Lysophosphatidic acid improves development of porcine somatic cell nuclear transfer embryos

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Abstract

This study was conducted to investigate whether lysophosphatidic acid (LPA) could improve the development of porcine somatic cell nuclear transfer (SCNT) embryos. Porcine SCNT-derived embryos were cultured in chemically defined polyvinyl alcohol (PVA)-based porcine zygote medium (PZM)-4 without or with LPA, and the development, cell proliferation potential, apoptosis, and expression levels of pluripotent markers were evaluated. LPA significantly increased the rates of cleavage and blastocyst formation compared to those seen in the LPA un-treatment (control) group. The expression levels of embryonic development-related genes (IGF2R, PCNA and CDH1) were higher ($P < 0.05$) in the LPA treatment group than in the control group. LPA significantly increased the numbers of total, inner cell mass and EdU (5-ethynyl-2'-deoxyuridine)-positive cells in porcine SCNT blastocysts compared to those seen in the control group. TUNEL assay showed that LPA significantly reduced the apoptosis rate in porcine SCNT-derived embryos; this was confirmed by decreases ($P < 0.05$) in the expression levels of pro-apoptotic genes, BAX and CASP3, and an increase ($P < 0.05$) in the expression level of the anti-apoptotic gene, BCL2L1. In addition, LPA significantly increased Oct4 expression at the gene and protein levels. Together, our data suggest that LPA improves the quality and development of porcine SCNT-derived embryos by reducing apoptosis and enhancing cell proliferation and pluripotency.

Keywords: Lysophosphatidic acid, Somatic cell nuclear transfer, Apoptosis, Cell proliferation, Oct4

Introduction

Somatic cell nuclear transfer (SCNT) is a promising technology with potential applications in basic life science research, agriculture and regenerative medicine. The production of live offspring by SCNT has been achieved in many mammalian species [1], including sheep [2], cow [3], mouse [4], pig [5], horse [6], dog [7], monkey [8] etc. The development of SCNT technology in pigs is producing transgenic pigs with specific genetic modifications of somatic cells [9]. As pigs and humans are similar in their anatomy, physiology and body size, the transgenic pigs are readily considered the primary alternative source for xenotransplantation and human disease model [10, 11]. Great progress has been made in cloning techniques, and the first successful cloned pig was achieved by SCNT technology in the year 2000 [5]. However, the efficiency of pig cloning by SCNT is still very low, with a success rate of less than 5% [12-14]. Numerous factors are responsible for poor cloning efficiency, including recipient oocyte quality, cell cycle stage of the donor cells, reconstructed embryo activation methods and culture condition. Of these, suboptimal culture conditions have been suggested as one of the most important factors responsible for the low success rate of SCNT techniques.

Lysophosphatidic acid (LPA) is a small glycerophospholipid (molecular mass: 430-480 Da), has been identified as a key signaling molecule participating in the regulation of reproductive functions in mammals [15, 16]. LPA shows growth factor-like and hormone-like functions on various animal cells [17], and it can give rise to various cellular responses, including cell proliferation, survival, and differentiation etc. [18-20]. The beneficial effect of LPA on oocyte maturation and/or development of preimplantation embryos has been evaluated in many species such as golden hamster [21], mice [22], pig [23, 24], bovine [25-27] and human[28]. For instance, LPA supplementation of the relevant *in vitro* maturation medium was shown to improve the nuclear and cytoplasmic maturation of golden hamster oocytes [21] and enhance bovine oocyte maturation and embryo development while decreasing apoptosis in these species [25, 29]. In pig, previous studies showed that LPA improved the development and quality of porcine *in vitro* fertilization (IVF) [24]- and parthenogenetic activation (PA) [23]-derived embryos. Collectively, the above reports suggest that LPA could be an interesting addition to an *in vitro* embryo culture system. However, whether LPA can enhance the development of porcine SCNT embryos, and its possible mechanisms are still not completely comprehended.

To investigate the possible mechanisms of LPA influence the development and quality of porcine SCNT-derived embryos, we cultured porcine SCNT embryos in the chemically defined

PVA-based medium, porcine zygote medium-4 (PZM-4), with or without LPA, and examined the cleavage, blastocyst formation, cell proliferation potential, ROS formation, mitochondrial membrane potential and apoptosis index, as well as the expression levels of pluripotent markers and genes involved in development and apoptosis.

Materials and Methods

Chemicals and animal ethics

All chemicals or reagents used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chungnam National University.

Oocyte collection

Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory within 2-3 h in PBS solution supplemented with 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate. Follicular contents were aspirated from antral follicles (3 to 6 mm in diameter) visible on the ovarian surface using an 18-gauge needle attached to a 10-ml disposable syringe. Porcine cumulus-oocyte complexes (COCs) with homogenous cytoplasm and more than two uniform layers of compact cumulus cells were selected for *in vitro* maturation (IVM).

Oocyte IVM

For porcine oocyte IVM, groups of 50-60 COCs were placed into each well of a four-well multi dish (Nunc, Roskilde, Denmark) containing 500 μl maturation medium (TCM 199) supplemented with 10% porcine follicular fluid, 3.5 mM D-glucose, 0.57 mM L-cysteine, 0.91 mM sodium pyruvate, 75 μg/ml penicillin, 50 μg/ml streptomycin, 10 ng/ml epidermal growth factor, 10 IU/ml pregnant mare serum gonadotropin and 10 IU/ml human chorionic gonadotropin. The COCs were cultured at 38.5℃ in saturated-humidity air containing 5% CO_2. After 22 h of maturation incubation, the porcine COCs were transferred to the same maturation medium lacking hormones and incubated for an additional 22 h.

Preparation of porcine fetal fibroblasts

Porcine fetal fibroblasts were isolated from fetuses at day 35 of gestation [30]. Cells were cryopreserved upon reaching confluence. Before SCNT experiments, the cells were thawed and cultured in DMEM (Gibco, 11995-073) supplemented with 10% fetal bovine serum (Gibco, 16000-044), 1% MEM-NEAA (Gibco, 11140-050) and 0.1% gentamicin reagent solution (Gibco, 15750-060), at 38.5℃ in a humid 5% CO_2 atmosphere.

Cloned embryo reconstruction by SCNT

For SCNT, denuded oocytes were subjected to enucleation, nuclear transfer, fusion and cytoplast activation procedures. Briefly, porcine oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm using an enucleation needle (ORIGIO Inc., Charlottesville, VA, USA) in enucleation medium. A single donor cell was transferred into the perivitelline space of each enucleated oocyte using the same enucleation pipette. The reconstructed embryos were fused and activated with two 100-μs DC pulses of 1.1 kV/cm, using an Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA) in 0.3 M D-mannitol supplemented with 1.0 mM $CaCl_2 \cdot H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM HEPES. The activated embryos were washed with PZM-4 (PVA based) and 8-10 embryos were transferred into 100-μl micro drops of PZM-4, covered with mineral oil, and incubated at 38.5℃ in a 5% CO_2 atmosphere. The day of nuclear transfer was designated as day 1.

Dual differential staining

The numbers of inner cell mass (ICM) and trophoblast (TE) cells in blastocysts were determined using dual differential staining. Briefly, the zona pellucida (zp) was removed from blastocysts by incubation in 0.5% pronase solution. The zp-free embryos were washed in PBS containing 0.1% (w/v) PVA (PBS-PVA) and transferred into a 1:5 dilution of rabbit anti-pig whole serum (P3164; Sigma) for 1 h at 38.5℃. After being briefly rinsed with PBS-PVA, the samples were incubated with guinea pig complement (S1639; Sigma) diluted 1:10 with PBS-PVA containing 10 μg/ml propidium iodide (PI) and 10 μg/ml Hoechst 33342, for 1 h in the dark at 38.5℃. After being briefly rinsed in PBS-PVA, the samples were mounted on slides with coverslips and observed under epifluorescence microscopy (BX51, Olympus Tokyo, Japan). Blue (Hoechst 33342) and pink (both Hoechst 33342 and PI) colors were taken as representing ICM and TE cells, respectively.

ROS (reactive oxygen species) measurement

Porcine SCNT-derived embryos were incubated with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, Eugene, OR, USA) for 30 min in the dark at 38.5℃. After being briefly rinsed in PBS-PVA, the samples were examined under an epifluorescence microscope (Olympus BX51). The same instrument parameters were used to normalize the results across the replicates. The fluorescence intensity in each embryo was measured using the ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD, USA) after background subtraction.

JC-1 assay

JC-1 dye (T3168; Thermo Fisher Scientific, Eugene, OR, USA) was used to assess the mitochondrial membrane potential. Samples were exposed to 10 μg/ml JC-1 at 38.5℃ in the dark for 15 min, washed in PBS-PVA and examined under an epifluorescence microscope (Olympus BX51). Fluorescence images were captured and saved as graphic files in TIEF format. The ratio of red florescence (J-aggregates, corresponding to activated mitochondria) to green florescence (J-monomers, corresponding to less-activated mitochondria) was used to calculate mitochondrial membrane potential using the ImageJ software after background subtraction.

Immunofluorescence staining

The porcine SCNT-derived blastocysts were fixed in 4% paraformaldehyde for 30 min, and then permeabilized in 0.5% (v/v) Triton X 100 for 30 min. After permeabilization, the samples were washed with PBS-PVA, blocked with 3% BSA in PBS for 1 h, washed in PBS containing 0.5% BSA and 0.1% gelatin (PBG) for 20 min and incubated overnight at 4℃ with anti-Oct-3/4 (1:200, sc-5279; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The samples were then washed in PBG for 20 min and then reacted with goat anti-mouse IgG-R (1:200, sc-2092; Santa Cruz Biotechnology) in the dark for 1 h. Negative control samples were processed as described above, except that no anti-Oct-3/4 antibody was added. Finally, all samples were mounted on slides using VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and examined with a Zeiss laser-scanning confocal microscope (LSM5 Live, Carl Zeiss, Germany).

EdU labeling

For EdU staining, a Click-iT EdU Imaging kit (C10337; Invitrogen) was used according to the provided instructions. Briefly, porcine SCNT-derived blastocysts were incubated in a final

concentration of 10 μM EdU solution for 5-6 h at 38.5℃. Blastocysts were fixed for 15 min in 4% paraformaldehyde solution. After being briefly rinsed in PBS containing 3% BSA, the samples were permeabilized with 0.5% Triton X-100 for 20 min, washed in PBS containing 3% BSA, and then reacted with a Click-iT reaction cocktail containing Alexa Fluor 488 azide. After EdU labeling, samples were mounted on slides using VECTASHIELD mounting medium containing DAPI and examined under a Zeiss laser-scanning confocal microscope.

TUNEL assay

Apoptosis in blastocysts were examined using a TUNEL assay kit (TMR Red; Roche, Germany). Porcine SCNT-derived blastocysts were washed in PBS-PVA and fixed in 4% paraformaldehyde for 30 min. The samples were permeabilized with 0.5% Triton X-100 for 30 min, and then reacted with TUNEL reaction solution for 1 h at 38.5℃ in the dark. The samples were then washed in PBS-PVA and mounted on slides using VECTASHIELD mounting medium containing DAPI. A negative control test was performed as described above, except that no terminal deoxynucleotidyl transferase was added.

Real-time PCR

Porcine blastocysts were collected and stored at -70℃ until analysis. The expression levels of embryo development-related genes (*IGF2R*, *SLC2A1*, *DNMT3A*, *PCNA*, *CDH1* and *DSC2*), apoptosis-related genes (*BAX*, *CASP3* and *BCL2L1*) and pluripotent marker genes (*Oct4*, *Sox2* and *Nanog*) were analyzed using quantitative real-time PCR. Total RNA was extracted from each sample (10 blastocysts) using an RNeasy Mini Kit (Cat. No. 74104; Qiagen, Valencia, CA, USA), and cDNA was prepared with a TOPscript™ RT DryMIX kit (Enzynomics, Daejeon, Republic of Korea) in accordance with the manufacturer's instructions. Real-time PCR was conducted with a TOPreal™ qPCR 2X PreMIX (*SYBR Green with low ROX*) kit (Enzynomics) on a CFX96 Touch Real Time PCR Detection System (Bio-Rad, Laboratories Inc., Singapore). PCRs with no template controls were performed for each primer pair. Relative mRNA expression levels were analyzed using the $2^{-\triangle\triangle Ct}$ method. *ACTB* was used as an internal standard. The sequences of the primers used to amplify each gene are presented in Table 1.

Experimental design

In Experiment 1, we examined the effects of LPA on the development (cleavage and blastocyst formation rates) and embryonic development-related gene expression (*IGF2R*, *SLC2A1*, *DNMT3A*, *PCNA*, *CDH1* and *DSC2*) of porcine SCNT-derived embryos.

In Experiment 2, we examined the effects of LPA on the cell proliferation potential (the numbers of total, ICM, TE and EdU-positive cells) of porcine SCNT-derived blastocysts.

In Experiment 3, we tested the effects of LPA on ROS formation, mitochondrial membrane potential, apoptosis index and apoptosis-related gene expression (*BAX*, *CASP3* and *BCL2L1*) in porcine SCNT-derived blastocysts.

In Experiment 4, we tested the effects of LPA on the expression levels of a pluripotency marker protein (Oct4) and pluripotency marker genes (*Oct4*, *Sox2* and *Nanog*) in porcine SCNT-derived embryos.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were compared using one-way analysis of variance (ANOVA) and *t* tests. Percentage data were subjected to arcsine transformation prior to analysis. Each experiment consisted of at least three replicates. The data are presented as the mean ± SEM. $P < 0.05$ was considered significantly different.

Results

1. Effect of LPA on the development of porcine SCNT-derived embryos

To determine the appropriate concentration, we pre-tested the influence of different concentrations of LPA on porcine SCNT-derived blastocyst formation rate. We found that 10 μM LPA is the optimal concentration for porcine SCNT-derived embryo development (Fig 1A). Thus, 10 μM LPA was employed in the subsequent study.

As shown in Fig 1, LPA significantly increased the cleavage rate at day 2 of culture and increased the blastocyst formation rates at days 6 and 7 of culture (Fig 1B, C). LPA also increased the cleavage rate at day 3 when compared to that of the control (Fig 1C), but this difference was not significant ($P = 0.068$). In addition, LPA significantly increased the gene expression levels of *IGF2R*, *PCNA* and *CDH1* when compared to control levels (Fig 1D). In contrast, LPA did not alter the gene expression levels of *SLC2A1*, *DNMT3A* or *DSC2* (Fig 1D).

2. Effect of LPA on the cell proliferation potential of porcine SCNT-derived embryos

LPA significantly increased the numbers of total cells (TC) and inner cell mass (ICM) cells when compared to the corresponding numbers of the control group (Fig 2A, B). LPA did not influence the trophectoderm (TE) cell number or the ICM-to-TE cell ratio (Fig 2B). However, the EdU-positive cell number and the EdU-positive-to-TC ratio were significant higher in the LPA treatment group versus the control group (Fig 2C, D).

3. Effect of LPA on ROS generation, mitochondrial membrane potential and apoptosis in porcine SCNT-derived embryos

LPA significantly reduced the level of ROS generation (Fig 3A), increased the mitochondrial membrane potential (Fig 3B, C), and decreased the apoptosis rate (numbers of TUNEL-positive cells/numbers of TC; $P < 0.05$) compared to the corresponding values for the control group (Fig 3D, E). The expression levels of the pro-apoptotic genes, *BAX* and *CASP3*, were significant lower ($P < 0.05$) (Fig 3F) whereas the expression level of the anti-apoptotic gene, *BCL2L1*, was upregulated ($P < 0.05$) (Fig 3F) in the LPA treatment group compared to the control group.

4. Effect of LPA on the expression levels of pluripotency markers in porcine SCNT-derived embryos

Fluorescence staining showed that the Oct4 expression level was significantly higher in the LPA treatment group than in the control group (Fig 4A, B). In addition, LPA significantly increased the gene expression level of *Oct4* when compared to the control (Fig 4C). However, LPA did not influence the gene expression levels of *Sox2* and *Nanog* in porcine SCNT-derived blastocysts (Fig 4C).

Discussion

Previous studies showed that LPA could improve porcine PA- and IVF-derived embryos [23, 24, 31]. Our present results newly demonstrate that LPA supplementation of a chemically defined PVA-based medium (PZM-4) could improve the development and quality of porcine SCNT-derived embryos.

The number of cells in the blastocyst is an important parameter for embryo quality, as blastocysts with numerous cells possess greater cell proliferation and better implantation potential after embryo transfer [32]. The ability of LPA to increase cell proliferation was previously reported in a porcine preadipocyte cell line [19], and the addition of LPA to a BSA-based undefined medium was shown to increase the cell numbers in porcine PA and/or IVF blastocysts [24, 31]. In the present study, we found that LPA increased the total, ICM and EdU-positive cell numbers in porcine SCNT-derived blastocysts. Thus, we speculate that the ability of LPA to positively influence porcine cloned embryo development and quality might at least partly reflect its ability to improve the cell proliferation potential.

The timing of cleavage and blastocyst formation can be as indicator of developmental potential and quality of embryos [33, 34]. A previous study showed that early-cleaved embryos have greater potential to develop to blastocysts, compared to late-cleaved embryos [35]. In addition, early-formed blastocysts (formed on day 6) were found to be of generally higher quality than late-formed blastocysts (day 7 or 8) in pig [32]. LPA has been shown to facilitate the compaction of embryos in mice [36] and accelerate blastocoel formation in pig [23]. Here, we found that the proportion of early-cleaved embryos (at day 2 of culture) and early-formed blastocysts (at day 6) were significantly higher in LPA treatment group than the control group, indicating that LPA accelerated cleavage and blastocyst formation in porcine SCNT embryos.

It is well known that the utilized *in vitro* culture conditions can dramatically alter the gene expression patterns of embryos [32]. In the current study, we evaluated several embryonic development-related genes (*IGF2R*, *SLC2A1*, *DNMT3A*, *PCNA*, *CDH1* and *DSC2*) after LPA treatment. We observed that LPA did not significantly influence the gene expression levels of *SLC2A1*, *DNMT3A* and *DSC2*, indicating that these genes may not participate in the activities of LPA on porcine embryos. However, the expression levels of *IGF2R*, *PCNA* and *CDH1* genes in porcine SCNT embryos were significantly changed after LPA treatment. Among them, IGF2R (insulin-like growth factor 2) has been shown to play very important roles in fetal growth and placental function in mammals [37]. CDH1 (cadherin 1), which is a classical member of the cadherin superfamily, is responsible for intercellular connectivity [38]. Our observation that LPA significantly elevated the expression levels of *IGF2R* and *CDH1* is consistent with previous reports that LPA significantly increased the gene expression levels of *IGF2R* in bovine embryos [25, 29] and that of *CDH1* in porcine embryos [23]. PCNA (proliferating cell nuclear antigen) is involved in the DNA replication and repair machinery that can be used to evaluate embryonic development [39]. Here, we found that LPA significantly increased the gene expression of *PCNA*,

304 which would logically contribute to the ability of LPA supplementation to improve the quality of
305 porcine cloned embryos.
306 Apoptosis occurs during the preimplantation embryo development of *in vitro*- and *in vivo*-
307 produced embryos and may contribute to embryonic loss [40]. ROS and mitochondrial
308 membrane potential can play essential roles in apoptosis. ROS can directly induce oxidative
309 injury or trigger a mitochondrial permeability transition in cells [41]. Here, we found that the
310 ROS level and mitochondrial membrane potential were significantly lower and higher,
311 respectively, in the LPA treatment group compared to the control group. This suggests that LPA
312 could effectively reduce oxidative stress and maintain the mitochondrial membrane potential in
313 porcine SCNT-derived embryos. Furthermore, and consistent with previous reports that LPA can
314 reduce apoptosis in the embryos of many species [15, 23-26], we found that LPA significantly
315 reduced the rate of apoptosis in porcine SCNT-derived blastocysts compared to the control.
316 Similarly, previous reports found that the apoptosis index was decreased in LPA-treated porcine
317 PA- and IVF-derived embryos [23, 24]. In terms of apoptosis-related gene expression, LPA
318 supplementation during *in vitro* culture was previously reported to significantly increased the
319 gene expression of *BCL2L1* and/or reduce those of *BAX* and *CASP3* in bovine IVF embryos [29]
320 or porcine PA [23] and IVF [24] embryos. In the present study, LPA supplementation was found
321 to significantly increase the gene expression of *BCL2L1* gene and reduce those of *BAX* and
322 *CASP3* in porcine SCNT-derived embryos. These data suggest that LPA blocks early apoptosis
323 during porcine SCNT embryo development by modulating the expression of *BCL2* family genes.
324 The blastocyst includes TE and ICM cells. TE cells will form extraembryonic tissues,
325 whereas ICM cells give rise to the fetus. In the ICM cells of the blastocyst, the transcription
326 factor Oct4 collaborates with Sox2 and Nanog form a circuit that maintains pluripotency [42-44].
327 These factors are usually involved in transcription regulation during early preimplantation
328 embryo development and cell differentiation [45]. Thus, the expression levels of these factors
329 can often be used as indicators of embryo quality and development. In the pig, endogenous Oct4
330 expression is maintained in all nuclei of the blastocyst, Nanog is not detected in any nucleus of
331 the blastocyst [46], and Sox2 is specifically expressed in ICM cells of porcine blastocysts [43].
332 Our immunostaining results confirmed that Oct4 is expressed the TE and ICM cells of porcine
333 blastocysts. Notably, high-level expression of Oct4 in porcine cloned embryos was previously
334 suggested to be important for improving embryo quality and cloning efficiency [46]. In the
335 present study, LPA significantly increased the expression level of Oct4 compared to that seen in

the control group. Thus, the ability of LPA to improve the quality of porcine cloned embryos might be partially due to its upregulation of Oct4.

In conclusion, our results collectively suggest that LPA can improve the development of porcine SCNT-derived embryos in terms of reducing apoptosis and enhancing blastocyst formation, cell proliferation and pluripotency.

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Table

Table 1. Primers used in this study

Genes	Primer sequence (5'->3')	Product size (bp)	Accession number
Oct4	F: GTGTTCAGCCAAACGACCAT	200	NM_001113060
	R: TTGCCTCTCACTCGGTTCTC		
Sox2	F: ACCAGAAGAACAGCCCAG	159	NM_001123197
	R: CCGTCTCCGACAAAAGTT		
Nanog	F: AACTGTGTTCTCGCAGACC	154	NM_001129971
	R: ACATTTCATTCGCTGGTTC		
BAX	F: ACACCTCATAGCCATGAAAC	232	YA_55048
	R: ATGGCTGACATCAAGATACC		
CASP3	F: CCTCACCATCATCACACTGG	279	AF_098067
	R: AGCTCTCGGAACATCTCGAA		
BCL2L1	F: AGAGCTTTGAGCAGGTATTG	253	NM_214285
	R: GCATTGTTTCCGTAGAGTTC		
IGF2R	F: AGGTCTCACCTCTTCAGGTT	120	AF_342812
	R: CTGTGCAAATTAAGGCTTCT		
PCNA	F: CCTGTGCAAAAGATGGAGTG	187	XM_003359883
	R: GGAGAGAGTGGAGTGGCTTTT		
CDH1	F: CTGTATGTGGCAGTGACTAAC	174	EU_805482
	R: AGTGTAGGATGTGATCTCCAG		
SLC2A1	F: GGTGCTCCTGGTCCTGTTCT	125	XM_013977359.1
	R: CGGGTGTCTTGTCGCTTT		
DNMT3A	F: GGACAAGAATGCCACCAAATC	185	XM_005662686.2
	R: CGAACCACATGACCCAACG		
DSC2	F: GTGAAAGGAGGGCACCAGA	174	XM_005674194.1
	R: ACGGGGCTGCGTGTAACTGT		
ACTB	F: GTGGACATCAGGAAGGACCTCTA	137	U_07786
	R: ATGATCTTGATCTTCATGGTGCT		

F, forward; R, reverse.

Figures and figure legends

Fig 1.

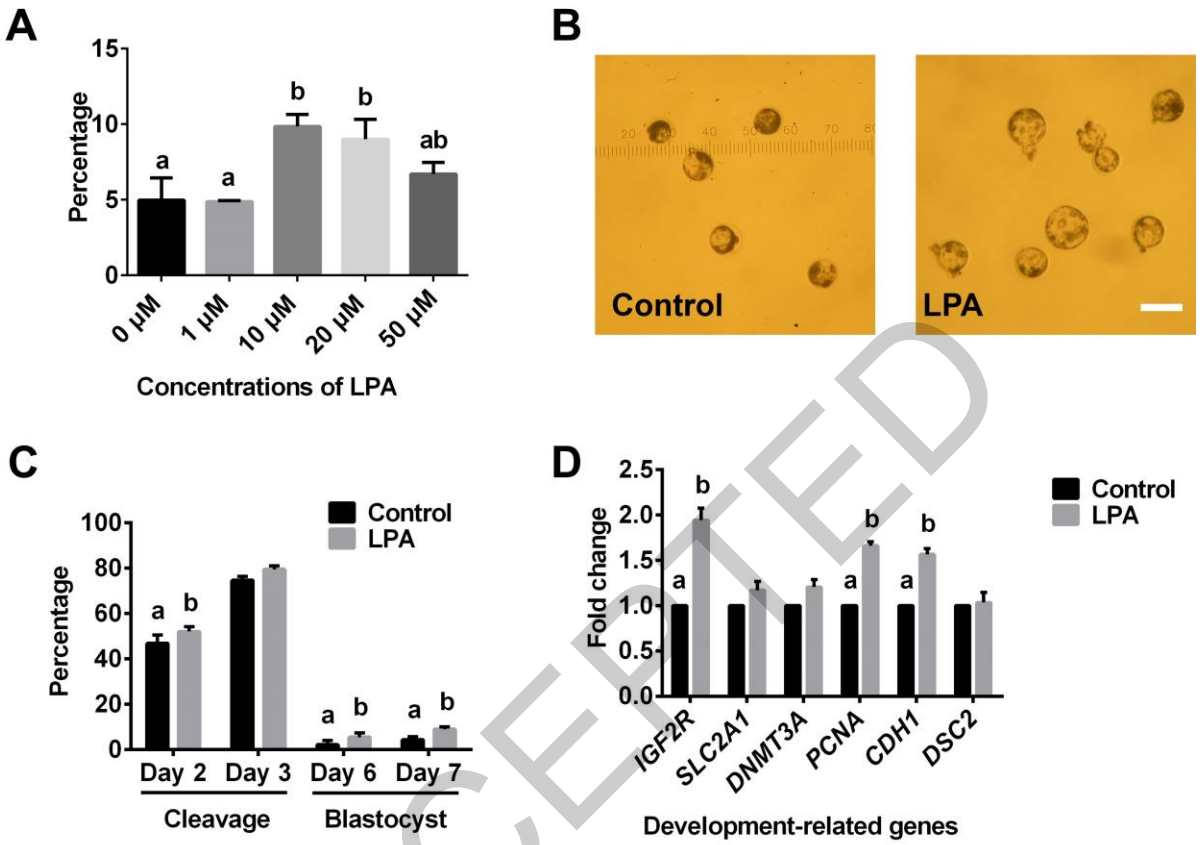


Figure 1. Effect of LPA on the development of porcine SCNT-derived embryos. A: The influence of different concentrations (0 μM, n=121; 1 μM, n=123; 10 μM, n=121; 20 μM, n=121; 50 μM, n=119) of LPA on porcine SCNT-derived blastocyst formation. 10 μM LPA was used in the next study, because 10 μM LPA is the optimal concentration for porcine SCNT-derived embryo development. B: Images of porcine SCNT blastocysts after LPA treatment. C: The development of porcine SCNT-derived embryos cultured with LPA (10 μM). Experiment was conducted at least thrice, and a total of 137 (Control) and 146 (LPA group) embryos were used for the assay, respectively. D: The expression levels of embryonic development-related genes in SCNT-derived embryos cultured with LPA. Different letters indicate a significant difference ($P < 0.05$). Scale bar = 200 μm in A.

Fig 2.

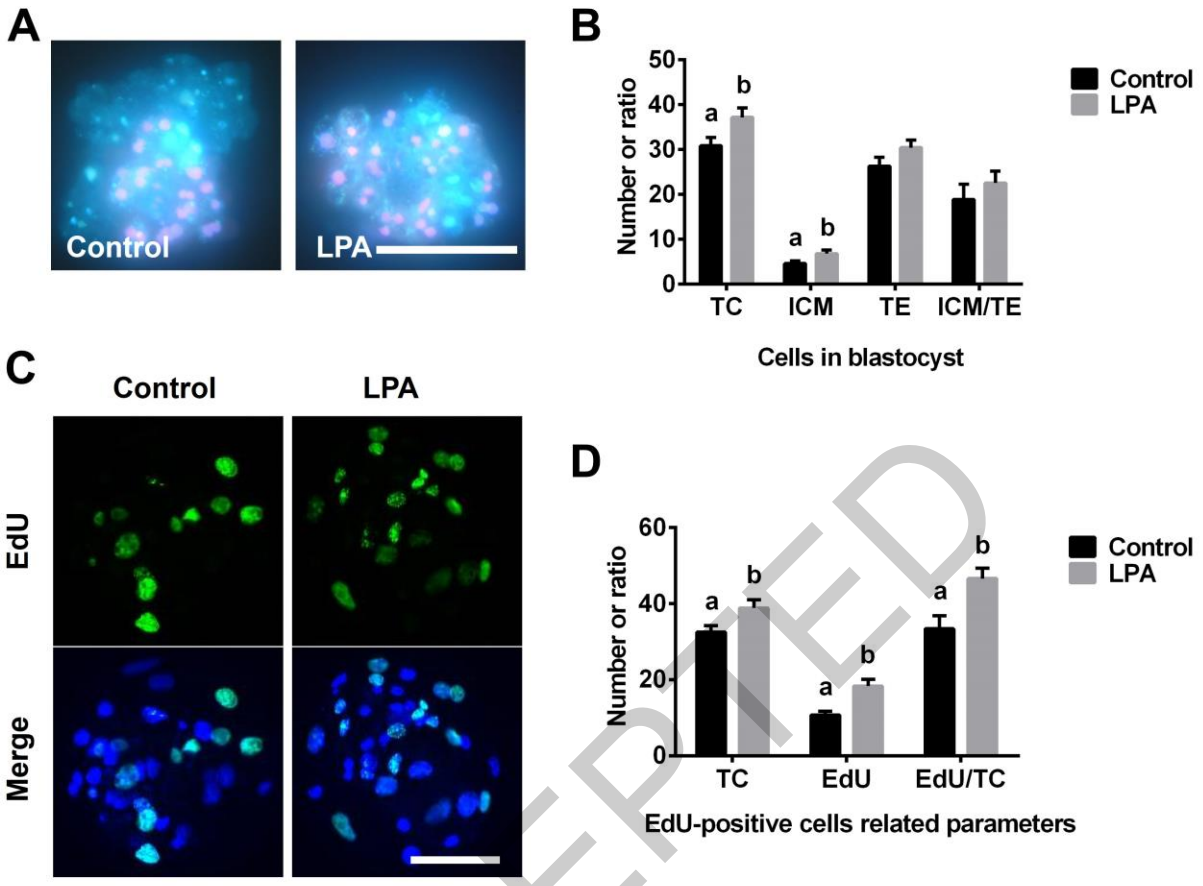
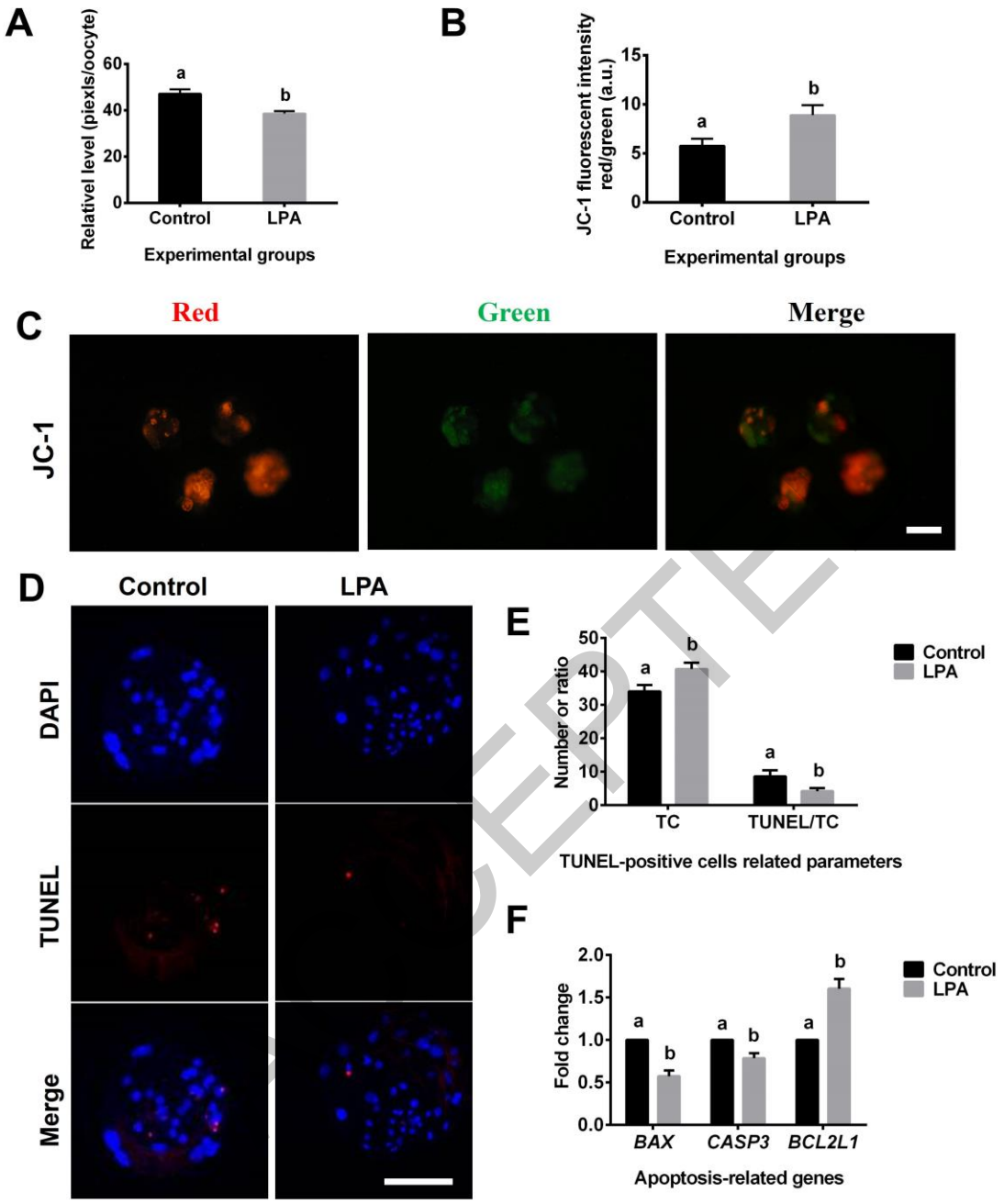


Figure 2. Effect of LPA on the cell proliferation potential in porcine SCNT-derived blastocysts. A: Differential staining of porcine SCNT blastocysts. Blue (Hoechst 33342) and pink (Hoechst 33342 plus PI) show inner cell mass (ICM) and trophoblast (TE) cells, respectively. B: Numbers of total cells (TC), ICM cells, TE cells and the ratio of ICM cells to TE cells in porcine SCNT-derived blastocysts. Experiment was conducted at least thrice, and a total of 19 (Control) and 21 (LPA group) embryos were used for the assay. C: Porcine SCNT-derived blastocysts were stained with EdU to detect DNA replication (green), and nuclei were stained with DAPI (blue). D: The numbers of EdU-positive cells and the ratio of EdU-positive cells to TC. Experiment was conducted at least thrice, and a total of 23 (Control) and 24 (LPA group) embryos were used for the assay. Different letters indicate a significant difference ($P < 0.05$). Scale bar = 200 μm in A and 100 μm in C.

513 **Figure 3.**



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516 **Figure 3. Effect of LPA on ROS level, mitochondrial membrane potential and apoptosis in**
517 **porcine SCNT-derived blastocysts.** A: ROS levels in porcine SCNT-derived embryos exposed
518 to LPA treatment. Experiment was conducted at least thrice, and a total of 41 (Control) and 47
519 (LPA group) embryos were used for the assay. B and C: Fluorescence pixel ratio (red/green) and
520 representative images of JC-1 staining (red, J-aggregates; green, J-monomers) in porcine SCNT
521 embryos. Experiment was conducted at least thrice, and a total of 18 (Control) and 26 (LPA

group) embryos were used for the assay. D: Images of apoptotic cells in porcine SCNT blastocysts evaluated by TUNEL assays. Red indicates apoptotic cells and blue indicates nuclei. E: Apoptosis rates (TUNEL-positive cell number/total cell number) in porcine SCNT-derived blastocysts. Experiment was conducted at least thrice, and a total of 21 (Control) and 24 (LPA group) embryos were used for the assay. F: The expression levels of apoptosis-related genes (*BAX*, *CASP3* and *BCL2L1*) in SCNT blastocysts. Different letters indicate a significant difference ($P < 0.05$). Scale bars = 100 μm in C and D.

Figure 4.

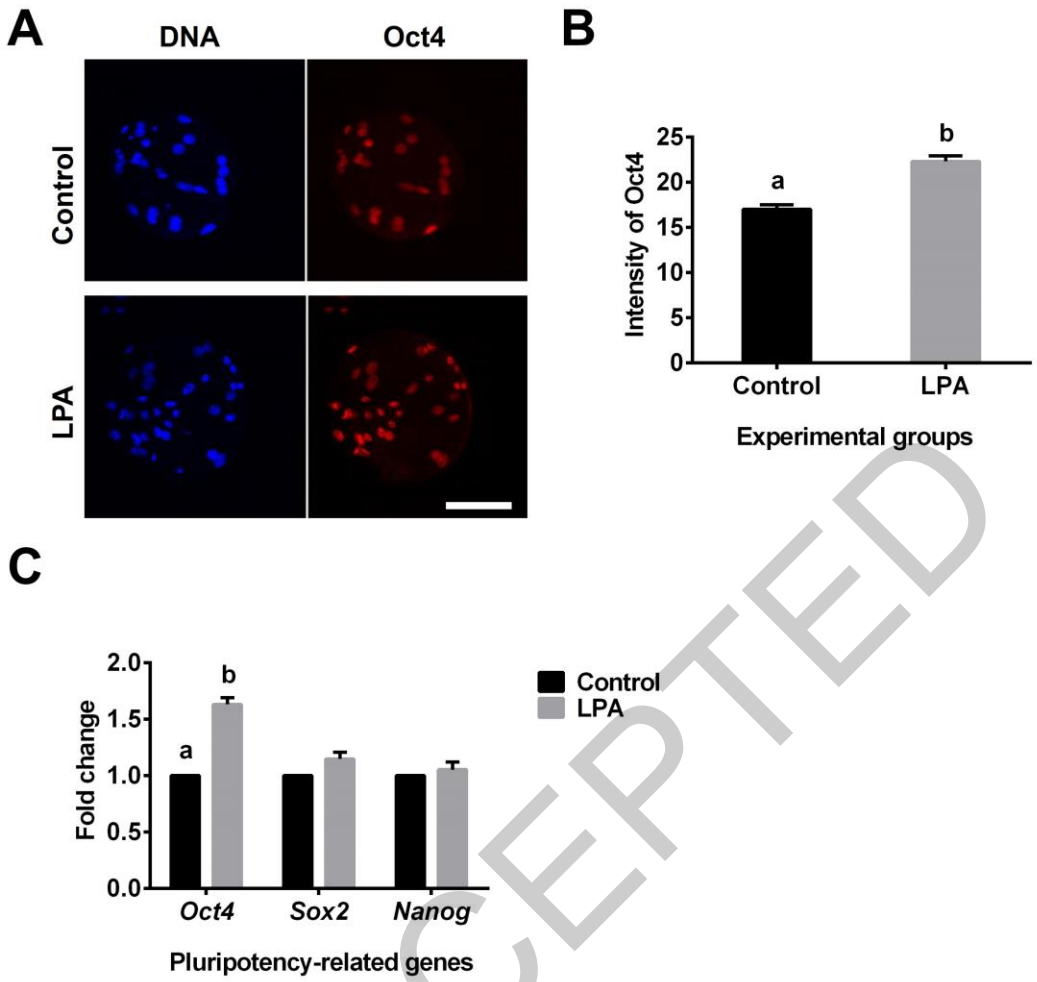

Figure 4. Effect of LPA on the expression levels of pluripotency markers in porcine SCNT-derived blastocysts. A: The expression of Oct4 protein in porcine SCNT-derived blastocysts. B: Quantification of Oct4 expression. Experiment was conducted at least thrice, and a total of 18 (Control) and 22 (LPA group) embryos were used for the assay. C: The gene expression levels of *Oct4*, *Sox2* and *Nanog*. Different letters indicate a significant difference ($P < 0.05$). Scale bar = 100 μm in A.