Master's Thesis

Cell synchronization by rapamycin improves the developmental competence of porcine SCNT embryos

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GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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Cell synchronization by rapamycin improves the developmental competence of porcine SCNT embryos

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ABSTRACT

The cell cycle stage of donor cells influences the success of somatic cell nuclear transfer (SCNT). This study investigated the effects of rapamycin treatment on the synchronization of porcine fibroblasts, in comparison with control and serum-starved cells, SCNT donor cell viability, and SCNT-derived embryo development. Porcine fibroblasts were treated with 0.1, 1, 10, and 100 μM rapamycin for 1 or 3 days. The proportion of cells in G_0/G_1 phase was significantly higher among cells treated with 1 μM rapamycin for 3 days (D3-1R) than among control and serum-starved cells ($P < 0.05$). In comparison with control cells, rapamycin-treated cells exhibited reduced proliferation, similar to serum-starved cells. The viability (as assessed by the MTT assay) of D3-1R-treated cells was good, similar to control cells, showing their quality was maintained. To confirm nutrient regulation by rapamycin treatment, we checked the transcript levels of nutrient transporter genes (*SLC2A2*, *SLC2A4*, *SLC6A14*, and *SLC7A1*). These levels were significantly lower in D3-1R-treated cells than in control cells ($P < 0.01$). We performed SCNT with D3-1R-treated cells ($SCNT^{D3-1R}$) to confirm the effect of cell cycle synchronization by rapamycin treatment. Although $SCNT^{D3-1R}$ embryos did not have an increased fusion rate, their cleavage and blastocyst formation rates were significantly higher than those of control embryos ($P < 0.05$). Regarding embryo quality, the numbers of total and apoptotic cells per blastocyst were increased and decreased, respectively, in $SCNT^{D3-1R}$ blastocysts. The mRNA levels of developmental (*CDX2* and *CDH1*) and pro-apoptotic (*FAS* and *CASP3*) genes were significantly higher and lower, respectively, in $SCNT^{D3-1R}$ blastocysts than in control blastocysts ($P < 0.05$). These results demonstrate that rapamycin treatment affects the cell cycle synchronization of donor cells and enhances the developmental potential of porcine SCNT embryos.

Key words: *cell cycle synchronization, porcine, rapamycin, SCNT*

1. INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a valuable technique for cloning livestock and transgenic animals (Francois et al., 2012). Pigs (Hammer et al., 1985), sheep (Campbell et al., 1996), cattle (Yoko et al., 1998), mice (Wakayama et al., 1998), and goats (Alexander et al., 1999) have been produced by SCNT. Transgenic animals have also been generated such as a human α-lactalbumin-expressing goat (Feng et al., 2015) and an enhanced green fluorescent protein-expressing pig (Hyun et al., 2003) and cow (Sezen et al., 2001) by SCNT. Although SCNT has successfully been used for cloning, its efficiency is low.

Many studies attempted to improve the *in vitro* development of SCNT embryos via various approaches such as cell synchronization (Hayes et al., 2005), use of different donor cell types (Rosen et al., 1999; Li et al., 2013; Pan et al., 2015), and treatment of donor cells with a histone deacetylase inhibitor (Zhao et al., 2010). The cell cycle stage of donor cells is one of the most important considerations. Its importance has been noted since the first SCNT animal (Dolly) was produced using a donor cell in G_0 phase (Campbell et al., 1996). Only nuclei in G_0 (David et al., 1999) or G_1 (Zou et al., 2002) phase of the cell cycle can be transplanted into enucleated metaphase II (MII) oocytes. Several methods have been used for synchronization at G_0/G_1 phase of the cell cycle such as serum starvation (Yin et al., 2007) and contact inhibition (Sun et al., 2008).

Additionally, mimosine (Vacková et al., 2003), roscovitine (Park HJ et al., 2010), and cycloheximide (Goissis et al., 2007) can synchronize porcine cells at G_0/G_1 phase. Mimosine, a non-protein plant amino acid, reportedly blocks cell cycle progression in late G_1 phase prior to the onset of DNA synthesis (Lalande, 1990; Krude, 1999). Roscovitine, an inhibitor of Cdk2 and maturation-promoting factor, effectively arrests human fibroblasts in G_0/G_1 phase of the cell cycle and, following its removal, cells arrested in G_0/G_1 resume cycling and

enter S phase as expected (Alessi et al., 1998; Sun et al., 2008). Cycloheximide, a protein synthesis inhibitor, was reported to effectively induce activation of porcine MII oocytes when combined with electric stimulation (Mario et al., 2003).

Rapamycin is a bacterial macrolide with antifungal and immunosuppressant activities (Dumont et al., 1990). It forms a complex with FK506-binding protein 12 and then binds to mammalian target of rapamycin (mTOR), selectively inhibiting its kinase activity and function related to acquire by a gain-of-function mechanism (Guertin et al., 2007). Rapamycin reportedly has an array of effects that suggest it could be a useful antitumor agent (Guertin et al., 2007). It inhibits the proliferation of many cancer cell lines grown in culture (Sarbassov et al., 2006).

Rapamycin suppresses progression of the cell cycle from G_1 to S phase by blocking and inhibiting several transduction pathways downstream of growth factor-induced activation of mTOR (Sehgal et al., 1998). Rapamycin mimics a starvation signal. Rapamycin treatment and amino acid deprivation both down-regulate genes involved in protein synthesis, turnover, and folding (Tao et al., 2002). By contrast, they have opposing effects on the regulation of genes involved in amino acid biosynthesis, amino acid transport, and tRNA synthesis (Tao et al., 2002), with rapamycin treatment and amino acid/glucose deprivation inhibiting and increasing their function, respectively (Tao et al., 2002).

The objective of this study was to investigate the effects of treatment with various concentrations of rapamycin for 1 or 3 days on the cell cycle synchronization and quality of donor cells. To understand how rapamycin treatment induces arrest at G_0/G_1 phase, we examined nutrient transporter gene expression in cells treated with 1 μM rapamycin for 3 days (D3-1R). Furthermore, the developmental competence of reconstructed SCNT porcine embryos was analyzed. We hypothesize that rapamycin treatment improves synchronization

of the donor cell cycle and the developmental competence of SCNT blastocysts. This study may enhance donor cell quality and the efficiency of SCNT embryo development.

2. MATERIALS & METHODS

2.1.Oocyte collection and *in vitro* maturation

Pre-pubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in saline supplemented with 75 mg/mL penicillin G and 50 mg/mL streptomycin sulfate within 2 h at 32–35℃. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2–8 mm in diameter with an 18-gauge needle and a disposable 10 mL syringe. COCs were washed three times in tissue culture medium (TCM)-199-HEPES containing 0.1% (w/v) bovine serum albumin (BSA). Groups of 50 COCs were matured in 500 μL of TCM-199 (Gibco, Grand Island, NY, USA) containing Earle's salts, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 0.5 μg/mL follicle-stimulating hormone, 0.5 μg/mL luteinizing hormone, and 10% (v/v) porcine follicular fluid under mineral oil for 38 h at 38.8℃ in 5% CO_2 and air.

2.2. Donor cell preparation and rapamycin treatment

Donor fibroblasts for SCNT were derived from a Jeju Black Pig. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 1% penicillin/streptomycin. Cells at passage 10–12 were cultured for 2–3 days until they reached confluency and expanded by passage at 38.8℃ in 5% CO_2 and humidified air for further use. Rapamycin (Santa Cruz, CA, USA) was added to the culture medium at a concentration of 0, 0.1, 1, 10, and 100 μM (0, 0.1, 1, 10, and 100 R, respectively) for 1 or 3 days (D1 and D3, respectively). Rapamycin-treated cells were compared with control cells in

DMEM containing 10% FBS and positive control cells cultured in DMEM containing 0.5% FBS (0.5 F, serum starvation). Media were replaced with the same media at day 2.

2.3. SCNT and *in vitro* culture

Following maturation, cumulus cells were removed by gently pipetting in the presence of 1 mg/mL hyaluronidase for 2–3 min. After recovery for 30 min, the first polar body and nucleosome were removed using a 20 μm glass pipette in HEPES-buffered TCM-199 supplemented with 0.4% (w/v) BSA and 7.5 μg/mL cytochalasin B (CB) with the Oosight imaging system. The donor cell was inserted into the perivitelline space adjacent to the cytoplasm. The karyoplast-cytoplast complexes were fused in fusion medium containing 0.3 M D-mannitol, 0.5 mM HEPES, 0.05% fatty acid-free (FAF)-BSA, 0.05 mM $CaCl_2$, and 0.1 mM $MgSO_4$. Inserted donor cells were aligned to the northern wire in a fusion chamber (Lf201, Nepagene, Chiba, Japan) with a direct current impulse of 110 V/cm for 60 μsec. After fusion, reconstructed embryos were activated in 7.5 μg/mL CB for 3 h. SCNT embryos were transferred to PZM-5 medium supplemented with 0.4% FAF-BSA. On day 4, the media was replaced with 10% human adipose tissue-derived mesenchymal stem cell bioactive material medium (Park et al., 2013). On days 2 and 7, the cleavage and blastocyst formation rates were recorded. The cleavage rate was counted by the number of 2- to 4-cell embryos derived from the fused embryos at day 2 post SCNT. The blastocyst formation rate was calculated by the number of blastocysts produced from the cleaved embryos at day 7 after SCNT.

2.4. Flow cytometry

Cells were seeded at a density of 1×10^6 cells in 100 mm^2 culture plates. The following day, 1×10^5 cells were treated with test compounds or 0.1% dimethyl sulfoxide (DMSO). After incubation for 24 h, the cell culture supernatant and centrifuged at 2,000 rpm for 2 min at room temperature. The supernatant was discarded and pelleted cells were washed twice by repeated suspension in phosphate-buffered saline (PBS) and were centrifuged at 2,000 rpm for 2 min at room temperature. Pelleted cells were carefully suspended in 500 μg/mL RNase A and 50 μg/mL propidium iodide (Sigma, St. Louis, MO, USA) at 37°C for 45 min. Fixed cells were analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer until 20,000 cells were counted. The cell cycle distribution was analyzed using WinMDI 2.9 software.

2.5. MTT assay

Cell death was measured using the MTT cell viability assay with a slight modification as described previously (Mossman, 1983). Porcine fibroblasts were plated in triplicate in 96-well plates at a density of 1×10^5 cells/well in 200 μL of DMEM. After treatment with rapamycin for D1 or D3, metabolically active cells were detected by adding MTT to a final concentration of 0.5 mg/mL and incubation for another 4 h at 37℃. DMSO was added (200 μL/well) and mixed to solubilize the purple product. Optical densities were read using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a reference wavelength of 590 nm (Kim et al., 1999).

2.6. TUNEL assay

To detect fragmented DNA, 14 blastocysts control and D3-1R treated group, respectively, were fixed with 4.0% (w/v) paraformaldehyde prepared in PBS overnight at 4℃ and then incubated with 0.1% Triton X-100 at 38.8℃ for 30 min. Blastocysts were incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit, Roche, Manheim, Germany) in the dark for 1 h at 38.8℃. The total numbers of mitotic and apoptotic cells were scored. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and embryos were washed with PBS containing 0.1% BSA. Blastocysts were mounted onto glass slides and examined under an inverted Olympus IX-71 (Japan) microscope. At least ten blastocysts were examined per group.

2.7. Real-time RT-PCR with SYBR Green

For real-time reverse-transcriptase-PCR analysis, mRNA was prepared from blastocyst using magnetic beads (Dynabeads mRNA Purification Kit; Dynal, Oslo, Norway) according to the manufacturer's instructions. For each treatment group, 15 *in vitro*-produced, Day 7 blastocyst were resuspended in 100 μL of lysis/ binding buffer (100 mMTris-HCl, pH 7.5, 500 mMLiCl, 10 mM EDTA, pH 8.0, 1% LiDS, and 5 mM DTT) and vortexed at room temperature for 5 min to lyse the tissue. A 50-μL aliquot of an oligo-(dt)-25 magnetic-bead suspension was added, and the samples were incubated at room temperature for 5 min. the hybridized mRNA and oligo-(dT) beads were washed twice using wash buffer A (10 mMtris-HCl, pH 7.5, 0.15M LiCl, and 1 mM EDTA). mRNA was eluted from beads in 15 μL of double-distilled, DEPC- Treated water (Lee et al., 2014).

Extraction of mRNA was performed as described above, and standard cDNA was

synthesized using an oligo-(dT) primer and SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using the primer sets listed in Table 1 in a Step One Plus Real-time PCR System (Applied Biosystems, Warrington, UK) with a final reaction volume of 20 μL containing the SYBR Green PCR Master Mix (Applied Biosystems). The PCR conditions were as follows: 10 min at 94℃, followed by 39 cycles of 30 sec at 60℃ and 55 sec at 72℃, and a final extension of 5 min at 72℃. Samples were then cooled to 12℃. Relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$method (Livak et al., 2001) after normalization against the GAPDH mRNA level.

Table 1. Primers used for real-time PCR

Gene	GeneBank accession no.	Primer sequence*	Annealing temperature (℃)	Product size (bp)
GAPDH	AF017079	F:GGGCATGAACCATGAGAAGT R:AAGCAGGGATGATGTTCTGG	54	230
SLC2A2	EF140874.1	F:CTCTCATTAGTTGGAGCTCTCT R:TAGAGTCCTGATATGCCTCTTC	54	92
SLC2A4	NM_001128433.1	F:CTTCTTCATCTTCACCTTCC R:TGCTCTAAAAGAGAGGGTGT	54	82
SLC6A14	NM_001166042.1	F:GGGTGGTTTAGTTGCTCTATC R:CACACTAGTAAGGCAGTTTGTC	54	89
SLC7A1	NM_001012613.1	F:GAACGTCTATCTCATGATGC R:TAGATGAAGAAGCCTATCAGC	54	99
CDX2	AM778830	F:AGCCAAGTGAAAACCAGGAC R:TGCGGTTCTGAAACCAGATT	60	178
CDH1	EU805482	F:CTGTATGTGGCAGTGACTAAC R:AGTGTAGGATGTGATCTCCAG	55	174
FAS	AJ001202	F:AAGTTCCCAAGCAAGGGATT R:AATTTCCCATTGTGGAGCAG	60	207
CASP3	NM_214131.1	F:AAGTTCCCAAGCAAGGGATT R:ACAAAGTGACTGGATGAACC	55	93

2.8.Statistical analysis

The general linear model procedure within the Statistical Analysis System (SAS User's Guide 1985, Statistical Analysis System Inc., Cary, NC, USA) was used to analyze data from all experiments. A paired Student's t-test was used to compare relative gene expression. P-values of < 0.01 and 0.05 were considered significant.

3. Results

3.1. Rapamycin treatment induces cell cycle arrest at G_0/G_1 phase

To determine the optimal concentration of rapamycin for cell synchronization, porcine fibroblasts were cultured with 0.1 R, 1 R, 10 R, 100 R, 0.5 F, or control for D1 or D3. We performed fluorescence-activated cell sorting to confirm the effect of rapamycin on the cell cycle (Figure 1A). M1, M2 and M3 indicated that G_0/G_1 phase, G_2/M phase and S phase respectively. The percentage of G_0/G_1 phase synchronization following treatment for D1 was as follows: 0.1 R, 84.9 ± 2.5%; 1 R, 83.8 ± 1.3%; 10 R, 80.0 ± 3.5%; 100 R, 52.6 ± 1.5%; 0.5 F, 85.9 ± 2.6%; and control, 56.2 ± 1.6%. The percentage of G_0/G_1 phase synchronization following treatment for D3 was as follows: 0.1 R, 79.3 ± 2.1%; 1 R, 93.6 ± 1.9%; 10 R, 58.0 ± 3.8%; 100 R, 60.5 ± 1.5%; 0.5 F, 82.0 ± 3.2%; and control, 61.0 ± 2.2%). At D1, there were significantly higher proportion of G_0/G_1 phase cells in 0.5 F, 0.1 R, 1 R, and 10 R treated cells than that of control cells (Figure 1B) ($P < 0.05$). At D3, the proportion of G_0/G_1 phase cells in 1 R group was the highest among treatment groups and 0,5 F and 0.1 R treatment groups were also significantly higher proportion of G_0/G_1 phase cells than control or other higher R treated cell group ($P < 0.05$).

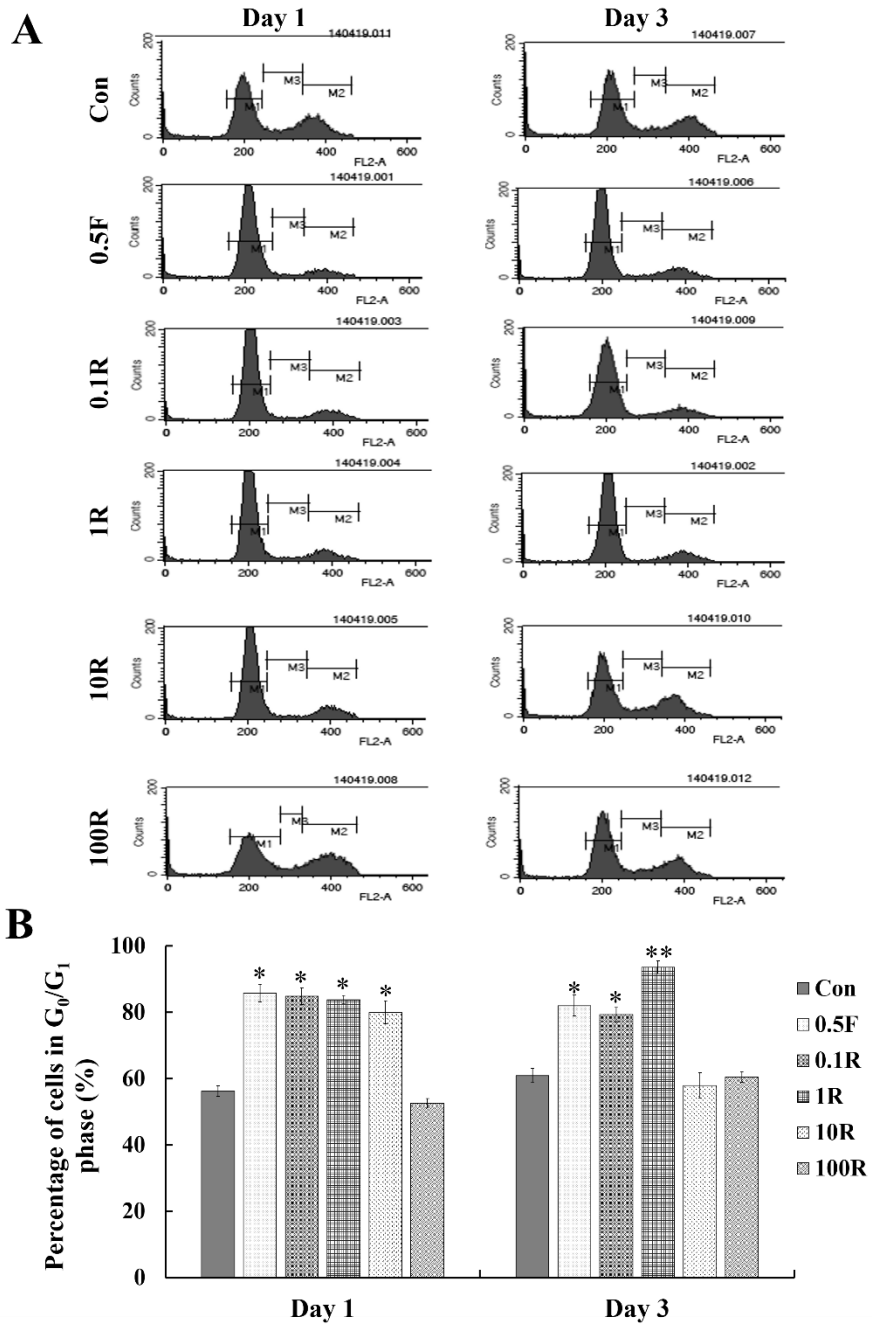

Figure. 1. The effect of rapamycin treatment on cell synchronization at G_0/G_1 phase. DNA histograms **(A)** and percentages of cells in G_0/G_1 phase **(B)** for each group of cultured porcine fibroblasts. Con, control; 0.5 F, serum starvation; 0.1 R, 0.1 μM rapamycin; 1 R, 1 μM rapamycin; 10 R, 10 μM rapamycin; and 100 R, 100 μM rapamycin; M1, G_0/G_1 phase;

M2: G_2/M phase; M3: S phase.The percentages of cells in G0/G1 phase were calculated by the WinMDI 2.9 software. Significant differences from the control are indicated (*-**P < 0.05). Values are means ± standard error of the mean of independent experiments.

3.2. Rapamycin treatment maintains the quality of porcine fibroblasts

To investigate the effect of rapamycin treatment on cell proliferation and viability, cell counting and the MTT assay were performed. The numbers of cells in each group are shown in Figure 2A. At D1, the number of cells did not vary among samples treated with 0.1 R, 1 R, 10 R, 100 R, and 0.5 F, but was higher in the control group (3.333 ± 0.572, 3.433 ± 0.351, 2.927 ± 0.411, 2.773 ± 0.337, 4.013 ± 0.311 vs 5.687 ± 0.300, respectively). At D3, the number of cells was higher in samples treated with 0.1, 1, and 10 R than in those treated with 100 R and 0.5 F, but was highest in the control sample (14.500 ± 0.572, 13.850 ± 0.577, 12.217 ± 0.723, 7.447 ± 0.561, 4.440 ± 0.699 vs 27.133 ± 0.441, respectively; Figure 2A). In Figure 2B, cell proliferation was normalized against that of control cells at the same time point. The relative proliferation rate of cells treated with 0.1 R, 1 R, 10 R, 100 R, and 0.5 F was significantly lower than that of control cells (set to 1) at D1 (0.415 ± 0.056, 0.489 ± 0.068, 0.416 ± 0.100, 0.360 ± 0.044, and 0.676 ± 0.087, respectively) and D3 (0.467 ± 0.008, 0.549 ± 0.016, 0.475 ± 0.031, 0.311 ± 0.010, and 0.161 ± 0.047, respectively, Figure 2B) (P < 0.05). At D1, the proliferation of cells treated with 0.1, 1, 10, and 100 R was similar. The proliferation of cells treated with 1 R was sustained at D3 in comparison with D1. At D1, cell viability did not differ among the groups, as assessed by measuring absorbance at 590 nm (control, 0.201 ± 0.011; 0.5 F, 0.146 ± 0.005; 0.1 R, 0.151 ± 0.016; 1 R, 0.179 ± 0.013; 10 R, 0.176 ± 0.012; and 100 R, 0.177 ± 0.002; Figure 2C). At D3, however, as assessed by

measuring absorbance at 590 nm, the viability of 1 R-treated cells (0.281 ± 0.016) was similar to that of control cells (0.269 ± 0.008) in comparison with cells treated with 0.5F, 0.1, 10, and 100 R (0.146 ± 0.001, 0.203 ± 0.004, 0.224 ± 0.000, and 0.210 ± 0.011, respectively; Figure 2C).

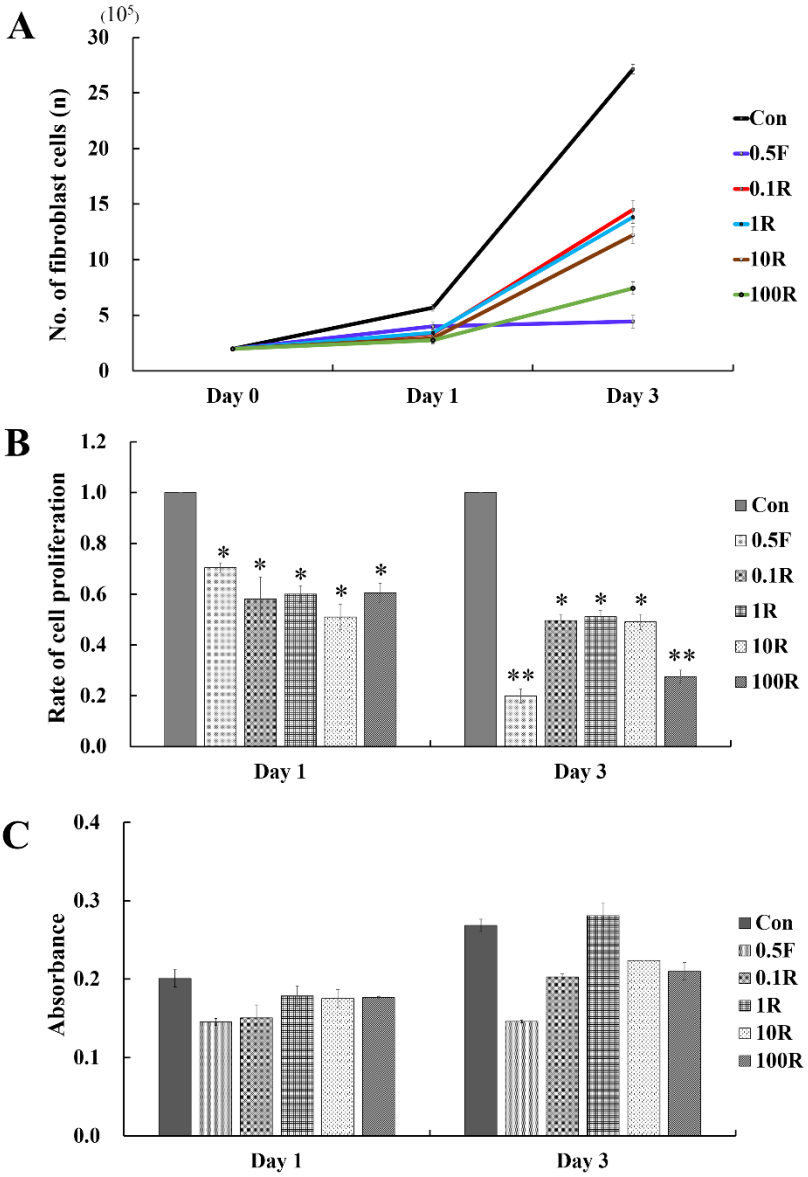

Figure. 2. The effect of rapamycin treatment on cell proliferation **(A)**, relative cell proliferation **(B)**, and cell viability **(C)**. In (B), data are normalized against those of control

cells (set to 1) at the same time point. Cell viability (C) was analyzed by MTT assay. The value of absorbance means live cells. Con, control; 0.5 F, serum starvation; 0.1 R, 0.1 μM rapamycin; 1 R, 1 μM rapamycin; 10 R, 10 μM rapamycin; 100 R, 100 μM rapamycin; D1, day 1; and D3, day 3. Significant differences from the control are indicated (*-**P < 0.05). Values are means ± standard error of the mean of independent experiments.

3.3. Rapamycin treatment down-regulates nutrient transporter expression in fibroblasts

To investigate the mechanisms underlying the beneficial effect of rapamycin, we assessed mRNA expression of glucose and amino acid transporter genes in porcine fibroblasts by real-time quantitative RT-PCR (Figure 3). mRNA expression of glucose transporter (*SLC2A2* and *SLC2A4*) and amino acid transporter (*SLC6A14* and *SLC7A1*) genes was significantly down-regulated in cells treated with 1 R for D1 and D3 in comparison with control cells (Figure 3A and 3B) (P < 0.01).

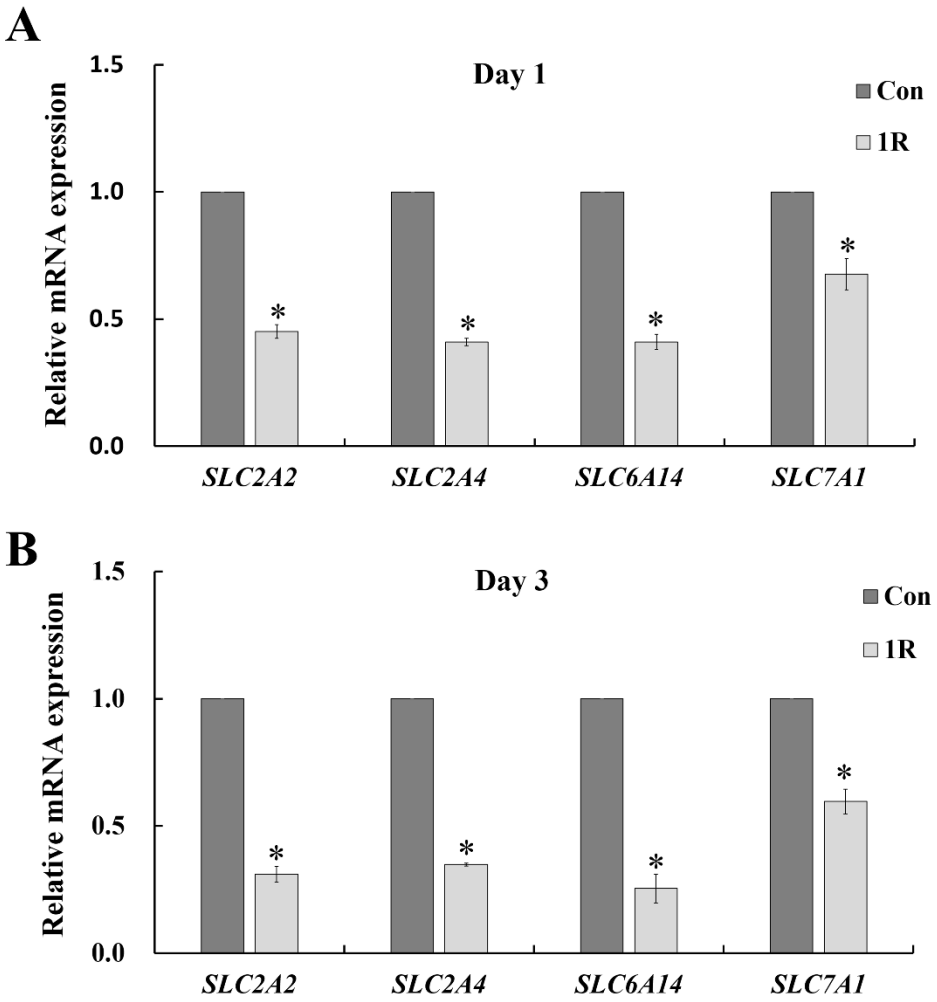

Figure. 3. Relative mRNA expression of glucose transporter (*SLC2A2* and *SLC2A4*) and amino acid transporter (*SLC6A14* and *SLC7A1*) genes in cells treated with 1 μM rapamycin for 1 day **(A)** and 3 days **(B)**. Con, control and 1 R, 1 μM rapamycin. Significant differences from the control are indicated (*P < 0.01). Values are means ± standard error of the mean of independent experiments.

3.4. Rapamycin treatment of donor cells improves the quality of SCNT embryos

SCNT was performed with D3-1R-treated cells ($SCNT^{D3\text{-}1R}$) to confirm the effect of cell synchronization by rapamycin treatment. We analyzed $SCNT^{D3\text{-}1R}$ embryo development (Table 2). The fusion rate did not differ between $SCNT^{D3\text{-}1R}$ and control embryos (71.1 ± 9.2% and 69.9 ± 6.9%, respectively). The cleavage rate of $SCNT^{D3\text{-}1R}$ embryos was significantly higher than that of control embryos (61.7 ± 1.8% and 40.0 ± 3.3%, respectively) ($P < 0.05$). The blastocyst formation rate of $SCNT^{D3\text{-}1R}$ embryos was significantly higher than that of control embryos (46.6 ± 2.2% and 20.3 ± 1.0%, respectively) ($P < 0.05$).

The morphology of blastocysts developed from $SCNT^{D3\text{-}1R}$ embryos was better than that of blastocysts developed from control embryos (Figure 4A, a and b). The total cell number per blastocyst was significantly higher in $SCNT^{D3\text{-}1R}$ blastocysts than in control blastocysts (69.8 ± 9.33 and 48.3 ± 7.04, respectively; Figure 4A, a'' and b''; and Figure 4B) ($P < 0.05$). Relative mRNA expression of genes important for development was quantified by real-time PCR (Figure 5). Expression of development-related genes (*CDX2* and *CDH1*) was significantly higher in $SCNT^{D3\text{-}1R}$ blastocysts than in control blastocysts ($P < 0.05$).

When genomic DNA fragmentation was measured in individual embryos using the TUNEL assay, the index was significantly lower in $SCNT^{D3\text{-}1R}$ embryos than in control embryos (5.4% ± 1.2% and 11.8% ± 2.1%, respectively; Figure 4A, a' and b'; and Figure 4C) ($P < 0.05$). mRNA expression of pro-apoptotic genes (*FAS* and *CASP3*) was significantly lower in $SCNT^{D3\text{-}1R}$ blastocysts than in control blastocysts ($P < 0.05$).

Table 2. *In vitro* development of SCNT$^{D3\text{-}1R}$ embryos (r = 3)

Group	No. of nuclear transfer oocytes	No. (%) of fused oocytes	No. (%) of embryos	
			Cleaved at day 2	Developed at day 7
Control	229	160 (69.9 ± 6.9)	64 (40.0 ± 3.3)	13 (20.3 ± 1.0)
SCNT$^{D3\text{-}1R}$	235	167 (71.1 ± 9.2)	103 (61.7 ± 1.8)*	48 (46.6 ± 2.2)*

*P < 0.05.

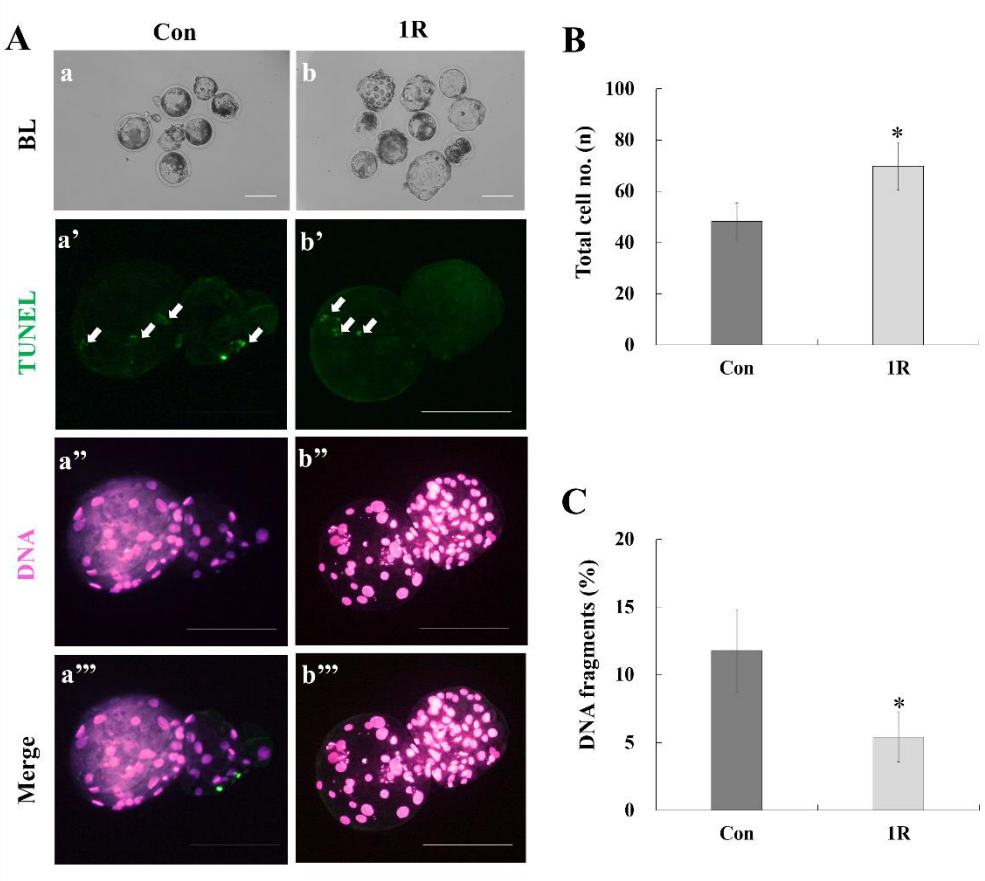

Figure. 4. Evaluation of the morphology **(A)**, total cell number **(B)**, and percentage of apoptotic cells **(C)** in blastocysts derived from SCNT$^{D3\text{-}1R}$ embryos at day 7. Con, control;

$SCNT^{D3\text{-}1R}$, SCNT embryo with D3-1R-treated cells; a and b, morphology of blastocysts; a' and b', TUNEL assay; a'' and b'', Hoechst staining; and a''' and b''', merge. Significant differences from the control are indicated (*P < 0.05). Values are means ± standard error of the mean of independent experiments. Bar, 100 μm.

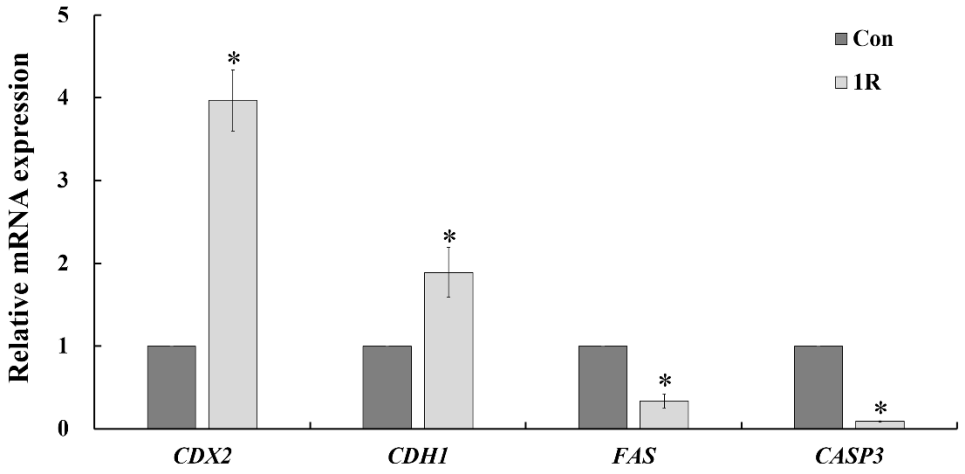

Figure. 5. Relative mRNA expression of developmental (*CDX2* and *CDH1*) and pro-apoptotic (*FAS* and *CASP3*) genes in blastocysts derived from $SCNT^{D3\text{-}1R}$ embryos. Con, control and $SCNT^{D3\text{-}1R}$, SCNT embryo with D3-1R-treated cells. Significant differences from control blastocysts are indicated (*P < 0.05). Values are means ± standard error of the mean of independent experiments.

4. DISSCUSTION

In the present study, we investigated whether rapamycin treatment of donor porcine fibroblasts is useful for cloning. Our data show that rapamycin treatment of donor cells induces cell cycle arrest at G_0/G_1 phase and maintains cell quality. Nutrient transporter gene expression(*SLC2A2*, *SLC2A4*, *SLC6A14*, and *SLC7A1*) was down-regulated by rapamycin treatment. $SCNT^{D3\text{-}1R}$ embryos had significantly improved cleavage and blastocyst formation rates.

D3-1R treatment effectively induced cell cycle arrest at G_0/G_1 phase. For successful reprogramming, donor cells must be in G_0/G_1 phase when transferred into matured oocytes (Sugimura et al., 2010). Use of donor cells in G_0/G_1 phase reduces the possibility of chromosomal aberrations during development of nuclear-transplanted cells and can improve SCNT embryo development (Hayes et al., 2005). Synchronization reagents such as roscovitine (Naresh et al., 2012) and mimosine (Vacková et al., 2003) have been widely used to induce the arrest of donor cells at G_0/G_1 phase. Roscovitine blocks the proliferation of vascular smooth muscle cells (Zhang et al., 2014) and causes little apoptosis in porcine fetal fibroblasts (Park et al., 2010). Mimosine, as a cell cycle blocker, inhibits the proliferation of carcinoma cells and causes little apoptosis in rat cells (Stephen et al., 1996). Rapamycin suppresses progression of the cell cycle from G_1 to S phase by blocking and inhibiting several transduction pathways (Sehgal et al., 1998). It triggers phosphorylation-mediated activation of phosphatases or kinases through mTOR. This action of rapamycin may regulate cell proliferation (Sehgal et al., 1998). In the present study, the quality of D3-1R-treated cells was maintained similar to control cells. Rapamycin was previously reported to block apoptosis driven by known death inducers (Calastretti et al., 1999; Johnson and Lawen, 1999) or to not affect cell survival (Marx and Marks, 1999; Minshall et al., 1999). Although

rapamycin only slightly reduces the cell culture growth rate, it maintains cell viability at a higher level than that of control cultures by delaying cell death (Robert et al., 2001; Lee et al., 2012). Our results show that rapamycin treatment induced cell synchronization and suppressed cell proliferation, and that the quality of D3-1R-treated cells was maintained similar to control cells. Therefore, we suggest that rapamycin treatment arrests the cell cycle at G_0/G_1 phase without having harmful effects.

A previous study indicated that rapamycin mimics the nutrient starvation signal (Diane et al., 2004), however, it synchronizes the cell cycle via a completely different mechanism to serum starvation. Rapamycin-induced cell cycle arrest at G_0/G_1 phase occurs via inhibition of nutrient transporter genes. Recently, rapamycin has been known the inducer of autophagy (Li et al., 2013). Autophagy could increase production of nutrients from intracellular stores in removal of nutrient transporter genes (Chang et al., 2009). Coupling of autophagy and ubiquitin-proteasome system also reduced cell death through protection of ER (Endoplasmic Reticulum) stress and ER decompensation (Ding et al., 2007). In nutrient starvation conditions, such as glucose and amino acid deprivation, these genes are expressed normally (Tao et al., 2002). However, rapamycin treatment inhibits expression of the glucose transporter genes *SLC2A2* (Michau et al., 2013) and *SLC2A4* (Furuya et al., 2013) and the amino acid transporter genes *SLC6A14* (Elina et al., 2003) and *SLC7A1* (Wang et al., 2014). Based on the phenotype of the knockout animal, Slc2a2 is a contender for the β cell glucose sensor (Efrat, 1997). The *Slc2a4* gene encodes an insulin-responsive glucose transporter protein in Xenopus oocytes (Birnbaum, 1989). The *Slc6a14* gene encodes a Na- and Cl-dependent membrane protein that transports neutral and cationic amino acids across the plasma membrane in Xenopus oocytes (Sloan et al., 1999). Certain amino acids, such as glutamine and arginine, are important precursors for nucleotide synthesis and are essential for tumor growth (Senthil et al., 2011). SLC7A1 encodes a high-affinity cationic amino acid

transporter (CAT1), which facilitates uptake of arginine and lysine in mammalian cells (Hatzoglou et al., 2004). These genes are responsible for uptake of glucose and amino acids by cells. Nutrient uptake occurs under appropriate conditions in untreated cells; however, it is suppressed via down-regulation of nutrient transporter genes in rapamycin-treated cells (Tao et al., 2002). Our results indicate that rapamycin triggered repression of *SLC2A2*, *SLC2A4*, *SLC6A14*, and *SLC7A1* expression in comparison with control cells. We performed Real-time RT PCR at three times. Although we didn't show the protein levels, the relative expressions ofthe transporter geneswere significantly down regulated (3 to 4 folds)in D3-1R treated group than in control group. Moreover, there was little standard deviation among the repeat experiments. Therefore, it would be the reasonable result for rapamycin treatment trigger repression of transporter gene expression. Taken together, these results suggest that D3-1R-treated cells are arrested at G_0/G_1 phase via inhibition of nutrient transporter gene expression.

We performed SCNT using D3-1R-treated donor cells. Although the fusion rate was not affected in $SCNT^{D3\text{-}1R}$ embryos, the cleavage and blastocyst formation rates were significantly increased. Furthermore, the total cell number per blastocyst was higher in $SCNT^{D3\text{-}1R}$ blastocysts than in control blastocysts. The total number of cells in an embryo is an important measure of embryonic development and quality (Hao et al., 2004). Blastocysts containing many cells are more likely to successfully implant and give rise to live offspring (Van et al., 1997). Relative mRNA expression of *CDX2* and *CDH1* was also increased in blastocysts developed from $SCNT^{D3\text{-}1R}$ embryos. Cdx2 plays a key role in embryo placental development and mouse early embryo development, and determines the level of trophectoderm differentiation induced by Oct3/4 (Chawengsaksophak et al., 1997). E-cadherin (CDH1) functions in the establishment and stabilization of cellular junctions in armadillo embryos (Aberle et al., 1996). *CDH1* is a marker for evaluating nuclear

reprogramming in cloned porcine embryos (Lee et al., 2006; Wolf et al., 2011). Collectively, these results indicate that rapamycin treatment improves the embryonic developmental competence of porcine SCNT oocytes.

Apoptosis is generally defined as programmed cell death that is an indicator of abnormal embryo development and stress. In the present study, DNA fragmentation was significantly decreased in blastocysts developed using rapamycin-treated donor cells. We investigated relative mRNA expression of the pro-apoptotic genes *FAS* and *CASP3*. FAS is a ligand that induces apoptosis in normal cells. When it binds to death receptors such as CD95 and TNFRSF6, apoptosis is dependent on caspase 8 (Wiley et al., 1995; Almasan et al., 2004). *CASP3* is a member of the caspase family that plays an essential role in the initiation of apoptosis (Alan et al., 1999). In this study, mRNA expression of pro-apoptotic genes was decreased in blastocysts developed from $SCNT^{D3\text{-}1R}$ embryos, showing that apoptosis was decreased in these blastocysts. Thus, the quality of blastocysts derived from $SCNT^{D3\text{-}1R}$ embryos was enhanced.

In conclusion, our results show that rapamycin treatment of porcine fibroblasts induced cell cycle arrest at G_0/G_1 phase while maintaining the quality of donor cells. Through the optimized rapamycin treatment, cell proliferation was inhibited via down-regulation of nutrient transporter gene, but cell viability was similar to control cells. However, this function is very complicated and it needs to investigate more profound activity forthe rapamycin treated cells as further study. This study demonstrated that the quality of blastocysts derived from $SCNT^{1R\text{-}D3}$ embryos was improved, with effects on the developmental rate, total cell number per blastocyst, and expression of the developmentally important genes *CDX2* and *CDH1*. $SCNT^{1R\text{-}D3}$ blastocysts exhibited decreased DNA fragmentation and decreased expression of the pro-apoptotic genes *FAS* and *CASP3*. Thus, rapamycin is a useful chemical for donor cell synchronization and these findings may be

applicable to improve the efficiency of SCNT for the production of cloned animals.In the future, the expression of transporter gene, SLC2A2, SLC2A4, SLC6A14, SLC7A1 in protein-level will be confirmed by western blot analysis. Besides, we hope to better understand the relationship (Figure 6, Stanislaw MS) between positive or negative effect of rapamycin and cell cycle progression and to move further downstream to determine how mTOR couples these fundamental biological processes.

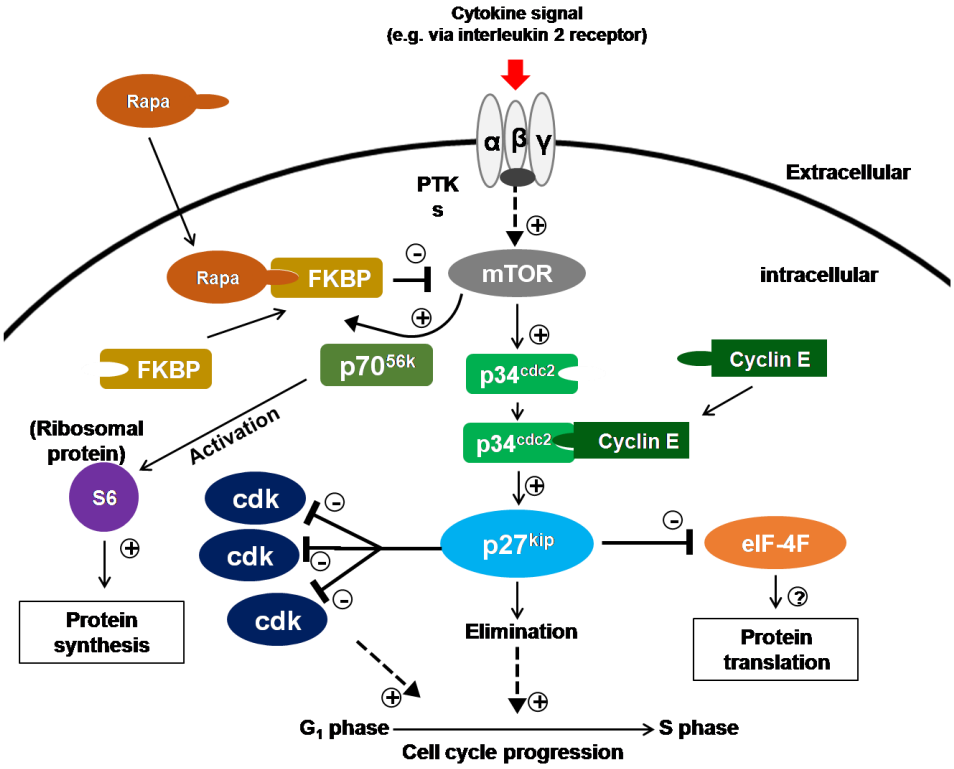

Figure. 6. Mechanism of action of rapamycin (Rapa). Although the pre-drug rapamycin binds to FK506-binding protein (FKBP, which is the same molecule that is bound by FK506), the complex that is formed between SRL and FKBP binds to the mammalian target of rapamycin (mTOR). The SRL–FKBP–mTOR complex inhibits biochemical pathways that are required for cell progression through the late G1 phase or entry into the S phase of the cell cycle. Thus, unlike cyclosporine (CsA) and FK506 (which block the production of

cytokines), SRL blocks cytokine signal transduction. SRL is thought to target: (1) the 70-kD S6 protein kinase p70S6K; (2) the eukaryotic initiation factor eIF-4F; (3) the G1-controlling cyclin-dependent kinase (cdk) proteins, such as the D2 cycline cdk2, the D2 cycline cdk6 or the E cycline cdk2 and (4) the kinase inhibitory protein Kip1 (p27kip), which blocks cell progression to the S phase. Abbreviations used: p34cdc2 = a kinase; PTKs = protein tyrosine kinases.

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ABSTRACT IN KOREAN

체세포 핵 이식 기술은 질환모델동물을 생산하여 현재 개발중인 치료제의 임상실험동물로 사용할 수 있다. 질환모델동물에는 헌팅턴 질환 돼지 모델, 당뇨병 질환 돼지 모델, EGFP 형질전환 돼지 모델 등이 연구되어 왔다. 형질전환동물을 생산하기 위해 체세포 핵 이식 기술은 필수적이며 매우 유용한 기술이지만, 복제효율은 매우 낮은 단점을 갖고 있다. 이를 개선하기 위해 공여세포 종류를 달리한다거나, 공여세포에 히스톤 탈아세틸화 효소 억제제를 처리하여 재편성(reprogramming)을 증대시키는 등 많은 연구가 진행되고 있다. 본 논문에서는 복제효율에 영향을 미치는 많은 요인 중 하나인 공여세포의 세포주기를 조절하여 체세포 핵 이식 배아의 발달률을 증가시키고자 하였다. 세포주기를 G_0/G_1 주기로억제하는 기능이 있는 라파마이신 (rapamycin)을 공여세포에 처리하여 세포 동기화율, 활성, 증식률을 관찰하였으며 이 물질의 세포주기 억제기능이 어떤 메커니즘으로 일어나는지 알아보기 위해 영양소 운반 단백질의 발현율을 확인하였다. 그 결과, 공여세포는 G_0/G_1 주기에서 동기화된 것을 관찰하였으며,세포활력 또한 대조군과 유사하게 나타났다. 영양소 운반 단백질 (*SLC2A2, SLC2A4, SLC6A14, SLC7A1*)들은 대조군과 비교했을 때 유의적으로 낮게 발현되었다. 라파마이신 처리된 공여세포를 체세포 핵 이식 기술에 사용했을 때의 체세포 핵이식 배아의 양적 및 질적조사를 실시한 결과, 라파마이신 처리된 공여세포를 사용한 배아가 대조군보다 발달률이 유의적으로 증가하였다. 결론적으로 라파마이신은 공여세포의 세포동기화에 매우 효과적인 물질이며, SCNT 효율을 증대시켜 추후 복제동물생산에 적합할 것으로 사료된다.

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