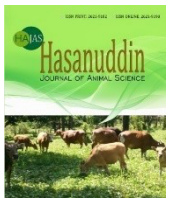

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In Vitro Development and Gene Expression of Pig Embryos Cloned from Induced Pluripotent Stem Cells and Embryonic Fibroblasts

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ABSTRACT

Somatic cell nuclear transfer (SCNT) in pigs remains inefficient, mainly due to incomplete nuclear reprogramming of donor cells. Induced pluripotent stem (iPS) cells have been proposed as promising nuclear donors, but their developmental competence in porcine SCNT is unclear. This study compared the in vitro developmental ability of embryos reconstructed with porcine embryonic fibroblasts (PEFs) or porcine iPS (piPS) cells. Developmental rates, total and epiblast cell numbers were assessed in SCNT, in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and parthenogenetic activation (PA) embryos. The effect of oocyte activation status on piPS-derived SCNT embryos was also examined. Expression of well-known pluripotency transcription factor genes OCT4, SOX2, NANOG, c-MYC, and KLF4 were quantified by quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Results showed cleavage and blastocyst formation rates of piPS-derived embryos (55.0% and 14.6%) were comparable to PEF-derived SCNT, IVF, and ICSI, while PA embryos showed

significantly higher rates (67.8% and 44.5%). Non-activated oocytes yielded higher development than activated ones. Blastocyst cell numbers were similar across groups; however, SCNT embryos contained fewer epiblast cells than IVF and PA. qRT-PCR showed markedly reduced OCT4, comparable SOX2, NANOG, and c-MYC, and higher KLF4 in NT and PA embryos than IVF. The results conclude that piPS cells can serve as nuclear donors but do not improve cloning efficiency compared with fibroblasts. Aberrant lineage allocation and dysregulated gene expression suggest incomplete reprogramming, highlighting the need for improved piPS quality.
Keywords: Induced pluripotent stem cells (iPSCs), pig, nuclear transfer

INTRODUCTION

Although nuclear transfer (NT) has been successfully established in mice and several domestic animals, the efficiency of producing cloned animals varies considerably across species. In pigs, cloning efficiency remains particularly low compared with other livestock species [1]. This poor outcome is largely attributed to the insufficient or inaccurate reprogramming of the donor nucleus by the recipient ooplasm [2] [3]. The type of donor cell also plays a crucial role in determining the success of cloning [1]. In pigs, fetal fibroblasts are reportedly more effective donors than adult fibroblasts, cumulus, or oviduct cells [4], whereas in buffalo, cumulus cells have been shown to be superior to skin fibroblasts and granulosa cells for NT [5].

The generation of induced pluripotent stem (iPS) cells in mice using defined factors, namely, OCT4, SOX2, KLF4, and c-MYC (OSKM) [6], has stimulated interest in their application as donor nuclei for NT. Compared with differentiated somatic cells, iPS cells may improve the developmental competence of cloned embryos. Following this breakthrough, iPS cells have been established from several domestic animals, including cattle [7] and pigs [8], by reprogramming fibroblasts from different genetic backgrounds. However, although multiple groups have reported piPS cells with embryonic stem (ES)-like characteristics in pigs, capturing and maintaining a stable naïve pluripotent state remains challenging [8] [9]. Naïve iPS cells are particularly valuable because they can efficiently integrate into blastocysts and contribute to germline chimeras [9].

The lack of authentic porcine ES cells has limited the use of pigs as biomedical models and in preclinical transplantation research [10]. To overcome this, porcine iPS (piPS) cells have been developed and applied in transgenesis, gene targeting, and cloning. Notably, piPS cells have been used as nuclear donors to generate live cloned piglets, although the overall cloning efficiency remains low [11]. Recently, transgene-free piPS cells have been derived using episomal vectors and miR-302/367, providing greater stability and eliminating concerns regarding persistent transgene expression [12]. In parallel, advances in culture systems have promoted naïve-like pluripotent states and the establishment of stable porcine embryonic stem cells (pESCs), highlighting the importance of species-specific optimization [13]. Despite these advances, the developmental efficiency of piPS cell-derived SCNT embryos remains low [14], and further evaluation is required to clarify whether piPS cells can improve nuclear reprogramming and developmental competence in pigs.

Therefore, in the present study, the in vitro developmental ability of porcine cloned embryos reconstructed with piPS cells as donor nuclei was evaluated and compared with the performance of embryos derived from porcine embryonic fibroblasts (PEFs), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and parthenogenetic activation (PA).

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all chemicals used in the experiments were purchased from Sigma Chemical Co., St. Louis, MO, USA.

In Vitro Maturation Culture of Oocytes

Pig ovaries were obtained from a slaughterhouse and transported to the laboratory within 3 h of collection in 0.9% physiological saline at 30°C. The cumulus-oocyte complexes (COCs) were recovered by aspiration from 3–6 mm diameter ovarian follicles using an 18-gauge needle firmly attached to a 10 ml disposable syringe and then allowed to settle down by gravitation at the bottom of 15 ml conical tubes at 39°C for 5 min. The supernatant was discarded, and the precipitate was washed three times by sedimentation with modified Tyrode Lactate 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (TL-HEPES)-polyvinylalcohol (TLH-PVA), according to previously reported procedures [15] [16]. After washing, the precipitate was re-suspended in TLH-PVA medium and observed under a stereo microscope to recover the COCs. COCs with three or more uniform layers of compact cumulus cells and a homogeneous cytoplasm were selected, and 50–70 COCs were matured in each well of a four-well Nunc dish containing 500 µL of modified North Carolina State University (mNCSU37) medium [17] supplemented with 0.6 mM L-cysteine, 10% porcine follicular fluid, 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), and hormones; 1.3 µg/ml Follicle Stimulating Hormone (FSH) and 0.6 µg/ml Luteinizing Hormone (LH) for 20 h, and then cultured without dbcAMP and hormones for another 20 h under an atmosphere of 5% CO_2 in air at 39°C [18]. After 40 h of in vitro maturation (IVM), the COCs were denuded of cumulus cells in NCSU37 supplemented with 0.1% hyaluronidase. Maturation to metaphase II (MII) was confirmed by the presence of polar bodies.

Donor Cells

Porcine embryonic fibroblasts (PEFs) and porcine iPS (piPS) cells were used as donor cells. The piPS cell line was clone 2, derived from PEFs, as previously reported [19] [20]. Porcine iPS cells were maintained on mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder layers in porcine iPS medium [21] at 39°C under 5% CO_2 in air. The medium was changed daily, and the cells were passaged every 2–3 days. PEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) at 39°C under 5% CO_2 in air.

Nuclear Transfer (NT)

For NT, a piezo-actuated micromanipulator was used, as previously described [18] [21] [22]. Briefly, matured oocytes were treated with 0.4 µg/ml demecolcine for 1 h, and then enucleated in NCSU37 supplemented with 5 µg/ml cytochalasin B, 0.4 µg/ml demecolcine, 0.05 M sucrose, and 0.4% BSA. Enucleated oocytes were incubated for 1 h in NCSU37 with 0.4% Bovine Serum Albumin (BSA) at 39°C under 5% CO_2. Donor cells were injected into enucleated oocytes, followed by electrical fusion and activation. Oocytes were chemically activated in modified porcine zygote medium (mPZM)-5 with 5 µg/mL Cytochalasin B (CB), 10 µg/ml Cycloheximide (CHX), and 50 nM Trichostatin A (TSA) for 2 h, and then cultured in PZM-5 with 50 nM TSA for 22 h [23]. Thereafter, the reconstructed embryos were cultured in mPZM-5 for 4 d, then in mPZM-5 + 10% FBS for 2 d under 5% CO_2 in air at 39°C [18].

In vitro Fertilization (IVF)

Liquid semen was obtained weekly from a commercial swine supplier (Swine Genetic Co., Ltd., Shizuoka, Japan) and stored at 15°C. Semen was centrifuged at 500 × g for 5 min, and the pellet was resuspended in a 1× Modena extender (SEM-5x; Research Institute for Functional Peptides, Yamagata, Japan). After one wash, motile spermatozoa were separated using a Percoll gradient (50%/80%; GE Healthcare, Uppsala, Sweden) and centrifuged at 700 × g for 20 min. The recovered sperm cells were washed twice with porcine fertilization medium (PFM) and resuspended. Groups of 15–20 COCs were co-incubated with sperm (2×10^6/ml) in 100-µl PFM droplets for 20 h at 39°C under 5% CO_2 in air. Presumptive zygotes were freed from cumulus cells by vortexing for 4 min in porcine oocyte embryo-collection medium (POE-CM) and washed twice in POE-CM and twice in PZM-5 before being cultured in 40-µl PZM-5 droplets (20–25 embryos/drop) covered with mineral oil. Embryos were cultured until Day 6 (IVF = Day 0), and cleavage (≥2 cell) and blastocyst formation were assessed 48 h and 168 h post-insemination, respectively.

Parthenogenetic Activation (PA)

Parthenogenetic activation was performed as previously described [18, 22], with minor modifications. Metaphase II oocytes were activated with a direct current (DC) pulse of 150 V/mm for 100 µs in 0.28 M Mannitol (Nacalai Tesque, Kyoto, Japan) containing 0.01% Polyvinyl Alcohol (PVA), 0.1 mM $MgSO_4$, and 0.25 mM $CaCl_2$. Activated oocytes were cultured in mPZM-5 with 5 µg/ml CB for 4 h [24], washed three times, and then cultured in 30-µl droplets of mPZM-5 (10 embryos/drop) under mineral oil for 7 d at 39°C in 5% CO_2. Cleavage and blastocyst development were evaluated at 48 h and 168 h.

Intracytoplasmic Sperm Injection (ICSI)

ICSI was performed using a piezo-micromanipulator attached to an inverted microscope (IX70; Olympus, Tokyo, Japan). Frozen semen was thawed at 37°C for 30 s, centrifuged briefly, and resuspended in HEPES-buffered NCSU-37 containing 10% Polyvinylpyrrolidone (PVP). A single spermatozoon was aspirated into an injection pipette and injected into the cytoplasm of an oocyte placed in a 5-µl droplet of NCSU-37 under mineral oil. Injection was performed at the 3 o'clock position, with the polar body located at 6 or 12 o'clock. The injected oocytes were washed thrice in mPZM-5, incubated for 1 h, and activated using the same procedure used for PA embryos.

Cell Number Counting

After culture, the blastocysts were washed three times in Dulbecco's phosphate-buffered saline (PBS) containing 0.03% PVA, stained with 10 µg/ml Bisbenzimide (Hoechst 33342; Sigma-Aldrich) for 5 min, and mounted on glass slides. Cell nuclei were counted under ultraviolet (UV) illumination (filter set 365/10 nm) using a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Gene expression was analyzed by qPCR, as described by Li et al. [23], with modifications. Briefly, cyclic Deoxyribose Nucleic Acid (cDNA) was synthesized from blastocysts using the Cells-to-cDNA II kit, and qPCR was performed using the Thunderbird SYBR qPCR mix. The expression levels of OCT4, SOX2, NANOG, c-MYC, and KLF4 were normalized to that of YWHAG. Relative quantification was performed using the ΔΔCT method [18]. Table 1 presents the primers used for quantitative real-time PCR analysis.

Table 1. Primers used for quantitative real-time PCR analysis

Genes	Primers	Sequence (5'-3')	Product Size (bp)	Accession Number
Oct4	Forward	GCTGACAACAACGAGAATCTGC	99	NM_001113060.1
	Reverse	ACGCGGACCACATCCTTCTCTAG		
Nanog	Forward	TTCCTTCCTCCATGGATCTG	214	NM_001129971.1
	Reverse	ATCTGCTGGAGGCTGAGGTA		
Sox2	Forward	ACAGCCCAGACCGAGTTAAGC	84	NM_001123197.1
	Reverse	GGTTCTCTTGGGCCATCTTG		
Klf4	Forward	GAGGAGCCAAAGCCAAAGA	60	XM_005660316.2
	Reverse	GTGAGTGGCCGTCCTTTTC		
c-MYC	Forward	ACGGCTTGATGTGTCCTGAG	70	NM_001044610.2

Statistical analysis

All experiments were performed three times. Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, La Jolla, CA, USA). All data are presented as the mean ± SEM. Statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSIONS

The *in vitro* developmental potential of porcine embryos reconstructed with PEFs or piPS cells and embryos produced by IVF, ICSI, and PA are summarized in Table 2. The cleavage and blastocyst formation rates of NT embryos reconstructed with piPS cells (55.00% and 14.60%, respectively) were comparable to those reconstructed with PEFs (59.40% and 19.50%). Both NT groups showed developmental outcomes similar to those of IVF (46.40% cleavage; 13.00% blastocyst) and ICSI embryos (38.40% cleavage; 15.70% blastocyst). In contrast, PA embryos exhibited significantly higher cleavage and blastocyst rates (67.80% and 44.50%, respectively).

The total cell numbers of blastocysts did not differ significantly among the groups, ranging from 29.8±5.6 in the ICSI group to 40.5±25.7 in the IVF group. To further evaluate the effect of oocyte activation status, piPS-derived NT embryos reconstructed with non-activated or artificially activated oocytes were compared. Non-activated recipients yielded higher cleavage (45.0%) and blastocyst formation per cleaved embryo (46.9%) than activated recipients (36.0% and 30.0%, respectively), although the total cell numbers remained similar (39.7±8.8 vs. 37.4±5.0).

These results suggest that piPS cells are a viable donor cell type for somatic cell nuclear transfer (SCNT). The similarity of results to those observed in IVF and ICSI embryos indicated that NT-derived embryos can achieve developmental milestones comparable to those observed in embryos produced by conventional fertilization techniques. This aligns with findings from Chen et al. [25], who emphasized that while NT embryos often lag behind IVF embryos in developmental efficiency, optimized protocols and donor cell selection can narrow this gap. Moreover, the comparable blastocyst cell numbers across NT, IVF, and ICSI groups (ranging from ~30 to ~40 cells) suggest that embryo quality, in terms of cellular composition, is not significantly compromised by the method of production.

Table 2. Developmental potential of porcine embryos reconstructed by SCNT or produced by IVF, ICSI, and PA

Embryo type/condition	No. cultured	Cleavage n (%)	Blastocyst n (%)	Blastocyst cell number (mean ± SD)
NT piPS	230	122 (55.00)	32 (14.60)	30.60±15.90
NT pEF	237	140 (59.40)	46 (19.50)	35.80±12.10
IVF	492	226 (46.40)	63 (13.00)	40.50±25.70
ICSI	172	66 (38.40)	27 (15.70)	29.80±5.60
PA	223	151 (67.80)	99 (44.50)	32.90±7.90
NT piPS (non-activated)	98	44 (45.00)	21 (46.90*)	39.70±8.80
NT piPS (activated**)	111	40 (36.00)	12 (30.00*)	37.40±5.00

* Blastocyst rate calculated per cleaved embryos

** Activated with electrical stimulation + cycloheximide for 4 h

Parthenogenetically activated embryos demonstrated significantly higher cleavage (67.80%) and blastocyst formation (44.50%) rates. This is consistent with previous reports that parthenogenetic activation can yield high developmental rates due to the absence of sperm-derived epigenetic reprogramming challenges [26]. However, PA embryos are not viable for full-term development due to their uniparental origin, limiting their utility to research contexts.

Interestingly, NT embryos reconstructed with non-activated oocytes showed higher cleavage (45.00%) and blastocyst formation per cleaved embryo (46.90%) than those with artificially activated oocytes (36.00% and 30.00%). This suggests that premature or artificial activation may disrupt the reprogramming window necessary for successful nuclear remodeling and embryonic genome activation.

These findings echo the observations of Chen et al. [25], who noted that the timing and method of oocyte activation are critical for SCNT success, as they influence chromatin remodeling and epigenetic reprogramming.

Immunostaining analysis revealed differences in the contribution of epiblast cells among the embryo groups (Table 3; Fig. 1). Blastocysts derived from piPS and PEF NT embryos contained only ~3 epiblast cells on average, corresponding to <10% of the total blastocyst cells, whereas IVF and PA blastocysts contained significantly more epiblast cells (8.10±1.50 and 6.10±0.50, respectively), accounting for ~15–18% of the total cells. These findings indicate that NT-derived embryos, regardless of the donor cell type, exhibit impaired epiblast cell lineage allocation compared to IVF and PA embryos.

The epiblast is the pluripotent cell population within the inner cell mass (ICM) that gives rise to all somatic lineages of the embryo. Its specification is a critical milestone in early embryogenesis, marking the embryo's capacity for gastrulation and subsequent organogenesis [27] [28]. In this study, blastocysts derived from nuclear transfer (NT) using piPS and PEF cells showed significantly fewer epiblast cells (~3 cells; <10% of total), compared to IVF (8.10±1.50) and PA (6.10±0.50) embryos, which had ~15–18% epiblast contribution. The reduced numbers of epiblast cells in NT embryos suggest compromised lineage segregation and pluripotency establishment. This impairment may stem from incomplete nuclear reprogramming of donor somatic cells, epigenetic dysregulation, or suboptimal activation protocols [27] [29]. Despite

achieving blastocyst formation, NT embryos may lack the cellular architecture and molecular cues necessary for robust epiblast lineage commitment.

Table 3. Effect of embryo production type on epiblast cell numbers

Embryos origins	No.of embryos	Mean Cell Number±SD		
		Epiblast	Total	Epiblast: Total
piPS blastocysts	20	3.30±1.20	35.70±9.50	0.08±0.11
PEF blastocysts	22	3.50±2.20	37.20±8.80	0.09±0.15
IVF blastocysts	20	8.10±1.50	44.50±4.50	0.18±0.14
PA blastocysts	21	6.10±0.50	41.20±8.60	0.15±0.24

Values in the same column are not different, P>0.05

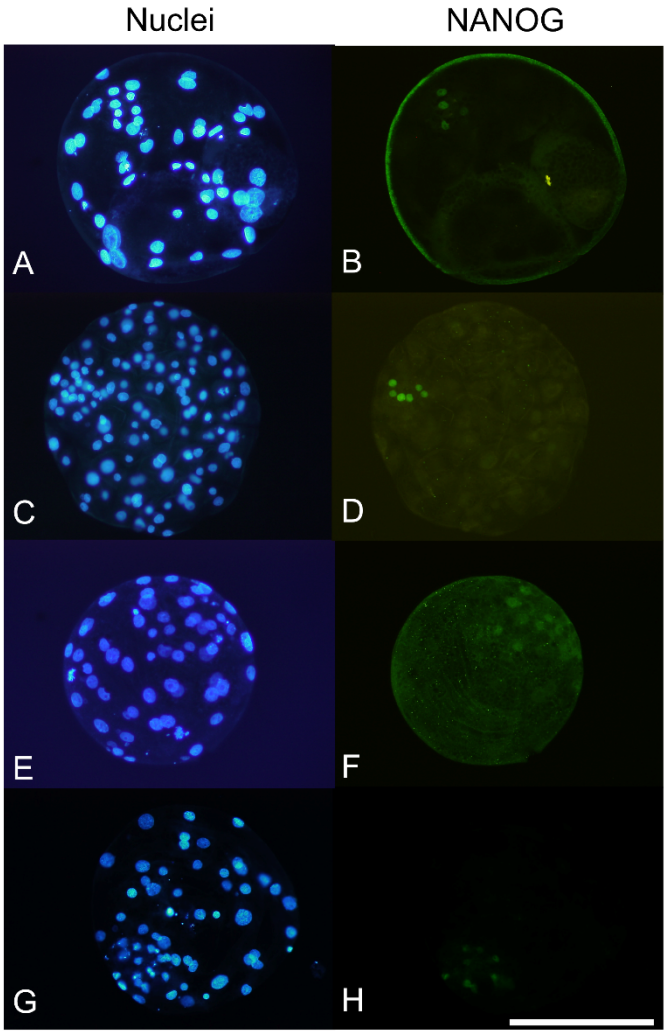

Figure 1. Quantification of Epiblast Cells in NT, Parthenogenetic, and IVF Embryos by Immunostaining (A.B) piPS blastocysts, (C.D); PEF blastocysts, (E.F) PA blastocysts, (G.H) IVF blastocysts. Scale bar is 100um.

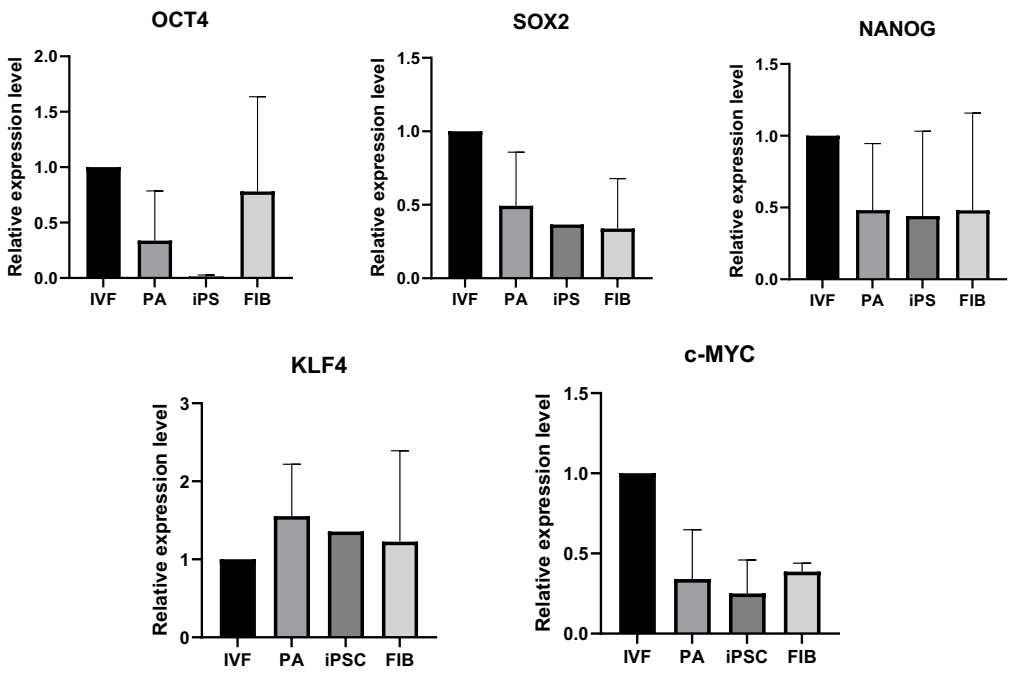

Figure. 2. qRT-PCR analysis of Oct4, Sox2, Nanog, c-Myc and Klf4 in porcine embryos; Error bars = standard error of mean

IVF and PA embryos, which undergo natural or chemically induced activation without nuclear transfer, exhibited significantly higher epiblast cell counts. This supports the notion that intact gamete-derived zygotes possess superior intrinsic programming for lineage specification. PA embryos, although not viable for full-term development, often serve as models for studying early lineage dynamics due to their consistent developmental trajectories [29]. The findings underscore a key limitation in SCNT-based embryo production: while morphological blastocyst formation may occur, functional pluripotency, as indicated by epiblast cell abundance, is often compromised. This has implications for deriving embryonic stem cells from NT embryos and for improving cloning efficiency in livestock biotechnology.

To evaluate the molecular characteristics, the expressions of OCT4, SOX2, NANOG, c-MYC, and KLF4 were quantified using qRT-PCR (Fig. 2). Both PEF- and piPS-derived NT blastocysts expressed all pluripotency-related genes; however, PA and piPS-derived embryos showed markedly reduced OCT4 and SOX2 expression. The NANOG and c-MYC expression levels were largely comparable among the groups. KLF4 expression was higher in PEF-, piPS-, and PA-derived embryos than in IVF blastocysts. Collectively, these results indicate that the gene expression profiles of NT embryos differ from those of IVF embryos, suggesting incomplete or abnormal reprogramming.

The above results showed aberrant lineage allocation and dysregulated gene expression in the resulting embryos, suggesting incomplete reprogramming and highlighting the need for improved piPS quality. Several studies have aimed to improve the reproductive efficiency of animals cloned using NT [26]. Nevertheless, embryo yield and developmental success remain low, particularly in pigs [1] [27]. This inefficiency is largely attributed to the incomplete

reprogramming of donor nuclei by the recipient cytoplasm [2] [3]. Improving NT outcomes in pigs would strengthen their role as biomedical models and potential organ donors for xenotransplantation.

Among the factors influencing NT efficiency, the choice of donor cell type is critical [1] [28], and artificial activation methods can also affect developmental competence [29]. In this study, the developmental potential of embryos reconstructed using PEFs and piPS cells was evaluated and compared with IVF, ICSI, and PA controls. The cleavage and blastocyst formation rates of piPS-derived embryos were similar to those of embryos derived from PEFs, IVF, or ICSI, confirming previous reports that piPS cells do not improve developmental competence compared with fibroblasts [14] [19] [20]. In contrast, PA embryos exhibited superior developmental rates. Notably, when non-activated oocytes were used as recipients, both cleavage and blastocyst formation per cleaved embryo improved compared to activated oocytes, although the total cell numbers remained similar. This suggests that the oocyte activation status can modulate reprogramming efficiency.

Lineage allocation analysis revealed that NT embryos contained significantly fewer epiblast cells than IVF and PA embryos, indicating a defect in the establishment of the pluripotent inner cell mass. Similar abnormalities have been reported by others and attributed to incomplete lineage-specific reprogramming [20]. At the molecular level, piPS-derived NT embryos exhibited characteristic dysregulation of pluripotency genes. OCT4 and SOX2 expressions were markedly reduced, which may compromise the establishment of the inner cell mass and maintenance of pluripotency. In contrast, NANOG and c-MYC were expressed at comparable levels across groups, without significant differences. KLF4 expression was consistently higher in NT and PA embryos than in IVF embryos, and such elevated expression may reflect aberrant regulation during the reprogramming process. These findings are consistent with previous reports that residual exogenous factors and epigenetic memory in piPS cells can interfere with the proper regulation of endogenous gene expression [19] [28], even though our established piPS cell line displayed naïve-like features [9] [21].

Recent advances have offered possible solutions. Transgene-free piPS cells [12] eliminate the problem of persistent factor expression, and epigenetic interventions, such as KDM4A/D mRNA supplementation [30], BRG1 overexpression [31], and XIST suppression [24], have improved porcine SCNT outcomes. Incorporating these approaches may help overcome the deficits in lineage allocation and gene expression observed in the present study.

CONCLUSIONS

Although piPS cells can serve as nuclear donors, they do not improve cloning efficiency compared to fibroblasts and are associated with aberrant lineage allocation and dysregulated pluripotency gene expression. Future optimization should focus on donor cell quality, activation protocols, and targeted epigenetic rescue strategies.

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AUTHORS' CONTRIBUTIONS

All authors participated in the research and writing of the manuscript.

COMPETING INTERESTS

The authors declared that there is no conflict of interest

ETHICAL CLEARANCE

In this study, ovaries from slaughterhouses were used as slaughter residue.

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