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Three-dimensional suspension induction of porcine EPSCs into PGCs

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ABSTRACT

Primordial germ cells (PGCs), the precursors of germ cells, originate from the post-implantation epiblast under the regulation of signals from other germ layers. Elucidating the origin and differentiation mechanisms of PGCs is essential for understanding embryonic development and germ cell formation. This study delineated the cell fate characteristics and key molecular expression patterns during the differentiation of porcine extended pluripotent stem cells (pEPSCs) into epiblast-like cells (EpiLCs) and subsequently into PGCs, while also revealing the phenomenon and regulatory mechanisms of spontaneous PGC specification during this process. pEPSCs were first induced into EpiLCs using N2B27 medium with Activin A and bFGF, then further differentiated via 3D suspension culture. Analysis of key markers revealed a complex cell fate transition. During EpiLC induction, the core marker OTX2 was highly expressed, alongside upregulation of other lineage markers. Notably, even without exogenous BMP4, the PGC marker PRDM1 was detectable at 36 h, indicating spontaneous PGC specification. In subsequent embryoid bodies (EBs), PRDM1 expression increased alongside pluripotency gene reactivation, confirming advanced PGC differentiation. This work establishes that pEPSCs can differentiate into PGCs via an “EpiLCs→EB” pathway and demonstrates spontaneous PGC specification during the EpiLC stage. These findings provide a foundation for understanding the regulatory mechanisms of PGC differentiation in vitro and for optimizing PGCs induction systems.

1. Introduction

Primordial germ cells (PGCs), the precursors of sperm and oocytes, originate in humans during the 2nd to 3rd week of early post-implantation development. They then migrate along the endoderm to reach the embryonic genital ridge. This highly reproducible motor phenomenon relies on the proper spatiotemporal arrangement of other tissues to ensure that the PGCs reaches its final destination [1,2]. The arrival of PGCs at the genital ridge during migration is crucial. Incorrect localization of these cells prevents the formation of germ cells and leads to teratoma development, resulting in severe consequences [3,4].

In recent years, with the deepening understanding of PGC biology, methods for generating primordial germ cell-like cells (PGCLCs) in vitro have proven to be effective tools for studying PGC development. Although multiple starting cell types and treatment protocols have been

developed, the most advanced methods use a two-step approach to induce PGCLCs from embryonic stem cells (ESCs): first, inducing mouse embryonic stem cells (mESCs) into epiblast-like cells (EpiLCs), then aggregating them into embryoid bodies (EBs), and subsequently promoting PGCLC formation with a combination of signaling factors [5]. The first step involves culturing ESCs for 48 h in the presence of basic fibroblast growth factor (bFGF) and Activin A to induce EpiLC conversion; the second step involves culturing 800–2000 EpiLCs with leukemia inhibitory factor (LIF), stem cell factor (SCF), epidermal growth factor (EGF), and bone morphogenetic protein 4 (BMP4) for 4–6 days, achieving highly efficient PGCLC induction, with an efficiency about 30 % higher than that from epiblast stem cells (EpiSCs) [6,7]. Furthermore, transcriptomic analysis has confirmed that PGCLCs generated in vitro closely resemble in vivo PGCs. This technique has been applied to analyze genetic and epigenetic changes in mouse PGCs, and similar

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strategies are used to obtain human PGCLCs. However, studies have found significant differences in PGC specification mechanisms between humans and mice, making mouse *in vivo* and *in vitro* PGC models difficult to use as references for human PGC research [5].

The early developmental stages of porcine primordial germ cells (pPGCs) are highly similar to humans, indicating a conserved developmental pattern in the human PGC lineage [8]. Similarly, porcine embryos, like their human counterparts, develop into bilaminar embryonic discs [2]. Expression of early PGC markers—OCT4 (a pluripotency marker), BLIMP1 (PRDM1), and SOX17—has been detected in embryos from embryonic days 12 (E12) to E14 [9]. By E15, a stage equivalent to E8.5 in mice, pPGCs begin migrating toward the genital ridge, and by E18, they initiate colonization of the genital ridge [2].

In 2016, Jian Yong Han's team successfully induced porcine PGCLCs (pPGCLCs) from porcine induced pluripotent stem cells (iPSCs) [7]. The induction protocol mirrored that used for generating PGCs from mESCs. Specifically, porcine iPSCs were first cultured for two days in the presence of basic fibroblast growth factor (bFGF) and Activin A to induce their differentiation into EpiLCs. Subsequently, the dissociated EpiLCs were exposed to a combination of four cytokines—BMP4, LIF, SCF, and EGF—to induce their differentiation into pPGCLCs.

In 2019, Pengtao Liu established a porcine extended pluripotent stem cells (EPSCs) system and successfully generated PGCs by overexpressing four core transcription factors: NANOG, TFAP2C, SOX17, and PRDM1, confirming their similarity to human PGCs at the transcriptional level, suggesting high conservation of PGCs across different species [10]. This study, based on their induction protocol, optimized it to improve the efficiency of porcine PGC induction and also explored the developmental trajectory from pluripotent stem cells to PGCs.

The precise migration and differentiation of PGCs serve as the “golden window” for reproductive system development, and research into their regulatory mechanisms holds significant scientific value. Furthermore, generating PGCs *in vitro* from pluripotent stem cells has become a key model for studying their developmental trajectory. Studies have demonstrated that *in vitro* induced PGCLCs can produce fully functional mature gametes and yield healthy offspring, and the ability of pluripotent stem cells to differentiate into germ cells has been verified, promoting the potential application of this technology in humans and common livestock, providing a theoretical basis for the clinical translation of stem cell technology. This study established an efficient and rapid induction and differentiation system from pluripotent stem cells (PSCs) to pPGCs, successfully obtaining EpiLCs and PGCs through stepwise induction. The induced PGCs exhibited typical EB spherical structures with irregular surface morphology and expressed PGC-related marker genes.

2. Methods

2.1. Culture of mouse STO and preparation of FR4 feeder cells

Sandos inbred strain mouse-derived 6-thioguanine and ouabain-resistant cells (STO, a type of mouse embryo feeder layer cell line) were cultured at 37 °C, 5 % CO₂ humidified environment for 3–4 days. When cell density reached 70–80 %, they were treated with 1 µg/mL mitomycin (MCE, catalog number (Cat. No.) HY-13316) for 12 h to inhibit mitosis, thereby preparing FR4 feeder layer cells. After removing residual mitomycin, they were digested with 0.05 % trypsin/EDTA (Thermo Fisher Scientific, Cat. No. 25300062) at 37 °C, 5 % CO₂ for 5 min to obtain a single cell suspension. An equal volume of STO medium was used to neutralize the digestate, centrifuged at 1000 rpm for 5 min, resuspended, and seeded at a density of 8×10^5 cells/dish onto 0.1 % gelatin (Solarbio, Cat. No. 9000-70-8) pre-treated 35 mm culture dishes.

The FR4 feeder layer cells were maintained with medium changes every 3 days and used within a period of 3–15 days. The STO/FR4 medium is composed of DMEM (Thermo Fisher Scientific; Cat. No. C11995500BT) supplemented with 10 % fetal bovine serum (FBS)

(Vistech, New Zealand) and 1 % penicillin–streptomycin (P/S) (Thermo Fisher Scientific; Cat. No. 15140122).

2.2. Culture of porcine EPSCs

As previously reported by Wu [11], porcine extended pluripotent stem cells (pEPSCs) were seeded onto FR4 feeders and cultured in specialized medium (pEPSC medium) at 37 °C, 5 % CO₂ for 3–4 days. During subculture, cells were resuspended in pEPSC medium supplemented with 5 % FBS and 0.5 µM Y27632 (Selleck, Cat. No. S1049), cultured for 2 days, then replaced with standard pEPSC medium. After cells reached confluence, they were subcultured at a 1:3 ratio, using the same digestion method as FR4 cells.

pEPSC medium composition: KO DMEM (Thermo Fisher Scientific, Cat. No. 10829018), 0.3 % FBS, 0.5 % N2 supplement (Thermo Fisher Scientific, Cat. No. 17502048), 1 % B27 supplement (Thermo Fisher Scientific, Cat. No. 17502048), 1 % P/S, 2 mM GlutaMAX (Thermo Fisher Scientific, Cat. No. 35050061), 1 % non-essential amino acids (NEAA, Thermo Fisher Scientific, Cat. No. 11140050), 0.55 % β-mercaptoethanol (Thermo Fisher Scientific, Cat. No. 21985023), 65 µg/mL ascorbic acid (Vitamin C, Sigma-Aldrich, Cat. No. 49752), 2.5 µM XAV939 (Sigma-Aldrich, Cat. No. X3004), 0.2 µM CHIR99021 (Toris, Cat. No. 4423), 0.15 µM WH-4-023 (Toris, Cat. No. 5413), 20 ng/mL Activin A (PeproTech, Cat. No. 120-14E), 10 ng/mL LIF (PeproTech, Cat. No. 300-05).

The pEPSC medium was composed of KO DMEM (Thermo Fisher Scientific; Cat. No. 10829018), 0.3 % FBS, 0.5 % N2 supplement (Thermo Fisher Scientific; Cat. No. 17502048), 1 % B27 supplement (Thermo Fisher Scientific; Cat. No. 17502048), 1 % P/S, 2 mM GlutaMAX (Thermo Fisher Scientific; Cat. No. 35050061), 1 % non-essential amino acids (NEAA) (Thermo Fisher Scientific; Cat. No. 11140050), 0.55 % β-mercaptoethanol (Thermo Fisher Scientific; Cat. No. 21985023), and 65 µg/mL vitamin C (Sigma-Aldrich; Cat. No. 49752), 2.5 µM XAV939 (Sigma-Aldrich; Cat. No. X3004), 0.2 µM CHIR99021 (Toris; Cat. No. 4423), 0.15 µM WH-4-023 (Toris; Cat. No. 5413), 20 ng/mL Activin A (PeproTech; Cat. No. 120-14E), 10 ng/mL LIF (PeproTech; Cat. No. 300-05).

2.3. 2D plane induction of pEPSCs to EpiLCs

Once the pEPSCs exhibit optimal growth—characterized by a raised, rounded morphology and full confluency—induction into EpiLCs is initiated. Following enzymatic digestion according to the standard EPSC passaging protocol, the cells are transferred to a 12-well plate and subjected to differential adhesion on the FR4 feeder layer by static incubation for 10 min. The supernatant is then collected and centrifuged at 1000 rpm for 5 min. The resulting cell pellet is resuspended in EpiLCs induction medium, seeded into a 12-well plate, and supplemented with 1 µM Y27632. Cells are cultured for 2 days at an induction ratio of 1:2. The progression of EpiLCs induction is subsequently monitored daily using phase-contrast microscopy.

N2B27 basal medium N2B27 base medium consisted of 50 % F12/DMEM medium (Thermo Fisher Scientific; Cat. No. C11330500BT) and 50 % Neurobasal medium (Thermo Fisher Scientific; Cat. No. 21103049), 5 % FBS, 1 % N2 supplement (Thermo Fisher Scientific; Cat. No. 17502048), 1 % B27 supplement (Thermo Fisher Scientific; Cat. No. 17502048), 1 % P/S (Thermo Fisher Scientific; Cat. No. 15140122), 2 mM GlutaMAX (Thermo Fisher Scientific; Cat. No. 35050061), 0.55 % β-mercaptoethanol (Thermo Fisher Scientific; Cat. No. 21985023). The porcine EpiLCs culture medium is N2B27 basal medium supplemented with 20 ng/mL Activin A and 12 ng/mL bFGF (PeproTech; Cat. No. 300-05).

2.4. 3D levitation induction of porcine EpiLCs to PGCs

After 2 days of porcine EpiLCs culture, they were treated using the

FR4 cell digestion method, resuspended in PGC medium supplemented with 1 μ M Y27632, and seeded into 24-well low-adhesion plates (Corning, Cat. No. CLS3473) for three-dimensional suspension culture. The medium was supplemented with 1 μ M Y27632, and the cells were cultured for 3 days, followed by daily half-medium changes. The cell seeding ratio was maintained at 1:2. The induction of PGCs was monitored daily using phase-contrast microscopy (Nikon).

The porcine PGC culture medium was composed of N2B7 basal medium supplemented with 50 ng/mL BMP4 (R&D; Cat. No.314-BP), 50 ng/mL BMP2 (SnioBiological; Cat. No. 10426-HNAE), 50 ng/mL BMP8a (R&D; Cat. No. 1073-BP), 50 ng/mL SCF (SnioBiological; Cat. No. 10451-HNAE), 50 ng/mL EGF(SnioBiological; Cat. No. 10605-HNAE), 50 ng/mL LIF (PeproTech; Cat. No. 300-05).

2.5. RT-qPCR assay

Total RNA was extracted using RNAiso Plus reagent (Takara; Cat. No. 9108) via the guanidinium isothiocyanate–phenol–chloroform method, and quality was assessed with a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc). Complementary DNA (cDNA) was synthesized from 2 μ g of RNA using the HiScript III RT SuperMix for qPCR (containing a gDNA wiper) (Vazyme; Cat. No. R323), following the manufacturer's protocol. RT-qPCR was carried out using the ChamQ SYBR qPCR Master Mix (Vazyme; Cat. No. Q311) with a three-step cycling protocol (Chen et al., 2025). Primers were designed using Primer Premier 5, and amplification was performed on a CFX Connect system (BioRad; Cat. No.1855200). The primer sequences are listed in Table 1.

2.6. Immunofluorescence staining

After 3 days of suspension culture, PGCs were collected into 1.5-mL centrifuge tubes, allowed to stand for 10 min, and the supernatant was carefully removed. All subsequent steps were performed using the same procedure for handling the suspension. Cells were washed three times with PBS and then fixed with 4 % paraformaldehyde (pH 7.4) at room temperature for 2 h. Permeabilization was carried out using 0.2 % Triton X-100 (Solarbio; Cat. No. 9002-93-1) at room temperature for 1 h. Blocking was performed with 10 % FBS for 2 h, followed by incubation with primary antibodies at 4 °C for 12 h. The primary antibodies used included SOX17 (R&D; Cat. No. AF1924-SP, Polyclonal Goat IgG), TFAP2C (R&D; Cat. No. AF5059-SP, Polyclonal Goat IgG), VASA (abcam; Cat. No. ab13840, Polyclonal Rabbit IgG), and PRDM1 (eBioscience; Cat. No.14-5963, Polyclonal Rat IgG). Subsequently, cells were incubated with secondary antibodies—rabbit anti-goat (ZSGB-BIO; Cat. No. ZF-0514), goat anti-rabbit (ZSGB-BIO; Cat. No. ZF-0516), and rabbit anti-rat (Immunoway; Cat. No. RS23240) IgG (H + L)—conjugated with Alexa Fluor 488 (1:500) at room temperature for 2 h. Nuclear staining was conducted using Hoechst 33342 (Sigma-Aldrich; Cat. No. B2261,

1:1000) at room temperature for 5 min. Finally, cells were transferred to 35 mm glass-bottom cell culture dishes (NEST; Cat. No. 801022), and fluorescence images were acquired using an EVOS M5000 microscope (Thermo Fisher Scientific; Cat. No. AMF5000).

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate differences between groups. All data are expressed as mean $\pm$ standard deviation (SD). Statistical significance was defined as $p < 0.05$. Significance levels are denoted as follows: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$ respectively.

3. Results

3.1. EpiLCs were generated through 2 D planar induction differentiation of porcine EPSCs

Based on the established pEPSCs culture system, and rat and mouse PGC differentiation induction protocols [10,12,13], this study used a two-step method for the induction of PGCs. Initially, pEPSCs were subjected to planar induction for 2 days to differentiate into 2D cells. Subsequently, the resulting EpiLCs were designated as Day 0. and on this basis, suitable protein factors were optimized and added for suspension induction towards 3D cell differentiation, ultimately obtaining PGCs (Fig. 1-A).

pEPSCs (Fig. 1-B) were transdifferentiated into EpiLCs after 48 h of induction, with cell morphology changing from tight, domed clusters to loose, spindle-shaped, flattened morphology, consistent with epiblast cell characteristics (Fig. 1-C). RNA samples were extracted from cells at each differentiation stage, and mRNA expression was analyzed (Fig. 1-D). A gradual decrease in the expression of the pluripotency gene NANOG was observed in pEPSCs, whereas the core marker OTX2 of EpiLCs remained consistently highly expressed, showing no significant difference compared to the initial pEPSCs. The high expression of OTX2 in both porcine EPSCs and EpiSCs has been previously reported, aligning with findings in the literature [12,14]. Based on these results, cells differentiated from pEPSCs for 12–48 h are considered to represent EpiLCs (Fig. 1-E). In rats and mice, PGCs are induced from the post-implantation epiblast, with the transcription factor OTX2 serving as a key regulator of this process [13]. This indicates a high level of conservation from PSCs to EpiLCs.

3.2. Primitive streak and trophoblast cells were generated through 2D planar induction differentiation of porcine pEPSCs

At 12 h of induced differentiation, significant expression of the early primitive streak (PS) marker MIXL1 was detected, peaking at 24 h and becoming undetectable by 36 h (Fig. 2-A). The late PS marker T (TBXT), which also serves as an early mesoderm marker, exhibited a concurrent increase in expression with MIXL1 and reached its maximum level at 36 h (Fig. 2-B). These findings indicate that the PS emerges during the 12–24-h induction period and subsequently differentiates into mesoderm between 24 and 36 h post-induction.

During the 12–48 h induction period, TFAP2C and HAND1 expression levels continued to rise (Fig. 2-C). At this stage, some cells were still in the early primitive streak stage, ruling out the possibility that high HAND1 expression originated from primitive streak-derived mesoderm cells. Given that TFAP2C and HAND1 are key markers for trophoblast cells, it is speculated that the early expression of HAND1 at the 12-h point might be attributable to the trophoblast lineage. At 36 h of induction, expression of the late PS and early mesoderm marker T (TBXT) peaked, followed by a decline at 48 h. In contrast, HAND1, another mesoderm marker, remained highly expressed and continued to increase progressively. These findings indicate that between 36 and 48 h of induction, the trophoblast undergoes further differentiation into

Table 1
Primer information involved in the experiment.

Gene	Forward Primer	Reverse Primer
β -ACTIN	GCAAGGACCTCTACGCCAA	CACATCTGCTGGAAGGTGGA
OTX2	CTCTTTGTCCGACGCCA	TTTCAGTGGCCACTCTTCCC
NANOG	CCGTGTGGGGTAATCAGAGC	TCCCCAGCAGTTTCCAAGAC
MIXL1	GTCCACCACTCTGCTGTGA	CTGAGATGCTCCCTCCAACC
T	ATGATGGAAGAGCCGGGAGA	AGGGCAGGATACCTCTCACA
TFAP2C	GCTGCTCATGTAAACCTCT	TTTACACACTTGCTGTGGCG
HAND1	CTACCTGATGGACGTGTGG	GCAGCTCTCTTTCCGCTTG
CER1	TCCAGAGGCAGCGAGAAAAG	CCATCCTTCAGCCGAGTGAG
SOX17	CCCTCGGGGACATGAAGATGAAG	CTTCCAAGACTTGCCAGCATC
FGF8	GGAGGGAGCTAGGGAACAGA	ACCTGTTGGGAAACACCCTG
PRDM1	CGTGAACCCAGCACACTCT	GGCTCTGGATTGTGTGTCATT
PRDM14	ATGTTTGGTGAAGTGCACAC	CCCAGCACCTCTTTTCCAT
KIT	ACCGAAGGAGGCACTTACAC	GCGAAGCGAAGCACTCTGCT
DPPA3	CGACCTCAACCGACTATCCG	ACAACGTCCTACCCCTTTC
WNT3	CATCTGTGGCTGTGATTCCG	CTATTCTCACGTGCGTCTGC

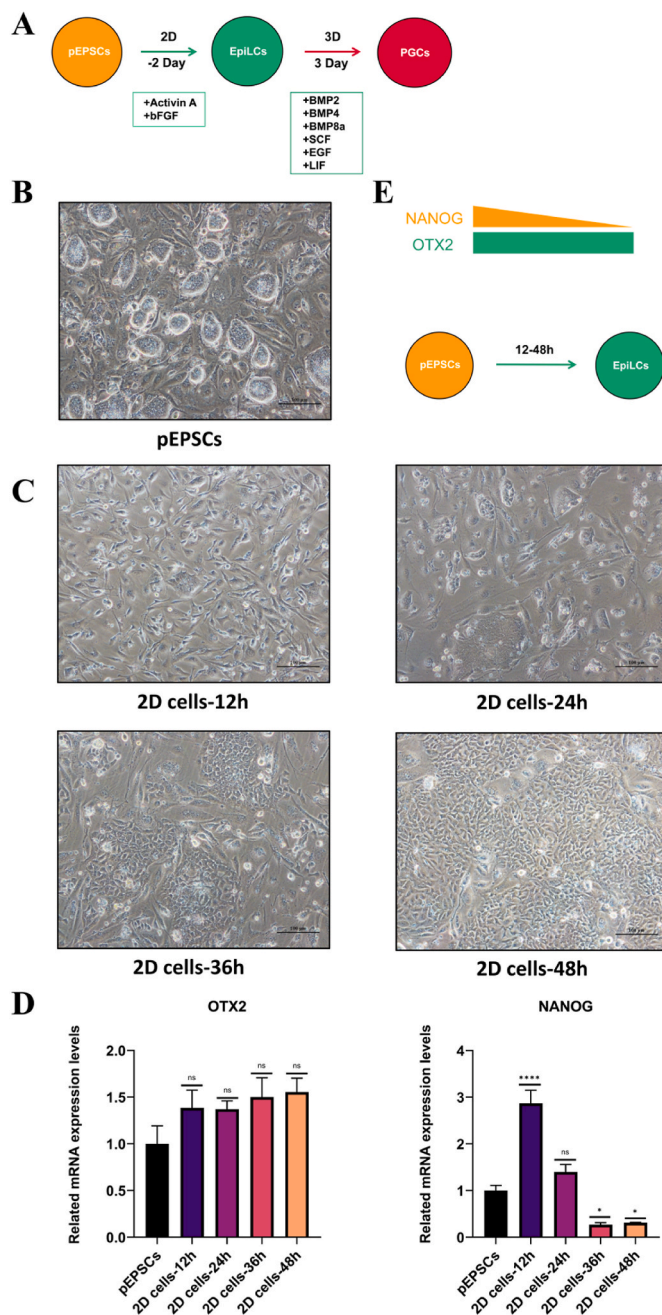


Fig. 1. Induction of porcine EPSCs into EpiLCs cells.

(A) Schematic illustration of the differentiation pathway from pEPSCs to porcine PGCs. (B) A bright-field image of pEPSCs after 3 days of culture, with a scale bar of 100 μ m. (C) Bright-field images showing the 2D planar induction differentiation from pEPSCs to 2D cells at various time points. Scale bar: 100 μ m. (D) The expression levels of OTX2 and NANOG genes in EpiLCs were measured by RT-qPCR; $n = 3$. (E) Schematic diagram of pEPSCs-induced differentiation into EpiLCs.

extraembryonic mesoderm (ExM). This process is likely accompanied by the production of BMP, which may suppress the visceral endoderm and consequently lead to a downregulation of endoderm-associated markers.

3.3. Visceral endoderm cells are generated through 2D planar induction differentiation of porcine EPSCs

The BMP family as key markers of mesoderm, plays a central role in initiating PGC specification. High expression levels of the BMP

antagonist CER1 were observed in 24 h 2D cells [15]. Concurrently, the endoderm lineage marker SOX17 also exhibited elevated expression at this stage (Fig. 3). As an early marker of PGCs, SOX17 promotes the upregulation of downstream CER1, which subsequently inhibits BMP signaling and further suppresses mesodermal lineage development. CER1 is considered a core marker of the endoderm [15,16], while the central transcription factor for the endoderm lineage, SOX17, works synergistically with OTX2 to maintain the gene expression program characteristic of endoderm. By the 12 h induction point, some cells had already entered the early primitive streak stage, suggesting that by 24 h, some cells might have transformed into the endoderm lineage, a process possibly originating from primitive streak differentiation. Over time, CER1 expression gradually decreased, while ectoderm markers FGF8 and WNT3 continued to decline, and OTX2 remained highly expressed. This expression pattern might be due to the addition of exogenous factors bFGF and Activin A, which might inhibit further differentiation of EpiLCs, thus maintaining their pluripotent state.

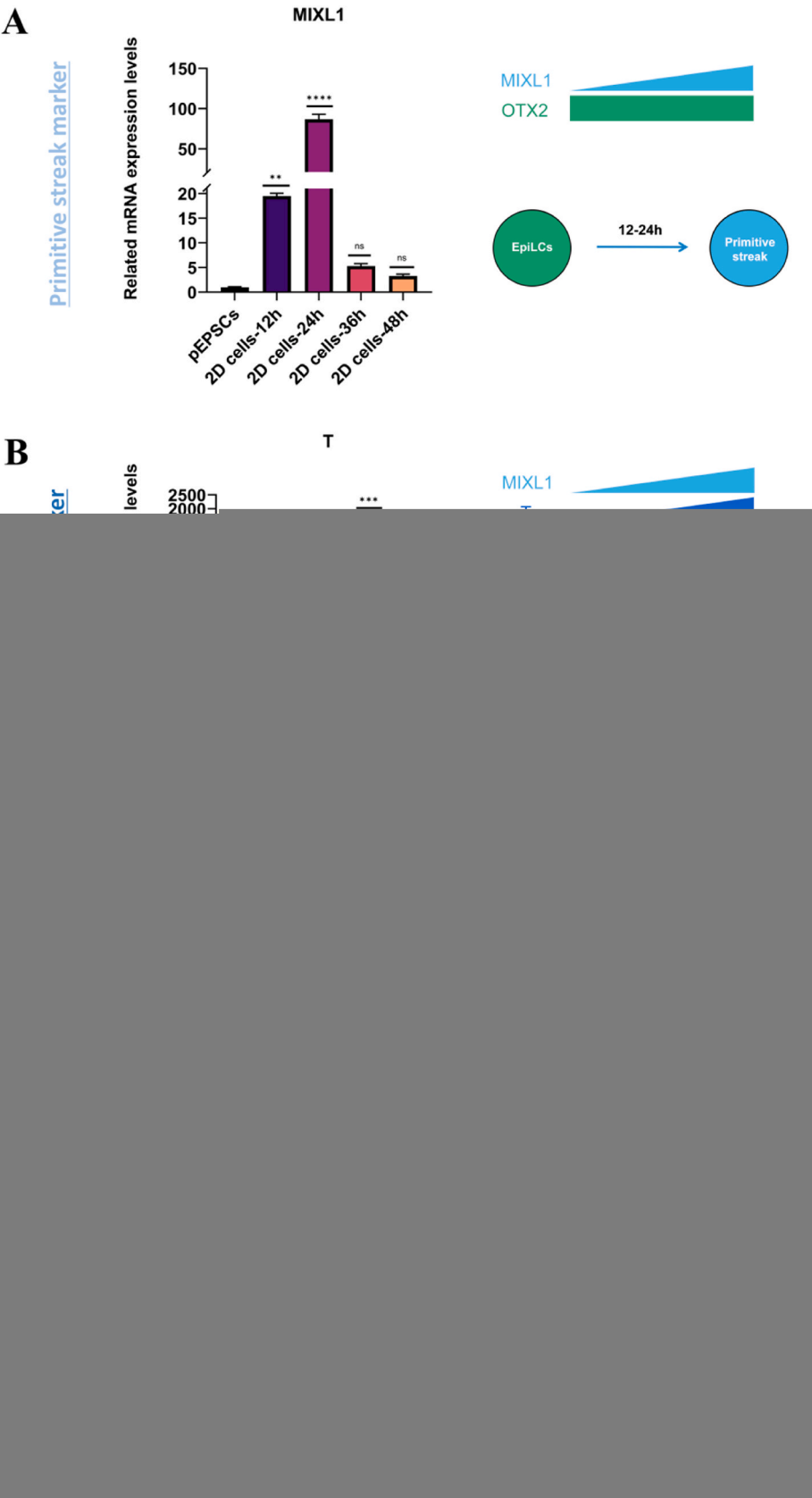
3.4. Early PGCs were generated through 2D planar induction differentiation of porcine EPSCs

Results shows that high SOX17 expression can not only upregulate endoderm lineage genes but also activate the PGC marker PRDM1. In human PGCs, SOX17 and TFAP2C initiate the core germ cell program, including the transcription repressor PRDM1 and pluripotency factors POU5F1 and NANOG, through an enhancer activation mechanism. This indicates that SOX17 and TFAP2C have a synergistic effect in regulating germ cell development-related genes. The induced visceral endoderm and trophoblast lineages collaboratively contribute to the proper development and differentiation of germ cells. Monitoring of early PGC markers revealed that SOX17 expression peaked at 36 h in 2D cells (Fig. 3), while TFAP2C remained highly expressed throughout the entire induction and differentiation process (Fig. 2-B). PGC marker analysis showed that PRDM1 increased significantly only at 36 h, while pluripotency markers PRDM14 and NANOG expression levels gradually decreased, and KIT expression remained consistently low.

PGC marker analysis showed that PRDM1 increased significantly only at 36 h while pluripotency markers PRDM14 and NANOG expression levels gradually decreased, and KIT expression remained consistently low (Fig. 4-A, Fig. 2-B). Only a notable increase in DPPA3 expression was observed after 48 h of induction (Fig. 4-A). Meanwhile, expression of the gonadal cell marker FGF8 showed no significant change in the early phase and subsequently decreased (Fig. 4-B). Partial early PGCLCs, rather than mature PGCs that colonize the gonads, were observed during the induction phase of 2D cells, with the expression of PGC marker being most pronounced at 36 h. Throughout the entire induction and differentiation process, the expression level of WNT3 was found to decrease continuously (Fig. 4-C). Therefore, 2D cells at 36 h (2D cells-36h) of induction were selected for 3D suspension-based induction of PGCs.

3.5. Induction of porcine EpiLCs into PGC-like cells in suspension

Under the condition of N2B27 basal medium supplemented with 5 % FBS and protein factors including BMP family members, SCF, EGF, and LIF, EpiLCs were transdifferentiated into PGCs over a 3-day induction period following 36 h of EpiLC induction (Fig. 1-A). In the suspension culture, the induced PGCs formed EBs with irregular surfaces (Fig. 5-A). RNA samples were collected from cells at Day 1 and Day 3 of induction to assess mRNA expression levels. Notably, on Day 1 of PGC induction, early PGC markers such as SOX17 and TFAP2C were found to be highly expressed, followed by a gradual decline (Fig. 5-B). Similarly, PGC markers including PRDM1, DPPA3, and KIT also showed high expression on Day 1, which subsequently decreased. Among them, PRDM1 expression remained markedly higher compared to that in EpiLCs-36h (Fig. 5-C). The expression levels of pluripotency genes NANOG and



(caption on next page)

Fig. 2. Porcine EPSCs were induced and differentiated into primitive streak and Trophoblast cells.

(A) The expression levels of primitive streak-related genes at various stages in pEPSCs and 2D cells were analyzed by RT-qPCR, accompanied by a schematic diagram illustrating the induction and differentiation of the primitive streak in 2D cells; $n = 3$. (B) The expression levels of mesoderm-related genes at various stages in pEPSCs and 2D cells were assessed by RT-qPCR, along with a schematic diagram depicting mesoderm induction and differentiation from the primitive streak; $n = 3$. (C) The expression levels of trophoblast cell-related genes at various stages in pEPSCs and 2D cells were examined by RT-qPCR, and a schematic diagram illustrates the induction and differentiation of trophoblast cells into extraembryonic mesoderm; $n = 3$.



Fig. 3. Differentiation of porcine EPSCs resulted in the formation of primitive streak and trophoblast cells.

(A) The expression levels of visceral endoderm-related genes were measured by RT-qPCR in pEPSCs and 2D cells at different stages; $n = 3$. (B) A schematic diagram illustrating the induction and differentiation of visceral endoderm from pEPSCs. (C) The expression levels of the FGF8 gene were assessed by RT-qPCR in pEPSCs and 2D cells at various stages; $n = 3$.

PRDM14 were gradually upregulated, aligning with the activation of late-stage pluripotency genes observed in PGCs.

Simultaneously the PS late-stage and early mesoderm marker T was highly expressed on Day 1 of induction, followed by a subsequent decline in expression (Fig. 5-B). It was previously found that MIXL1 was strongly expressed in EpiLCs induced for 24–36 h, suggesting that PGCs and the PS might appear concurrently, or there might be an alternating transformation between PS and PGC precursors. This finding consistent with reported characteristics of human PGCs and primitive streak appearing simultaneously [17]. In immunofluorescence validation, early PGC markers including SOX17, PRDM1, and TFAP2C all exhibited

detectable fluorescence signals, and the late-stage marker VASA protein was also expressed (Fig. 5-D), indicating that the protocol effectively induced porcine PGCs.

4. Discussion

With the continuous expansion of the livestock breeding industry, technologies based on stem cell gene editing and their conversion into gametes via PGCs have become increasingly refined. These approaches have been shown to significantly accelerate and shorten the breeding cycle compared to traditional methods, thereby facilitating the efficient

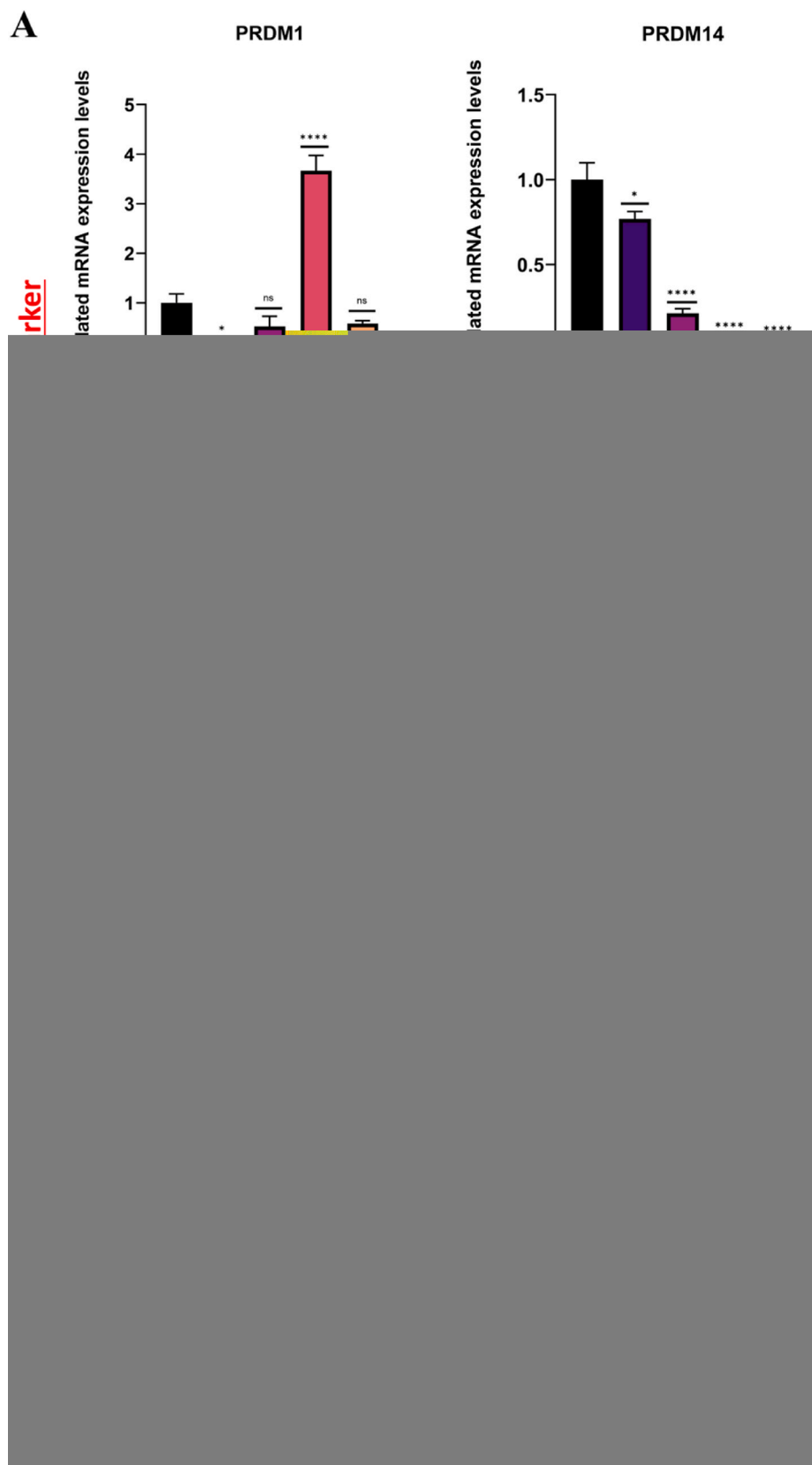


Fig. 4. Porcine EPSCs were induced to differentiate into primordial germ cells.
(A) The expression levels of PGC-related genes in pEPSCs and 2D cells at various stages measured by RT-qPCR; n = 3. (B) Schematic diagram of 2D cells induced differentiation into PGCs. (C) The expression levels of the WNT3 gene in pEPSCs and 2D cells at various stages measured by RT-qPCR; n = 3.



Fig. 5. Induction of EpiLCs into PGCs

(A) Cell morphology of porcine EpiLCs undergoing suspension induction toward PGCs on days 1, 2, and 3; scale bar = 100 μ m. (B) Expression levels of early PGC-related genes at different stages in pEPSCs and PGCs, as determined by qRT-PCR; $n = 3$. (C) Expression levels of PGC-related genes at various developmental stages in pEPSCs and PGCs, as assessed by qRT-PCR; $n = 3$. (D) Expression levels of pluripotency-associated and related genes at different stages in pEPSCs and PGCs, analyzed via qRT-PCR; $n = 3$. (E) Schematic representation of PGC differentiation induced from EPSCs. (F) Immunofluorescence staining of porcine PGCs induced from EpiLCs for 3 days, showing expression of SOX17, TFAP2C, PRDM1, and VASA; scale bars = 300 μ m, 300 μ m, 300 μ m, and 125 μ m, respectively.

generation of disease-resistant breeding pigs through gene editing. Moreover, pigs, as a representative large animal disease model, exhibit developmental patterns closely resembling those of humans [2]. The in vitro induction of porcine PGCs is therefore of great significance for advancing research on human PGC induction and gametes maturation.

In mice, the inner cell mass (ICM) of the pre-implantation blastocyst at E3.5–E4.5, and the epiblast of the post-implantation embryo at E5.5–E6.5, have been shown to give rise in vitro to two distinct types of PSCs, namely ESCs and EpiSCs [18,19]. The initiation of mouse germ cell fate is triggered in the embryonic epiblast through BMP4 signal transduction, with germ cell fate being induced around E6 [20]. PGCLCs derived in vitro from the epiblast have been shown to generate functional sperm when transplanted into neonatal testes lacking endogenous germ cells [21]. Therefore, EpiLCs serve as a pivotal fate transition stage for the induction and differentiation of PGCs both in vivo and in vitro.

A detailed analysis of the temporal progression from porcine EPSCs to EpiLCs revealed that this transition occurs within an exceptionally narrow time window. During this differentiation process, Activin A plays a regulatory role in directing pluripotent stem cells toward the EpiLC state. As a member of the TGF- β superfamily, Activin A binds to serine/threonine kinase receptors on the cell surface, thereby activating the SMAD signaling pathway. This activation modulates the transcription of target genes and governs key aspects of cell differentiation and development. A precise understanding of this signaling mechanism is essential for fine-tuning the concentration and exposure time of Activin A, since variations in these parameters can alter both the intensity and duration of pathway activation, ultimately influencing the trajectory and efficiency of cell differentiation [22]. Additionally, different types of pluripotent stem cells may exhibit distinct sensitivities to Activin A.

It has been demonstrated that culturing ESCs in a medium supplemented with Activin A and bFGF can induce their differentiation into EpiLCs and subsequently promote their progression toward PGC fate. The most effective induction of EpiLCs has been observed at an Activin A concentration of 20 ng/mL, indicating that Activin A provides critical signaling support during the transition to the EpiLC state [5]. When Activin A and bFGF were added to direct the differentiation of pEPSCs, the resulting cell population was a composite type, not a homogeneous EpiLC population.

Expression of the early PS marker MIXL1 was detected between 12 and 24 h after induction, followed by its downregulation, marking the

exit from the primitive streak cells stage (Fig. 2-A). Between 24 and 36 h, further differentiation into mesoderm was observed (Fig. 2-B). The mesoderm derived from the primitive streak is likely to produce elevated levels of BMP4 signaling, which may facilitate the generation of PGCs. Differentiation of PGCLC populations was induced by EpiLCs-36h in the absence of exogenous BMP protein factors, thereby supporting the hypothesis that PGCs can arise spontaneously during the early induction phase of EpiLCs. Expression of the PGC marker PRDM1 was detected at 36 h following pEPSC induction (Fig. 4-A, B), but subsequently declined. This suggests that precise regulation of the EpiLC induction duration may represent a critical checkpoint in PGC induction. However, the expression level of PRDM1 at 48 h of EpiLC induction was insufficient to drive complete transdifferentiation into PGCs.

As induction and differentiation progressed over time, the resulting cell populations became increasingly distinct, indicating that cell fate transitions occur rapidly within a short time frame. At 24 h post-induction, following the emergence of the PS, high expression levels of the endoderm markers SOX17 and CER1 were observed, which may have contributed to the generation of the definitive endoderm. In vitro induction studies of PGCLCs have demonstrated that SOX17 serves as a critical regulator of hPGC fate. Loss of SOX17 impairs hPGC induction, while PRDM1 acts downstream of SOX17 to suppress endoderm and somatic genes [23,24]. These findings align with our observations of differential expression levels of PRDM1 and SOX17, as well as the reduced expression of CER1. Attenuation of WNT3 leads to a corresponding decrease in the expression of its downstream effector T. The mesodermal protein T, being a downstream target of WNT3 signaling, is essential for mouse PGC specification but not for human PGCs. Given the high conservation between pigs and humans in PGC development processes, the in vitro induction of porcine PGCLCs might differ from the response patterns observed in mouse models [2,25].

The differentiation of pEPSCs into EpiLCs is a complex and transient process yielding heterogeneous cell populations. Early EpiLCs and trophoblast are generated within the first 12 h. Between 12 and 24 h, EpiLCs form the PS, which subsequently gives rise to mesoderm and endoderm. BMP4 derived from mesoderm and SOX17 from endoderm contribute to the specification of early PGC fate, while CER1 produced by endoderm antagonizes the development of the mesoderm lineage. The fate specification of early PGCs is jointly regulated by TFAP2C produced by trophoblast and SOX17 derived from endoderm, leading to

the subsequent formation of ExM. The composite cell population generated at 36 h further differentiates into PGCs and promotes their maturation under the conditions of 3D suspension culture and exposure to exogenous BMP4.

5. Conclusion

The differentiation of pEPSCs into EpiLCs is an extremely rapid and complex process. By monitoring the state of induced cells every 12 h, it was observed that besides the EpiLC lineage, there existed a mixed population comprising visceral endoderm trophoblast, primitive streak, and PGC lineages, and there was signaling crosstalk between these different cell populations. To enhance the efficiency of porcine PGC induction in vitro, the time point at which SOX17 and PRDM1 expression peaked—EpiLCs-36h—was selected for subsequent 3D suspension induction of PGCs. Quantitative analysis and immunofluorescence assays confirmed the successful generation of PGCs from EpiLCs-36h under suspension culture conditions.

CRediT authorship contribution statement

Liming Yuan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiaolong Wu:** Writing – review & editing, Writing – original draft, Resources, Formal analysis. **Xiaoxiao Fu:** Writing – review & editing, Writing – original draft. **Zuo Lei:** Investigation. **Hongzhao Shi:** Software. **Wenjie Xu:** Supervision, Software, Project administration. **Yaya Zhao:** Supervision, Software. **Jiannan Li:** Methodology. **Xueting Li:** Methodology. **Lexi Gao:** Project administration, Data curation. **Na Li:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Jinlian Hua:** Writing – review & editing, Writing – original draft, Visualization, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of interest statement

The authors declare no conflict of interest.

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