

# Effects of activin A on the pluripotency of induced pluripotent stem cells derived from porcine Sertoli cells

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## *Abstract*

The Activin/Nodal signaling pathway is necessary for promoting the self-renewal and pluripotency in several species. However, these effects in porcine stem cells have yet to be examined. We hypothesized that Activin/Nodal signaling could be an important factor for maintenance of porcine induced pluripotent stem cells (iPSCs). The effects of activin A on pluripotency of porcine iPSCs derived from Sertoli cells were examined. Two iPSC cell lines were cultured with different culture conditions (control, activin A, TGF- $\beta$ 1 inhibitor and activin A combined with TGF- $\beta$ 1 inhibitor). The Sertoli iPSCs treated with different conditions were compared for morphology, alkaline phosphatase (AP) activity, OCT4 protein expression and quantitative expression of genes (endogenous pluripotency genes and cell differentiated genes). This study revealed that the activin A treated iPSCs increased intensity of OCT4 protein expression. Furthermore, expressions of the pluripotent genes including *OCT4* and *NANOG* were significantly upregulated in the activin A treated group ( $P < 0.05$ ). In contrast, the selective TGF- $\beta$ 1 inhibitor adversely affected to iPSCs morphology and patterns of the AP and OCT4 protein expression. The TGF- $\beta$ 1 inhibitor also resulted in significant upregulation and downregulation for *DES* (mesoderm differentiation) and *GATA6* (endoderm differentiation), respectively ( $P < 0.05$ ) when compared with other groups. In conclusion, activin A significantly upregulated the endogenous expression of *OCT4* and *NANOG* pluripotent genes, thereby improving networks of the pluripotency in porcine iPSCs.

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**Keywords:** activin A, induced pluripotent stem cells, pluripotency, porcine, Sertoli cells, TGF- $\beta$ 1 inhibitor

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## Introduction

Pigs have become an effective large animal model for regenerative medicine as their organ size, physiology and functions are comparable to human (Ye *et al.*, 2014; Shiue *et al.*, 2016). They have been widely used for research in cardiovascular diseases, wound healing, diabetes, spinal cord injuries and xenotransplantation (Cibelli *et al.*, 2013). Pluripotent stem cells including embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs) essentially require two important properties of self-renewal and pluripotency (Romito and Cobellis, 2016). Although the ES cells, isolated from the inner cell mass of blastocyst stage embryos, have been described as pluripotent stem cells, the isolation and culture of porcine ES cells remain difficult (Plusa and Hadjantonakis, 2014). In addition, none of authentic porcine ES cells have been established (Hou *et al.*, 2016). More recently, the iPSCs are initially generated by introduction of ectopic transcription factors: *OCT4*, *SOX2*, *KLF4* and *c-MYC* (OSKM or Yamanaka factors) into adult somatic cells (Takahashi and Yamanaka, 2006). The advantages of iPSCs are lesser ethical issues (King and Perrin, 2014), and the generation of pig iPSCs has been more successful compared to the ES cells (Esteban *et al.*, 2009; Wu *et al.*, 2009; Montserrat *et al.*, 2011; Fujishiro *et al.*, 2013; Gao *et al.*, 2014; Du *et al.*, 2015; Ma *et al.*, 2018). However, the signaling pathway for maintenance of porcine iPSCs is still obscure (Fujishiro *et al.*, 2013). The pluripotent states of the porcine iPS-cell lines were derived from cell culture with additions of leukemia inhibitory factor (LIF) (Cheng *et al.*, 2012; Rodriguez *et al.*, 2012; Fujishiro *et al.*, 2013; Ma *et al.*, 2018), basic fibroblast growth factor (bFGF) (Ezashi *et al.*, 2009; West *et al.*, 2010; Hall *et al.*, 2012) or both LIF and bFGF (Montserrat *et al.*, 2011; Zhang *et al.*, 2015; Setthawong *et al.*, 2019). Optimization of cell culture conditions for porcine iPSCs is therefore necessary to improve the consensus generation and maintenance of porcine pluripotent stem cells (Gao *et al.*, 2014). Previous study demonstrated that mouse ES cells required LIF for the maintenance of the naïve state of pluripotency (Saunders *et al.*, 2013; Onishi and Zandstra, 2015). However, mouse epiblast stem cells and human ES cells required bFGF and Activin/Nodal signaling for self-renewal and maintaining the pluripotency (Vallier *et al.*, 2005; Vallier *et al.*, 2009). The importance of these signals for maintaining the porcine iPSCs remained to be entirely elucidated.

Activin/Nodal growth factors are important to control biological activities, including cell cycle progression, progenitor proliferation, differentiation during organogenesis (Gritsman *et al.*, 2000; Brennan *et al.*, 2001) and adult tissue homeostasis (Strizzi *et al.*, 2012). Activins bind to serine/threonine kinase receptors, type I and type II activin receptors, leading to the phosphorylation and activation of the Smad2 and Smad3 downstream pathway. The Smad2 and Smad3 subsequently form a complex with the Smad4 and translocate to the nucleus (Pauklin and Vallier, 2015). This complex activates the target genes which are bound with the transcriptional networks of OCT4 and NANOG proteins for the maintenance of

pluripotency genes. Of the key pluripotency factors including *OCT4*, *NANOG* and *SOX2* genes (Vallier *et al.*, 2009), the *OCT4* is one of the most important transcription factors required for maintaining an undifferentiated state and pluripotency (Niwa *et al.*, 2000; Pesce and Scholer, 2001; Kellner and Kikyo, 2010). *SOX2* acts synergistically with *OCT4* to activate *OCT-SOX* enhancers, which regulate expression of specific genes in the pluripotent stem cells (Masui *et al.*, 2007). Moreover, expression of *NANOG* has been recognized to be upregulated in iPSCs and is necessary to block endoderm differentiation (Vallier *et al.*, 2009; Hackett and Fortier, 2011). Alberio *et al.* (2010) first demonstrated that porcine epiblast stem cells (EpiSC), derived from days 10.5-12 embryos required Activin/Nodal signaling for the expression of the core pluripotent genes and self-renewal maintenance. Porcine EpiSC cultured with ALK5 inhibitor differentiated into neuronal lineage (Alberio *et al.*, 2010). However, knowledge on the importance of Activin/Nodal pathways for maintaining pluripotency of porcine iPSCs is fairly limited.

In the current study, we used porcine Sertoli iPSCs (Setthawong *et al.*, 2019) for investigating the effects of activin A on pluripotency of porcine iPSCs cultured either with or without TGF- $\beta$ 1 inhibitor. We hypothesized that supplementation of activin A into porcine iPSCs culture would improve the maintenance properties and also increase the expression levels of pluripotency genes in a defined culture condition.

## Materials and Methods

**Animal ethics and biosafety:** Animal maintenance, care and use protocol were performed according to the Animal Ethics Approval of Chulalongkorn University (No. 1673028). The biosafety was performed following the guidelines of the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC1631022).

**Generation and characterization of porcine Sertoli induced pluripotent stem cells:** Porcine Sertoli iPSCs were generated and characterized as previously reported (Setthawong *et al.*, 2019). Two Sertoli iPSC-like cell lines (PS-R8 and PS-R29) were used in this study. Prior to use, the Sertoli iPSCs were characterized by alkaline phosphatase staining (AP), OCT4 immunofluorescent staining and *in vitro* differentiation.

**Activin A and TGF- $\beta$ 1 inhibitor treatments during iPSCs cultivation:** The Sertoli iPSCs (PS-R8 and PS-R29, passage 15) were divided into 4 groups (control, activin A, TGF- $\beta$ 1 inhibitor and activin A combined with TGF- $\beta$ 1 inhibitor). The Sertoli iPSCs were cultured in a defined medium containing DMEM F-12 (Gibco) supplemented with 20% (v/v) of knockout serum replacement (Gibco), 1% (v/v) of a non-essential amino acid solution (Sigma), L-glutamine and an antibiotic (1 mM, Gibco),  $\beta$ -mercaptoethanol (0.1 mM, Gibco), 10 ng/ml human leukaemia inhibitory factor (hLIF, Millipore) and 10 ng/ml basic fibroblast growth factor (bFGF, BioVision). For activin A and TGF- $\beta$ 1 inhibitor treated groups, the Sertoli iPSCs were treated

with 10 ng/ml activin A (338-AC, R&D Systems) and 1 $\mu$ M TGF- $\beta$ 1 inhibitor (A83-01, Biovision), respectively. Sertoli iPSCs were cultured in the treatment conditions for 48 h (Tojo et al., 2005; Tomizawa et al., 2011; Yang et al., 2017). The Sertoli iPSCs cultured in iPS medium without activin A and TGF- $\beta$ 1 inhibitor served as a control group. The Sertoli iPSCs with different treatments were compared for morphology, AP activity, OCT protein expression and quantitative expression of genes (endogenous pluripotency and differentiation of three germ layers).

**Alkaline phosphatase staining and immunofluorescent microscopy:** Activity of AP in the Sertoli iPSCs was performed using the AP kit (Sigma-Aldrich). For immunofluorescent staining, the Sertoli iPSCs were fixed in 4% (w/v) paraformaldehyde in a phosphate-buffered saline (Gibco) and permeabilized with 0.1% Triton X-100 and 2% bovine serum albumin for 15 min. The cells were then incubated with a polyclonal antibody goat against OCT4 protein (IgG; 1:150) at 37°C for 1 h. The samples were then incubated with a secondary antibody labelled with Fit-C fluorescence at 37°C for 1 h. The DAPI was used to label DNA/nucleus.

**Determination of pluripotency and differentiation of three germ layers:** The levels of expression of pluripotency markers (OCT4, SOX2 and NANOG) and three germ layer markers (ectoderm: *NES*, mesoderm: *DES*, endoderm: *GATA6*) were investigated after 48 h

of treatment. Quantitative analysis of gene expression was analyzed by using relative real-time polymerase chain reaction (qPCR) in control group, activin A treated group, TGF- $\beta$ 1 inhibitor treated group and activin A combined with TGF- $\beta$ 1 inhibitor treated group. Total RNA was extracted from PS-R29 passage 15 treated cells using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of RNA was measured by Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, USA) and immediately stored at -80°C until used. The DNA contamination was treated by using DNase I (Promega, WI, USA). The cDNA was synthesized by ImProm-II™ Reverse Transcription System (Promega, WI, USA). Nuclease-free water was used in qPCR reaction as no template control. The qPCR was performed on ABI 7300 Real-time cycler (Applied Biosystems, Foster City, CA, USA). The qPCR reactions (15  $\mu$ l) were amplified with KAPA SYBR® FAST qPCR Kits (KK4600). Target forward and reverse primers (200 nM) were added into master mix reaction. The relative expression levels of *OCT4*, *SOX2*, *NANOG*, *NES*, *DES* and *GATA6* genes were normalized to an endogenous control gene ( *$\beta$ -ACTIN*). The qPCR condition was performed as the following: stage 1 of 50°C for 2 min and stage 2 of 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and 60°C for 30 s and annealing/extension 72°C for 1 min. Data were reported as the relative expression difference in relation to the control group. The specific primers are listed in Table 1.

**Table 1** Porcine specific primer sequences used for quantitative polymerase chain reaction

| Primer name                     | Sequence                                            | Accession Number | Amplicon size (bp) |
|---------------------------------|-----------------------------------------------------|------------------|--------------------|
| <i><math>\beta</math>-ACTIN</i> | F: TCCCTGGAGAAGAGCTACGA<br>R: CGCACTTCATGATCGAGTTG  | AJ312193         | 159                |
| <i>OCT4</i>                     | F: GGAAGAGAAAGCGGACAAGT<br>R: CAGCAGCCTCAAAATCCTCT  | NM001113060.1    | 216                |
| <i>SOX2</i>                     | F: CTACAGCATGTCCTACTCGC<br>R: GGGCAGTGTACCGTTGATG   | EU503117         | 274                |
| <i>NANOG</i>                    | F: CGAATGAAATGTAAGAGGT<br>R: CCAGCTCTGATTACCCAC     | DQ447201.1       | 162                |
| <i>NESTIN</i>                   | F: GGCTTCTCTCAGCATCTTGG<br>R: AAGGCTGGCATAGGTGTGTC  | XM_013996960.1   | 150                |
| <i>DESMIN</i>                   | F: CCTCAACTTCCGAGAAACAAGC<br>R: TCACTGACGACCTCCCATC | NM_001001535.1   | 108                |
| <i>GATA6</i>                    | F: CTCCATTGACAGCCGCTAT<br>R: CTGAGGCCGTTTCATCTTGCT  | NM_214328.2      | 109                |

**Statistical analysis:** The experiments were replicated three times and performed using two iPSC cell lines (PS-R8 and PS-R29). All data is expressed as mean  $\pm$  SD. Normal distribution was tested by Shapiro test. One-way ANOVA and Tukey's Multiple Comparison Test analysis were used to test the differences in gene expression between treatment groups. All statistical analyses were performed with SPSS version 22.0.0 (IBM, Armonk, NY, USA). *P* values less than 0.05 were considered statistically significant.

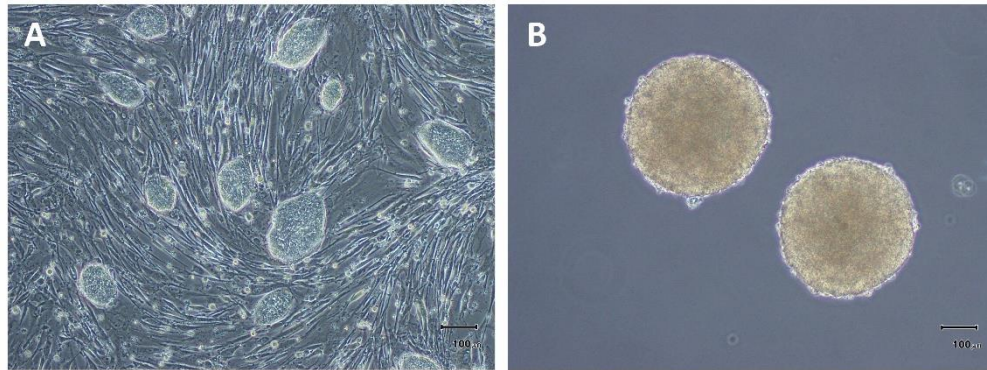
## Results

Sertoli iPSCs were generated by retrovirus transduction with OSKM. The iPSC colonies had a clear border, flattened cells and high nucleus-to-

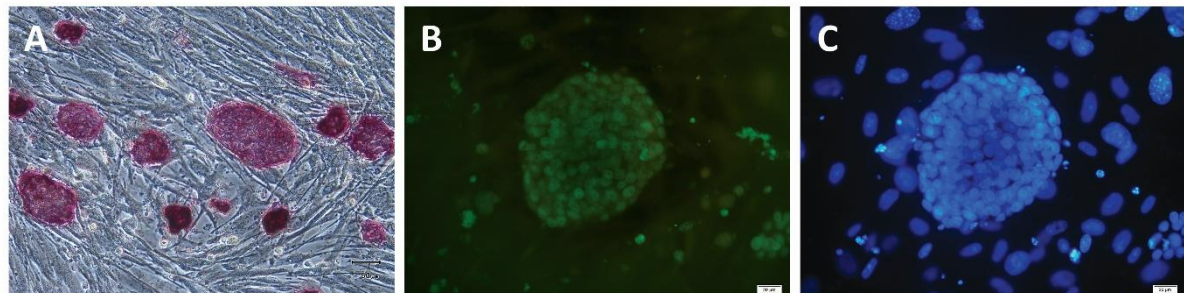
cytoplasm ratio with prominent nucleoli (Fig. 1A). These colonies were positive for AP staining (Fig. 2A) and OCT4 protein (green) observed in the cell nuclei (Fig. 2B and 2C). Differentiation property of the Sertoli iPSCs was examined by *in vitro* embryoid body formation (Fig. 1B). Two iPSC cell lines (PS-R8 and 29) were cultured in control and treatment groups (activin A, TGF- $\beta$ 1 inhibitor, and activin A combined with TGF- $\beta$ 1 inhibitor). In control group, Sertoli iPSC colonies showed round morphology, clear border and positive to AP and OCT4 staining (Fig. 3A-D). The porcine iPSCs treated with 10 ng/ml activin A had morphology and were positive to AP staining similar to that of the control group. However, activin A treated iPSCs increased intensity of OCT4 expression when compared with the control (Fig. 3E-H). Furthermore,

addition of TGF- $\beta$ 1 inhibitor adversely affected to iPSCs morphology. These iPSC colonies had loose morphology, increased intercellular spaces and tended to differentiation. In addition, the intensity of AP and OCT4 staining were subjectively decreased, especially for OCT4 protein (Fig. 3I-L). It was noticeable for activin A combined with TGF- $\beta$ 1 inhibitor group that the iPSCs demonstrated morphology similar to the control group but the mixture patterns of AP and OCT4 positive staining was found (Fig. 3M-P). To further investigate the effects of activin A and TGF- $\beta$ 1 inhibitor on gene expression, qPCR revealed that the expression of pluripotency genes (*OCT4* and *NANOG*)

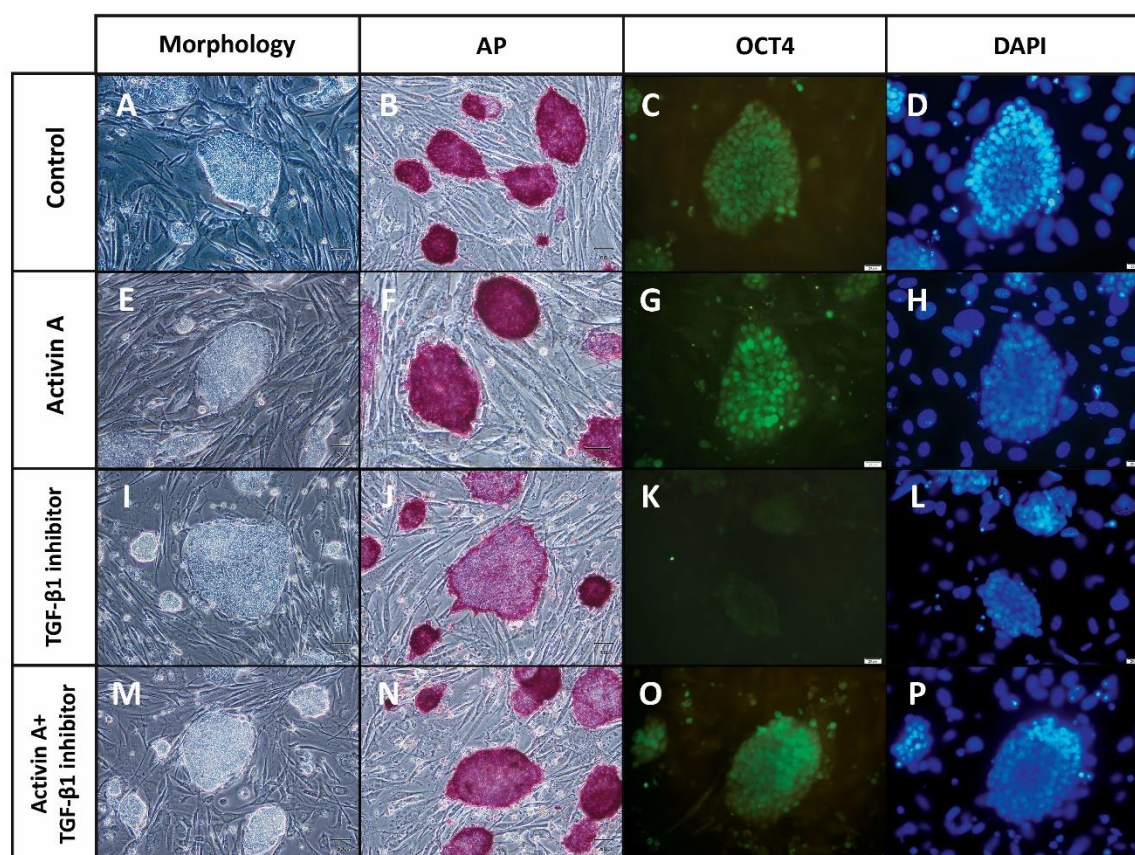
in the porcine iPSCs were significantly upregulated in activin A treated group (Fig. 4A and 4C). There was no significant difference in level expressions of *SOX2* in activin A, TGF- $\beta$ 1 inhibitor and activin A combined with TGF- $\beta$ 1 inhibitor groups (Fig. 4B). For expression of differentiation of the three germ layer genes, the expression of *DES* (mesoderm gene) was significantly upregulated in TGF- $\beta$ 1 inhibitor treated group (Fig. 4E), while *GATA6* (endoderm gene) was significantly downregulated in this group (Fig. 4F). However, there was no difference in the expression levels of *NES* (ectoderm gene) in each group (Fig. 4D).



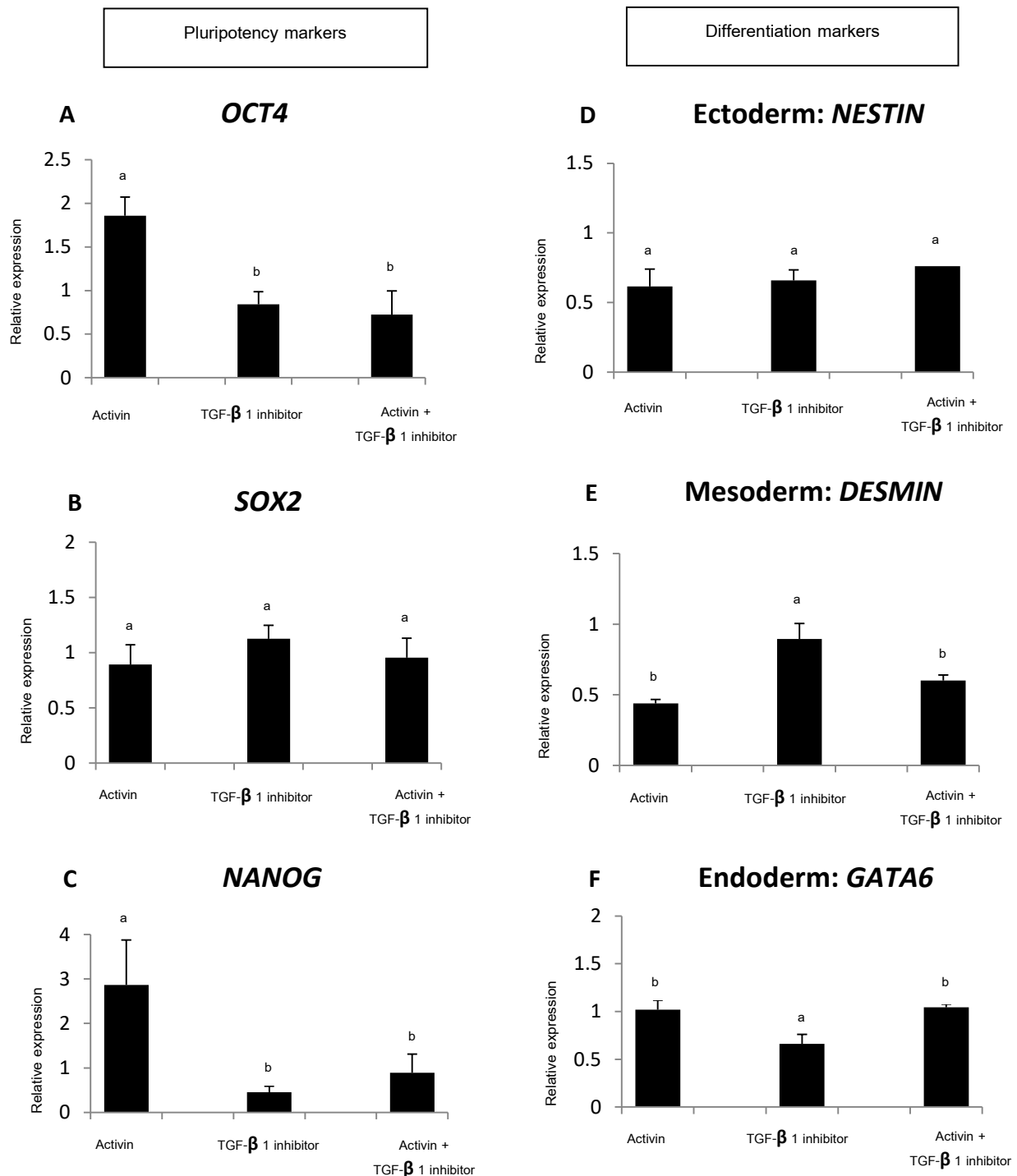
**Figure 1** The morphology of porcine iPSCs derived from Sertoli cells at passage 15 (A). *In vitro* differentiation of Sertoli iPSCs showed EB formation on day 3 (B). Scale bar represents 100  $\mu$ m.



**Figure 2** The iPSC colonies strongly expressed AP (A, scale bar =50  $\mu$ m). The pluripotency marker of OCT4 protein was indicated using immunofluorescent staining (B). DAPI was used as a counterstaining (C). The scale bars (B and C) represent 20  $\mu$ m.



**Figure 3** Characterization of Sertoli iPSC-like cells derived from different treatment condition (PS-R29, passage 15). The morphologies, AP (Scale bar represents 50  $\mu$ m) and immunofluorescent staining (pluripotency markers OCT4, Scale bar represents 20  $\mu$ m) were demonstrated after treated with activin A, TGF- $\beta$ 1 inhibitor or activin A combined with TGF- $\beta$ 1 inhibitor for 48 h.



**Figure 4** Quantitative RT-PCR analysis of mRNA expression levels of pluripotency markers (*OCT4*, *SOX2* and *NANOG*) and differentiation markers (ectoderm: *NESTIN*, mesoderm: *DES*, endoderm: *GATA6*). <sup>a,b</sup> Different letters on bars indicate values that are significantly different ( $P < 0.05$ ) when compared among treatment groups.

### Discussion

Porcine iPSCs have become an alternative choice of porcine ES cells in terms of its similarity in pluripotent properties (Ezashi *et al.*, 2012) while they have less ethical concerns. These cells have been proposed as a model for cell therapy in human (Koh and Piedrahita, 2014). However, the different culture conditions induced non-uniformity of porcine iPS-cell lines that were maintained in either naïve or primed stage

(Fujishiro *et al.*, 2013; Park *et al.*, 2013). The Sertoli cells are indeed specialized cells of the testis. These cells have been demonstrated to efficiently reprogram in several species such as mouse and pig (Sun *et al.*, 2014; Wang *et al.*, 2014; Setthawong *et al.*, 2019). In the present study, we examined the effects of activin A on porcine iPSCs on certain gene expressions (pluripotency and differentiation genes) following activin or its inhibitor treatments. According to previous study, Activin/Nodal signaling is important

for porcine EpiSC self-renewal and maintains pluripotency (Alberio *et al.*, 2010). We considered that porcine iPSCs might also rely on Activin/Nodal pathway. This study used a defined serum-free replacement (KSR) instead of FBS as we aimed at examining the effects of activin A on porcine iPSCs. Animal proteins such as fetal bovine serum have been demonstrated to interact and induce differentiation of pluripotent state (Vackova *et al.*, 2007). This defined condition is ideal for examining the cellular responses in activin A and its inhibitor treatments. The optimal doses of 10 ng/ml activin A and 1  $\mu$ M TGF- $\beta$ 1 inhibitor (A83-01) used in this study were chosen according to previous publications (Telugu *et al.*, 2010; Tomizawa *et al.*, 2011; Klincumhom *et al.*, 2014; Hou *et al.*, 2016; Yang *et al.*, 2017). As expected, activin A improved pluripotency of porcine iPSCs in terms of AP activity and OCT4 protein expression. This coincided with the findings that activin A significantly upregulated the endogenous pluripotent genes such as OCT4 and NANOG. Indeed, Activin/Nodal pathway plays an active role to maintain the expression of core pluripotency factors including OCT4 and NANOG (Vallier *et al.*, 2009). These two genes are co-regulatory of maintenance of pluripotency and inhibition of differentiation (Boyer *et al.*, 2005). In porcine pluripotent stem cells, activin A signaling could promote OCT4 and NANOG expression via Smad2 and 3 cascades, thereby increasing the pluripotency and inhibition of differentiation (Brevini *et al.*, 2012). This transduction pathway appeared to conserve among species (Vallier *et al.*, 2009; Alberio *et al.*, 2010). However, there was no significant difference in the level expression of SOX2 between treatment groups. The possible reason for this may link to the fact that the upstream SOX2 expression is not directly controlled by the activin signaling (Dabdoub *et al.*, 2008). When the activin A is inhibited using a selective TGF- $\beta$ 1 inhibitor with small molecule A83-01, the porcine iPSCs changed in colony morphology, pattern of AP activity, and OCT4 protein expression. Moreover, the TGF- $\beta$ 1 inhibitor treated group significantly downregulated the OCT4 and NANOG genes, while a gene associated with differentiation (DES) were upregulated. These findings are contradictory with other studies that the inhibition of activin signaling led the pluripotent stem cells to differentiate toward the ectoderm lineage (Alberio *et al.*, 2010; Gafni *et al.*, 2013). However, TGF- $\beta$ 1 inhibitor treated group significantly downregulated the endoderm GATA 6 gene similar to a previous report indicating that the TGF- $\beta$ 1 inhibitor blocked the expression of endoderm markers and promoted the expression of mesoderm markers in the presence of BMP4 *in vitro* (Pauklin and Vallier, 2015). This adverse effect of the inhibitor on pluripotency could be partially reversed if the TGF- $\beta$ 1 inhibitor was simultaneously added into the medium with activin A. It is therefore suggested that the TGF- $\beta$ 1 inhibitor used in the current study could not completely inhibit the actions of activin A or full effect of the inhibition may require a longer time of exposure.

In conclusion, Activin/Nodal signaling is important for self-renewal and maintenance of pluripotency in porcine iPSCs. The activin A promoted the expression of endogenous pluripotent genes including OCT4 and

NANOG genes. We suggested that addition of activin A would be beneficial for maintenance of self-renewal in porcine iPSCs. Further research is still needed to prove the long-term effects of the activin A on cell pluripotency.

**Conflict of interest:** The authors declare that they have no conflicts of interest.

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