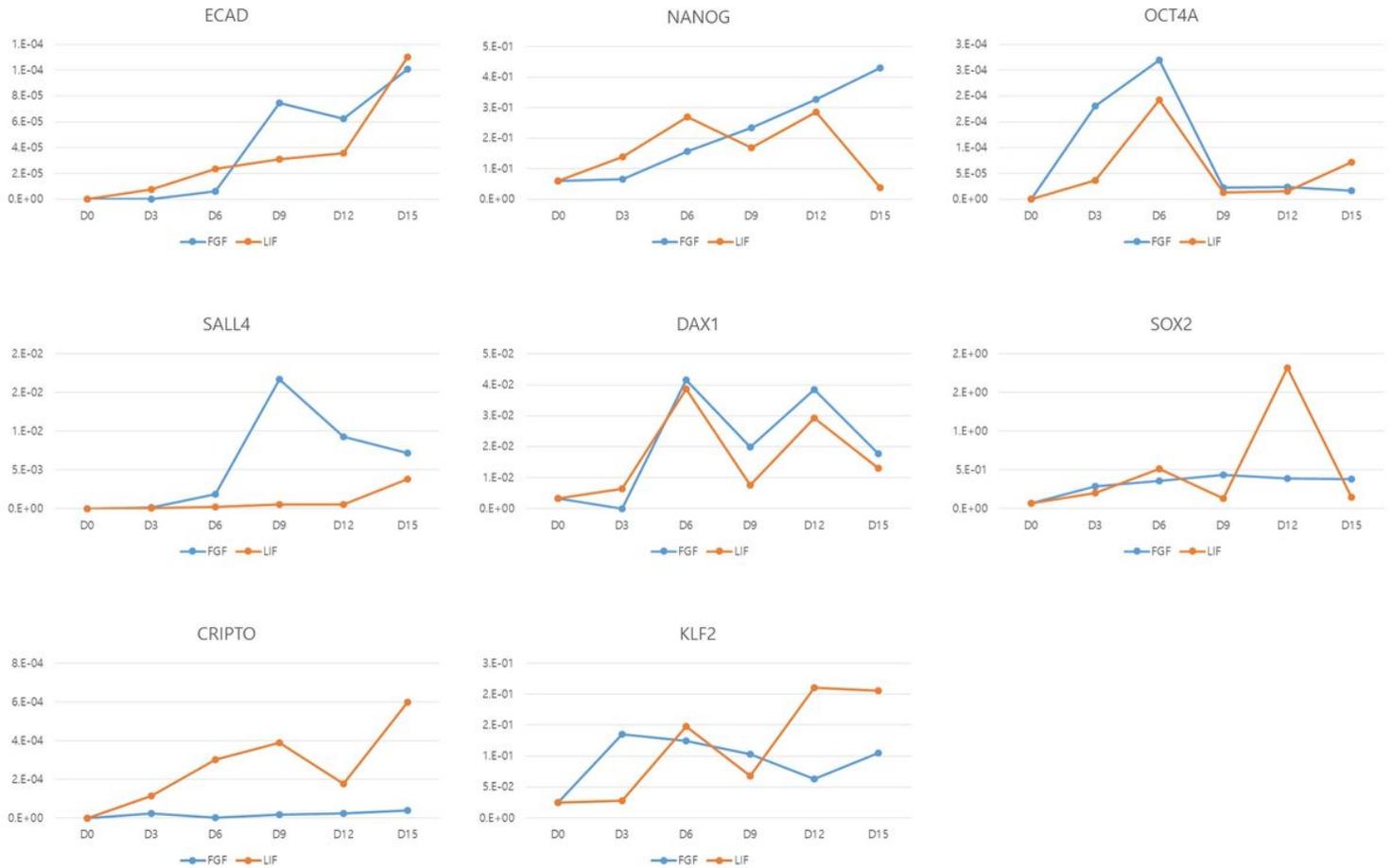
## Figures



**Figure 1**

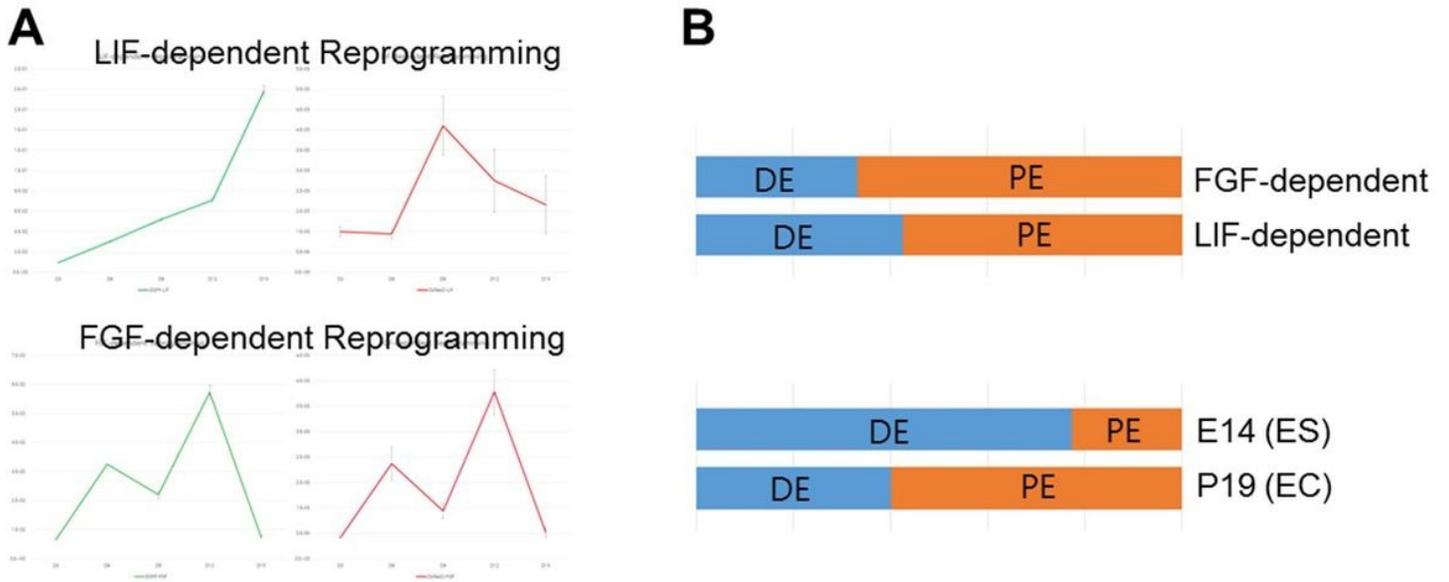
Attempt to obtain porcine induced pluripotent stem cells A. Schematic illustration of the generation of LIF- or FGF-dependent induced pluripotent stem cells with the porcine *OCT4* promoter-derived reporter system. B. Transfection of DE-GFP and PE-RFP checked using RT-PCR. T.G; Transfection of GFP, T.R;

Transfection of RFP, C.G, Control of GFP; C.R, Control of RFP; V.G, Vector (positive control) of GFP; V.R, Vector of RFP; C. Representative bright-field cell morphology of Dox-induced reprogramming of porcine embryonic fibroblasts in LIF- or FGF-dependent culture conditions on D0, 3, 6, 9, 12 and 15. D. Immunostaining of OCT4 expression in LIF- or FGF-dependent induced pluripotent stem cells on D0 and D15. Scale bar = 200  $\mu$ m.



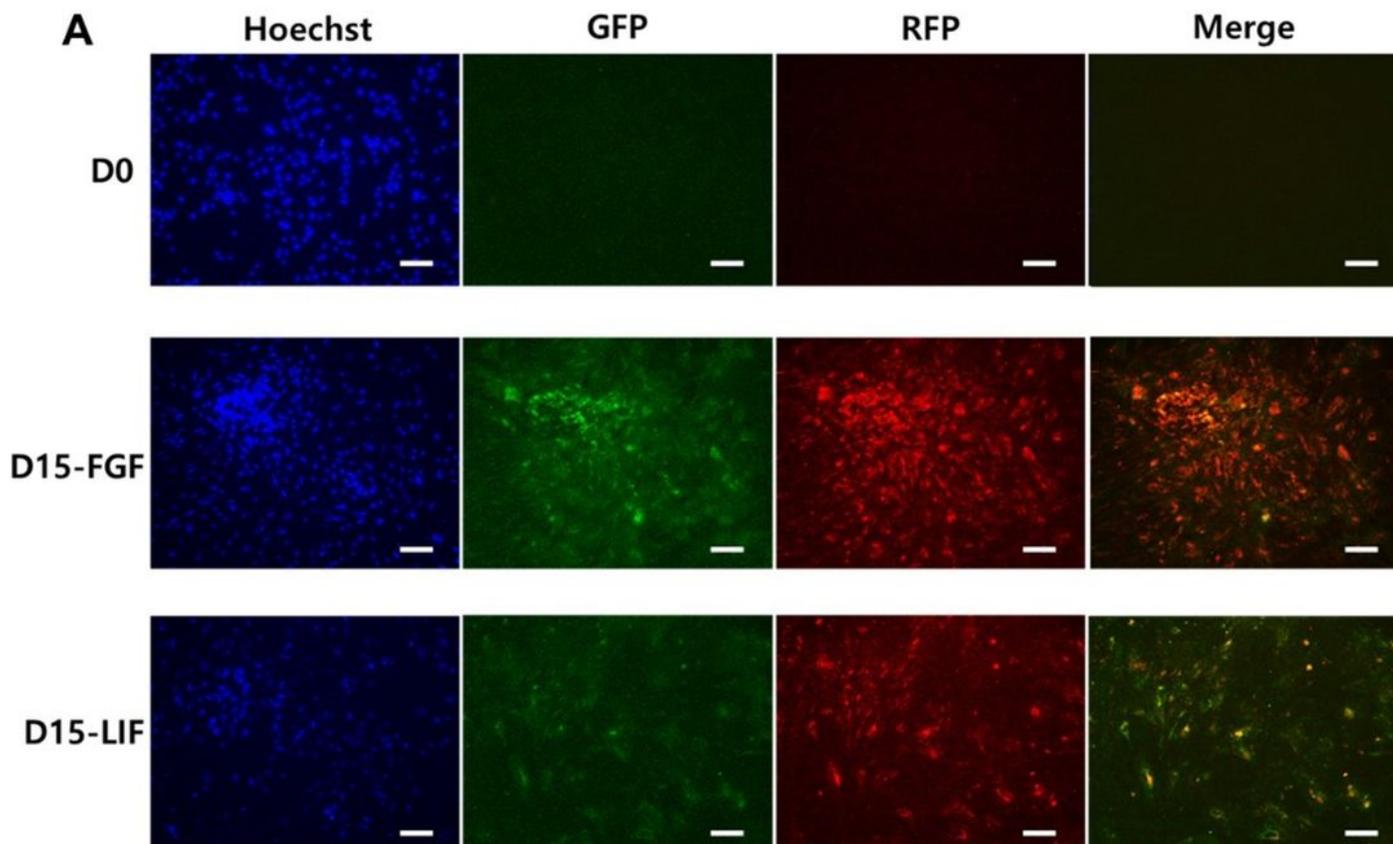
**Figure 2**

Expression levels of pluripotent marker genes in FGF- or LIF-dependent reprogramming. Comparison of pluripotency marker expression in FGF- or LIF-dependent reprogramming conditions using quantitative PCR analyses of pluripotent markers. Expression of pluripotent markers was normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

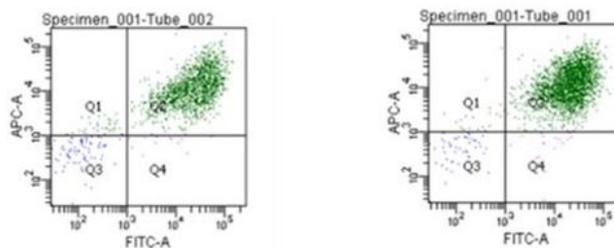


**Figure 3**

Expression levels of pluripotent marker genes in LIF- or FGF-dependent reprogramming. A. Analysis of expression patterns of distal and proximal enhancers using two previously constructed reporter system, pOCT4- $\Delta$ PE-eGFP vector (DE-GFP) and pOCT4- $\Delta$ DE-eGFP vector (PE-GFP) [29] in LIF- or FGF-reprogramming conditions. In the LIF-dependent reprogramming condition, the activity of the distal enhancer increased continuously and the proximal enhancer increased until D9 and decreased after that point. In the FGF-dependent reprogramming condition, the activity of distal and proximal enhancers fluctuated. B. Comparison of the active rate of the porcine OCT4 distal and proximal enhancers in FGF- or LIF- dependent reprogramming condition after reprogramming in mouse embryonic stem cells (E14) and mouse embryonic carcinoma (P19).



**B**



	<b>FGF-condition</b>	<b>LIF-condition</b>
Double positive	90.1%	96.1%
GFP only	1.0%	0.8%
RFP only	2.0%	0.8%
Double negative	6.9%	2.3%
Positive rates of fluorescence	93.1%	97.7%

**Figure 4**

Activation of porcine OCT4 distal and proximal enhancers after LIF- or FGF-dependent reprogramming. A. Hoechst signal (blue) is a nuclear counterstain. Distal enhancer (Green) and proximal enhancer (Red) were not activated on D0, but they were activated on D15 under LIF- or FGF-dependent reprogramming culture conditions. Merged signal (Red and Green) indicated that various pluripotent cells exist, such as double positive, only GFP-positive, only RFP-positive and double negative after reprogramming. Scale bar

= 200  $\mu$ m. B. Fluorescent cell rates in FGF- or LIF-dependent reprogramming conditions were identified using FACS analysis-positive cell. FITC, GFP-Alexa 488; APC, RFP-Alexa 647.