Original Paper

The HDAC Inhibitor LAQ824 Enhances Epigenetic Reprogramming and *In Vitro* Development of Porcine SCNT Embryos

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Key Words

Pig, Histone deacetylase inhibitor • Somatic cell nuclear transfer • Reprogramming • *In vitro* development

Abstract

Background/Aims: Hypoacetylation caused by aberrant epigenetic nuclear reprogramming results in low efficiency of mammalian somatic cell nuclear transfer (SCNT). Many epigenetic remodeling drugs have been used in attempts to improve *in vitro* development of porcine SCNT embryos. In this study, we examined the effects of LAQ824, a structurally novel histone acetylase inhibitor, on the nuclear reprogramming and *in vitro* development of porcine SCNT embryos. **Methods:** LAQ824 treatment was supplemented during the culture of SCNT embryos. The reprogramming levels were measured by immunofluorescence and quantified by image J software. Relative expression levels of 18 genes were analyzed by quantitative real-time PCR. **Results:** 100 nM LAQ824 treatment of post-activation SCNT embryos for 24 h significantly improved the subsequent blastocyst formation rate. The LAQ824 treatment enhanced histone 3 lysine 9 (H3K9) levels, histone 4 lysine 12 (H4K12) levels, and reduced global DNA methylation levels as well as anti-5-methylcytosine (5-mC) at the pseudo-pronuclear and 2-cell stages. Furthermore, LAQ824 treatment positively regulated the mRNA expression of genes for histone acetylation (*HAT1*, *HDAC1*, 2, 3, and 6), DNA methylation (*DNMT1*, 3a and 3b), development (*Pou5f1*, *Nanog*, *Sox2*, and *GLUT1*) and apoptosis (*Bax*, *Bcl2*, *Caspase 3* and *Bak*) in blastocysts. **Conclusion:** Optimum exposure (100 nM for 24 h) to LAQ824 post-activation improved the *in vitro* development of porcine SCNT embryos by enhancing levels of H3K9 and H4K12, reducing 5-mC, and regulating gene expression.

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Introduction

Porcine somatic cell nuclear transfer (SCNT) is a powerful technique for basic and biomedical research [1]. Cloned pigs may provide suitable donor organs for regenerative

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medicine and xenotransplantation [2]. However, despite the fact that offspring of several mammalian species have been produced via SCNT, the efficiency of porcine SCNT remains very low, less than 5% [3]. This is generally attributed to abnormal reprogramming, such as histone hypoacetylation and DNA hypermethylation, rather than to any genetic mutations [4]. It is well known that the majority of the reprogramming of donor cell nuclei occurs in the recipient oocyte cytoplasm or in the early embryo before embryonic genome activation (EGA) [5, 6]. Histone acetylation, a type of epigenetic modification, which seems to be a key to successful reprogramming, is controlled by the activity of two groups of enzymes, including histone acetyltransferases (HATs) and histone deacetylase (HDACs) [7]. It occurs within a short period of time, causing unwinding of the chromatin structure to increase nuclear volume, facilitating the regulation of gene expression in SCNT embryos [8].

It has been demonstrated that a histone deacetylase inhibitor (HDACi) improves the levels of acetylation in nuclear donor cells or early SCNT embryos [9]. Recently, there were several reports that HDACi treatment positively regulates histone acetylation levels, epigenetic reprogramming, gene expression, full term embryo development and DNA methylation levels in a manner similar to these events taking place in *in vitro* fertilized embryos. The development of porcine SCNT embryos was improved by treatment with HDACi, such as trichostatin A (TSA) [10, 11], valproic acid (VPA) [12], scriptaid [13, 14], oxamflatin [15], sodium butyrate (NaBu) [16], LBH589 [17], CUDC-101 [18], m-carboxycinnamic acid bishydroxamide (CBHA) [3], PXD101 [19], suberoylanilide hydroxamic acid (SAHA) [20] and PCI-24781 [21].

LAQ824 (Dacinostat, (E)-N-hydroxy-3-[4-[[2-hydroxyethyl-[2-(1H-indol-3-yl) ethyl] amino]-methyl]phenyl]prop-2-enamide) is a structurally novel HDACi belonging to a class of hydroxamic acid analogs known to inhibit class I, IIa, and IIb histone deacetylases [22, 23]. However, the effect of LAQ824 on *in vitro* development of SCNT embryos has not been studied. Hence, in the present study porcine SCNT embryos were treated with LAQ824 to investigate whether it improved their developmental competence.

The objective of this study was to find the optimum conditions for LAQ824 treatment for improving *in vitro* development of porcine SCNT embryos, and to determine the global acetylation levels of histone 3 at lysine 9 (H3K9), histone 4 at lysine 12 (H4K12), and global DNA methylation levels of anti-5-methylcytosine (5-mC) using immunofluorescence, at different stages of porcine SCNT embryos. Then, we analyzed the effects of LAQ824 treatment on gene expression related to histone acetylation, DNA methylation, development and apoptosis in blastocyst.

Materials and Methods

The protocol for animal use was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-151019-4) in accordance with the Guide for the Care and Use of Laboratory Animals of Seoul National University. LAQ824 was purchased from Selleck Chemicals (Houston, TX, USA). Other chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Oocyte collection and in vitro maturation

Porcine ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory at 30–32 °C. The contents of follicles 3–6 mm in diameter were recovered by aspiration with an 18-gauge needle. Cumulus-oocyte complexes (COCs) were pooled and washed three times with tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA, USA), 2 mM of sodium bicarbonate, 10 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 5 mM sodium hydroxide, 1% Pen-Strep (Invitrogen), and 0.3% polyvinyl alcohol (PVA). Then, 50 COCs were placed into *in vitro* maturation (IVM) medium comprising TCM-199 supplemented with 2 mM sodium pyruvate, 5 µl/mL insulin transferrin selenium solution (ITS) 100X (Invitrogen), 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 10% porcine follicular fluid (vol/vol), 10 IU/mL human chorionic gonadotropin (hCG), and 10 IU/mL

equine chorionic gonadotropin (eCG). The selected COCs were incubated at 38.5 °C under 5% CO₂ in 95% humidified air for IVM. Following 22 h of maturation with hormones, the COCs were washed twice in fresh IVM medium and then cultured in hormone-free IVM medium for an additional 22 h.

Donor cell preparation for SCNT

Porcine fibroblasts were isolated from ear tissue of a Korean native adult pig. The tissue was cut into small pieces and cultured at 38 °C in an atmosphere of 5% CO₂ in air in Dulbecco's modified Eagle's medium (DMEM; Gibco, culture medium) containing 10% fetal bovine serum (FBS; Gibco, culture medium) (v/v), 1 mM sodium pyruvate, and 100 IU/mL each of penicillin and streptomycin. Cells from passages 3 to 7 were used as donors for SCNT. A single cell suspension was prepared by standard trypsinization procedures immediately before SCNT.

Somatic cell nuclear transfer

After IVM, COCs were denuded by gently pipetting with 0.1% hyaluronidase, and washed three times in Tyrode's albumin lactate pyruvate (TALP) medium using mouth pipette. Denuded oocytes were incubated in TALP containing 5 µg/mL Hoechst 33342 for 10 min and observed under an inverted microscope equipped with epifluorescence. An oocyte was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. Enucleation was performed by aspirating the first polar body and adjacent cytoplasm containing the metaphase II chromosomes with an aspiration pipette in TALP medium containing 5 µg/mL cytochalasin B. A single donor cell was inserted into the perivitelline space of an enucleated oocyte, and the couplets were equilibrated in fusion solution (0.28 M mannitol solution containing 0.5 mM HEPES and 0.1 mM MgSO₄), and then fused in a 20 µL droplet of fusion solution with a single DC pulse of 1.2 kV/cm for 30 µs using an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). After 1 h, fused couplets were equilibrated with activation solution (0.28 M mannitol solution containing 0.5 mM HEPES, 0.1 mM CaCl₂ and 0.1 mM MgSO₄), and then transferred to a chamber containing two electrodes overlaid with activation solution, and activated with a single DC pulse of 1.5 kV/cm for 30 µs using a BTX ElectroCell Manipulator 2001 (BTX Inc., San Diego, CA). SCNT embryos were washed three times with fresh porcine zygote medium-5 (PZM-5; Funakoshi Corporation, Tokyo, Japan), and transferred into 30 µL *in vitro* culture (IVC) droplets covered with mineral oil, and then cultured at 38.5 °C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. To count total cell numbers of blastocysts, they were collected on Day 7, washed in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA)-PVA (DPBS-PVA) and stained with 25 µg/mL of Hoechst 33342 for 10 min. After a final wash in DPBS-PVA, blastocysts were mounted on glass slides in a drop of 100% glycerol, compressed gently with a cover slip, and observed under a fluorescence microscope.

Post-activation treatment and embryo culture

Stock solutions of LAQ824 were dissolved in dimethyl sulfoxide (DMSO) at 20 mM and stored at -80°C. Following electrical activation, the SCNT embryos were treated with various concentrations (0-1000 nM) of LAQ824 for different duration (0-48 h) during IVC. After treatment, the embryos were washed three times with fresh PZM-5 medium and then transferred into LAQ824-free medium. Cleavage and blastocyst formation rates were evaluated on days 2 and 7, respectively, with the day of SCNT designated as day 0.

Immunofluorescence staining of SCNT embryos

SCNT embryos were washed three times in PBS containing 0.2% PVA, and fixed with 4% paraformaldehyde (w/v) in PBS for 30 min. All steps were performed at room temperature unless otherwise stated. Embryos were transferred into PBS containing 1% Triton X-100 (v/v) for 30 min. After blocking nonspecific sites with 2% bovine serum albumin (BSA) in PBS overnight at 4 °C, embryos were then incubated with primary antibodies (rabbit polyclonal antibody against histone H3K9 and H4K12 [Upstate Biotechnology, Lake Placid, NY, USA], diluted 1:200) at 37°C for 3 h. Then, goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was applied for 3 h at room temperature.

For staining of global methylation, fixed and permeabilized SCNT embryos were incubated with 50 µg/mL RNase A for 1 h at 37°C in the dark, then treated with 3 M HCl for 30 min at 37°C to denature DNA and neutralized with 100 mM Tris for 10 min. After washing three times, samples were blocked overnight

and incubated with primary antibodies, 5-mC mouse monoclonal antibody (mAb; Calbiochem, NA81, USA, diluted 1:200), and secondary antibodies Alexa Fluor 488-labeled goat anti-mouse IgG (1:200). After washing three times in PBS, DNA was counterstained with 25 µg/mL propidium iodide for 20 min.

Stained embryos were then mounted on glass slides and evaluated under an epifluorescence microscope (TE2000-S; Nikon) with the same exposure times and adjustments. The intensities of H3K9, H4K12 and 5-mC (green) were measured by analyzing the embryos pictures with Image J software (version 1.46r; National Institutes of Health, USA).

Analysis of gene expression by quantitative real-time PCR

Quantitative real-time PCR was performed according to a previous report [24]. Total RNAs were extracted to analyze gene expression, using TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and the total RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Total RNA transcribed into complementary DNA (cDNA) was produced using amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, Barker, TX, USA). A PCR plate (Micro-Amp Optical 96-Well Reaction Plate, Singapore) was made by adding 1 µL cDNA, 0.4 µL (10 pmol/µL) forward primer, 0.4 µL (10 pmol/µL) reverse primer, 10 µL SYBR Premix Ex Taq (TaKaRa, Otsu, Japan), and 8.2 µL of Nuclease-free water (NFW; Ambion, Austin, TX, USA) and then amplified on Applied Biosystems StepOne™ Real-Time PCR Systems (Applied Biosystems, Waltham, MA, USA). The amplification protocol included an initial denaturation step for 10 min at 95 °C followed by 40 cycles consisting of denaturation for 15 s at 95 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C. All oligonucleotide primer sequences are presented in Table 3. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*) using the equation, $R = 2^{-[\Delta C_t \text{ sample} - \Delta C_t \text{ control}]}$. For ease of comparison, the average expression level of each gene from control group was set as 1.

Statistical analysis

Each experiment was repeated at least three times. The data are expressed as the mean values ± standard error of the mean (SEM). The data were analyzed using univariate analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software. Differences in gene expression and fluorescence intensity were compared by Student's t-test. $P < 0.05$ was considered statistically significant.

Results

LAQ824 treatment improved in vitro developmental competence of porcine SCNT embryos

We treated porcine SCNT embryos with various concentrations (0, 10, 100 and 1000 nM) of LAQ824 and statistically analyzed subsequent developmental rates at the 2-cell and blastocyst stages (Table 1). The proportion of cleaved embryos on Day 2 was significantly lower in the group treated with 1000 nM compared with 10 nM of LAQ824 (77.6 ± 4.2 vs. 85.0 ± 0.8 , respectively, $P < 0.05$), but not compared with the control and 100 nM groups. On Day 7, the 100 nM LAQ824 treated group showed a significantly increased blastocyst formation rate compared to the control group (22.0 ± 2.8 vs. 11.9 ± 2.2 , respectively, $P < 0.05$).

Table 1. *In vitro* development of porcine SCNT embryos with different concentrations of LAQ824 for 24 h. a,b Values with different superscript letters within a column differ significantly ($P < 0.05$). Experiment was replicated at least three times

LAQ824 Concentration (nM)	No. of embryos		No. of embryos developed to (mean ± SEM, %)		Total cell number (mean ± SEM) in
	Cultured	≥ 2-cells	Blastocyst	Blastocysts	
0	99	84 (85.0 ± 0.8) ^{ab}	12 (11.9 ± 2.2) ^b		42.0 ± 2.0 ^b
10	99	87 (88.0 ± 1.0) ^a	15 (14.6 ± 3.9) ^{ab}		47.8 ± 4.0 ^{ab}
100	100	85 (84.6 ± 1.9) ^{ab}	22 (22.0 ± 2.8) ^a		52.9 ± 2.5 ^a
1000	99	77 (77.6 ± 4.2) ^b	10 (10.0 ± 0.3) ^b		41.7 ± 1.7 ^b

Table 2. *In vitro* development of porcine SCNT embryos with 100 nM of LAQ824 for different durations. a,b,c Values with different superscript letters within a column differ significantly ($P < 0.05$). Experiment was replicated at least three time

LAQ824 Duration (h)	No. of embryos Cultured	No. of embryos developed to ≥ 2 -cells	No. of embryos developed to Blastocyst	(mean $\pm$ SEM, %)	Total cell number (mean $\pm$ SEM) in Blastocysts
0	133	118	19	(88.1 $\pm$ 1.9) (14.2 $\pm$ 1.0) ^c	43.3 $\pm$ 2.7 ^b
6	133	114	24	(85.1 $\pm$ 1.8) (18.0 $\pm$ 0.5) ^b	47.0 $\pm$ 2.3 ^{ab}
24	133	112	41	(83.5 $\pm$ 2.4) (29.9 $\pm$ 2.9) ^a	53.8 $\pm$ 1.8 ^a
48	134	118	25	(87.4 $\pm$ 2.1) (20.8 $\pm$ 0.9) ^{bc}	44.4 $\pm$ 2.7 ^b

Then, porcine SCNT embryos were treated with 100 nM LAQ824 for 0, 6, 24 and 48 h (Table 2). Treatment with LAQ824 had no effect on the subsequent embryo cleavage rates, but treatment for 24 h significantly improved development to the blastocyst stage compared with the control group (29.9 $\pm$ 2.9 vs. 14.2 $\pm$ 1.0, respectively, $P < 0.05$). Moreover, treatment with 100 nM LAQ824 for 24 h significantly increased the total cell number/blastocyst vs. the control group ($P < 0.05$).

Detection of immunofluorescence at various SCNT embryo development stages

To find how LAQ824 treatment improved the *in vitro* developmental competence of porcine SCNT embryos, histone acetylation (H3K9 and H4K12) and DNA methylation (5-mC) levels of epigenetic markers were studied at the pseudo-pronuclear, 2-cell, 4-cell and blastocyst stages. The immunofluorescence signals for histone acetylation (H3K9 and H4K12) and DNA methylation (5-mC) were positively upregulated in the LAQ824 treatment group at the pseudo-pronuclear (Fig. 1) and 2-cell stages (Fig. 2), while the level of H4K12 was also enhanced at the 4-cell stage (Fig. 3). However, at the blastocyst stage there was no significant differences between the control and treatment groups (Fig. 4).

Table 3. Primer sequences used for real-time PCR. F, Forward primer; R, Reverse primer

Genes	Primer sequences (5'-3')	Product size (bp)	Accession No.
<i>GAPDH</i>	F: GTCGGTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
<i>HAT1</i>	F: ACGCCTCGCTCAAGAGTAGA R: AGTCATCATCTGATTTCACA	90	XM_013984281
<i>HDAC1</i>	F: CGCATGACTCACAATTGCT R: AGCCATCAATACCGGACAG	211	XM_013999116
<i>HDAC2</i>	F: ACAGGAGACTTGAGGAT R: CACATTAGCGTGACCTT	232	XM_001925318
<i>HDAC3</i>	F: GCTGCTGGACGGATGAGA R: CTGGATGGAGCGTGAAGT	108	NM_001243827
<i>HDAC6</i>	F: CCGCCGAAGTGTAAT R: AGACCTGCCAGTCATCCC	187	XM_003360315
<i>Sirt1</i>	F: GCTGCAACCCTGTAAAGCTT R: ACTGAAGAAGCTGGTGGTGA	151	NM_001145750
<i>DNMT1</i>	F: TCGAACCAAAACGGCAGTAC R: CGGTCAGTTTGTGTTGGACA	215	NM_001032355
<i>DNMT3a</i>	F: CTGAGAAGCCCAAGGTCAAG R: GTACTGATACGGCAGTCCA	200	NM_001097437
<i>DNMT3b</i>	F: AGTGTGTGAGGAGTCCATTG R: GCTTCGGCAATCACCAGT	133	XM_013985277
<i>Bax</i>	F: TGCCTCAGGATGCATCTACC R: AAGTAGAAAAGCGGACCCAC	199	XM_003127290
<i>Bcl2</i>	F: AGGGCATTTCAGTGACCTGAC R: CGATCCGACTCACCAATACC	193	NM_214285
<i>Caspase3</i>	F: CGTGCTTCTAAGCCATGGTG R: GTCCCACTGTCCGTCTCAAT	186	NM_214131
<i>Bak</i>	F: GCGGAAAACGCCTATGAGTA R: GCAGTGATGCAGCATGAAGT	189	XM_001928147
<i>Pou5f1</i>	F: TTTGGGAAGGTGTTACGCCAAACG R: TCGGTTCTCGATACTGTCCGCTT	198	NM_001113060
<i>Nanog</i>	F: GGTTTATGGGCTGAAGAAA R: GATCCATGGAGGAAGGAAGA	98	NM_001129971
<i>Sox2</i>	F: ATGCACAACCTCGGAGATCAG R: TATAATCCGGGTGCTCCTTC	130	NM_001123197
<i>CDX2</i>	F: TGTGCGAGTGGATGCGGAAG R: CCGAATGGTGATGTAGCGAC	149	NM_001278769
<i>GLUT1</i>	F: GCTTCCAGTATGTGGAGCAA R: AAGCAATCTCATCGAAGGTC	132	XM_013977359

Gene expression levels in LAQ824 treatment and control groups in SCNT-derived blastocysts

Relative expression levels of 18 genes were analyzed between the LAQ824 treatment and control groups in SCNT-derived blastocysts using quantitative real-time PCR (Fig. 5). In histone acetylation-related genes, LAQ824 treatment significantly increased *HAT1*, and decreased *HDAC1*, 2, 3 and 6. The levels of mRNA expression for DNA methylation-related genes (*DNMT 1*, 3a and 3b) were decreased in the LAQ824 treatment group. Expression levels of development-related genes (*Pou5f1*, *Nanog*, *Sox2* and *GLUT1*) in the LAQ824 treatment group were significantly higher than in the control group. Transcript levels for apoptosis-related genes (*Bax*, *Bcl2*, *Caspase3* and *Bak*) were positively regulated in the LAQ824 treatment group.

Fig. 1. Levels of H3K9, H4K12, and 5-mC in pseudo-pronuclear stage embryos. (A) Immunofluorescence staining at the pseudo-pronuclear stage of H3K9, H4K12, and 5-mC (green) in the control group and after 100 nM LAQ824 treatment for 24 h post-activation. Each sample was counterstained with propidium iodide (PI) to visualize DNA (red). Merged images of H3K9, H4K12, and 5-mC and DNA staining are shown. The merged images of H3K9 and DNA are yellow. Scale bar indicates 50 μ m. (B) Quantifications of H3K9, H4K12, and 5-mC are represented by signal intensities per group. Fluorescence intensity was measured using Image J software 1.46r. Bars and error bars show mean values and SEM, respectively. Different asterisk above columns of the same development stage indicate statistically significant differences ($P < 0.05$).

Discussion

To date, somatic cells have been reprogrammed into pluripotency using various approaches, the most common methods being induced pluripotent stem cells (iPSCs) [25-27] or nuclear transfer into the enucleated cytoplasm of metaphase II oocytes using SCNT [28-40]. Embryonic stem cells (ESCs) have greater therapeutic potential than iPSCs because they have fewer genetic and epigenetic defects [41]. Porcine SCNT is also a promising technology, with potential applications in regenerative medicine and xenotransplantation [2]. Therefore, we need to address the low efficiency of porcine SCNT, which is mainly attributed to abnormal epigenetic modifications.

HDAC inhibitors are being increasingly used in IVC treatment for improving the efficiency of SCNT and parthenogenetic activation [42]. In this study, we sought to improve the development of porcine SCNT embryos with LAQ824 (class I, IIa and IIb HDAC inhibitor) treatment. We found that LAQ824 treatment (100 nM for 24 h) positively regulated epigenetic reprogramming and gene expression in SCNT embryos. Moreover, it significantly increased blastocyst formation rate and blastocyst quality as measured by development-related genes and total cell numbers.

Histone acetylation, a key epigenetic factor, modifies and regulates chromatin configuration and also plays a crucial role in nuclear reprogramming. It is well accepted that HDACi suppress the expression of HDACs, resulting in reduced deacetylation and increased acetylation levels. In the present study, we tested the acetylation level of SCNT embryos. Compared to the control group, LAQ824 treatment enhanced the levels of H3K9 and H4K12

Fig. 2. Levels of H3K9, H4K12, and 5-mC in 2-cell stage embryos. (A) Immunofluorescence staining at the 2-cell stage of H3K9, H4K12, and 5-mC (green) in the control group and after 100 nM LAQ824 treatment for 24 h post-activation. Each sample was counter-stained with propidium iodide (PI) to visualize DNA (red). Merged images of H3K9, H4K12, and 5-mC and DNA staining are shown. The merged images of H3K9 and DNA are yellow. Scale bar indicates 50 μ m. (B) Quantifications of H3K9, H4K12, and 5-mC are represented by signal intensities per group. Fluorescence intensity was measured using Image J software 1.46r. Bars and error bars show mean values and SEM, respectively. Different asterisk above columns of the same development stage indicate statistically significant differences ($P < 0.05$).

at the pseudo-pronuclear and 2-cell stages, but there was no significant difference at the blastocyst stage. Moreover, LAQ824 treatment produced a higher level of H4K12 at the 4-cell stage. In addition, we also detected increased gene expression related to histone acetylation in blastocysts. The LAQ824 treatment not only upregulated gene expression of *HAT1*, but downregulated gene expression of *HDAC1*, 2, 3 and 6. Therefore, one of the ways in which LAQ824 treatment improved the *in vitro* developmental competence of porcine SCNT embryos may be that increased histone acetylation levels, caused by promotion of HATs and inhibition of HDAC activities, may facilitate chromatin opening. DNA methylation, as another epigenetic marker [43], also plays an important role in the process of reprogramming and influences *in vitro* developmental competence of SCNT embryos via the activity of DNA methyltransferases (DNMTs). In this study, we revealed that LAQ824 treatment downregulated the mRNA expression of *DNMT1*, 3a and 3b in blastocysts. Moreover, the protein level of 5-mC was significantly reduced at the pseudo-pronuclear and 2-cell stages with LAQ824 treatment, but not at the 4-cell and blastocyst stages. The EGA occurs at the 4-cell stage in porcine embryos. Accordingly, immunostaining results demonstrated that H3K9 and 5-mC have close relationships with EGA, but H4K12 may not. In addition, these findings illustrated that LAQ824 treatment improved epigenetic reprogramming in porcine SCNT embryos.

To evaluate the quality of blastocysts, we measured the mRNA expression levels of genes related to development (*Pou5f1*, *Nanog*, *Sox2*, *CDX2*, and *GLUT1*) and apoptosis (*Bax*, *Bcl2*, *caspase3*, and *Bak*) in SCNT blastocysts with LAQ824 treatment.

The first distinct lineage differentiation in the mammalian embryo occurs at the blastocyst stage; when blastomeres are segregated into inner cell mass (ICM) and trophectoderm (TE) [44]. *Pou5f1* plays an important role in determining early steps in embryogenesis and differentiation [45]. Transcription factors, *Pou5f1* and *Nanog*, are

Fig. 3. Levels of H3K9, H4K12, and 5-mC in 4-cell stage embryos. (A) Immunofluorescence staining at the 4-cell stage of H3K9, H4K12, and 5-mC (green) in the control group and after 100 nM LAQ824 treatment for 24 h post-activation. Each sample was counterstained with propidium iodide (PI) to visualize DNA (red). Merged images of H3K9, H4K12, and 5-mC and DNA staining are shown. The merged images of H3K9 and DNA are yellow. Scale bar indicates 50 μ m. (B) Quantifications of H3K9, H4K12, and 5-mC are represented by signal intensities per group. Fluorescence intensity was measured using Image J software 1.46r. Bars and

error bars show mean values and SEM, respectively. Different asterisk above columns of the same development stage indicate statistically significant differences ($P < 0.05$).

expressed in ICM cells and act to maintain pluripotency. However, it has been reported that *Pou5f1* is not only expressed in ICM, but also in TE of porcine blastocysts [46]. In the present study, the expression of *Pou5f1* was significantly increased in the LAQ824 treatment group. *Nanog*, which is considered to be another ICM marker [47], was more highly expressed in the LAQ824 treatment group. *Sox2* in cooperation with *Pou5f1* has also been shown to maintain the pluripotent ESCs phenotype [48]; its expression level was also enhanced with LAQ824 treatment. In contrast, *CDX2* is dispensable for TE differentiation and essential for trophectoderm stem cell self-renewal [49], however, there was no significant difference between the control and LAQ824 treatment groups. *GLUT1* is a major glucose transporter in the ICM, which contains the ESCs; it is also expressed in TE cells [50]. The mRNA expression of *GLUT1* was enhanced by LAQ824 treatment at the blastocyst stage. In addition, there is a relationship between blastocyst quality and apoptosis [51]. LAQ824 treatment significantly reduced expression of pro-apoptotic genes (*Bax*, *Caspase3* and *Bak*) and increased anti-apoptotic gene expression (*Bcl2*) in blastocysts. These results demonstrated that LAQ824 treatment not only increased the expression of development-related genes, but also improved ICM development. Therefore, LAQ824 treatment may be beneficial for supporting ESCs. Moreover the total cell number/blastocyst and blastocyst quality were improved in the LAQ824 treatment group by positively regulating apoptotic genes.

In conclusion, the present study indicated that 100 nM LAQ824 treatment for 24 h post-activation, improved the *in vitro* developmental competence of porcine SCNT embryos by positively regulating epigenetic reprogramming levels, and gene expression related to histone acetylation and DNA methylation. Furthermore, LAQ824 treatment enhanced total cell number/blastocyst and the quality of blastocysts by increasing expression of *Pou5f1*, *Nanog*, *Sox2*, *GLUT1* and *Bcl2*, and decreasing expression of *Bax*, *Caspase3* and *Bak*.

Fig. 4. Levels of H3K9, H4K12, and 5-mC in blastocyst stage embryos. (A) Immunofluorescence staining at the blastocyst stage of H3K9, H4K12, and 5-mC (green) in the control group and after 100 nM LAQ824 treatment for 24 h post-activation. Each sample was counterstained with propidium iodide (PI) to visualize DNA (red). Merged images of H3K9, H4K12, and 5-mC and DNA staining are shown. The merged images of H3K9 and DNA are yellow. Scale bar indicates 50 μ m. (B) Quantifications of H3K9, H4K12, and 5-mC are represented by signal intensities per group. Fluorescence intensity was measured using Image J software 1.46r. Bars and error bars show mean values and SEM, respectively.

Fig. 5. Messenger RNA (mRNA) expression levels (mean $\pm$ SEM) of (A) histone acetylation, (B) DNA methylation, (C) development and (D) apoptosis-related genes in blastocysts derived from fusion couplets treated with 100 nM LAQ824 for 24 h. Within the same mRNA group, bars with different superscript letters are significantly different ($P < 0.05$). Cas3, Caspase3.

Abbreviations

SCNT (somatic cell nuclear transfer); HAT (histone acetyltransferases); HDAC (histone deacetylase); EGA (embryonic genome activation); COCs (cumulus-oocyte complexes);

IVM (*in vitro* maturation); IVC (*in vitro* culture); ITS (insulin transferrin selenium); EGF (epidermal growth factor); hCG (human chorionic gonadotropin); eCG (equine chorionic gonadotropin); DMEM (Dulbecco's modified Eagle's medium); FBS (fetal bovine serum); PBS (phosphate-buffered saline); TALP (Tyrode's albumin lactate pyruvate); PZM-5 (porcine zygote medium-5); DMSO (dimethyl sulfoxide); cDNA (Complementary DNA).

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Disclosure Statement

The authors have no conflict of interest to disclose.

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