Effects of Combined Treatment of MG132 and Scriptaid on Early and Term Development of Porcine Somatic Cell Nuclear Transfer Embryos

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Abstract

Although improving, the efficiency of producing offspring by somatic cell nuclear transfer (SCNT) is still low (<1.5%). Our laboratory has demonstrated that histone deacetylase inhibitor (Scriptaid) treatment of re-constructed embryos enhances blastocyst formation and cloning efficiency in pigs. It has also been shown that proteasomal inhibitor MG132 treatment for 2 h after activation of oocytes increases blastocyst rate and pregnancy rate. The current experiment was carried out to determine the effects of combined MG132 and Scriptaid treatment on early embryo development *in vitro* and on term development *in vivo*. Immediately after electrofusion and activation, SCNT oocytes were treated with 0, 1, or 10 μ M MG132 for 2 h in the presence of 500 nM Scriptaid, washed and treated with Scriptaid for an additional 14 to 15 h, then cultured in porcine zygote medium 3 (PZM3) until day 6. There was no difference in percent cleavage ($58.1 \pm 7.2\%$, $62.7 \pm 7.2\%$, and $62.5 \pm 7.2\%$) on day 2, or total cell number (23.1 ± 2.2 , 24.0 ± 2.0 , and 24.5 ± 2.3 for the 0, 1, and 10 μ M MG132 groups, respectively) on day 6 among the three groups. Interestingly, there was no difference in percentage of blastocysts between the 0 ($18.5 \pm 4.7\%$) and 1 ($25.1 \pm 4.7\%$) μ M MG132 treatment groups; however, compared with the 10 μ M MG132 group ($14.0 \pm 4.7\%$), more embryos from the 1 μ M MG132 group developed into blastocysts ($p < 0.05$). To determine the effects on term development *in vivo*, two MG132 groups were included (0 and 1 μ M MG132), and embryos were treated as above and transferred into synchronized surrogates after treatment. There was no difference in the oocyte–donor cell fusion rate, number of embryos transferred, pregnancy rate at days 28, 60, and at term, pigs delivered per embryo transfer, litter size, body weight at birth, nor cloning efficiency between the Scriptaid-alone control and MG132+Scriptaid combined groups. In summary, the combined treatment of MG132 and Scriptaid did not improve term development compared to Scriptaid treatment alone.

Introduction

SOMATIC CELL NUCLEAR TRANSFER (SCNT) technology holds many promises to produce genetically modified pigs as biomedical models to study human diseases, as sources of organs in xenotransplantation research, and for production agriculture as well. However, its efficiency for producing offspring is low and can be influenced by many factors. The main reason for low cloning efficiency is thought to be inadequate nuclear remodeling and reprogramming of the donor nucleus (Whitworth et al., 2010). Treatment of re-constructed embryos with histone deacetylase (HDAC) in-

hibitors facilitates nuclear reprogramming by potentially opening up the DNA architecture, thus allowing proteins like transcription factors and RNA polymerases to gain access to the DNA and begin transcription. Scriptaid [6-(1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide] is an HDAC inhibitor that results in an increase in the global acetylation of histones. Scriptaid treatment of SCNT embryos increases cloning efficiency in pigs (Zhao et al., 2009, 2010) and can correct expression of some aberrantly expressed genes (Whitworth et al., 2011). Thus, Scriptaid has been used as a standard postfusion treatment of reconstructed embryos in our laboratory.

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Maturation-promoting factor (MPF) in the recipient oocyte is an important factor for regulating nuclear remodeling and reprogramming of donor nucleus (Kawahara et al., 2005; You et al., 2010), thus permitting normal embryo development (Kwon et al., 2008). MPF is composed of two subunits—cyclin-dependent kinase p34^{cdc2} and cyclin B (Dekel, 1996). Cyclin binding to p34^{cdc2} forms the pre-MPF complex, the activation of which is achieved by dephosphorylation of Thr-14 and Tyr-15 on p34^{cdc2} (Solomon et al., 1990). Metaphase II (MII) oocytes show high MPF activity, and high MPF activity induces nuclear envelope breakdown and premature chromosome condensation of somatic cell nuclei (Kawahara et al., 2005; Tani et al., 2001). An intracellular Ca²⁺ signal triggered by the fertilizing sperm is the endogenous signal for MPF destruction in MII-arrested oocytes (Jones, 2004). In SCNT, electrical and/or chemical treatments have been used to induce fusion of donor cell membrane with oocytes and activation of reconstructed embryos (Lai and Prather, 2003). Electrical stimulation induces Ca²⁺ influx in oocytes (Sun et al., 1992), and the increased Ca²⁺ prevents dephosphorylation of p34^{cdc2} kinase and consequently decreases MPF activity (Jones, 2004). After the donor cell has fused with enucleated oocyte and activated, MPF activity in SCNT embryos decreases (You et al., 2010), which may be detrimental to reprogramming of donor nuclei and subsequent embryo development.

Chemicals such as *N*-benzyloxycarbonyl-leucylleucyl-Leucinal (MG132) have been found to regulate MPF activity in oocytes (Gao et al., 2005; Kikuchi et al., 2000). MG132 is a reversible proteasomal inhibitor that maintains MPF activity by preventing proteasomal degradation of cyclin (You et al., 2010). Postactivation treatment with MG132 improves the development of murine (Gao et al., 2005), bovine (Tani et al., 2007), and porcine (You et al., 2010) SCNT embryos. In addition, MG132 treatment after simultaneous fusion and activation increased the overall pregnancy rate in pigs (Whitworth et al., 2009). Thus, as first proposed by Sutovsky and Prather (2004), we further hypothesized that the proteasome inhibitor MG132 would inhibit premature degradation of MPF and/or other reprogramming-related factors, which would facilitate nuclear remodeling and reprogramming and improve the developmental competence of SCNT embryos. Although our laboratory has demonstrated that Scriptaid (Zhao et al., 2009, 2010) and MG132 (Whitworth et al., 2009) treatment alone improved cloning efficiencies, the combined treatment using both inhibitors has not been evaluated. The objective of the present study was to determine the effects of combined Scriptaid and MG132 treatment on early embryo development *in vitro* and on full-term developmental competency of SCNT embryos after embryos were transferred to synchronized gilts.

Materials and Methods

Media and reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The cell culture medium was Dulbecco's modified Eagle medium (DMEM) supplemented with 15% (vol/vol) fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and gentamicin (APP Pharmaceutical, Schaumburg, IL, USA). The embryo culture medium was porcine zygote medium 3 (PZM3) (108.0

mmol/L NaCl, 10.0 mmol/L KCl, 0.35 mmol/L KH₂PO₄, 0.4 mmol/L MgSO₄·7H₂O, 25.07 mmol/L NaHCO₃, 0.2 mmol/L sodium-pyruvate, 2.0 mmol/L Ca(lactate)₂·5H₂O (Fisher Scientific, Pittsburg, PA, USA), 1.0 mmol/L glutamine, 5.0 mmol/L hypotaurine, 20 ml/L Eagle basal medium amino acid solution, 10 ml/L modified Eagle medium amino acid solution, 0.05 mg/mL gentamicin, and 3 mg/mL bovine serum albumin (BSA, pH 7.3). Stock solution of Scriptaid (S7817, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at 1 mM and stored at -20°C. A stock solution of MG132 (Z-Leu-Leu-Leucinal, Enzo Life Sciences, Plymouth Meeting, PA, USA) was dissolved in 100% ethanol at 10 mM and stored at -80°C. Scriptaid and MG132 were added to the embryo culture medium in specific amounts according to the protocol of experiment.

Porcine oocytes and in vitro maturation

Oocytes from sows were purchased from Applied Reproductive Technology, LLC (Madison, WI, USA) and shipped overnight in maturation Medium No. 1 [medium 199 supplemented with 25 mM HEPES, 22 µg/mL pyruvate, 1 µg/mL insulin, 10 ng/mL epidermal growth factor (EGF), 10 µg/mL porcine follicle-stimulating hormone (FSH), 0.1 mg/mL cysteine, 20 µg/mL gentamicin, and 10% (vol/vol) porcine follicular fluid) at 38.5°C (Zhao et al., 2009). Twenty-two hours after maturing in Medium No. 1, the oocytes were moved to Medium No. 2 (same maturation medium without HEPES and FSH). After a total of 40 h of maturation, oocytes were vortexed in 0.05% hyaluronidase in HEPES-buffered Tyrode medium containing 0.01% polyvinyl alcohol (PVA) for 4 min to remove the cumulus cells. Matured oocytes having an extruded first polar body with uniform cytoplasm were used for SCNT.

Primary cells establishment and donor cell preparation

Three lines of genetically modified and one line of wild-type, fetal-derived donor cells were used to measure *in vivo* development. One of the genetically modified cell lines (line A) was used to monitor *in vitro* development. These cell lines were established as previously described (Lai and Prather, 2003; Whitworth et al., 2009). Primary cultures were frozen in FBS containing 10% (vol/vol) DMSO. The day before nuclear transfer, fibroblasts were thawed and cultured in four-well plates overnight. A suspension of single cells was prepared by trypsinization of the cultured cells, followed by resuspension in manipulation medium (25 mM HEPES-buffered TCM-199 with 3 mg/mL BSA) before SCNT.

SCNT and embryo culture

SCNT was performed as previously described (Zhao et al., 2010). Briefly, denuded oocytes were enucleated by aspirating the first polar body and MII chromosomes and a small amount of surrounding cytoplasm using a beveled glass pipette with an inner diameter of 17–20 µm. The medium for micromanipulation consisted of HEPES-buffered TCM-199, 0.3% BSA, and 7.5 mg/mL of cytochalasin B for enucleation and no cytochalasin B for injection.

A single intact donor cell was injected into the perivitelline space and placed adjacent to the recipient cytoplasm.

Oocyte–donor cell couplets were placed into embryo culture medium PZM3 (Yoshioka et al., 2002) until fusion and activation. The fusion of oocyte–donor cell couplets and cloned embryo activation was accomplished with two direct current pulses of 1.2 kV/cm for 30 μ sec with a 1-sec interval between provided by a BTX Electro Cell Manipulator 200 (BTX, San Diego, CA, USA) in fusion medium (0.3 M mannitol, 1.0 mM CaCl_2 , 0.1 mM MgCl_2 , and 0.5 mM HEPES, pH 7.3). Oocytes were then incubated for 30 min in PZM3 and evaluated for fusion under a stereomicroscope. Only the fused embryos were cultured in 500 μ L of PZM3 at 38.5°C in humidified atmosphere of 5% CO_2 air.

Postactivation treatment and embryo culture

Immediately after electrical activation, the SCNT embryos were treated with 500 nM Scriptaid and 0, 1, or 10 μ M MG132 for 2 h. Embryos were washed for three times and treated with 500 nM Scriptaid for another 14–15 h. After treatment, embryos were washed three times in fresh PZM3 and cultured at 38.5°C in 5% CO_2 in humidified air until day 6. Cleavage and blastocyst formation were evaluated on days 2 and 6, respectively, with the day of SCNT designated as day 0.

Embryo transfer

For embryo transfer, SCNT embryos were treated in the same way as described above, but with two MG132 concentrations at 0 or 1 μ M. On the day after treatment, embryos were transferred as described below. The production of SCNT embryos was undertaken twice a week, and embryos were treated with 0 (treatment 1, Scriptaid only) or 1 μ M MG132 (treatment 2, MG132 and Scriptaid treatments combined) randomly on one of the 2 days. Day-1 SCNT embryos were transferred into the oviducts of surrogates on day 0 or 1 of the estrous cycle. Day 0 is the first day of estrus for the recipient gilts. Pregnancy was diagnosed by ultrasound examination at days 28 and 60 of pregnancy. The cloned piglets were delivered by cesarean section on day 117 of gestation if they did not start to farrow on their own and were hand raised. All animal procedures were performed in accordance with Animal Care Guidelines and with the approval of the University of Missouri Animal Care and User Committee.

Number of nuclei in blastocysts

On day 6 of culture, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and mounted on slides in mounting medium containing 4,6-diamidino-2-phenylindole (DAPI). Slides were analyzed using an epifluorescence microscope (Nikon, Natick, MA, USA) equipped with a digital camera. Images were captured and processed using Nikon NIS-Elements software.

Statistical analysis

All dependent variables were analyzed for normality using the Wilk–Shapiro test (Statistical Analysis Systems, 2008). Data for the dependent variables, percent oocyte meiotic maturation, percent cleavage, percent blastocyst formation, number of nuclei within the blastocysts, percent oocyte–donor cell fusion, number of pigs born, piglet body weight at birth, and cloning efficiency were analyzed by the GLM procedure of SAS® software (v9.2) (Statistical Analysis Systems, 2008), with treatment and day in class as the main effects. Fisher's exact chi-squared test was used to analyze pregnancy rate between treatment groups at days 28, 60, and at delivery.

Results and Discussion

The effects of postfusion treatment with different dosage of MG132 (0, 1, and 10 μ M) on embryo development *in vitro* were studied and summarized in Table 1. There was no difference in the percentage of cleaved embryos and in cell number of day 6 blastocyst-stage embryos. Compared to the 0 μ M MG132 control group, the fraction of SCNT embryos developing into blastocysts in the 1 μ M MG132 group was numerically higher ($25.1 \pm 4.7\%$ vs. $18.5 \pm 4.7\%$), but they were not different ($p=0.3$). Interestingly, treatment with 1 μ M MG132 showed a significantly higher percentage of blastocyst formation than the 10 μ M MG132 group ($p<0.05$). Thus, for the embryo transfer experiment, 1 μ M MG132 was used in the present study, although 10 μ M was used in a study by Whitworth et al. (2009) where no Scriptaid was introduced. The discrepancy in MG132 concentrations between studies may be caused by the combined treatment of MG132 and Scriptaid in the current study.

To determine the effect of MG132 treatment on term development, two MG132 treatment groups (0 and 1 μ M) were included. Percentage of oocyte meiotic maturation, percentage of oocyte–donor cell fusion, percentage of blastocyst formation, estrous stage of surrogate at embryo transfer, and number of embryos transferred were not different between the two groups (Table 2). Pregnancy was monitored by ultrasound. Sixteen and 9 surrogates (72.7% and 40.9%) from the treatment 1 control (Scriptaid only) group and 10 and 5 from the treatment 2 (MG132+Scriptaid) group (58.8% and 29.4%) were pregnant by days 28 and 60 of pregnancy, respectively (Table 2 and Supplementary Table 1) (Supplementary Data are available at www.liebertpub.com/cell/). One surrogate from the treatment 1 group returned at 110 days of pregnancy, resulting in 8 and 5 term pregnant sows for treatment 1 and 2 groups, respectively. But the pregnancy rate was not different at any pregnancy stage between the two groups. Eight treatment 1 control pregnant surrogates delivered 0, 2, 2, 2, 3, 4, 5, and 6 pigs by cesarean sections

TABLE 1. PERCENTAGE OF CLEAVED EMBRYOS, BLASTOCYST FORMATION, AND CELL NUMBER OF DAY-6 EMBRYOS

Treatment	n	Cleaved (%)	Blastocysts (%)	Cell number
Scriptaid	107	66 ($58.1 \pm 7.2\%$)	22 ($18.5 \pm 4.7\%$) ^{a,b}	23.1 ± 2.2
1 μ M MG132+Scriptaid	110	73 ($62.7 \pm 7.2\%$)	29 ($25.1 \pm 4.7\%$) ^a	24.0 ± 2.0
10 μ M MG132+Scriptaid	111	74 ($62.5 \pm 7.2\%$)	17 ($14.0 \pm 4.7\%$) ^b	24.5 ± 2.3

^{a,b}Means with different letters differ ($p<0.05$).

TABLE 2. PERCENTAGE OF OOCYTE MATURATION, FUSION, SURROGATES ESTROUS CYCLE AT TRANSFER, TOTAL EMBRYOS TRANSFERRED, DAY 28 AND 60 PREGNANCY RATE IN THE TREATMENT 1 (SCRIPTAID ALONE) AND TREATMENT 2 (MG132+SCRIPTAID) GROUPS

Treatment	Oocyte maturation, (%)	Fusion rate (%)	Surrogate estrous cycle (day)	No. of transfers	No. of embryos per transfer	Day-28 pregnancy rate	Day-60 pregnancy rate
Treatment 1	55.3±2.2	87.7±1.0	0.4±0.1	22	188.0±8.9	16 (72.7%)	9 (40.9%)
Treatment 2	58.4±2.5	86.9±1.1	0.6±0.1	17	196.0±10.1	10 (58.8%)	5 (29.4%)

(average litter size=3) and 5 sows in the MG132+Scriptaid treatment 2 group delivered 0, 1, 3, 5, and 5 pigs (average litter size=2.8). There was no difference in litter size. The piglet body weight at birth (703.7±69.4 vs. 767.5±87.4 g), average term-sized pigs per embryo transfer (1.09±0.38 vs. 0.82±0.44), and cloning efficiency (pigs born out of total embryos transferred, 0.50±0.17% vs. 0.36±0.20%) were not different between the treatment 1 control and MG132+Scriptaid treatment 2 groups. Furthermore, four cell lines were tested in the current study, and no difference was observed among the cell lines in pregnancy rate and numbers of pigs delivered. Taken together, combined treatments of MG132 and Scriptaid did not improve overall term development compared to Scriptaid treatment alone.

It was demonstrated that protease activity affects some components involved in nuclear reprogramming, such as the transition of H1 linker histones in the mouse (Gao et al., 2005). However, the MG132 treatment did not improve overall term development, despite the fact that MG132 treatment significantly enhanced blastocyst formation as reported by You et al. (2010), and it was numerically higher in the present study. This suggests that, although MG132 treatment of SCNT embryos might help to overcome the suboptimal culture conditions *in vitro*, it could not overcome other inherent limitations. The timely degradation of some proteins might be an essential prerequisite for successful development. Inhibiting the degradation process, even for a brief period, might have activated stress kinases and induced apoptosis (Ishizawa et al., 2004), thus offsetting the advantage of permitting a prolonged reprogramming period. Whitworth et al. (2009) demonstrated that 10 μ M MG132 treatment increased overall pregnancy rate, the current study did not show a similar increase when 1 μ M MG132 treatment was combined with Scriptaid, which may again be explained by the compound effects.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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