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Chemically Defined Media Can Maintain Pig Pluripotency Network *In Vitro*

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SUMMARY

Pig embryonic stem cells (pESCs) have been considered an important candidate for preclinical research on human therapies. However, the lack of understanding of pig pluripotent networks has hampered establishment of authentic pESCs. Here, we report that FGF2, ACTVIN, and WNT signaling are essential to sustain pig pluripotency *in vitro*. Newly derived pESCs were stably maintained over an extended period, and capable of forming teratomas that contained three germ layers. Transcriptome analysis showed that pESCs were developmentally similar to late epiblasts of preimplantation embryos and in terms of biological functions resembled human rather than mouse pluripotent stem cells. However, the pESCs had distinct features such as coexpression of SSEA1 and SSEA4, two active X chromosomes, and a unique transcriptional pattern. Our findings will facilitate both the development of large animal models for human stem cell therapy and the generation of pluripotent stem cells from other domestic animals for agricultural use.

INTRODUCTION

Derivation of pluripotent cells can be accomplished by *in vitro* culture of early embryos (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Pluripotent stem cells (PSCs), represented by embryonic stem cells (ESCs) and epiblast stem cells (EpiSCs), can differentiate into various cell types and body tissues and thus show promise for regenerative medicine and cell therapy. Because of the physiological and immunological similarities between pigs and humans, porcine pluripotent cell lines have been identified as important candidates in preliminary studies on human disease (Hall, 2008). Since the 1990s, much effort has focused on deriving genuine pig ESCs from early embryos; however, the cell lines produced did not meet the required criteria, especially *in vivo* developmental competency such as chimera and teratoma formation (Ezashi et al., 2016), possibly due to the lack of optimized culture medium. During development of the early embryo, which has the inner cell mass (ICM), considered as pluripotent founder population, the pig has a longer preimplantation period compared with mouse and human (Alberio and Perez, 2012). Therefore, in the pig embryo the cell-signaling network that governs pluripotency has different patterns compared with the mouse embryo (Hall and Hyttel, 2014; Liu et al., 2015).

Mouse and human ESCs were first produced using undefined culture conditions composed of feeder cells, fetal bovine serum (FBS), and embryonal carcinoma-derived conditioned media (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). It was subsequently verified that leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) signaling pathways play crucial roles in maintaining the pluripotency of mouse ESCs (Hanna et al., 2010). Moreover, inhibitors of extracellular signal-regulated kinase (ERK) and glycogen synthase kinase (GSK)-3b signaling support pluripotency and enhance the ground state by reducing heterogeneity in mouse ESCs (Guo et al., 2016). In human, unlike mouse, pluripotency is sustained through the ERK and ACTIVIN/NODAL signaling pathways, which are activated by fibroblast growth factor 2 (FGF2) and transforming growth factor β (TGF-β) (Pera and Tam, 2010). To derive the pig ESCs, various combinations of interleukins, oncostatin M, ciliary neurotrophic factor, epidermal growth factor, activin A (ActA), and stem cell factor—as well as LIF and FGF2—have been used for *in vitro* culture of the pig ICM (Vackova et al., 2007). However, although some culture conditions supported short-term *in vitro* culture of the pig ICM (Alberio et al., 2010; Puy et al., 2010; Vassiliev et al., 2010), the ICM cells differentiated if culture was prolonged. Instead of pluripotent cells, during *in vitro* culture multipotent stem cells, so-called ES-like cells, have been spontaneously obtained by several groups (Park et al., 2013).

PSCs are divided into naive and primed states depending on their developmental status (Hanna et al., 2010). The pluripotency of cells in these two states is sustained by different signaling pathways with mutually exclusive functions. The understanding of the roles of cytokines has been improved, and novel molecules have been discovered for

Table 1. Effects of Serum Replacement on the Outgrowth of Primary Pig ICM Colonies

Serum Replacement[a]	Replication No.	Seeded Blastocyst No.	Attachment Efficiency (%)	Expansion Efficiency (%)	Sox2-Expressed Colony (%)	Colonies Maintained over Two Passages
1	3	30	83.3 ± 8.8[b]	56.7 ± 8.8	40.0 ± 5.8	ND[c]
2	3	30	76.7 ± 12.0	46.7 ± 3.3	30.0 ± 0.0	ND
3	3	30	66.7 ± 16.7	46.7 ± 14.5	33.4 ± 8.8	ND
4	3	30	70.0 ± 12.0	56.7 ± 8.8	40.0 ± 5.8	ND

[a]1: 20% knockout serum replacement (KSR); 2: N2/B27 supplements; 3: 5% KSR + N2/B27 supplements; 4: 20% KSR + 0.1% chemically defined lipid concentrate.
[b]No significant differences existed in all indices among experimental groups.
[c]Not detected, The colonies cultured with those conditions were not able to expand after subculture.

retaining pluripotency (Kim et al., 2013; Ma et al., 2013). From the new findings, we would like to find the solutions for optimizing culture conditions to derive bona fide pig ESCs. Therefore in this study, because additional or different combinations of signaling molecules would be needed to support pluripotent networks in the pig, we attempted to define essential factors for deriving pig ESCs. The undifferentiated features of newly derived pig PSCs from hatched blastocysts were assessed by quantifying the expression of pluripotency marker genes and evaluating the capacity for differentiation. The developmental status of pig PSCs was evaluated by comparative transcriptome analysis with pig preimplantation embryos and human and mouse PSCs. Finally, pig pluripotency was maintained *in vitro* by chemically defined media, with pig PSCs having mouse EpiSC- or human ESC-like pluripotent state in terms of developmental status and biological functions.

RESULTS

Optimization of Culture Conditions for Pig PSCs from Blastocysts

To optimize the culture medium for pig PSCs from blastocysts, we examined basic components of the medium such as serum replacement and signaling molecules. Although FBS has been widely used in cell culture, it is unsuitable for optimizing culture medium because of variation between batches and the presence of undefined factors. Therefore, KnockOut Serum Replacement (KSR), N2/B27, KSR + lipid concentrate (LC), and KSR + N2/B27 supplements were evaluated as alternatives to FBS. The attachment and expansion efficiencies of all groups were similar without significant differences (Table 1). Moreover, when we examined the expression of SOX2, known as ICM markers of early blastocyst in pig (Figure S1A) (Liu et al., 2015), no significant differences in the numbers of SOX2-expressing primary colonies were observed among the four groups. However, the KSR and KSR + LC supplements enhanced the growth of SOX2-expressing cells more than did the N2/B27 and KSR + N2/B27 supplements (Figure 1A). Especially in the KSR + LC group, all cells were SOX2 positive, whereas SOX2-negative cells were observed in the other groups. Although KSR and KSR + LC supported growth of ICM-derived cells during culture, long-term cultured cell lines could not be obtained using only FGF2-supplemented media.

Next, we identified the signaling molecules required for extended *in vitro* culture of the ICM. Pig preimplantation development differs from those of mouse and human, making it tempting to think that activation of additional signaling is pivotal in maintaining pluripotency *in vitro*. Because FGF2 enhanced pig ICM outgrowth (Figure S1B), and pig is considered a nonpermissive species (Choi et al., 2016), we selected FGF2, activin A (ActA), and the WNT signaling activator CHIR99021 (CH), known as extrinsic regulators for primed PSCs (Pera and Tam, 2010) for culture of pluripotent ICM *in vitro*. Four combinations—FGF2, FGF2 + ActA, FGF2 + CH, and FGF2 + ActA + CH—were treated with media containing KSR or KSR + LC, as presented in Tables 2 and 3. There were no significant differences among the combinations of signaling molecules in any of the indices and no considerable differences in the growth patterns of SOX2-positive cells among the groups (Figure 1B). Of note, two stable cell lines were established in KSR + LC medium supplemented with FGF2 + ActA + CH (Table 3; Figures 1C and S1C). However, the cells gradually differentiated during extended culture and developed morphological heterogeneity (Figure S1D). Activation of WNT signaling by CH reportedly has effects on both maintenance of pluripotency and induces differentiation (Kim et al., 2013). Therefore, to enhance effects on maintaining pluripotency we added IWR-1, an inhibitor of the canonical WNT signaling pathway, to the culture medium. In medium containing IWR-1, cells were stable over the long term without differentiation and exhibited an epithelial morphology with a high nucleus-to-cytoplasm ratio (Figure 1D; parthenogenetic ESC line 3 [PG-ES-3]).

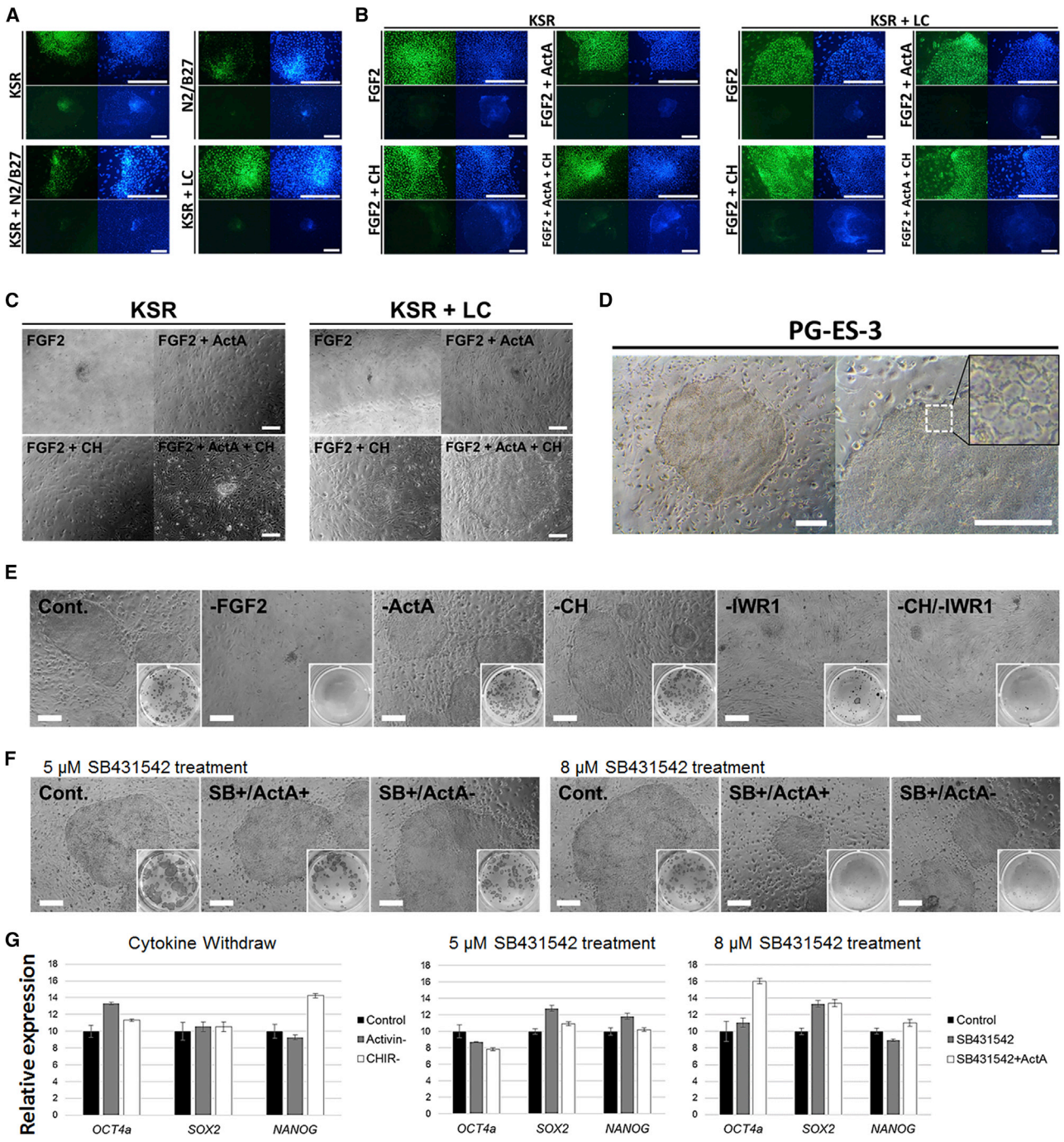

Figure 1. Optimizing Culture Conditions for Pig Embryonic Stem Cells

(A) Expression of SOX2 in primary porcine inner cell mass (ICM) outgrowths cultured with various serum replacements (green, SOX2; blue, DNA). Scale bars: top, 300 μm; bottom, 200 μm. See also Figures S1A and S1B.

(B) Expression of SOX2 in primary porcine ICM outgrowths cultured in KnockOut Serum Replacement (KSR) or KSR + lipid concentrate (LC)-supplemented medium containing cytokines (green, SOX2; blue, DNA). Scale bars: top, 300 μm; bottom, 200 μm.

(C) Long-term culture of pig ICM outgrowth in various culture media. Scale bars, 400 μm. See also Figures S1C and S1D.

(D) Typical morphology of pig embryonic stem cells (ESCs) (passage 2). Scale bars, 400 μm.

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We verified the above by culturing blastocyst-derived pig stem cells in the absence of the factors identified. Removal of FGF2 or IWR-1 for two passages (10 days; the cells of all experimental groups were subcultured by same splitting ratio) reduced proliferation dramatically and induced differentiation of ESCs (Figure 1E). Removal of ActA had no significant effect, possibly due to a paracrine effect of ActA produced by the feeder cells. Indeed, application of the ACTIVIN/NODAL pathway inhibitor SB431542 to exclude the effects of feeder cells reduced the proliferation rate in a dose-dependent manner, but did not affect the pluripotency of pig PSCs (Figures 1F and 1G). In addition, it was reported that ACTIVIN signaling is required for self-renewal of reprogrammed pig cells (Yang et al., 2017). These observations indicate that ActA promoted the growth of pig PSCs but did not maintain their undifferentiated state. Accordingly, we concluded that FGF2, ActA, and WNT signaling are essential for maintaining the pluripotency and developed a culture medium for deriving and maintaining pig PSCs from blastocysts.

Characterization of Pig ESCs

To characterize the pig PSCs, we additionally established seven parthenogenetic ESC (PG-ES) and 11 IVF-derived ESC (IVF-ES) lines from 32 parthenogenetic and 42 IVF blastocysts, respectively. From these, PG-ES-7, PG-ES-8, IVF-ES-11, and IVF-ES-12 were selected for further analysis. Colonies of these cell lines were single-layered and flattened, similar to those of primed PSCs, as represented by mouse EpiSCs and human ESCs, and exhibited alkaline phosphatase (AP) activity (Figures 2A and S2A). The cell lines were stably maintained over extended periods (over 50 passages in 1 year) with a normal karyotype (Figures 2B and S2B). However, IVF-ES-12 had an extra copy of chromosome 17 (37 + XY). Immunostaining showed that the pig ESCs expressed the pluripotency markers OCT4, SOX2, NANOG, SSEA1, SSEA4, TRA-1-60, and TRA-1-81 (Figures 2C and S2C). Of note, naive and primed pluripotency markers were simultaneously observed in pig ESCs. Although they have the primed PSC-like morphology, the pig ESCs showed coexpression of SSEA1 and SSEA4, markers of naive and primed PSCs, respectively. TRA-1-60 and TRA-1-81 were also expressed in the pig ESCs.

To verify features of pig ESCs at the transcriptome level, we next performed RNA-sequencing (RNA-seq) analysis of the PG-ES-3, PG-ES-7, and IVF-ES-11 lines, and pig fetal fibroblasts (Table S1). Unlike fibroblasts, the pig ESC lines showed high transcriptomic correlation irrespective of their embryonic origin (Figure 2D). Through identifying differentially expressed genes, we found that 3,518, 3,728, and 3,645 genes were upregulated in the PG-ES-3, PG-ES-7, and IVF-ES-11 lines, respectively, compared with fibroblasts. These genes corresponded to 95, 95, and 100 gene ontology (GO) terms, respectively (Table S2). The expression levels of genes associated with pluripotency were higher in the PG-ES-3, PG-ES-7, and IVF-ES-11 lines than in fibroblasts (Figure 2E). Epigenetically, bisulfite sequencing showed that the *OCT4* core promoter region in pig ESCs was hypomethylated compared with that of fibroblasts (Figure 2F). To assess the status of X chromosomes, which is one of the criteria for developmental stage (Hanna et al., 2010), we evaluated the expression level of *XIST*, a regulator of X chromosome inactivation, using bisulfite sequencing and qPCR. Despite the transcriptomic similarity, there were variations in X chromosome status among the cell lines. Expression of *XIST* was suppressed in the PG-ES-3, PG-ES-8, and IVF-ES-11 lines (IVF-ES12 was the male cell line), indicating the presence of two active X chromosomes (Figures 2G and S2D). However, *XIST* expression was upregulated in the PG-ES-7 line, indicating that one X chromosome was inactivated. It seems that culture conditions such as oxidative stress affect the X chromosome status in pig ESCs (Lengner et al., 2010).

To evaluate the differentiation ability of pig ESCs, we conducted spontaneous differentiation, direct differentiation, and teratoma formation assays. Firstly, when pig ESCs were cultured in suspension, embryoid bodies (EBs) were formed and subsequently underwent spontaneous differentiation upon being plated on culture plates (Figure S2E). During differentiation, three germ-layer markers (*NES*, *PAX2*, and *PAX6* [ectoderm]; *ALB*, *AFP*, *SOX17*, and *GATA4* [endoderm]; and *BMP4* and *DES* [mesoderm]) were upregulated while the pluripotency markers *OCT4a*, *NANOG*, *LIN28*, and *ZFP42* were downregulated (Figure 3A). In contrast, *SOX2*, also known as neural lineage marker, was expressed throughout differentiation. To test the potential of direct differentiation into specific cell lineages in pig ESCs, we used commercially available media and a differentiation method previously applied in human PSC research. Following culture in differentiation media, the pig ESCs differentiated into neural cells and pancreatic progenitor

(E) Effects of cytokine withdrawal on pig ESCs (passage 47, seeding density: approximately 3.2 × 10^3/cm^2; bottom right, alkaline phosphatase [AP]-stained images). Scale bars, 400 μm.

(F) Effects of SB431542 on pig ESCs (passage 50; bottom right, AP-stained images). Scale bars, 400 μm.

(G) Effects of cytokine withdrawal and SB431542 on the expression of pluripotency marker genes by qPCR. Data are presented as mean ± SEM, n = 3 technical replicates.

Table 2. Effects of Cytokines on the Outgrowth of Primary Pig ICM Colonies in KSR-Supplemented Medium

Cytokines[a]	Replication No.	Seeded Blastocyst No.	Attachment Efficiency (%)	Expansion Efficiency (%)	Sox2-Expressed Colony (%)	Subcultured Colony No.	Colonies Maintained over Two Passages
1	3	30	66.7 ± 12.0[b]	30.0 ± 5.8	30.0 ± 5.8	5	ND[c]
2	3	30	60.0 ± 10.0	40.0 ± 5.8	26.7 ± 6.7	5	ND
3	3	30	70.0 ± 10.0	46.7 ± 13.3	33.3 ± 12.0	5	ND
4	3	30	70.0 ± 11.5	43.3 ± 24.0	26.7 ± 14.5	5	ND

[a]1: 10 ng/mL FGF2; 2: 10 ng/mL FGF2 + 5 ng/mL activin A; 3: 10 ng/mL FGF2 + 0.5 μM CHIR99021; 4: 10 ng/mL FGF2 + 5 ng/mL activin A + 0.5 μM CHIR99021.
[b]No significant differences existed in all indices among experimental groups.
[c]Not detected, The colonies cultured with those conditions were not able to expand after subculture (related to Figure 1C).

cells. The pancreatic progenitor cells were derived from pig ESCs via definitive endoderm. The definitive endoderm had a distinctive petal-like morphology and expressed the endodermal markers *SOX17*, *GATA4*, and *CXCR4* (Figure 3B). During differentiation, endodermal and pancreatic markers *CXCR4*, *HNF4A*, *NKX6.1*, and *PDX1* were gradually increased whereas pluripotency markers *OCT4A*, *SOX2*, and *NANOG* were downregulated (Figure 3C). Finally, PDX1 expression in the differentiated cells was determined by immunostaining (Figure 3D). The neurons and astrocytes were differentiated from pig ESCs through the neural rosettes. The neural rosettes displayed polarized neuroepithelial structures containing neural stem-like cells. The deriving neurons spontaneously developed into nerve-like structures during extended culture. Immunostaining showed that the tight junction protein ZO-1 was localized to the lumen of the neural rosettes, and the neuron-specific marker TUJ1 and astrocyte-specific marker GFAP were expressed in the neurons and astrocytes, respectively (Figure 3E). The pig ESCs also differentiated into cardiomyocytes after 13 days according to a previous study (Kehat et al., 2001). The area of beating muscle cells increased over time, and the cardiac muscles contracted for more than 2 weeks (Videos S1 and S2). Pig ESCs could be induced into specific cell types using differentiation conditions for human PSCs, implying physiological similarities between the cells of pig and human. Finally, to examine their *in vivo* differentiation capacity, pig ESCs were subcutaneously transplanted into athymic nude mice. Three months later, teratomas derived from the injected pig ESCs were collected (Figure S2F); their identity was confirmed by genotyping analysis (Figure S2G). Teratomas derived from the PG-ES-7, PG-ES-8, and IVF-ES-11 lines were composed of multiple cell types came from three germ layers, whereas endodermal tissues were not detected in IVF-ES-12-derived teratomas (Figure 3F). Taking these data together, we confirmed that the optimized culture conditions facilitated long-term stable maintenance of pluripotent pig ESCs with *in vitro* and *in vivo* differentiation potential.

Comparative Transcriptome Analysis with Pig Preimplantation Embryos and Human/Mouse PSCs

RNA-seq analysis was used to compare the transcriptome of pig ESCs with pig preimplantation embryos and human/mouse PSCs. First, we tried to assess the developmental stage of pig ESCs by comparing them with pig preimplantation embryos. Transcriptomic comparisons with fetal fibroblasts, day 7/8 ICM, day 10/11 epiblasts, and day 12/13 epiblasts showed that the groups were distinctly clustered, and ICM had the highest level of transcriptomic variation between samples (Figure 4A). Next, to elucidate developmental aspects further, we performed gene expression analysis using 92 genes related to pluripotency and lineage differentiation, which were selected based on previous studies (Cao et al., 2014; Hall and Hyttel, 2014; International Stem Cell Initiative et al., 2007; Wei et al., 2018). Despite originating from day-7 hatched blastocysts, the pig ESCs were more similar to epiblasts than to 7/8 ICM (Figure 4B). Thus, the ICM may have developed into an epiblast-like state during derivation of ESCs. Heatmap analysis of 50 naive and primed pluripotency marker genes showed that pig ESCs were more similar to the epiblasts than to the ICM, indicating greater similarity to primed pluripotent cells (Figure S3A) (Hall and Hyttel, 2014).

We next performed a comparative analysis with human, mouse, and pig ESCs. Compared with the corresponding fibroblasts, 5,006 and 13,006 genes were upregulated in human and mouse PSCs, respectively. These genes corresponded to 105 and 71 GO terms, respectively (Table S2). Of these, 36 GO terms were shared by IVF-ES-11, and mouse and human PSCs. Of note, 28 GO terms related to nucleotide binding, ion transport, and extracellular matrix functions were shared by IVF-ES-11 and human PSCs, whereas six GO terms were shared by mouse PSCs and IVF-ES-11 (Figure 4C). The results for the PG-ES-3 and PG-ES-7 lines were similar to those of the IVF-ES-11 line (Figure S3B and Table S2). A greater number of KEGG terms related to pluripotency, Rap1, and cyclic AMP signaling were shared by human and pig ESCs than by mouse and

Table 3. Effects of Cytokines on the Outgrowth of Primary Pig ICM Colonies in KSR + LC-Supplemented Medium

Cytokines[a]	Replication No.	Seeded Blastocyst No.	Attachment Efficiency (%)	Expansion Efficiency (%)	Sox2-Expressed Colony (%)	Subcultured Colony No.	Colonies Maintained over Two Passages
1	3	29	75.2 ± 13.1[b]	51.5 ± 4.6	44.8 ± 2.9	4	ND[c]
2	3	29	82.6 ± 3.8	48.9 ± 10.6	45.6 ± 10.9	5	ND
3	3	29	85.9 ± 7.1	51.5 ± 4.6	48.1 ± 6.1	5	ND
4	3	29	79.2 ± 5.8	62.6 ± 11.5	52.6 ± 12.6	4	2 lines[d]

[a]1: 10 ng/mL FGF2; 2: 10 ng/mL FGF2 + 5 ng/mL activin A; 3: 10 ng/mL FGF2 + 0.5 μM CHIR99021; 4: 10 ng/mL FGF2 + 5 ng/mL activin A + 0.5 μM CHIR99021.
[b]No significant differences existed in all indices among experimental groups.
[c]Not detected, The colonies cultured with those conditions were not able to expand after subculture (related to Figure 1C).
[d]Derived cell lines were maintained over 3 months.

pig ESCs (Figure S3C and Table S3). Next, gene expression levels were analyzed by calculating fold-change values relative to fibroblasts to reduce interspecies differences. Notably, analysis of genes related to pluripotency and differentiation markers showed that the pig ESCs clustered separately from human and mouse PSCs (Figure S3D), and the mouse, human, and pig PSCs were well separated in the principal component analysis plot (Figure S3E). Moreover, the pig ESCs had distinct expression patterns of genes related to the lipid metabolism, JAK/STAT, MAPK, TGF-β, and WNT pathways (Figures S3F and S3G). Therefore, although pig ESCs have unique transcriptional features, they are functionally similar to human PSCs.

DISCUSSION

Pluripotency of Pig ESCs Requires More Support Than Human and Mouse ESCs

In this study, we derived primed pluripotent pig ESCs by defining cell-signaling molecules required for maintaining pluripotency in pigs. Numerous approaches have been used to generate bona fide pig PSCs including induced PSCs, embryonic germ cells, and ESCs. However, establishment of ESCs and reprogramming of somatic cells in pig has been hampered by differences in the molecular mechanisms of embryo development among mouse, human, and pig. During development of the early embryo, pig has a distinct features of preimplantation stages compared with mouse and human (Alberio and Perez, 2012). It has been surmised that unique cellular signaling networks are involved in establishing and maintaining pluripotency during pig embryo development. Accordingly, additional or different combinations of signaling molecules are required to support pluripotency networks in the pig.

Because it is outbred (only the highly inbred 129, C57BL/6, and BALB/c mouse strains are permissive [Hanna et al., 2010]), and inhibitors of ERK and GSK signaling repress the expression of endogenous pluripotency genes during reprogramming of pig cells (Choi et al., 2016; Petkov et al., 2014), pig may be a nonpermissive rather than a permissive species. Therefore, based on these observations, we prescreened several signaling molecules including FGF2, ActA, CH, BMP4, and Noggin, to improve *in vitro* survival rate of the pig ICM. Of these, only FGF2, ActA, and CH facilitated *in vitro* survival of the pig ICM, and application of these three factors in combination allowed generation of pig ESCs. Finally, we defined four essential factors, FGF2, ActA, CH, and IWR-1, for maintaining pig pluripotency *in vitro*; indeed, one or two of these factors alone is reportedly not sufficient (Hall and Hyttel, 2014). Thus, the combination defined in this study is essential to derive pig authentic ESCs. Recently, bovine ESCs were derived by treatment of FGF2 and IWR-1 (Bogliotti et al., 2018), indicating that inhibition of canonical WNT signaling is important for pluripotency of ungulate. However, absence of ActA and CH did not significantly influence the expression of pluripotency genes in pig ESCs. The ActA and Wnt produced by the feeder cells may be sufficient to maintain pig ESCs. ACTIVIN/NODAL signaling plays an important role in the maintenance of primed pluripotency (Hanna et al., 2010). WNT signaling is activated in human and mouse early embryos, and upregulates the expression of pluripotency genes in human and mouse ESCs (Sato et al., 2004). In addition, supplementation of high concentrations of ActA and a WNT activator enables feeder-free culture of human PSCs (Ludwig et al., 2006; Tomizawa et al., 2011). Therefore, ActA and CH would be important candidates in the development of a feeder-free culture system of pig PSCs.

From the metabolic aspect, lipid supplementation as a serum replacement is also an important for the derivation of pig ESCs. Energy metabolism plays a pivotal role in supporting stemness and pluripotency (Dahan et al., 2018). Of various energy sources, fatty acids involve ICM growth of mouse embryo and maintenance of PSCs by regulating mitochondrial function (Dunning et al., 2010; Wang et al., 2017). In domestic animals, fatty acid supplements

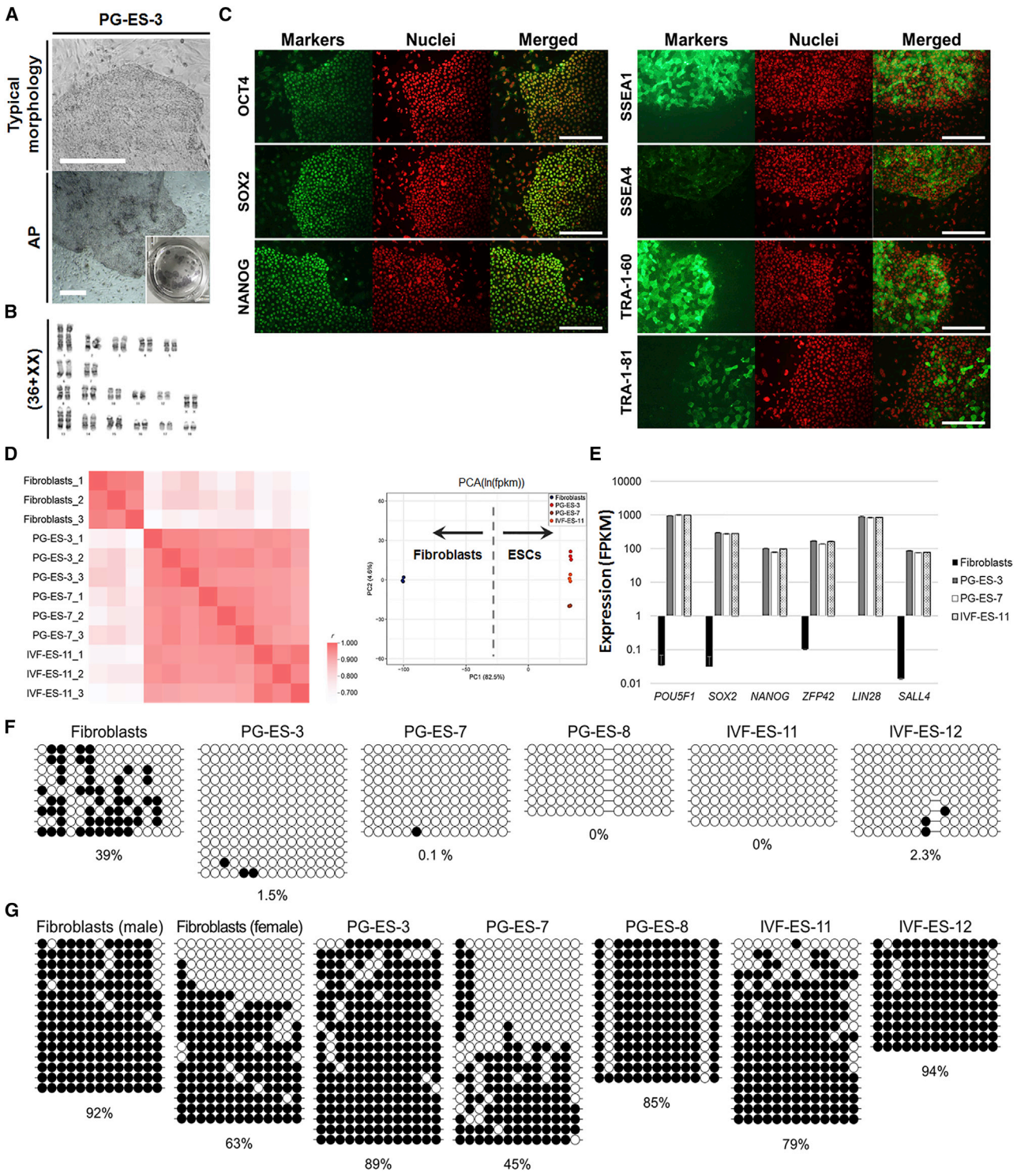

Figure 2. Characterization of Undifferentiated Pig ESCs

(A) Typical morphology and AP staining of pig ESCs (bottom right, AP-stained images). Scale bars, 400 μm. See also Figure S2A.

(B) Karyotype of pig ESCs. See also Figure S2B.

(C) Immunostaining for pluripotency markers in pig ESCs. Scale bars, 200 μm. See also Figure S2C.

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improve *in vitro* production of pig embryos (Lee et al., 2017) and maturation of bovine oocytes (Sanchez-Lazo et al., 2014). In pigs, a lipid supplement facilitated the mesenchymal-to-epithelial transition during cellular reprogramming by upregulating cyclic AMP signaling (Zhang et al., 2018). Of note, the expression pattern of genes related to lipid metabolism in pig ESCs differs from that in human and mouse ESCs. Indeed, ATP synthesis and fatty acid metabolism during embryo development differ between pig and mouse (Cao et al., 2014). The degrees of dependence on glucose metabolism for energy production also differ among pig, mouse, and human preimplantation embryos (Secher et al., 2016). Thus, it is important to further investigate the effect of energy metabolism on ESCs to improve the understanding of the pig pluripotency.

Pig ESCs Resemble Human PSCs in Terms of Physiology and Function, but Have Unique Features

Newly derived pig ESCs have unique molecular characteristics. Indeed, the expression pattern of pluripotency marker genes in pig preimplantation embryos differs from that in mouse and human embryos (Bernardo et al., 2018; Hall and Hyttel, 2014); this may also be the case for pig ESCs. Unlike in human ESCs, SSEA1, known as surface marker for naive PSCs, was highly expressed in pig ESCs, as assessed by immunostaining. Interestingly, expression of SSEA1 was detected in the ICM of pig early embryos but not in the ICM of human blastocysts (Hall, 2012). Along with SSEA1, the expression of human ESC markers such as TERF1 and GDF3 was detected in the ICM of pig blastocysts (Hall et al., 2010). Consistent with these observations, several studies reported that SSEA1 was expressed in pig *in vitro*-cultured ICM (Alberio et al., 2010; Vassiliev et al., 2010) and induced PSCs (Ezashi et al., 2009) instead of SSEA4. Pig ESCs also showed variation in their X chromosome status. In female human ESCs, X-linked genes are monoallelically expressed because of oxidative stress-induced expression of *XIST*, leading to X chromosome inactivation (Lengner et al., 2010; Shen et al., 2008). By contrast, human ESCs derived in the presence of a physiological concentration of oxygen possess two active X chromosomes, which demonstrated that oxygen concentration in culture affects the X chromosome status in human ESCs. The timing of X chromosome inactivation in pigs is unclear, although it does not occur in blastocysts (Hwang et al., 2015). Thus, further studies using embryos and ESCs are required for understanding pig XCI.

Despite distinct features, pig ESCs resemble human PSCs, having a primed-like pluripotent status. Colonies of pig ESCs displayed a flattened monolayer morphology and were maintained by MAPK/ERK, ACTIVIN/NODAL, and WNT signaling. Pig ESCs are more similar to late epiblasts of pig preimplantation embryos than early ICM as revealed by transcriptome analysis, suggesting that pig stem cells are in a developmentally primed state. Moreover, the physiological and biological functions of pig ESCs were more similar to those of human PSCs than to those of mouse PSCs, as determined by direct differentiation and GO/KEGG term analysis. Overall, our data indicate that pig ESCs are in a primed pluripotent state resembling human PSCs. Consistent with these findings, human PSCs can involve chimeric embryo development when injected into pig embryos (Wu et al., 2017), and pig germ cell development mimics human germ cell specification at the molecular and morphological levels (Kobayashi et al., 2017). In this study, the derived pig ESC lines were capable of forming teratomas in immunodeficient mice, which is one of the criteria for assessing *in vivo* differentiation; by contrast, the previously reported cells had incomplete differentiation capacity (Ezashi et al., 2016). In addition, the pig ESCs differentiated into specific cell lineages using methods typically applied to human PSCs. Therefore, pig ESCs may enable development of large animal models for use in human stem cell research. Preclinical testing using animal models including large animals as well as mouse is essential before the therapeutic use of human PSCs. Recently, engraftment of human PSCs into immunodeficient or immunosuppressed pigs to investigate stem cell transplantation in large-sized animals has been reported (Choi et al., 2017; Templin et al., 2012). However, it is challenging to overcome interspecies physiological differences, the so-called xenobarrier, by immunosuppression. Moreover, cell transplantation research excluding the immune systems is restricted in deriving significant outcomes. Therefore, derived pig ESCs would be a valuable candidate for an alternative large animal model in studies of cell therapy.

In summary, FGF2, ActA, and WNT signaling are essential for maintenance of pig pluripotency *in vitro*, and pig ESCs could be established by modulating the three cellular signaling pathways. These cells were capable of

(D) Heatmap based on pairwise Pearson correlation (r) of global gene expression levels and multidimensional scaling (MDS) plot of RNA-seq data for pig ESCs and fibroblasts. PCA, principal component analysis.

(E) FPKM (fragments per kilobase per million reads) values of pluripotency markers in pig ESCs, Data are presented as mean ± SEM, n = 3 independent experiments.

(F and G) Bisulfite sequencing of the promoter region of *OCT4* (F) and the promoter region of *XIST* (G). Circles, individual CpG dinucleotides; white and dark circles, unmethylated and methylated CpG, respectively. Each row represents an individual clone of a PCR product. See also Figure S2.

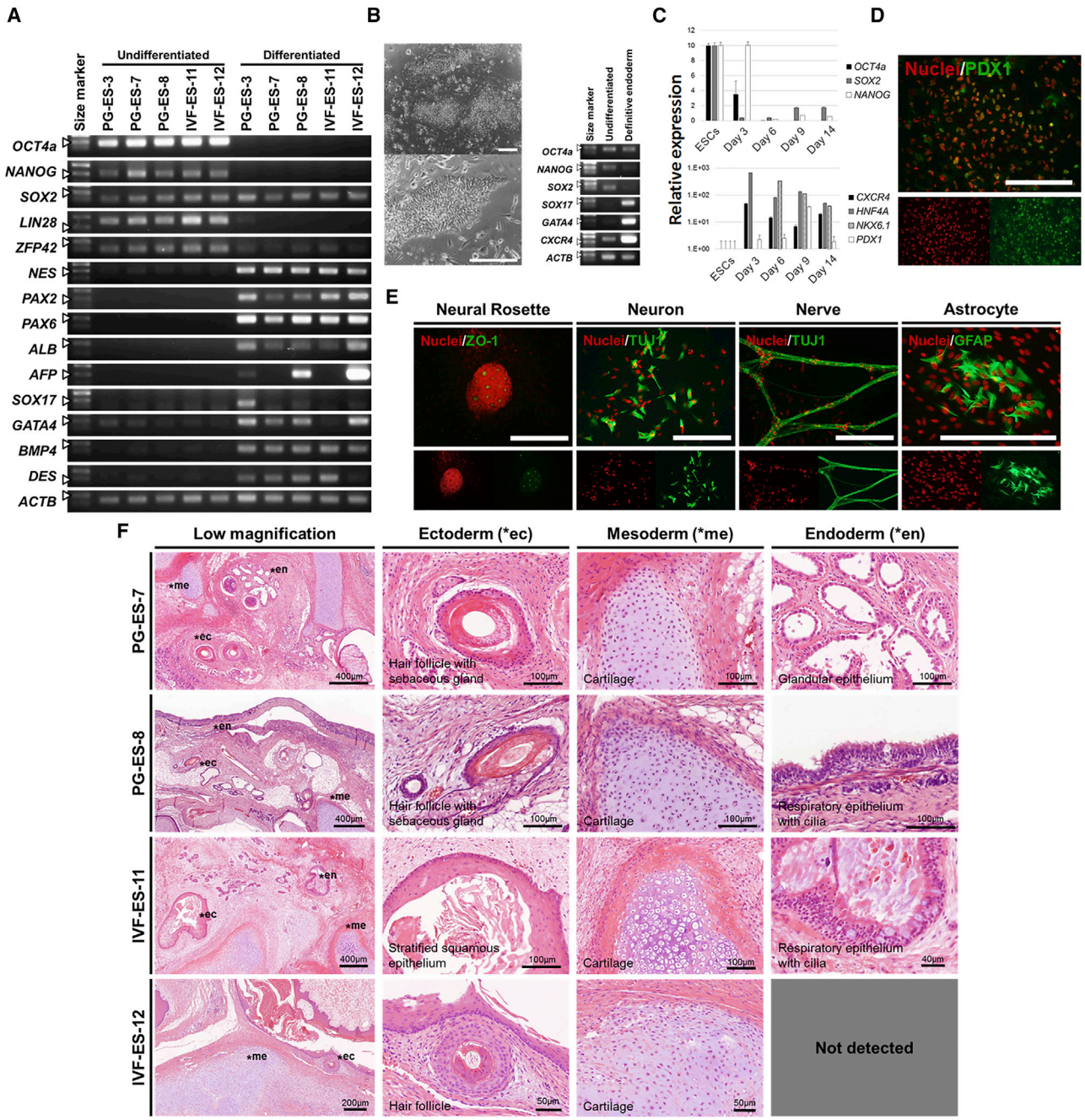

Figure 3. Analysis of the Differentiation of Pig ESCs

(A) RT-PCR analysis of the expression of pluripotency and differentiation marker genes in pig ESCs and differentiated cells (white arrowheads, 200-bp markers). See also Figure S2E.

(B) Morphology of definitive endoderm derived from pig ESCs and RT-PCR analysis of the expression of endodermal markers (white arrows, 200-bp markers). Scale bars, 400 μm.

(C) Expression pattern of endodermal marker genes during pancreatic differentiation. Data are presented as mean ± SEM, n = 3 technical replicates.

(D) Immunostaining for PDX1 in the differentiated cells. Scale bars, 200 μm.

(E) Morphology of neural cells derived from pig ESCs and immunostaining for ZO-1, TUJ1, and GFAP. Scale bars, 400 μm.

(F) Histology of teratomas formed from pig ESCs. See also Figures S2F and S2G.

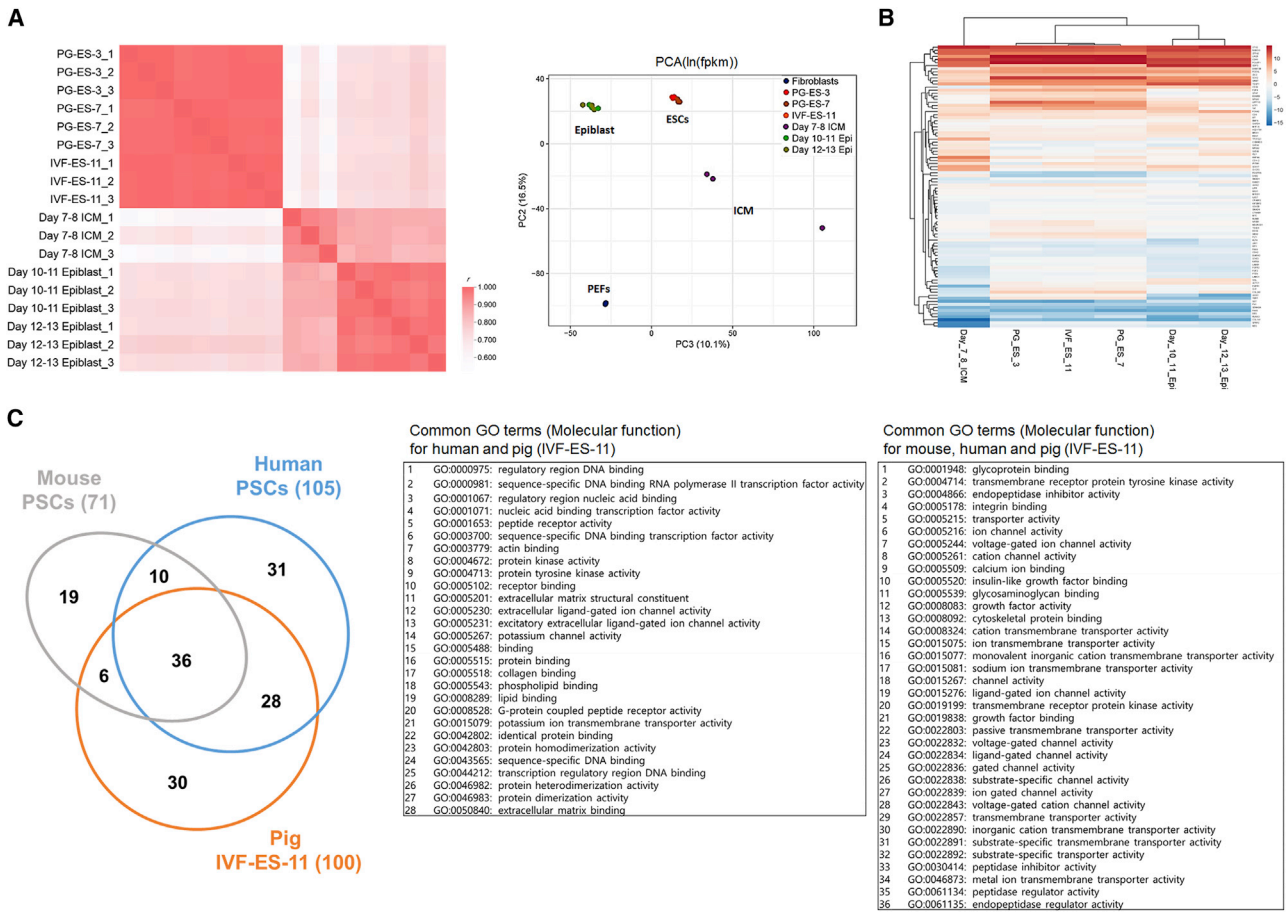

Figure 4. Comparative Transcriptome Analysis of Mouse, Human, and Pig PSCs

(A) Heatmap based on pairwise Pearson correlation (r) of global gene expression levels and MDS plot of RNA-seq data for pig preimplantation embryos and ESCs.

(B) Heatmap of the expression levels (fold-change values compared with fibroblasts) of pluripotency and differentiation marker genes in pig preimplantation embryos and ESCs. See also Figure S3A.

(C) Venn diagram of gene ontology (GO) terms among mouse, human, and PSCs (IVF-ES-11), and lists of common GO terms (human/pig and mouse/human/pig). See also Figures S3B–S3G; Tables S2 and S3.

differentiating into three germ layers *in vitro* and *in vivo*. The pig ESCs were in a primed pluripotent state, and thus resembled human PSCs in terms of physiology and biological functions. Therefore, pig PSCs may represent an alternative to the large animal models used in human stem cell research, and could be applied to produce transgenic and cloned animals and cultured meat. Finally, derived pig PSCs will improve our understanding of pluripotency across species and promote preclinical studies of human PSC-based therapeutics.

EXPERIMENTAL PROCEDURES

Animal Care

The care and experimental use of pigs and mice were approved by the Institutional Animal Care and Use Committee at Seoul National University (approval nos. SNU-160120-9-1, SNU-161114-3-1, and SNU-170223-2-1). The ovaries used were donated from a local slaughterhouse (Dodram, Korea) for research. Pregnant ICR mice and athymic nude mice were purchased from Samtaco Bio (Korea) and OrientBio (Korea), respectively. The mice were maintained according to the standard protocol of the Institute of Laboratory Animal Resources at Seoul National University.

Test of Serum Replacements and Signaling Molecules for Deriving Pig ESCs

To find suitable culture media for deriving pig ESCs, we seeded hatched parthenogenetic blastocysts on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells under various culture conditions. The tested media consisted of basal medium, replacements for FBS, and signaling molecules. The basal medium was composed of KnockOut Dulbecco's modified Eagle's medium (KO-DMEM) containing 1× Glutamax, 0.1 mM

β-mercaptoethanol, 1× minimal essential medium (MEM) nonessential amino acids, and 1× antibiotic-antimycotic (all from Gibco, Gaithersburg, MD, USA). The serum alternatives were 20% (v/v) KSR (KnockOut Serum Replacement; Gibco), 1× N2/B27 supplements (Gibco), 20% (v/v) KSR, and 0.1% (v/v) chemically defined LC (Gibco), and 5% (v/v) KSR and 1× N2/B27 supplements. The tested signaling molecules were 10 ng/mL human recombinant basic fibroblast growth factor (hrFGF2; R&D Systems, Minneapolis, MN, USA), 5 ng/mL ActA (R&D Systems), 0.5 μM CHIR99021 (CH; Cayman Chemical, Ann Arbor, MI, USA), and 1.5 μM IWR-1 (Sigma-Aldrich, St. Louis, MO, USA). At 7 days after embryo seeding, primary colonies of pig ESCs were observed and then fixed with 4% (w/v) paraformaldehyde. Cultures were performed under humidified conditions in an atmosphere containing 5% CO_2 and 5% O_2 at 38°C.

Derivation and Culture of Pig ESCs

The hatched blastocysts were seeded on mitotically inactivated MEFs in ESC derivation medium under humidified conditions in an atmosphere containing 5% CO_2 and 5% O_2 at 38°C. ESC derivation medium was KO-DMEM containing 20% (v/v) KSR, 0.1% (v/v) LC, 1× Glutamax, 0.1 mM β-mercaptoethanol, 1× MEM nonessential amino acids, and 1× antibiotic-antimycotic (all from Gibco) supplemented with 10 ng/mL hrFGF2 (R&D Systems), 5 ng/mL ActA (R&D Systems), 0.5 μM CH (Cayman Chemical), and 1.5 μM IWR-1 (Sigma-Aldrich). At 7 days after seeding, primary colonies of pig ESCs were maintained by subculturing.

The pig ESCs were cultured in ESC medium (KO-DMEM containing 15% [v/v] KSR, 0.1% [v/v] LC, 1× Glutamax, 0.1 mM β-mercaptoethanol, 1× MEM nonessential amino acids, and 1× antibiotic-antimycotic; all from Gibco) supplemented with 20 ng/mL FGF2 (R&D Systems), 5 ng/mL ActA (R&D Systems), 1.5 μM CH (Cayman Chemical), and 2.5 μM IWR-1 (Sigma-Aldrich)]. Pig ESCs were subcultured every 5–7 days. At 24 h before subculturing, pig ESCs were cultured with ESC culture medium containing 10 μM Y-27632 (Santa Cruz Biotechnology, Dallas, TX, USA). Next, expanded colonies were dissociated into small clumps using TrypLE Express (Gibco). These clumps were transferred onto new feeder cells and cultured with ESC medium containing 10 μM Y-27632 (Santa Cruz) for 24 h. Next, attached clumps were cultured with ESC medium lacking Y-27632 for 4–6 days. Medium was changed every 24 h and pig ESCs were cultured under humidified conditions in an atmosphere containing 5% CO_2 at 37°C.

Spontaneous Differentiation of Pig ESCs *In Vitro* Using the Embryoid Body Method

Cultured pig ESCs were dissociated into single cells using TrypLE Express (Gibco) and cultured on ultralow-attachment plates (Sigma-Aldrich) with DMEM containing 15% (v/v) FBS and 10 μM Y-27632 (day 1 only) without other cytokines for 5 days. After suspension culture, the dissociated cells aggregated and formed EBs, which were seeded on 0.1% (w/v) gelatin-coated plates and cultured for 2–3 weeks in DMEM containing 15% (v/v) FBS. The resulting differentiated cells were lysed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for further analysis.

Differentiation of Pig ESCs toward Neural Lineages, Cardiac Muscle, and Pancreatic Progenitor Cells

Pig ESCs were differentiated forward to neural lineages using STEMdiff Neural Induction Medium, Neuron Differentiation Kit, and Astrocyte Differentiation Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. In brief, cultured pig ESCs were dissociated into single cells using TrypLE Express (Gibco) and cultured on ultralow-attachment plates (Sigma-Aldrich) with STEMdiff Neural Induction Medium containing 10 μM Y-27632 (day 1 only) for 5 days. After suspension culture, dissociated cells formed aggregates, which were seeded on 0.1% (w/v) gelatin-coated plates and cultured for 5 days in STEMdiff Neural Induction Medium. The derived neural rosettes were additionally cultured using STEMdiff Neuron differentiation Kit and STEMdiff Astrocyte Differentiation Kit for induction into neurons and astrocytes. Differentiated cells were then fixed in 4% (w/v) paraformaldehyde for immunostaining.

Cardiac muscle was derived from pig ESCs as described previously (Kehat et al., 2001). In brief, cultured pig ESCs were dissociated into single cells using TrypLE Express (Gibco) and cultured on ultralow-attachment plates (Sigma-Aldrich) in DMEM containing 20% (v/v) FBS and 10 μM Y-27632 (day 1 only) for 10 days. The resulting EBs were seeded on 0.1% (w/v) gelatin-coated plates and cultured for 10 days in 20% FBS-containing DMEM. After the adhesive culture, beating cardiac muscle was observed and recorded using Infinity Analyze software (Lumenera, Ottawa, Canada).

Pancreatic progenitor cells was produced from pig ESCs using STEMdiff pancreatic progenitor kit (STEMCELL Technologies). In brief, cultured pig ESCs were dissociated into single cells using TrypLE Express (Gibco) and seeded on 0.1% (w/v) gelatin-coated plates in ESC culture medium containing 10 μM Y-27632. At 7 h after seeding, the culture medium was exchanged for STEMdiff definitive endoderm basal medium, and the attached cells were cultured for 14 days according to the manufacturer's manual. During pancreatic differentiation, the differentiated cells were collected for RNA extraction and immunostaining.

Teratoma Formation Assay

Pig ESCs at 70%–80% confluence were used for transplantation. Approximately 5–10 × 10^6 ESCs were resuspended in 200 μL of ESC culture medium containing 50% (v/v) BD Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ, USA) and 10 μM Y-27632. Next, the resuspended pig ESCs were injected subcutaneously into 5-week-old athymic nude mice (OrientBio). At 2–3 months after transplantation, 1- to 2-cm teratomas were collected, fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, and stained with H&E for light microscopic examination.

For genotyping of teratomas, genomic DNA was extracted from teratomas and the peritoneum of athymic nude mice using the G-spin Total DNA Extraction Kit (iNtRON Biotechnology, Korea). Genomic DNA samples were amplified using 10 pmol of species-specific primers (Table S4) and 2× PCR master mix solution (iNtRON Biotechnology). PCR reactions were performed in a thermocycler under the following conditions: 95°C for 5 min; 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).

Isolation of Undifferentiated Pig ESCs by Magnetic Activated Cell Sorting

Before RNA and DNA extraction from pig ESCs, SSEA1-positive undifferentiated pig ESCs were sorted using a magnetic activated cell sorting Cell Separation System (Miltenyi Biotec, Germany) to remove differentiated cells and feeder cells. The dissociated pig ESCs were reacted with an anti-SSEA1 antibody (1:200; MAB4301, Millipore) and anti-mouse immunoglobulin M microbeads (1:5; Miltenyi Biotec). SSEA1-positive cells were sorted on an LS column (capacity, 1×10^8 magnetically labeled cells; Miltenyi Biotec) according to the manufacturer's instructions. The identity of the sorted SSEA1-positive cells as undifferentiated pig ESCs was verified by immunostaining and genotyping (Figure S4).

Statistical Analysis

The data obtained in this study are presented as the mean ± standard error of the mean (SEM) and were analyzed using Prism 6 software (GraphPad Software, San Diego, CA, USA). The significance of differences was determined by one-way analyses of variance followed by Fisher's least significant difference test. Differences were considered significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in figures).

ACCESSION NUMBERS

The accession number for the RNA-seq date of pig ESCs reported in this paper is GEO: GSE120031. For comparative studies, datasets of pig preimplantation embryos, human PSCs, and mouse PSCs were obtained from the GEO database (GEO: GSE92889, GSE73211, and GSE106332, respectively).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.05.028.

AUTHOR CONTRIBUTIONS

K.-H.C. and D.-K.L. performed, analyzed and interpreted all cell experiments. K.-H.C., D.-K.L. and S.-W.K. performed, analyzed and interpreted RNA-sequencing analysis. S.-H.W. and D.-Y.K. performed histological analysis of teratomas. K.-H.C., D.-K.L., and C.-K.L. conceived all experiments and wrote the manuscript, with contributions from all other authors.

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