Pig Epiblast Stem Cells Depend on Activin/Nodal Signaling for Pluripotency and Self-Renewal

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Activin/Nodal signaling is required for maintaining pluripotency and self-renewal of mouse epiblast stem cells and human embryonic stem cells (hESC). In this study, we investigated whether this signaling mechanism is also operative in cultured epiblasts derived from Days 10.5–12 pig embryos. Pig epiblast stem cell lines (pEpiSC) were established on mouse feeder layers and medium supplemented with basic fibroblast growth factor (bFGF). pEpiSC express the core pluripotency factors *OCT4* (or *POU5F1*), *NANOG*, *SOX2*, and *NODAL*, but they do not express *REX1* or alkaline phosphatase activity. Blocking leukemia inhibitory factor (LIF)/JAK/STAT3 pathway by adding the specific JAK I inhibitor 420099 and an anti-LIF antibody over 3 passages did not affect pluripotency of pEpiSC. In contrast, cells grown with the Alk-5 inhibitor SB431542, which blocks Activin/Nodal pathway, differentiated readily toward the neural lineage. pEpiSC are pluripotent, as established by their differentiation potential to ectoderm, mesoderm, and endoderm. These cells can be induced to differentiate toward trophectoderm and to germ cell precursors in response to bone morphogenetic protein 4 (BMP-4). In conclusion, our study demonstrates that pig epiblasts express the core pluripotency genes and that the capacity for maintaining self-renewal in pEpiSC depends on Activin/Nodal signaling. This study provides further evidence that maintenance of pluripotency via Activin/Nodal signal is conserved in mammals.

Introduction

MOUSE EMBRYONIC STEM CELLS (mESC), conventionally derived from the inner cell mass (ICM) of preimplantation blastocysts, are pluripotent and can self-renew indefinitely in culture. mESC depend on the cytokines leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP-4) to maintain the undifferentiated state [1,2]. Human embryonic stem cells (hESC) are also derived from blastocysts; however, these cells depend on basic fibroblast growth factor (bFGF) and Activin A for pluripotency and self-renewal [3,4]. Interestingly, pluripotent cells derived from mouse epiblasts, a part of the ICM, also require bFGF and Activin A for pluripotency and self-renewal [5,6]. hESC share multiple features with mouse epiblast stem cells (mEpiSC), such as growing as flat and compact colonies [5–7], they respond to BMP-4 by differentiating to trophectoderm (TE) [8] and germ cell progenitors [9], and they do not require LIF/JAK/STAT3 signaling for self-renewal [4,10]. The functional and phenotypic similarities between these cell types suggest a developmental relationship [5,6], and demonstrate that

there are at least 2 signaling mechanisms involved in capturing pluripotency in vitro. Recent reports show that bFGF and Activin A are also necessary for maintaining rabbit ESC [11,12], indicating that this signaling pathway may be a conserved mechanism of pluripotency in mammals.

Here we tested this hypothesis in pigs, a representative of ungulates. Attempts to derive stem cells from pig embryos have traditionally relied in the isolation and culture of ICM using protocols established for mESC [13,14]. These strategies have not resulted in consistent results, suggesting that the blastocyst stage may not be amenable for stem cell derivation in this species. We show that pig epiblasts, however, can be isolated and cultured in vitro and that maintenance of the undifferentiated state requires bFGF and Activin A signaling instead of LIF and BMP-4. Pig epiblast stem cell lines (pEpiSC) can be cultured for extensive periods and upon growth factors withdrawal the cells can be induced to differentiate to 3 somatic germ layers, germ cell progenitors, and trophoblast. Our study supports the hypothesis that FGF/Activin/Nodal is a conserved signaling pathway for maintenance of pluripotency in mammals, and this can be

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exploited for developing strategies for the derivation of ESC from ungulate embryos.

Materials and Methods

Epiblast isolation, stem cell cultures, and in vitro differentiation

Pig epiblasts were isolated from in vivo-derived ovoid-early tubular stage embryos collected 10.5–12 days after insemination. Embryo development can vary greatly between individual animals; therefore, embryos showing advanced signs of development were not included in the isolations. The epiblasts used in our study correspond to late ICM-Pre-streak I/II stages, as described by Vejlsted et al. [15]. Embryos were flushed from uterine horns with phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS), and transferred to culture medium supplemented with 25 mM HEPES. Epiblasts were separated from trophoblast and underlying primitive endoderm (PE) using 21 G needles and fine forceps. Pure epiblasts were placed individually onto 4-well dishes containing mitomycin C-inactivated mouse feeder cells (50×10^4 cells/cm²) and were cultured in 5% CO₂ at 39°C. Mouse fibroblasts were derived from E13.5 fetuses from CD1 strain, and were passaged 2–3 times before mitomycin C inactivation. Feeder layers were used 2–3 days after mitomycin C inactivation. Stem cell culture medium (SCM) consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 containing 1% minimal essential medium (MEM) nonessential amino acids, 1% glutamine, 1% penicillin/streptomycin, and 0.007% β -mercaptoethanol supplemented with bFGF (R&D Systems, Minneapolis, MN) and either serum replacement (SR; Invitrogen, Carlsbad, CA) or FCS (see Table 1 for details). Plating efficiency of primary cultures was determined by scoring the number of attached colonies after 48 h. Attached epiblasts were grown initially for 5–7 days before manual splitting and transfer onto fresh feeders. These colonies were passaged another time before freezing by vitrification as described by Reubinoff et al. [16]. Survival of vitrified colonies was around 90% for 10 lines tested. Mouse LIF (1,000 IU/mL; Gibco, Grand Island, NY)

was used for initial isolations. Conditioned medium was prepared from primary mouse embryonic fibroblast inactivated with mitomycin C as described by Xu et al. [17]. Medium was changed daily and cells were passaged manually every 5–6 days. When enzymatic passaging was used, a mixture of 1 mg/mL collagenase type IV and 1 mg/mL dispase (Gibco) was added to the cultures for 15 min at 39°C. Colonies were disrupted by gentle pipetting and transferred into 15-mL tubes containing fresh culture medium and left for 5 min to sediment. The medium was removed and 5 mL of fresh medium was added before washing by centrifugation. Cell pellets were resuspended by gentle pipetting and distributed onto fresh feeders. mESC were grown as described by Bian et al. [18]. For spontaneous differentiation, pEpiSC at 50%–70% confluency were cultured in DMEM/F12 + 10% FCS without bFGF for 30 days. For neural differentiation, we used the protocol described by Conti et al. [19]. For differentiation to TE and to germ cell precursors, EpiSC at Passages 6–9 were manually cut into small pieces and aggregated into embryoid bodies (EB) using hanging drops for 2–3 days. EB were transferred to low attachment dishes and kept in suspension for another 4 days, after which they were transferred to gelatinized dishes with SCM containing 10% FCS and supplemented with 10–50 ng/mL (for trophoblast; for EpiSC8 and 21) and 50–100 ng/mL (for germ cell precursors; for EpiSC8, 21, and 42) of BMP-4 (R&D Systems). This medium was changed daily for up to 14 days.

Immunocytochemistry and karyotype

Cells grown on coverslips were washed in PBS before fixation with 4% paraformaldehyde for 20 min. After washing, the cells were permeabilized with 0.1% Triton and blocked in PBS + 5% bovine serum albumin (BSA) for 1 h. All primary antibody incubations were carried out overnight at 4°C, unless otherwise indicated. Antibodies used were: Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100), Nanog (AbCam, Cambridge, MA; 1:100), Tubulin (R&D Systems; 1:50), and MAP2 (R&D Systems; 1:300). Respective secondary antibodies were added for 1 h at room temperature. After washing with

TABLE 1. SUMMARY OF CONDITIONS USED FOR DERIVATION OF EPIBLAST STEM CELLS

Base medium	Supplement	Plating efficiency (%)	Cell lines frozen P2 (%)
DMEM/F12 + 20% KSR	a. 5 ng/mL bFGF	2/4 (44.4)	1/2 (50)
	b. 5 ng/mL bFGF + Activin A	2/5 (40)	1/2 (50)
	c. 10 ng/mL bFGF	7/10 (70)	4/7 (57.1)
	d. 10 ng/mL bFGF + LIF	7/11 (63.6)	4/7 (57.1)
	e. 20 ng/mL bFGF	3/5 (60)	1/3 (33.3)
	Total:	21/35 (60)	11/21 (52.3)*
Conditioned DMEM/F12 + 20% KSR	a. 5 ng/mL bFGF	5/9 (55.5)	3/9 (33.3)
	b. 10 ng/mL bFGF	3/6 (50)	1/3 (33.3)
	Total:	8/15 (53.3)	4/12 (33.3)*
DMEM/F12 + 20% FCS	a. 5 ng/mL bFGF	6/7 (85.7)	3/6 (50)
	b. 10 ng/mL bFGF	5/7 (71.4)	1/5 (20)
	Total:	11/14 (78.5)	4/11 (36.3)
Conditioned DMEM/F12 + 20% FCS	a. 5 ng/mL bFGF	7/7 (100)	5/7 (71.4)
	b. 10 ng/mL bFGF	5/7 (71.4)	3/5 (60)
	Total:	12/14 (85.7)	8/12 (66.6)*

*Indicates lines propagated for >12 passages.

TABLE 2. PRIMER SEQUENCES USED IN THIS STUDY

Primer Name	Sequence	Accession Number	Amplicon (bp)
OCT4	F: GCAAACGATCAAGCAGTGA R: GGTGACAGACACCGAGGGAA	NM_001113060	201
NANOG	F: CCCGAAGCATCCATTTC R: CGAGGGTCTCAGCAGATGACAT	DQ447201	101
SOX2	F: AAGAGAACCCCAAGATGCACAAC R: GCTTGGCCTCGTCGATGAAC	TC208722	219
NODAL	F: CGTCTCCAGATGGACCTGTT R: CTGCTCTGGAGAGAGGTTGG	AM072821	222
REX1	F: TTTCTGAGTACGTGCCAGGCAA R: GAACGGAGACACGCTTCTCAGAG	TC206552	201
GATA6	F: CAGGAAACGAAAACCTAAGAGCAT R: TTCTCGGGATTAGCGCTCTC	TC238300	201
SOX17	F: CGCACGGAGTTTGAACAATA R: CAGACGTCGGGGTAGTTACAG	TC248504	167
CDX2	F: AGAACCCCAAGTCTCTGTCTT R: CAGTCCGAAACACTCCCTCACA	TC206866	101
β -ACTIN	F: TCCCTGGAGAAGAGCTACGA R: CGCACTTCATGATCGAGTTG	AJ312193	159
LIF	F: AAAGGAGCCTCAATCCTGGT R: TGACAGCCCAGCTTCTTCTT	TC325249	190
LIF-r	F: GTCTTGGAGGGATCCTGTGA R: AGGCAGAGCAACGTGAGAAT	TC359558	188
NESTIN	F: TACCTGGAAGCGGAAGAGAA R: CTGATCCAGGTCTGCCTTGT	TC295480	201
BLBP	F: TTGGTGATGTGGTTGCTGTT R: TCACATTTTCCACCTCCACA	TC241634	174
PAX6	F: CAGCTTCACCATGGCAAATA R: GGGAAATGAGTCCTGTGGAA	TC269600	197
GLAST	F: GACGCTCCAGAGAAATACGC R: ACCCAAGGATTGTACCCACA	TC246095	217
GFAP	F: CCTGTGCAGACCTTCTCCA R: ACGGTCTTCACCACAATGTT	TC202557	101
KERATIN14	F: TCCAAGGACTACAGCCCCTA R: AGGTTCAAGCTCCGCTCTCGTA	TC242553	155
CARDIAC ACTIN	F: CAGGTATTGCTGATCGCATGCA R: ATTTGCGGTGGACGATGGA	TC270296	201
GOOSECOID	F: GAAGCCCTGGAGAACCTCTT R: GCTTTCGACGACGTCTTGTT	TC294022	200
PECAM	F: CATTTCCAAAGTCAGCAGCA R: ATCATCATGCCTCCCTTCTG	TC238302	171
MSX2	F: CTGGTCAAGCCTTTCGAGAC R: GCTTCCGATTGGTGTGTGT	TC322813	151
H19	F: CCGGTCAATTTGGTTTCAG R: RCAGGAGGCCAGAGAAACAAG	TC338021	178
HAND1	F: GCACGATTGGAGAAGGAGAG R: GGAAGGAGAGCCCATAGAGG	TC303337	169
PGr	F: AAGTCACTGCCAGGTTTTTCG R: TGCCACATGGTAAGGCATAA	TC371235	209
EOMES	F: TACCAACCACGTCTGCACAT R: GGAAGCGGTGTACATGGAGT	TC251524	210
DAZL	F: GCATTTTCACGACATCATGG R: TAGCCACCTATTGCCTGTCC	TC280269	219
VASA	F: ATGAAGCTGATCGCATGTTG R: CCTACATGCTCCACCCACTT	TC238380	203
SCYP3	F: GTTTGGAAAACCCAGCAAGA R: ACTTTTTGTTGCTGGCGAAA	TC304948	152

PBS + 1% BSA, the cells were mounted using Vectashield with 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). For SSEA-1 (Hybridoma bank, Iowa City,

IA; 1:50) and SSEA-4 (Hybridoma bank; 1:50), cells were treated the same except that they were not permeabilized. Fluorescent images were acquired using epifluorescence and

simple PCI image analysis software. Alkaline phosphatase (AP) activity was detected using an AP detection kit (Sigma, St. Louis, MO). For karyotyping, cells were incubated in 0.05 $\mu\text{g/mL}$ demecolcine for 30 min, trypsinized, and pelleted. The pellet was resuspended in a hypotonic solution (0.075 M KCl) for 30 min. After centrifugation, the cells were fixed in ice-cold ethanol for 1 h. The cell suspension was dropped onto humidified slides and air-dried. Following dehydration with 70%, 90%, and 100% ethanol, the metaphase spreads were stained with Giemsa. Metaphase spreads (20 per cell line) prepared from 3 cell lines at Passage 7 (EpiSC8, 21) and Passage 12 (EpiSC42) were visualized under phase contrast using 100 $\times$ objective under immersion oil.

Signaling inhibitors

The STAT3 inhibitor 420099 (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock and was added to cells at 0.6 μM as described by Tesar et al. [6]. SB431542 (Tocris, Ellisville, MO) was dissolved in DMSO to 100 mM and stored at -20°C . Cells were treated with 20 μM SB431542 for 6 days as described previously by Tesar et al. [6]. Anti-mLIF antibody was added to the cultures (100 ng/mL; R&D Systems) for 3 passages. Control groups were exposed to an equivalent concentration of DMSO. Plating efficiency was estimated according to Tesar et al. [6] in triplicate experiments. In brief, cell clumps were randomly placed onto 12-well plates and their numbers established after attachment. When colonies reached confluency, they were passaged using collagenase/dispase and all cells were plated onto new wells. After 3 passages, the total number of colonies was counted for each group and they were represented as percentage difference to the starting number of colonies plated.

Gene expression

RNA isolation was carried out using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Dissected epiblasts, trophoblast, and pEpiSC colonies were frozen in RLT buffer containing β -mercaptoethanol at -80°C until use. DNase-treated RNA was used for cDNA synthesis using Omniscript (Qiagen). Pig gene sequences were retrieved from TIGR project (<http://compbio.dfci.harvard.edu/tgi/>) and from gene bank (NCBI). Primers used in this study are presented in Table 2. Three cell lines (EpiSC8, 21, and 42) were used for multilineage differentiation experiments and similar results were obtained in each case.

Statistical analysis

Comparisons of plating efficiency and cell line derivations at Passage 2 using different media were performed using Fisher's exact test. Unpaired *t*-test was used for comparing the effects of JAK inhibitor and LIF addition/blocking to the cultures.

Results

Isolation and establishment of pig epiblast stem cells

Pig epiblasts were dissected manually from embryos recovered at Days 10.5–12 after insemination. In pig embryos,

the dark polar trophoblast cells (or Rauber's layer) shed off allowing good visualization of the epiblast under a stereomicroscope (Fig. 1A). During the dissection, the darker TE and the underlying globular PE layer were removed carefully using fine needles (Supplementary Fig. 1; Supplementary materials are available online at <http://www.liebertpub.com/>). The dissected epiblasts (Fig. 1B) were plated individually onto 4-well plates containing mitomycin C-inactivated mouse feeder cells. Parallel cultures using pig embryonic fibroblasts as feeder layers failed to support epiblast stem cell growth, and promoted rapid differentiation. Primary cultures were done using culture medium containing either 20% FCS or SR supplemented with bFGF and LIF (see Materials and Methods for details). Within 1–2 days, most epiblasts attached and grew as a homogeneous group of cells with evident high nuclear/cytoplasm ratio and distinctive nucleoli (Fig. 1C and 1D). During the following 3–6 days, cells began to differentiate into multiple cell types and most epiblasts were enclosed in a mass of cells with phenotypic characteristics and gene expression profiles of extraembryonic cells (Supplementary Fig. 2A–2C; Supplementary materials are available online at <http://www.liebertpub.com/>). These cells grew very rapidly resulting in the loss of many undifferentiated epiblast stem cell colonies. After sequential manual isolation of epiblast stem cell colonies from the surrounding differentiating cells, 27 cell lines were obtained at early passage (P2) using different culture conditions (Table 1 and Fig. 1E), and 3 lines were cultured long term. Plating efficiency and number of lines at P2 were not significantly different among the culture media used in the initial derivations. These cells grew as flat and compact colonies, presented a normal karyotype (Fig. 2E; EpiSC8: 19/20, EpiSC21: 20/20, and EpiSC42: 18/20) and were passaged routinely every 5–6 days (Fig. 1F and Supplementary Fig. 2D).

Isolation in medium containing either FCS or SR supported the establishment of cell lines when bFGF was included in the medium. Increasing concentrations of FGF did not improve the derivation efficiency; therefore, long-term expansion was carried out with medium containing 5 ng/mL bFGF. Addition of exogenous Activin A or LIF did not improve the efficiency of derivations; therefore, they were not included after P2. More consistent derivations with reduced extraembryonic differentiation were obtained when conditioned medium containing FCS was used instead of conditioned medium with SR or unconditioned medium. Selected lines were cultured long term on mouse feeder layers either in medium with 20% SR or FCS supplemented with 5 ng/ μL bFGF.

For initial characterization we tested AP activity, a conventional marker of mouse and hESC. Freshly isolated epiblasts and epiblasts plated for 12 h stained positive for AP. After P2, however, AP activity was no longer detectable in pEpiSC (Fig. 2A). This observation correlates with observations in mEpiSC that show no AP activity [6]. Surface marker expression shows that mEpiSC are SSEA-1-positive [6,19] and similar observations were reported for pig epiblasts [20]. We detected strong SSEA-1 expression in cultured epiblasts and weak or no expression of SSEA-4 (Fig. 2B). Expression of *NANOG* and *SOX2* is lower in blastocysts compared with pig epiblasts [21,22]. Here we show that cultured epiblasts express *OCT4*, *NANOG* (Fig. 2C and 2D), *SOX2*, *REX1*, and *NODAL* (Fig. 2D), demonstrating that pig epiblasts express

FIG. 1 Isolation and culture of pig epiblasts. (A) The epiblast of pig embryos appears as a clear area surrounded by darker trophoblast cells. Inset: whole embryo at the ovoid stage. (B) A dissected epiblast prior to culture and (C) 12 h after plating. (D) Higher magnification of C shows prominent nucleoli and high nuclear/cytoplasmic ratio. (E) Stem cell colony at P2 (arrows) surrounded by differentiated cells. (F) A stem cell colony at P6 grows as a flat and compact colony. Scale bar: 10 μ m, inset: 50 μ m. E: epiblast, TE: trophoblast.

FIG. 2 Characterization of pig epiblast stem cell lines (pEpiSC). (A) Alkaline phosphatase activity detected in isolated pig epiblasts and in mESC, used as positive controls. (B) Pig epiblasts are SSEA-1-positive and only weakly positive to SSEA-4. (C) OCT4 and NANOG immunostaining in pig epiblasts. (D) Gene expression of isolated epiblasts and pEpiSC at P5 and P22. (E) A representative metaphase spread showing a normal karyotype. Color images are available online at www.liebertonline.com/scd.

the core pluripotency genes. Interestingly, we also detected a number of extraembryonic markers such as *GATA6*, *SOX17*, and *CDX2* in freshly isolated epiblasts. Established pEpiSC lines (Epi8, 21, and 42) also maintained expression of these pluripotency markers, except for *REX1*, and lost expression of the extraembryonic genes detected in isolated epiblasts (Fig. 2D). It is worth mentioning that *REX1* is also a marker of PE in the pig [23], thus the expression in isolated epiblasts could be due to cells committed to this lineage.

The results from this initial characterization show that the expression profile of pEpiSC is similar to what was reported for mEpiSC [6], indicating that pEpiSC maintained epiblast-like characteristics after long-term culture. Furthermore, it suggests that under these culture conditions extraembryonic gene expression is repressed rapidly in these cultures.

Maintenance of self-renewal

Our initial cultures included LIF, although it was not clear whether these cells required this cytokine for self-renewal. pEpiSC express LIF and LIF-r (Supplementary Fig. 3; Supplementary materials are available online at <http://www.liebertpub.com/>), like hESC [11]; therefore, we decided to test the requirements of pEpiSC by culturing the cells with the JAK I inhibitor 420099 and the Alk-5 receptor inhibitor SB431542. Cells cultured for 3 passages with JAK I inhibitor remained morphologically similar to vehicle control (DMSO)-treated cells, and retained expression of *OCT4*, *NANOG*, and *NODAL* (Fig. 3A). Interestingly, more cells grew in the presence of JAK I inhibitor than untreated controls, suggesting that activation of STAT3 pathway may lead to reduced proliferation rate in pEpiSC (Fig. 3B). Inhibition of STAT3 in hESC reduces differentiation and apoptosis [4]

and increases plating efficiency in mEpiSC [5]. Thus, our results support the idea that STAT3 signaling is not required for self-renewal of pEpiSC. To confirm these results, we cultured pEpiSC in the presence of 100 ng/mL anti-mLIF antibodies for 3 passages. These cells showed no detectable loss of undifferentiated morphology, expression of pluripotency markers, and proliferation capacity, indicating that LIF/STAT3 signaling is not required for pEpiSC self-renewal (Fig. 3C; data not shown). Activin/Nodal use Activin receptor-like kinase 4/5/7 (Alk receptors) for signal transduction. When we inhibited this pathway using the Alk-5 inhibitor SB431542 for 6 days, we found that pEpiSC undergo clear morphological changes acquiring prominent neural phenotype. After staining with the neural marker tubulin (TuJ), large areas of neural differentiation were detected in these colonies (Fig. 3D). These experiments show that pEpiSC depend on Activin/Nodal signaling for self-renewal, and inhibition of this pathway promotes neuroectoderm differentiation.

Multilineage differentiation of pEpiSC

After having defined the growth conditions of undifferentiated pEpiSC, we tested their differentiation potential. First, we allowed spontaneous differentiation of 2 EpiSC lines (Epi21 (P7) and Epi42 (P9)) grown on feeder layers in medium without bFGF for 30 days. Cells with morphological characteristics of the 3 somatic germ layers were identified under phase contrast in different regions of the dishes (Fig. 4A). Because pEpiSC were differentiated on top of mouse feeder cells, we were not able to perform immunocytochemistry for identification of markers of somatic lineages, due to the false positives produced by the cross reactivity of the mouse cells to the specific antibodies. Instead, we extracted

FIG. 3 Signaling pathways required for pig epiblast stem cell lines (pEpiSC) pluripotency. (A) Gene expression of EpiSC treated with JAK I inhibitor. –RT: no reverse transcriptase control. (B) Plating efficiency of EpiSC grown with JAK I inhibitor and (C) with anti-LIF antibody for 3 passages. Asterisk indicates significant differences compared with control group ($P < 0.05$). Error bars: SEM ($n = 3$). (D) EpiSC grown with SB431542 stained for tubulin (TuJ). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Scale bar = 10 μ m.

RNA from these cells and we analyzed the expression of a panel of markers from ectoderm, mesoderm, and endoderm using reverse transcriptase polymerase chain reaction (RT-PCR). The results show that pEpiSC can spontaneously differentiate to cells representative of the 3 somatic germ layers (Fig. 4B) and confirm the morphological characteristics of the cells (Fig. 4A). Cells with neural morphology did not express markers of other lineages; however, endoderm cells expressed low levels of *PECAM*, and mesoderm cells expressed low levels of *KERATIN14* (data not shown), indicating that these cells formed mixed populations during differentiation. As further test for the differentiation potential of pEpiSC, we induced differentiation to the neural lineage using an established protocol [19]. pEpiSC colonies cut into small pieces were cultured in hanging drops to induce the formation of EB before plating and culturing in conditions that support differentiation of mouse and hESC to neural progenitors. Under these conditions, almost all cells arising from plated EB showed typical characteristics of neural progenitor cells and the neural markers TuJ and MAP2 were detected by immunocytochemistry (Fig. 4C).

We next investigated whether pEpiSC were able to differentiate into extraembryonic lineages following an induction protocol with BMP-4 as previously used with mEpiSC [5] and hESC [8]. EB made from pEpiSC for 6 days were plated onto gelatinized dishes and cultured for additional 8 days in medium supplemented with BMP-4 (Fig. 5A). Gene expression analysis of these cultures showed activation of

the trophoblast markers *EOMES*, *HAND1*, *H19*, *MSX2*, and progesterone receptor (*PGR*), indicating that pEpiSC initiate trophoblast differentiation in response BMP-4 (Fig. 5B). BMP-4 is also effective in inducing differentiation of hESC to germ cell precursors [20]. Using a similar protocol to that used for hESC, we tested whether germ cell markers were induced from BMP-4-treated pEpiSC. EB kept in suspension for 6 days were plated with or without BMP-4 and cultured for 8 days before gene expression analysis (Fig. 5A). Specific germ cell markers *DAZL* and *VASA* were detected in BMP-4-treated groups (Fig. 5C). The meiotic marker *SCYP3* [21], however, was not detected in these groups suggesting that extended cultures may be needed to initiate meiotic differentiation in vitro. These results indicate that pEpiSC have the potential to initiate the germ cell program upon induction with BMP-4. These results are in accord with reports in human and mESC differentiation toward germ cell lineage [9,22], indicating that the ability of BMP-4 to induce germ cells is conserved in mammals.

Discussion

Here we report the isolation and in vitro growth requirements for pEpiSC. In contrast to the mouse and human counterpart, pig epiblast-stage embryos are not implanted and can be retrieved by flushing the uterus of pregnant animals. In the pig, like in other ungulates such as sheep and cattle, the epiblast progresses through gastrulation before

FIG. 4 Differentiation potential of pig epiblast stem cell lines (pEpiSC). (A) Cells from 3 somatic germ layers visualized under phase contrast. (B) Gene expression analysis of cells from (A). RT± indicates with or without reverse transcriptase. (C) pEpiSC differentiated to the neural lineage visualized under phase contrast and after immunostaining for tubulin (TuJ) and MAP2. Color images available online at www.liebertonline.com/scd.

FIG. 5. Differentiation to trophoblast and germ cell lineage in response to BMP-4. **(A)** Diagram depicting the differentiation strategy through EB formation. **(B)** Gene expression analysis of trophoblast and **(C)** germ cell genes in BMP-4-induced pig epiblast stem cell lines (pEpiSC). TE: trophoblast tissue. -RT: representative no reverse transcriptase group used as control.

implantation of the conceptus [23–25]. The epiblast at this stage of development transitions from a round oblong disc to a developmentally advanced pear-shaped embryonic disc with a narrow posterior end [26]. Early round oblong epiblasts identified under a stereomicroscope can be easily separated from the remaining trophoblast cells on the basis of their contrasting appearance [27]. The underlying PE cells, which are transparent cells and have a globular phenotype, can be distinguished from the epithelial cells of the epiblast. Thus, the distinctive features of the bilaminar pig embryo allow the isolation of the individual components such as epiblasts, PE, and trophoblast.

Stem cells express a core group of genes that are critical for maintaining pluripotency [28]. Our analysis of pure epiblasts shows that these cells express the core genes *NANOG*, *OCT4*, and *SOX2* and the epiblast marker *NODAL*. Previous studies have shown that pig blastocysts express *OCT4*; however, its binding partner *SOX2* is expressed at low levels and *NANOG* is not expressed [29,30]. *OCT4*/*SOX2* proteins co-operate to activate the *NANOG* gene, suggesting that in pig blastocysts this interaction has not been established and therefore *NANOG* is not expressed. *NANOG* plays a critical role in establishing pluripotency in mammals [31]. In mouse blastocysts, *NANOG* is expressed in conjunction with *OCT4* and *SOX2* conferring pluripotent properties to the ICM [32,33]. In the pig the concerted expression of these factors is delayed until the epiblast stage [27] suggesting that the ICM is a transitional stage that has not yet acquired pluripotency. The lack of pluripotency in the pig ICM may explain the difficulty in establishing ESC from blastocysts. Previous reports have demonstrated that pig ICM derived from Day 8 blastocysts transit to an epiblast phenotype after 2–3 days in culture [34] and they rapidly differentiate [27]. Here, we cultured isolated epiblasts using conditions that support hESC and mEpiSC growth. We demonstrate that pEpiSC depend on Activin/Nodal signaling for self-renewal, and that the LIF/JAK/STAT3 pathway is not essential for pluripotency. The response to the Activin/Nodal inhibition, consisting in rapid differentiation to the neural lineage, is similar to the response of mEpiSC and hESC cultured under similar conditions [5,35]. In contrast, blocking of LIF/JAK/STAT3 pathway failed to interfere with self-renewal and pluripotency of pEpiSC, indicating that this pathway does not play a central role in sustaining pluripotency in pEpiSC. These

results are in agreement with previous findings with mEpiSC and hESC [4–6,10]. Interestingly, we found that pEpiSC have increased plating efficiency when grown with the specific JAK I inhibitor, similar to a previous report in mEpiSC [6], suggesting that inhibition of JAK/STAT3 signaling can have a beneficial effect in growth of EpiSC. Indeed, similar observations in hESC indicate that activation of STAT3 may promote differentiation and apoptosis in these cells [4,10], and therefore the inhibition of this pathway may be beneficial for the in vitro growth of Activin/Nodal-dependent stem cells. Blocking LIF signaling with an anti-mLIF antibody did not improve plating efficiency, suggesting that the small molecule inhibitor is more specific and robust in its inhibitory function. Conversely, addition of mLIF did not result in increased plating efficiency, suggesting that either mLIF is not biologically active in pig cells, or LIF signaling is not critical for maintaining pEpiSC. Furthermore, our functional tests confirm previous observations showing that addition of heterologous LIF does not prevent differentiation of pig ESC [36,37].

The surface marker SSEA-1 was readily detected in pig epiblasts, as shown previously [24,36] and SSEA-4 was very faint, indicating that the pattern of surface markers resembles that of mEpiSC [5] and is the opposite of hESC [7]. Alkaline phosphatase activity was detected in plated epiblasts, but not in pEpiSC after a few passages, similar to the observations in mEpiSC [5]. AP activity in isolated epiblasts was stronger in peripheral cells suggesting that these cells may have a different cellular identity to the rest of the epiblast. Indeed, analysis of isolated epiblasts shows expression of the extra-embryonic markers *GATA6*, *SOX17*, *REX1*, and *CDX2*, all of which are not expressed in pEpiSC. The expression of the extraembryonic genes in dissected epiblasts could either be due to the presence of few contaminating TE or PE cells left after the isolation procedure, or alternatively due to the presence of cells within the epiblast expressing these genes. Although we cannot exclude the first possibility we favor the latter, since the dissections resulted in consistently pure epiblast cells. Furthermore, during the establishment of the pEpiSC we systematically observed that plated epiblasts initially differentiated into extraembryonic cells that surrounded a center of stem cells. The epiblasts dissected from these outgrowths plated onto new feeders began to grow homogeneously, without differentiation, which suggests that the cells giving

rise to these extraembryonic cells were already committed when the epiblasts were plated. The systematic differentiation of the peripheral cells of the cultured epiblasts into extraembryonic cell types supports the idea that some cells within the margins of the epiblast may be primed to contribute to extraembryonic cell types. This is supported by observations that GATA6 is detected in some cells of pig ICM, as a prelude of the segregation of the PE [29]. It is tempting to speculate that the peripheral cells of the epiblast may be contributing to the sustained growth of the trophoblast and the PE during these stages of development, explaining the expression of markers of these tissues in our assays, and the rapid differentiation of plated epiblasts. The idea of marginal epiblast cells with different identity has been proposed recently for the rabbit [38] and our findings in the pig support these observations. Ongoing experiments in our laboratory are focused at establishing the identity of the cells in the pig epiblast.

We show that pEpiSC can spontaneously differentiate to cells expressing markers of 3 somatic germ layers and they can also respond to a differentiation protocol for inducing neural differentiation. Attempts to induce mesoderm and endoderm did not achieve consistent results, although this was not totally unexpected since results with hESC shows that directed differentiation into specific lineages can prove very challenging with some cell lines [39], and therefore further work is needed to establish the proper differentiation conditions for pEpiSC into these lineages.

Differentiation to the trophoblast lineage has been demonstrated for mEpiSC [5] and hESC [8] in response to BMP-4. Our results show that pEpiSC also activate trophoblast genes in response to BMP-4, demonstrating that these cells can give rise to all somatic germ layers and trophoblast. As a last test for the in vitro differentiation potential of pEpiSC, the contribution to the germ cell lineage was studied. We show that pEpiSC can reactivate the specific germ cell markers *DAZL* and *VASA* after induction with BMP-4. The capacity of BMP-4 to induce germ cells has been shown in vivo in the mouse [40] and in vitro with hESC [20]. Thus, our results with pEpiSC show that the role of BMP-4 as an inducer of the germ cell lineage is conserved.

In conclusion, our study shows that the epiblast of the pig embryo is pluripotent, and can be isolated and cultured long term. Cultured pig epiblasts depend on Activin/Nodal signaling for self-renewal, as previously shown for mEpiSC and hESC, indicating that maintenance of pluripotency by this signaling mechanism is conserved in mammals.

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Author Disclosure Statement

The author's declare that there is no conflict of interest that would prejudice the impartiality of this work.

References

- Smith AG, JK Heath, DD Donaldson, GG Wong, J Moreau, M Stahl and D Rogers. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688–690.
- Ying QL, J Nichols, I Chambers and A Smith. (2003). BMP induction of *Id* proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115:281–292.
- Vallier L, M Alexander and RA Pedersen. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci* 118(Pt 19):4495–4509.
- Dahéron L, SL Opitz, H Zaehres, MW Lensch, WM Lensch, PW Andrews, J Itskovitz-Eldor and GQ Daley. (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* 22:770–778.
- Brons IG, LE Smithers, MW Trotter, P Rugg-Gunn, B Sun, SM Chuva de Sousa Lopes, SK Howlett, A Clarkson, L Ahrlund-Richter, RA Pedersen and L Vallier. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448:191–195.
- Tesar PJ, JG Chenoweth, FA Brook, TJ Davies, EP Evans, DL Mack, RL Gardner and RD McKay. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448:196–199.
- Ginis I, Y Luo, T Miura, S Thies, R Brandenberger, S Gerecht-Nir, M Amit, A Hoke, MK Carpenter, J Itskovitz-Eldor and MS Rao. (2004). Differences between human and mouse embryonic stem cells. *Dev Biol* 269:360–380.
- Xu RH, X Chen, DS Li, R Li, GC Addicks, C Glennon, TP Zwaka and JA Thomson. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20:1261–1264.
- Kee K, VT Angeles, M Flores, HN Nguyen and RA Reijo Pera. (2009). Human *DAZL*, *DAZ* and *BOULE* genes modulate primordial germ-cell and haploid gamete formation. *Nature* 462:222–225.
- Humphrey RK, GM Beattie, AD Lopez, N Bucay, CC King, MT Firpo, S Rose-John and A Hayek. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* 22:522–530.
- Wang S, Y Shen, X Yuan, K Chen, X Guo, Y Chen, Y Niu, J Li, RH Xu, X Yan, Q Zhou and W Ji. (2008). Dissecting signaling pathways that govern self-renewal of rabbit embryonic stem cells. *J Biol Chem* 283:35929–35940.
- Honda A, M Hirose and A Ogura. (2009). Basic FGF and Activin/Nodal but not LIF signaling sustain undifferentiated status of rabbit embryonic stem cells. *Exp Cell Res* 315:2033–2042.
- Keefer CL, D Pant, L Blomberg and NC Talbot. (2007). Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. *Anim Reprod Sci* 98:147–168.
- Brevini TA, S Antonini, F Cillo, M Crestan and F Gandolfi. (2007). Porcine embryonic stem cells: facts, challenges and hopes. *Theriogenology* 68(Suppl. 1):S206–S213.
- Vejlsted M, H Offenberg, F Thorup and P Maddox-Hyttel. (2006). Confinement and clearance of OCT4 in the porcine embryo at stereomicroscopically defined stages around gastrulation. *Mol Reprod Dev* 73:709–718.
- Reubinoff BE, MF Pera, G Vajta and AO Trounson. (2001). Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. *Hum Reprod* 16:2187–2194.
- Xu C, MS Inokuma, J Denham, K Golds, P Kundu, JD Gold and MK Carpenter. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19:971–974.
- Bian Y, R Alberio, C Allegrucci, KH Campbell and AD Johnson. (2009). Epigenetic marks in somatic chromatin are remodelled to resemble pluripotent nuclei by amphibian oocyte extracts. *Epigenetics* 4:194–202.
- Conti L, SM Pollard, T Gorba, E Reitano, M Toselli, G Biella, Y Sun, S Sanzone, QL Ying, E Cattaneo and A Smith. (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3:e283.

20. Wianny F, C Perreau and MT Hochereau de Reviers. (1997). Proliferation and differentiation of porcine inner cell mass and epiblast in vitro. *Biol Reprod* 57:756–764.
21. Kuijk EW, L Du Puy, HT Van Tol, CH Oei, HP Haagsman, B Colenbrander and BA Roelen. (2008). Differences in early lineage segregation between mammals. *Dev Dyn* 237:918–927.
22. Hall VJ, J Christensen, Y Gao, MH Schmidt and P Hyttel. (2009). Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. *Dev Dyn* 238:2014–2024.
23. Talbot NC, CE Rexroad, Jr, VG Pursel, AM Powell and ND Nel. (1993). Culturing the epiblast cells of the pig blastocyst. *In Vitro Cell Dev Biol Anim* 29A:543–554.
24. Kee K, JM Gonsalves, AT Clark and RA Pera. (2006). Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. *Stem Cells Dev* 15:831–837.
25. Yuan L, JG Liu, J Zhao, E Brundell, B Daneholt and C Höög. (2000). The murine *SCP3* gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. *Mol Cell* 5:73–83.
26. Wei W, T Qing, X Ye, H Liu, D Zhang, W Yang and H Deng. (2008). Primordial germ cell specification from embryonic stem cells. *PLoS ONE* 3:e4013.
27. Guillomot M, A Turbe, I Hue and JP Renard. (2004). Staging of ovine embryos and expression of the T-box genes *Brachyury* and *Eomesodermin* around gastrulation. *Reproduction* 127:491–501.
28. Fléchon JE, J Degrouard and B Fléchon. (2004). Gastrulation events in the prestreak pig embryo: ultrastructure and cell markers. *Genesis* 38:13–25.
29. Hue I, SA Degrelle, E Campion and JP Renard. (2007). Gene expression in elongating and gastrulating embryos from ruminants. *Soc Reprod Fertil Suppl* 64:365–377.
30. Blomberg le A, WM Garrett, M Guillomot, JR Miles, TS Sonstegard, CP Van Tassell and KA Zuelke. (2006). Transcriptome profiling of the tubular porcine conceptus identifies the differential regulation of growth and developmentally associated genes. *Mol Reprod Dev* 73:1491–1502.
31. Blomberg LA, LL Schreier and NC Talbot. (2008). Expression analysis of pluripotency factors in the undifferentiated porcine inner cell mass and epiblast during in vitro culture. *Mol Reprod Dev* 75:450–463.
32. Boyer LA, TI Lee, MF Cole, SE Johnstone, SS Levine, JP Zucker, MG Guenther, RM Kumar, HL Murray, RG Jenner, DK Gifford, DA Melton, R Jaenisch and RA Young. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947–956.
33. Silva J, J Nichols, TW Theunissen, G Guo, AL van Oosten, O Barrandon, J Wray, S Yamanaka, I Chambers and A Smith. (2009). *Nanog* is the gateway to the pluripotent ground state. *Cell* 138:722–737.
34. Chambers I, D Colby, M Robertson, J Nichols, S Lee, S Tweedie and A Smith. (2003). Functional expression cloning of *Nanog*, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113:643–655.
35. Mitsui K, Y Tokuzawa, H Itoh, K Segawa, M Murakami, K Takahashi, M Maruyama, M Maeda and S Yamanaka. (2003). The homeoprotein *Nanog* is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113:631–642.
36. Smith JR, L Vallier, G Lupo, M Alexander, WA Harris and RA Pedersen. (2008). Inhibition of *Activin/Nodal* signaling promotes specification of human embryonic stem cells into neuroectoderm. *Dev Biol* 313:107–117.
37. Moore K and JA Piedrahita. (1997). The effects of human leukemia inhibitory factor (hLIF) and culture medium on in vitro differentiation of cultured porcine inner cell mass (pICM). *In Vitro Cell Dev Biol Anim* 33:62–71.
38. Blomberg L, K Hashizume and C Viebahn. (2008). Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. *Reproduction* 135:181–195.
39. Allegrucci C and LE Young. (2007). Differences between human embryonic stem cell lines. *Hum Reprod Update* 13:103–120.
40. Lawson KA, NR Dunn, BA Roelen, LM Zeinstra, AM Davis, CV Wright, JP Korving and BL Hogan. (1999). *Bmp4* is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* 13:424–436.

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