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Porcine somatic cell nuclear transfer using telomerase reverse transcriptase-transfected mesenchymal stem cells reduces apoptosis induced by replicative senescence

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ABSTRACT Mesenchymal stem cells (MSCs) have been widely used as donor cells for somatic cell nuclear transfer (SCNT) to increase the efficiency of embryo cloning. Since replicative senescence reduces the efficiency of embryo cloning in MSCs during *in vitro* expansion, transfection of telomerase reverse transcriptase (TERT) into MSCs has been used to suppress the replicative senescence. Here, TERT-transfected MSCs in comparison with early passage MSCs (eMSCs) and sham-transfected MSCs (sMSCs) were used to evaluate the effects of embryo cloning with SCNT in a porcine model. Cloned embryos from tMSC, eMSC, and sMSC groups were indistinguishable in their fusion rate, cleavage rate, total cell number, and gene expression levels of *OCT4*, *SOX2* and *NANOG* during the blastocyst stage. The blastocyst formation rates of tMSC and sMSC groups were comparable but significantly lower than that of the eMSC group ($p < 0.05$). In contrast, tMSC and eMSC groups demonstrated significantly reduced apoptotic incidence ($p < 0.05$), and decreased *BAX* but increased *BCL2* expression in the blastocyst stage compared to the sMSC group ($p < 0.05$). Therefore, MSCs transfected with telomerase reverse transcriptase do not affect the overall development of the cloned embryos in porcine SCNT, but enables to maintain embryo quality, similar to apoptotic events in SCNT embryos typically achieved by an early passage MSC. This finding offers a bioengineering strategy in improving the porcine cloned embryo quality.

Keywords: apoptosis, mesenchymal stem cell, porcine, somatic cell nuclear transfer, telomerase reverse transcriptase

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is an essential technique in biomedical research for the production of transgenic animals, conservation of endangered species, and establishment of embryonic stem cells (ESCs) (Lee et al., 2014). SCNT has been used to clone diverse animals such as pigs, mice, cattle, and non-human primates (Gouveia et al., 2020). Since pigs are not only favored for mass production but also due to their anatomical and physiological similarities to humans, for the establishment of disease models, production of bio-organs, and development

of new drug biotechnologies, they have been considered a highly valuable animal species in the field of animal cloning (Jeon et al., 2020). However, the cloning efficiency of pigs using SCNT is still relatively low in comparison to the high demand for cloned pigs in biomedical research. To overcome this, studies have aimed to reveal the molecular mechanisms underlying each stage of cloning such as donor cell manipulation, *in vitro* maturation (IVM) of oocytes, *in vitro* embryo culture, embryo transfer, and the preparation of surrogates (Ock et al., 2007; Lee et al., 2014).

In particular, the method of donor cell manipulation including the use of various cell types and transfected cells is a very efficient method as it does not affect the existing SCNT system. The donor cell used for SCNT takes advantage of the ability of the ooplasm to archive reprogramming and development into an embryo. However, since the reprogramming ability of the ooplasm is limited, researchers have attempted to use undifferentiated stem cells as donor cells which could be advantageous for successful reprogramming (Meissner and Jaenisch, 2006). However, stem cells such as ESCs or induced pluripotent stem cells (iPSCs) in a highly undifferentiated status show high proliferation rate making it difficult to synchronize recipient oocytes and donor cell cycles (Yuan et al., 2014). Further, expensive supplements such as leukemia inhibitory factor and basic fibroblast growth factor and extensive efforts are required to produce and maintain pluripotent stem cells which is often unpractical. On the contrary, the isolation and culture methods for MSCs are simple and easy, allowing the resources to focus on embryo manipulation (Lee et al., 2014). To select transfected cells or to secure large quantities of identical donor cells, long-term *in vitro* culture is required. However, MSCs could also enter replicative senescence known as Hayflick Limit similar to other somatic cells, where their proliferation ability and differentiation capacity decrease as expansion progresses (Turinetto et al.,2016). When these aged MSCs were used as donor cells for SCNT, cloning efficiency and embryo quality were reduced (Lee et al., 2014). Therefore, for the consistent production of cloned animals, a method of mass propagation while maintaining the stemness of MSCs is required.

To overcome the proliferation limit and/or to revert MSC aging, various studies have been conducted on the manipulation of telomerase reverse transcriptase (TERT), p53, octamer-binding transcription factor 4 (*OCT4*), sex determining region Y-box 2 (*SOX2*), and RB transcriptional corepressor 1 genes (Liu et al., 2013). In particular, TERT is an essential component of telomerase required to maintain telomere length; it has very low or no expression in most somatic cells, but is highly expressed in proliferative cells including stem cells and cancer cells (Hiyama and Hiyama, 2007). Therefore, TERT-transfection has been used to immortalize cells *in vitro*. Telomerase imparts genomic stability and cell viability to eukaryotic cells and is closely related to DNA damage-induced apoptosis (Del Bufalo et al., 2005). Although apoptosis can occur during normal process of embryo development, it increases when embryos are exposed to stress conditions or in the presence of cells that are unsuitable for normal development promoting embryonic death (Hao et al., 2003). Thus, apoptosis is an important criterion used to evaluate embryo quality (Lee et al., 2019). However, the effect of TERT-transfected MSCs (tMSCs) as donor cells on the apoptosis of cloned porcine embryo is currently unknown.

Therefore, this study was carried out to reveal the characteristics of porcine cloned embryos using tMSCs as donor cells compared to early passage MSCs (eMSCs) and sham-transfected MSCs (sMSCs). For the evaluation of embryos, fusion, cleavage, and blastocyst formation rates and the total cell number, apoptosis incidence, and expression level of early transcription factors and apoptosis-regulating genes in blastocyst stages were analyzed.

MATERIALS AND METHODS

All animal handling and experiments in this study were performed under guidance of the Research Ethics Committee of Gyeongsang National University Animal Center for Biomedical Experimentation (GNU-130308-P0022).

Chemicals and media

Unless otherwise stated, all chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Donor cell preparation

Bone marrow extracts were collected from 2-month-old male miniature pigs and MSC progenitors were isolated using density gradient centrifugation as previously de-

scribed (Jeon et al., 2019). MSC progenitors were seeded into 6-well plates with advanced Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% GlutaMax, 100 U/mL penicillin, and 100 μg/mL streptomycin. MSC progenitors were incubated at 38℃ with 5% CO2. The culture medium was changed every 2 days. When MSCs reached 70-80% confluence, cells were digested using 0.25% trypsin-EDTA and passaged at a 1:4 ratio. Passage 2 MSCs were transfected with pBabe-hygro-hTERT (Addgene, Watertown, MA, USA) and pBabe-hygro (Addgene) for the transfection of hTERT and control (sham), respectively, using electroporation (Neon, Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions. Subsequently, transfected MSCs were selected using 200 μg/mL Hygromycin B. TERT-transfected MSCs (tMSCs), early passage (passage 3-5) MSCs (eMSCs), and sham-transfected MSCs (sMSCs) were used as donors for cloning.

Oocyte preparation

Ovaries were collected from pre-pubertal pigs at a local slaughterhouse. Cumulus-oocyte complexes (COCs) were aspirated from 3-6 mm (diameter) follicles using an 18-gauge needle attached to a vacuum. COCs with uniform ooplasm and multilayered cumulus cells were used for *in vitro* maturation (IVM) and were incubated for 22 h in tissue culture medium-199 (TCM-199) supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL epidermal growth factor, 0.57 mM cysteine, 2.5 mM sodium pyruvate, 1 mM L-glutamine, 0.5 μg/mL follicle-stimulating hormone (FSH), and 0.5 μg/mL luteinizing hormone (LH). Then, COCs were further matured for an additional 20 h in the same medium without FSH and LH supplementations. IVM was conducted at 38.5℃ in a humidified atmosphere of 5% CO2 in air. COCs were dissociated through gentle pipetting for 1 min in Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium, supplemented with 0.1% hyaluronidase. Oocytes without cumulus cell but with a uniform ooplasm, intact cytoplasmic membrane, and visible first polar body (PB1) were used for embryo production.

Embryo production

Production of cloned embryos was performed using a previously described protocol with minor modifications (Jeon et al., 2012). Oocytes in the metaphase II stage were enucleated by aspirating PB1 and a small volume of adjacent ooplasm in HEPES-buffered TCM199 supplemented with 7.5 μg/mL cytochalasin B (CCB), 0.3% bovine serum albumin, and 12 mM sorbitol. Enucleation was confirmed by staining with 0.5 μg/mL Hoechst-33342 for 2 min at room temperature and examining cells under a fluorescence microscope. Donor cells were transferred into the perivitelline space of the enucleated oocyte. Oocyte-donor cell couplets were fused and activated simultaneously with two simultaneous DC pulses of 1.8 KV/cm for 30 μsec using an BTX Electro Square porator (ECM 830, BTX, Inc., San Diego, CA, USA) in 0.28 M mannitol solution containing 0.1 mM MgSO4, 0.05 mM CaCl2, and 0.01% BSA. Couplets were cultured in porcine zygote medium 5 (PZM5) supplemented with 7.5 μg/mL CCB for 3 h in PZM5 at 38.5℃ in a humidified atmosphere of 5% CO2 in air. Fused eggs were selected and further cultured in PZM5 without CCB. Cleavage rate and blastocysts formation rate were evaluated on Day 2 and 7, respectively.

Embryo evaluation of blastocysts stage

Total cell number and incidence of apoptotic body formation of blastocysts were evaluated using the TUNEL assay (In Situ Cell Death Detection Kit, Roche, Germany) using a previously described protocol (Jeon et al., 2012). Briefly, on day 7, blastocysts were fixed overnight in 4% formaldehyde at 4℃, then permeabilized with 0.5% Triton X-100 in 0.1% sodium citrate buffer for 1 h. Blastocysts were incubated in the TUNEL reaction cocktail at 37℃ for 1 h in the dark, and further incubated with RNase A (50 μg/mL) for 1 h, followed by counterstaining with propidium iodide (50 μg/mL) for 1 h. Blastocysts were mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA, USA) medium and examined under a fluorescence microscope (Nikon Ti-U, Japan).

Gene expression in blastocyst stage

Gene expression analysis was performed with minor modifications to a previously described protocol (Lee et al., 2017). Briefly, total RNA was extracted from pools (five replicates) of three blastocysts using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and residual genomic DNA was removed through RNase Free DNase (Qiagen) treatment for 15 min in room temperature. Since a low amount of total RNA was extracted from the 3 blastocysts which was below the usable concentration range of the

UV spectrophotometer, RNA could not be quantified. The RNA was converted to cDNA using the Sensiscript Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was carried out on a Rotor Gene Q qRT-PCR instrument (Qiagen) with RT2 SYBR Green ROX qPCR Mastermix (Qiagen) combined with 2 μL of cDNA and 0.5 μM forward and reverse primers (Table 1). Amplification was carried out using the following conditions: 95℃ for 10 min; 40 cycles at 95℃ for 15 s and 60℃ for 60 s; 60℃ to 95℃ at 1℃/s; 40℃ for 30 s. Ct values were analyzed using the Rotor-Gene Q Series Software (Qiagen) and *Succinate dehydrogenase complex, subunit A* was used for relative quantification of transcript levels.

Statistical analysis

Differences between groups were evaluated using the one-way analysis of variance (ANOVA) test followed by the Games-Howell post-hoc test using Prism 7 (GraphPad Inc., La Jolla, CA, USA). Data were expressed as mean ± standard error of mean, and $p < 0.05$ was considered statistically significant.

RESULTS

Embryo development: reconstruction and development rate of embryos

A total of 217, 204, and 233 cloned embryos were produced using tMSCs, eMSCs, and sMSCs as donor cells, respectively. Cloned embryos were used for subsequent analysis (Table 2). The fusion rate and cleavage rate of tMSC, eMSC, and sMSC groups did not differ. The blastocyst rate of the tMSC group did not differ from the sMSC group, however, the blastocyst rates of the tMSC group and sMSC group were significantly lower than eMSC group ($p < 0.05$).

Apoptosis and total cell number at the blastocyst stage

The total cell number and the apoptotic incidence were analyzed for evaluation of the embryo quality in the blastocyst stage (Fig. 1A). The total cell numbers of the tMSC, eMSC, and sMSC groups were not statistically different (Fig. 1B). The apoptotic incidence of the tMSC and eMSC groups was similar, however, significantly lower than that of sMSC group ($p < 0.05$) (Fig. 1C).

Table 1. Primer sequence for gene expression analysis

Gene	Sequence 5'–3'	Amplicon size (bp)	Reference
Octamer-binding transcription factor 4 (OCT4)	F: AGTCCCAGGACATCAAAGCG R: CCTCCCAAAGAGAACCCCC	129	NM_001113060.1
Sex determining region Y-box 2 (SOX2)	F: AGGACCAGCTGGGCTATCCG R: GCCCTGCTGCGAGTAGGACA	170	NM_001123197.1
NANOG homeobox (NANOG)	F: AACCAAACCTGGAACAGCCAGAC R: GTTTCCAAGACGGCCTCCAAAT	152	NM_001129971.1
B-cell lymphoma 2 (BCL2)	F: CTCCTGGCTGTCTCTGAAGG R: CCCGTGGACTTCACTTATGG	95	AJ606301.1
BCL2 associated X (BAX)	F: AAGCGCATTGGAGATGAACT R: AAAGTAGAAAAGCGCGACCA	147	XM_003121700.2
Succinate dehydrogenase complex, subunit A (SDHA)	F: CACACGCTTTCCTATGTCGATG R: TGGCACAGTCAGCTTCATTC	94	XM 003362140.1

Table 2. Fusion rate and developmental potential of porcine embryos cloned with tMSCs, eMSCs and sMSCs

Embryo groups	Oocytes used	mean% ± SEM (no. of embryos)		
		Fusion	Cleavage	Blastocyst
tMSC	217	94.0 ± 1.5 (204)	77.5 ± 1.8 (168)	13.8 ± 0.4 (30)
eMSC	204	94.7 ± 1.2 (193)	78.1 ± 1.0 (159)	15.7 ± 0.3 (32)*
sMSC	233	92.7 ± 1.3 (216)	76.6 ± 2.3 (179)	12.6 ± 1.2 (29)

Embryo groups: tMSC, embryos cloned with telomerase reverse trancriptase-transfected MSCs; sMSC, embryos cloned with sham-transfected MSCs; eMSC, embryos cloned with early passage MSCs. Asterisk indicates significant difference ($p < 0.05$). 6 replicates.

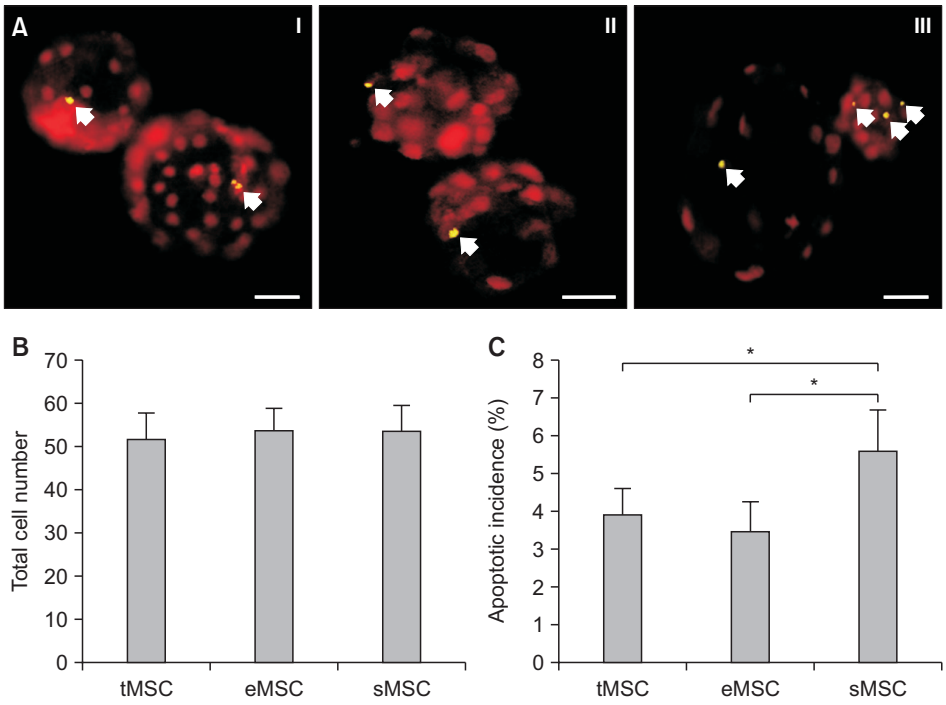

Fig. 1. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and propidium iodide (PI) staining of blastocyst stage embryos cloned with tMSC (I), eMSC (II) and sMSC (III). (A) Representative fluorescent microscope images. Apoptotic bodies and nuclei were labeled with TUNEL staining (white arrows) and PI (red), respectively. Scale bar = 50 μm. (B) Total cell number. (C) Apoptotic incidence. Graphs were presented as mean ± SEM. tMSC: telomerase reverse transcriptase-transfected MSC, sMSC: sham-transfected MSC, eMSC: early passage MSC. Asterisk indicates significant difference ($p < 0.05$). 4 replicates.

Gene expression analysis during the blastocyst stage

Apoptosis-related genes and early transcription factors were selected for the evaluation of essential gene expressions during blastocyst stage. In relation to apoptosis, *BAX* expression in tMSC and eMSC groups was not different, however, both groups showed lower *BAX* expression than sMSC group ($p < 0.05$). The *BCL2* expression in tMSC and eMSC groups was not different, however, both groups showed higher *BCL2* expression than sMSC group ($p < 0.05$). The gene expression of early transcription factors such as *OCT4*, *SOX2* and *NANOG* did not differ among tMSC, sMSC, and eMSC groups (Fig. 2).

DISCUSSION

This study was conducted to reveal the effect of using TERT-transfected MSCs as donor cells on porcine cloned embryos. The cloned embryos using TERT-transfected MSCs were not changed in their fusion rate, cleavage rate, and total cell number as well as in their expression of pluripotent genes including *OCT4*, *SOX2*, and *NANOG* at blastocyst stage. However, they showed reduced apoptotic incidence and changes in the expression of apoptosis-related genes at blastocyst stage. Therefore, TERT transfection of MSCs used for porcine cloning did not affect embryo development but affected the apoptosis-related quality of blastocysts.

Fusion of donor cell with oocyte membrane in SCNT is a necessary process for transferred DNA to be exposed to the ooplasm. This process is greatly affected by the donor cell membrane characteristics along with the electric field conditions (Daniel et al., 2008). In this study, the fusion rate did not differ according to the type of donor cells. This may be because there is no strong association between the cellular function of TERT and the synthesis as well as maintenance of proteins and lipids constituting the cell membrane (Hiyama and Hiyama, 2007). However, although there was no significant difference in fusion rate among the tMSC, sMSC, and eMSC groups, the fusion rate of the sMSC group was lower than that of tMSC and eMSC groups. This pattern was probably due to altered membrane properties resulting from enlarged cell size from replicative senescence or the accumulation of oxidative stress due to long-term *in vitro* culture (Zaim et al., 2012).

In terms of developmental rate, the cleavage rate was not affected by TERT transfection or replicative senescence. This was similar to the results from our previous study which showed that there was no difference in cloned embryos using MSCs of different aging levels as donors or cloned embryos using *OCT4*- or *SOX2*-transfected MSCs as donor cells, including parthenote embryos (Lee et al., 2014). The blastocyst rate of tMSC

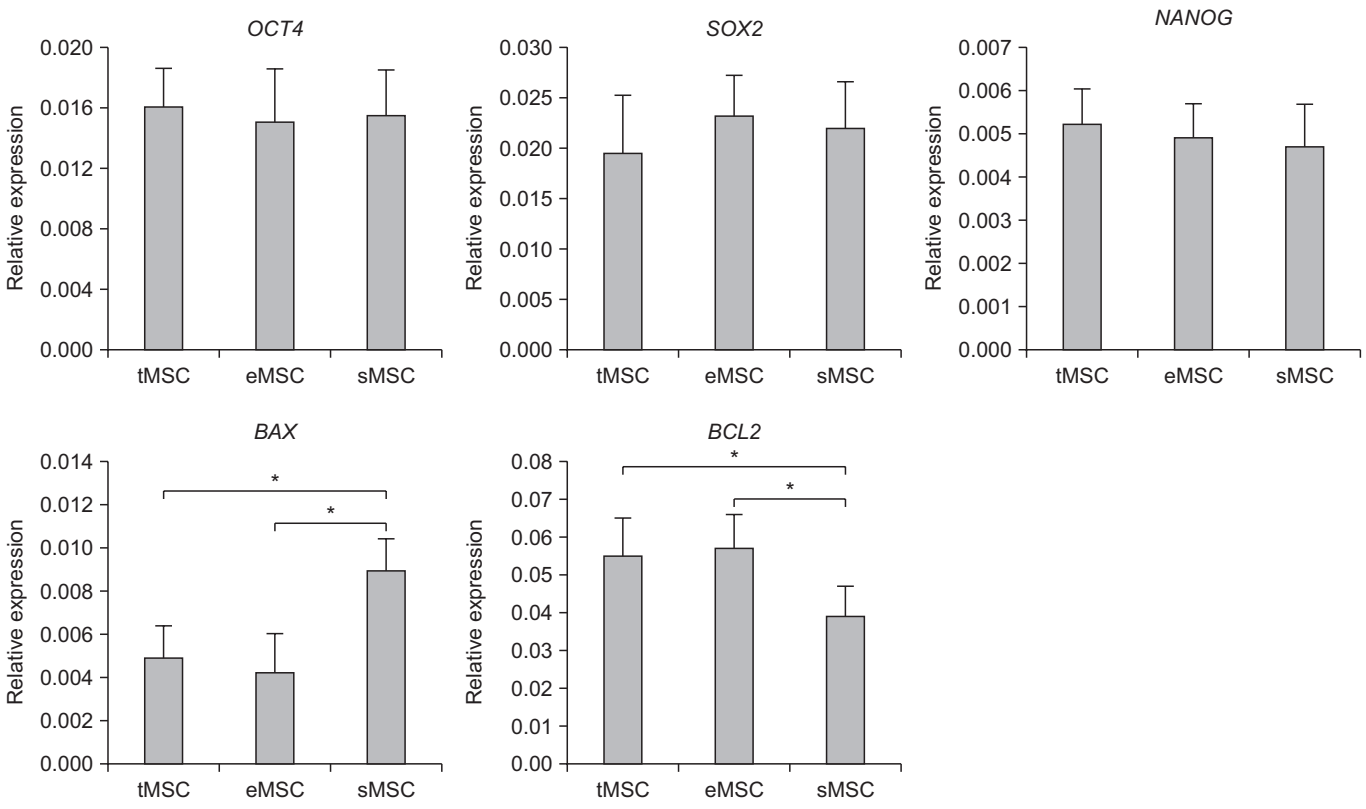

Fig. 2. Gene expression analysis of blastocyst stage embryos cloned with tMSC, eMSC, and sMSC. tMSC: telomerase reverse transcriptase-transfected MSC, sMSC: sham-transfected MSC, eMSC: early passage MSC. Graphs are presented as mean ± SEM. Asterisk indicates significant difference ($p < 0.05$). 5 replicates.

group, although not significant, was higher than that of sMSC group, a sham control that was passaged in the same manner as tMSC without TERT transfection. These observations are in line with previous studies where increased passage number of MSCs linked to occurrence of replicative senescence causing a decrease in proliferation ability, differentiation capacity, and cloning efficiency by SCNT (Zaim et al., 2012; Lee et al., 2014). However, considering that the blastocyst rate of the tMSC group was significantly lower than that of the eMSC group, the TERT transfection may still not be sufficient to maintain the characteristics of early passage MSCs. The deletion of *p53* affects TERT amplification. Additionally simultaneous overexpression of TERT and knockdown of p53 in MSCs makes them immortalize. Therefore, additional immortalization factors such as *c-Myc*, and *CDK4* may also need to be used for successful maintenance of early passage MSCs characteristics (Kanaya et al., 2000; Liu et al., 2013). On the contrary, a previous study reported that using MSCs as donor cells may decrease the variation of cloning efficiency compared fibroblasts However, this study showed that the variation of blastocyst rate in the tMSC group was similar to that of the eMSC group and was lower than that of sMSC group, showing that TERT transfection has a positive effect on development of cloned embryos (Kumar et al., 2012).

The total cell number, apoptotic incidence, and expression of early transcription factors in the blastocyst stage are important criteria for evaluating embryo quality because they affect the ratio of the inner cell mass to the trophectoderm as well as embryo development during post-implantation stages (Ock et al., 2007; Lee et al., 2019). In this study, the total cell number and gene expression of early transcription factors such as *OCT4*, *SOX2*, and *NANOG* were not altered among three groups studied, whereas the apoptotic incidence and the expression of apoptosis related genes such as *BAX* and *BCL2* in tMSC group was more similar to the eMSC group than the sMSC group. Therefore, we found that the advantage of using TERT-transfected MSCs as SCNT donor cells was that it improves the apoptosis-related quality of cloned embryos. These findings are similar to previous studies

where the expression of apoptotic gene *BAX* was lower while anti-apoptotic gene *BCL2* was higher in embryos cloned using MSCs as donor cells compared to the embryos cloned with fibroblasts (Kumar et al., 2007; Lee et al., 2014). Apoptosome, formed by cytochrome C released from the mitochondrial membrane during DNA damage, activates caspase-3 to begin apoptosis (Hao et al., 2003; Del Bufalo et al., 2005). Telomerase prevents DNA damage by preventing telomere shortening that occurs during DNA replication through telomere synthesis (Hiyama and Hiyama, 2007). Therefore, in this study, TERT-transfected MSCs may have improved DNA stability and reduced apoptosis from replicative senescence. However, this study was not consistent with a previous study that showed an increased apoptosis causes a decrease in the total cell number during the blastocyst stage (Kumar et al., 2007; Mulligan et al., 2012). This is probably due to the dynamic changes in the pattern of expression of genes according to the embryo development stages or the time gap of gene expression and cellular functions in the embryos (Kumar et al., 2012; Gouveia et al., 2020). Therefore, in order to better understand the effect of TERT transfection on embryo quality, further studies are needed to focus on the apoptosis-related gene expression during each developmental stage of pre-implantation as well as evaluating embryo development in post-implantation stages and in cloned offsprings.

In conclusion, the present study revealed that using TERT-transfected MSCs as a donor cell for SCNT reduces apoptosis induced by replicative senescence of donor cells. This result will be useful in establishing a strategy for securing large quantities of high-quality donor cells for SCNT and improving the quality of cloned pigs.

CONFLICSTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conceptualization: RJ, GJR
Data curation: RJ
Formal analysis: RJ
Funding acquisition: GJR
Investigation: RJ, GJR
Methodology: RJ, GJR
Project administration: GJR
Resources: GJR
Software: RJ
Supervision: GJR
Validation: RJ, GJR
Visualization: RJ
Writing - original draft: RJ
Writing - review & editing: GJR

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