Overexpression of miR-101-2 in donor cells improves the early development of Holstein cow somatic cell nuclear transfer embryos

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ABSTRACT

Accumulating studies have suggested that microRNA play a part in regulating multiple cellular processes, such as cell proliferation, apoptosis, the cell cycle, and embryo development. This study explored the effects of miR-101-2 on donor cell physiological status and the development of Holstein cow somatic cell nuclear transfer (SCNT) embryos in vitro. Holstein cow bovine fetal fibroblasts (BFF) overexpressing miR-101-2 were used as donor cells to perform SCNT; then, cleavage rate, blastocyst rate, inner cell mass-to-trophectoderm ratio, and the expression of some development- and apoptosis-related genes in different groups were analyzed. The miR-101-2 suppressed the expression of inhibitor of growth protein 3 (*ING3*) at mRNA and protein levels, expedited cell proliferation, and decreased apoptosis in BFF, suggesting that *ING3*, a target gene of miR-101-2, is a potential player in this process. Moreover, by utilizing donor cells overexpressing miR-101-2, the development of bovine SCNT embryos in vitro was significantly enhanced; the apoptotic rate in SCNT blastocysts was reduced, and the inner cell mass-to-trophectoderm ratio and *SOX2*, *POU5F1*, and *BCL2L1* expression significantly increased, whereas *BAX* and *ING3* expression decreased. Collectively, these findings suggest that miR-101-2 promotes BFF proliferation and vitality, reduces their apoptosis, and improves the early development of SCNT embryos.

Key words: somatic cell nuclear transfer, *ING3*, apoptosis, embryo development

INTRODUCTION

Since the time the first successful clone, Dolly the sheep, was created, many mammalian species have been successfully cloned by somatic cell nuclear transfer (SCNT; Wakayama et al., 1998; Baguisi et al., 1999; Woods et al., 2003). Somatic cell nuclear transfer technology brings good prospects for propagation of elite genetic merit livestock. Somatic cell nuclear transfer is also a promising technology because of its potential applications in biomedicine (Le et al., 2014). Low efficiency of SCNT clone embryos, however, is a significant barrier to the use of this technology. Many factors have been reported to influence the efficiency of SCNT, of which nuclear donor cells are one of the crucial factors. Tissue specificity, cell type, age, status, and the progression of the cell cycle of donor cells were shown to affect the development of cloned embryos (Inoue et al., 2007).

MicroRNA (miRNA) are small, highly conserved, noncoding RNA that usually suppress gene expression through partially complementary binding to the 3'-untranslated region (UTR) of target gene mRNA (Luo et al., 2013; Agarwal et al., 2015). Researchers found that decreasing miRNA-145 expression could improve the development of SCNT embryos in vitro (Li et al., 2016). Recent reports have also shown that the quality of SCNT embryos was enhanced via using donor cells expressing sperm-borne miR-449b or overexpressing miR-148a (Wang et al., 2017a,b). Furthermore, our previous research demonstrated that miR-125b promotes SCNT-mediated nuclear reprogramming by targeting *SUV39H1* (Zhang et al., 2017). These indicated miRNA play a part in the development of SCNT embryos.

We previously analyzed high-throughput sequencing data for differences between cloned and normal in vitro fertilized (IVF) preimplantation embryos (F. S. Quan, unpublished data) and found that miR-101-2 was aberrantly downregulated in cloned embryos compared with the IVF embryos. From a bioinformatics analysis, it is predicted that *ING3* is a high-ranking target gene

Received May 16, 2018.

Accepted January 22, 2019.

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of miR-101-2, which was abnormally highly expressed in cloned embryos compared with the IVF embryos. Ubiquitously expressed in mammalian tissues, *ING3* is particularly highly expressed in mouse, rhesus monkey, and human oocytes and is one of the candidate oocyte reprogramming factors (Awe and Byrne, 2013). In addition, numerous studies have demonstrated that miR-101 can regulate cell proliferation and apoptosis; it plays different roles in disparate kinds of cells by targeting several genes (Liang et al., 2014; Lin et al., 2014; Zhou et al., 2015). Therefore, it is hypothesized that overexpression of miR-101-2 would enhance the developmental potential of SCNT embryos in vitro.

To test this hypothesis, a stable cell line overexpressing miR-101-2 was established and effects on *ING3* expression, progression of the cell cycle, and apoptosis of bovine fetal fibroblasts (**BFF**) were examined. This stable cell line was subsequently used as a source of nuclear donor cells to produce SCNT embryos, and development rate, total cell numbers, apoptosis-related genes expression, apoptotic rate, and expression of development-related genes were examined.

MATERIALS AND METHODS

Ethics Statement

The experimental procedure followed was approved by the Animal Care and Use Committee of Northwest A&F University, China, and performed in accordance with the animal welfare and ethics guidelines. Bovine ovaries were collected from slaughtered mature cattle at a local abattoir in Xi'an, China. Frozen-thawed spermatozoa were bought from Bright Farming (Shanghai, China).

Reagents

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless specifically indicated otherwise. Disposable, sterile plastic ware was purchased from Corning (Corning, NY).

Experiment Design

The experiments undertaken will be briefly outlined in this section, and the details of each of the procedures undertaken will be described in the sections that follow. Experiment 1 was performed to determine whether miR-101-2 targets *ING3*. Experiment 2 was performed to determine whether miR-101-2 overexpression affects donor cell status and expression of *ING3*, *TP53*, *CDKN1A*, *BAX*, and *BCL2L1* mRNA. Experiment

3 was performed to measure the miR-101-2 expression in 2-cell, 8-cell, and Day-7 blastocysts from IVF, 513-B1-nuclear transfer (**513-B1-NT**; SCNT embryos produced using BFF transfected with empty vectors as donor cells), and **miR-101-NT** (SCNT embryos produced by using BFF with miR-101-2 overexpression as donor cells) treatments. Experiment 4 was performed to examine the effects of miR-101-2 overexpression on the quality of SCNT embryos and the expression of key developmental genes.

Cell Isolation and Culture

The BFF were established from the bovine fetal back skin tissue of approximately 90-d-old Holstein cow fetuses (Pan et al., 2015). Briefly, fetal skin tissue samples were rinsed several times with PBS containing penicillin and streptomycin and then minced into 1-mm³ pieces. Tissues were cultivated for 1 to 2 wk in 60-mm Petri dishes (Corning) containing Dulbecco's modified Eagle's medium/F12 high-glucose (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (**FBS**; Gibco, Grand Island, NY), 100 mg/mL of streptomycin, and 100 IU/mL of penicillin, in saturation humidity with 5% CO₂ at 37°C. When the BFF were at 90% confluence, they were trypsinized, rinsed, and recultivated in several new 60-mm Petri dishes with fresh culture medium for further passaging.

Human HEK293T cells were used for the luciferase reporter assay, and stored at the Key Laboratory of Animal Biotechnology, Ministry of Agriculture, Northwest A&F University, and cultured in Dulbecco's modified Eagle's high-glucose medium containing 10% FBS, in saturation humidity with 5% CO₂ at 37°C.

Vector Construction

The DNA fragments corresponding to the miR-101-2 precursor sequence were amplified by PCR from genomic DNA of BFF and cloned into the *Bam*HI and *Eco*RI restriction sites of pCDH-Promoter-MCS-EF1 Lentivector (CD513B-1, SBI, Mountain View, CA), resulting in the generation of the vector pCDH-miR101. The plasmids Psi-*ING3*-3'-UTR-wild type (**WT**) and Psi-*ING3*-3'-UTR-mutant (**Mut**) were generated by inserting *ING3*-3'-UTR-WT or *ING3*-3'-UTR-Mut into the Psi-check-2 *Xho*I and *Not*I restriction sites, respectively. All constructed plasmids were verified by DNA sequencing. Primer sequences are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15072>), and the sequences of the insert fragments are listed in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-15072>).

Cell Transfection and Clone Selection

Upon reaching 80% confluence on 60-mm Petri dishes, cells were resuspended in transfer buffer, placed in a 4-mm cuvette gap with 6 μ g of pCDH-miR101 or pCDH-513-B9 (empty vector), and then electroporated at 510 V with 3 pulses of 2-ms duration using a BTX Electro Cell Manipulator ECM2001 (BTX, Holliston, MA). The cells were placed in 90-mm Petri dishes to which fresh cell culture medium was added in advance at an average of 5×10^5 cells. After 24 h, the medium containing 2 μ g/mL of Puro (Sigma Aldrich) was replaced by a medium containing 1 μ g/mL of Puro when floating cells constituted approximately 50% of the total. Approximately 7 d later, Puro-resistant clone cells began to emerge and exhibited green fluorescence under UV light. When the clone cells became large enough for transfer, they were moved into new 60-mm Petri dishes and cultured in 500 ng/mL of Puro. Cells were passaged in several new 60-mm Petri dishes or frozen in 10% dimethylsulfoxide and 90% FBS upon reaching 90% confluence.

Luciferase Reporter Assay

For the luciferase reporter assay, 5×10^4 293T cells were plated in each well of a 24-well plate. The cells were co-transfected with 400 ng of pCDH-miR-101-2 or pCDH-513B and 40 ng of Psi-*ING3*-3'-UTR-WT or Psi-*ING3*-3'-UTR-Mut. Using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions, cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (TransGen Biotech, Beijing, China). Relative luciferase activity was normalized to *Renilla* luciferase activity. Transfections were performed in duplicate and repeated at least thrice in independent experiments.

Western Blot Analysis

Western blot was carried out as previously described with minor modifications (Wang et al., 2018). The cells were collected and washed with PBS, and then lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Inst. Biotech, Beijing, China) with 1% PMSF and protease inhibitor on ice for 30 min. A bicinchoninic acid protein assay kit (TransGen Biotech) was used for protein quantification. Proteins were separated by 10% SDS PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were then blocked in 10% nonfat milk (wt/vol in TBST) for 4 h and incubated with primary antibodies anti-ING3 (1:1,000, ab84645;

Abcam, Cambridge, UK) and internal reference anti- β -actin (1:1,000, ab8227; Abcam) overnight at 4°C. After washing 3 times in TBST, membranes were incubated with the secondary antibodies goat anti-rabbit (Beyotime Inst. Biotech, Beijing, China) for 2 h, which followed by washes for 3 times in TBST. After using the chemiluminescence reagent (Beyotime Inst. Biotech), immunoreactive proteins were visualized by autography using SuperSignal West Pico Substrate (Thermo Fisher Scientific, Waltham, MA). All steps were performed at room temperature unless stated otherwise. The gray level of the image was analyzed using Image J (<https://imagej.nih.gov/ij/>) software.

RNA Extraction, Reverse-Transcription PCR, and Quantitative Real-Time PCR

For measurement of the expression of mRNA in BFF, total RNA was extracted using Trizol reagent (TransGen Biotech) according to the manufacturer's instructions. First-strand cDNA were prepared from 1 μ g of RNA using a reverse transcription kit (TransGen Biotech). The miRNA samples were isolated using the mir-Vana miRNA Isolation Kit and reverse-transcribed using the miScript II RT Kit (Qiagen, Hilden, Germany).

For measurement of the expression of mRNA in embryos, a Cells-to-Signal kit (Ambion Co., Austin, TX) was used to isolate total RNA from IVF and SCNT embryos ($n = 18$ embryos per pool) according to the manufacturer's instructions. The M-MLV RT reagent included in the kit was used for reverse transcription. Samples of miRNA ($n = 55$ embryos per pool) were isolated using the miScript Single Cell qPCR Kit (Qiagen) and reverse transcribed using a miScript II RT Kit (Qiagen).

The expression levels of mRNA were measured using SYBR Premix Ex Taq (TaKaRa, Dalian, China) according to the manufacturer's instructions. The GAPDH was selected by GeNorm and NormFinder software as housekeeping gene to normalize mRNA expression levels (Supplemental Tables S3 and S4; <https://doi.org/10.3168/jds.2018-15072>; Perez et al., 2007). Mature miRNA expression was quantified using a miScript SYBR Green PCR Kit containing $10 \times$ miScript Universal Primer (Qiagen), according to the manufacturer's instructions. U6 was used as an internal control to normalize miRNA expression levels. The results were expressed as fold change = $2^{-\Delta\Delta C_t}$. ABI StepOnePlus PCR system (Applied Biosystems, Foster City, CA) was used to perform quantitative real-time PCR (qRT-PCR). The PCR cycle conditions were as follows: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C

for 30 s. Primer sequences were designed to span an intron splice site, and are summarized in Supplemental Table S5 (<https://doi.org/10.3168/jds.2018-15072>).

Cell Cycle and Apoptosis Analyses

The progression of the cell cycle and apoptosis were assessed by flow cytometry (Becton Dickinson, Oxford, UK). For analyzing the cell cycle, miR-101-2 and vector clone cells in a 60-mm dish were collected and rinsed with cold PBS and then fixed in 75% ethanol overnight at 4°C. Fixed cells were then washed and resuspended in 200 to 500 μ L of cold PBS supplemented with 20 μ L of RNase A and then incubated at 37°C for 30 min. This was followed by supplementation of 400 μ L of propidium iodide (100 mg/mL) staining solution into the resultant cell suspension and then incubated for 0.5 to 1 h at 4°C. Results were analyzed by flow cytometry. For analyzing cell apoptosis, miR-101-2 or vector clone cells were collected and washed twice with cold PBS. Then, they were supplemented with 400 μ L of 1 $\times$ Annexin V (BB-4101, BestBio, Shanghai, China) and 5 μ L of Annexin V-EGFP staining medium (BB-4101, BestBio). This cell suspension was mixed slightly and incubated in the dark for 15 min at 2 to 8°C. This mixture was then supplemented with 10 μ L of propidium iodide (10 mg/mL) for 5 min before flow cytometry.

Cell Proliferation Analysis

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Beyotime Inst. Biotech) according to the manufacturer's instructions. Briefly, the miR-101-2 clone cells, 513-B1 clone cells, and ordinary fibroblasts were seeded in 96-well plates, with each type of cell having the same density of 2,000 cells per well, being grown at 37°C for 24 h. The medium in each well was replaced with a new medium, which was then incubated with 10 μ L of CCK-8 for 2 h at 37°C. For each group, there were 3 replicates, along with the use of the medium at the same amount as in CCK-8 as a control. Finally, the absorbance was determined at 450 nm using a microplate reader.

Oocyte Collection, In Vitro Maturation, NT, and In Vitro Reconstituted Embryo Culture

Oocyte collection, in vitro maturation, and NT were performed as previously described with minor modifications (Wang et al., 2011). Briefly, slaughterhouse-derived ovaries were stored in sterile saline at 20 to 25°C, and transported to the laboratory within 6 h. Cumulus-oocyte complexes (COC) were aspirated

from antral follicles (diameter, 2–8 mm) with a 10-mL syringe, and only those with multilayered compact cumulus, uniformly dark, and evenly granulated cytoplasm were selected. These oocytes were rinsed thrice in PBS containing 3% FBS, and cultured for 20 h in bicarbonate-buffered tissue culture medium 199 (TCM-199, Gibco, BRL, Grand Island, NY) supplemented with 10% FBS, 1 mg/mL of 17 β -estradiol, and 0.075 IU/mL of human menopausal gonadotropin in saturation humidity with 5% CO₂ at 38.5°C.

The COC were then transferred into PBS with 0.1% bovine testicular hyaluronidase to disperse the cumulus cells from oocytes. Oocytes with uniform, granulated ooplasm and an extruded first polar body were selected for SCNT. Enucleation was carried out by using a 20- μ m (internal diameter) glass pipette. The first polar body and a small amount of surrounding cytoplasm were aspirated by the glass pipette in microdrops of PBS supplemented with 7.5 μ g/mL of cytochalasin B and 10% FBS. A single donor cell was then injected into the space between the zona pellucida and oocyte membrane. The oocyte-donor cell complex was transferred into a microdrop of Zimmermann's fusion medium, and sandwiched between 2 platinum electrodes, connected to the micromanipulator. Cell-oocyte fusion was performed by using a double electrical pulse of 35 V for 10 μ s. Reconstructed embryos were then cultured in modified synthetic oviductal fluid (mSOF) for 2 h, and activated in 5 μ M ionomycin for 4 min, followed by a 4-h exposure to 1.9 mM dimethylaminopyridine in mSOF. After activation, the activated embryos were transferred into drops of mSOF medium with 8 mg/mL of BSA and cultured in a humid atmosphere of 5% CO₂ at 38.5°C. 513-B1-BFF and miR-101-BFF cells from passage 3–5 were used as donor cells for SCNT. Serum deprivation was used to induce the G₀/G₁ phase. Embryos were cultured for 8 d to evaluate their developmental rate.

In Vitro Fertilization

In vitro fertilization was carried out according to previous descriptions with minor modifications (Zhang et al., 2017). Briefly, frozen-thawed spermatozoa were rinsed and washed in BO-IVF medium (IVF Bioscience, Falmouth, UK) for 30 min. Then, 20 to 25 COC were transferred into a 60- μ L microdrop of BO-IVF medium (IVF Bioscience) covered by mineral oil, after which 50 μ L of sperm suspension (2×10^6 spermatozoa/mL) was added into it. After incubating in a humid atmosphere of 5% CO₂ at 38.5°C for 18 h, they were transferred and washed in PBS with 0.1% bovine testicular hyaluronidase to remove cumulus cells and redundant spermatozoa from the zygotes, and then the

zygotes were transferred to be cultured in mSOF with 8 mg/mL of BSA for further use.

Apoptosis Assay

An apoptosis assay was carried out using the Dead-End Fluorometric TUNEL (Promega, Madison, WI) system as previously described with minor modifications (Chen et al., 2015). All steps were performed at room temperature, unless stated otherwise. After 7 d of culture, IVF blastocysts and in vitro cloned blastocysts from the miR-101-BFF nuclear transfer (miR-101-NT) group and the 513-B1-BFF nuclear transfer (513-B1-NT) group were cleaned for 5 min in PBS containing 0.2% polyvinyl acetate (PVA-PBS) and fixed for 2 h with 4% paraformaldehyde. After washes in PVA-PBS, fixed embryos were permeated by 0.2% Triton X-100 for 20 min, and transferred into E-buffer for 8 min. All the following procedures were carried out in the dark to prevent fluorescence bleaching. Embryos were incubated for 1 h at 37°C in 51 mL of apoptosis staining medium (45 mL of E-buffer, 1 mL of terminal deoxynucleotidyl transferase enzyme, and 5 mL of nucleotide mix), which was followed by terminating with 2 × saline sodium citrate for 15 min. Embryos were washed again and incubated for 5 min in 4,6-diamidino-2-phenylindole (DAPI; 5 mg/mL, C1002, Beyotime, Jiangsu, China). Stained embryos were mounted on a glass slide for observation under a Nikon (Tokyo, Japan) fluorescence microscope. Blastocyst cell numbers were estimated by counting the total number of nuclei using DAPI, and the number of apoptotic cells per blastocyst was determined by counting nuclei positive for green fluorescence.

CDX2 Staining in Blastocysts

The DAPI staining was performed to reveal the total cell number in blastocysts. The CDX2 staining was used to analyze trophectoderm (TE) cell number. Then, the inner cell mass (ICM) cell number was assessed by calculating the difference between the total cell number and TE cell number.

Blastocysts were collected and washed 3 times (5 min each) in PVA-PBS, after which they were fixed with 4% paraformaldehyde for 2 h at room temperature. Immunol Staining Blocking Solution (P0102, Beyotime, Jiangsu, China) was used to block blastocysts for 12 h at 4°C before washing them 3 times (5 min each) in PVA-PBS. Then, blastocysts were washed 3 times (5 min each) in PVA-PBS, which was followed by incubating them for 12 h at 4°C with anti-CDX2 mouse monoclonal antibody (BioGenex Inc., San Ramon, CA) diluted 1:300 in Immunol Staining Primary Antibody

Dilution Solution (P0103, Beyotime). After 3 cleanings, samples were incubated for 2 h at room temperature with Alexa Fluor 555-labeled goat anti-mouse IgG secondary antibody (A0459, Beyotime) diluted 1:600 in Immunol Staining Secondary Antibody Dilution Solution (P0108, Beyotime). Then we performed another 3 washes and used 5 mg/mL of DAPI (C1002, Beyotime) to stain the nucleus for 5 min. Samples were then cleaned and mounted on a glass slide with a drop of Antifade Mounting Medium (P0126, Beyotime) for analysis using a Nikon fluorescence microscope. The cell numbers in blastocysts were estimated by counting nuclei stained with DAPI, and the number of TE cells was determined by counting nuclei positive for CDX2. The cell number of ICM was estimated as the total number of nuclei minus the number of TE nuclei.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA). Data are presented as the mean ± standard deviation or mean ± standard error of the mean from at least 3 independent experiments. All embryos had been systematically produced from the same cohort of oocytes on the same day and were allocated randomly to each treatment group. The development rate, apoptosis rate, ICM/TE ratio, and total cell number were analyzed using one-way ANOVA. The relative gene expressions were established by testing the data for normal and equal variance using the Levene median test, ANOVA, followed by the Tukey's test for multiple pairwise comparisons. The 2-tailed Student's *t*-test was used for pairwise comparisons of relative luciferase activity. *P*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

ING3 Is a Direct Target of miR-101-2

Our analysis reveals that *ING3*, a member of the inhibitor of growth family, was a potential target of miR-101-2, as the 3'-UTR in *ING3* mRNA contained a complementary site for the seed region of miR-101-2 (Figure 1A). To confirm that *ING3* is a target of miR-101-2, we co-transfected the reporter plasmid psiCHECK2-*ING3*-3'-UTR WT or Mut and the miR-101-2 expression plasmid pCDH-miR-101-2 into the HEK293T cell line. As predicted, the relative luciferase activity of the WT 3'-UTR reporter significantly decreased in cells co-transfected with pCDH-miR-101-2 compared with that in cells co-transfected with empty pCDH vector. In contrast, the luciferase activity upon

the co-transfection of mutant 3'-UTR reporter and pCDH-miR-101-2 was no longer able to be suppressed compared with that in cells co-transfected with empty PCDH vector. These results indicate that miR-101-2 targets the 3'-UTR of *ING3*, directly suppressing its gene expression (Figure 1B). To test the hypothesis that miR-101-2 could downregulate *ING3* protein expression

in bovine fibroblasts, we first obtained the clone cells with pCDH-miR-101-2 or PCDH vector. We found that the overexpression of miR-101-2 caused significant decreases in *ING3* at both protein and mRNA levels (Figures 1C and 2). These findings indicate that the presence of miR-101-2 in bovine fibroblasts suppresses *ING3* expression.

Figure 1. The *ING3* gene is direct target of miR-101-2. (A) The part structure of miR-101-2 overexpression vector PCDH-miR-101-2. (B) Dual-luciferase assay of 293T cells co-transfected with the firefly luciferase constructs containing the *ING3* wild type (WT) or mutant (MUT) 3'-untranslated region (UTR) as well as miR-101-2 overexpression vector PCDH-miR-101-2 or the empty PCDH-513-B1 as the negative control. (C) Western blot analysis of the protein levels of *ING3* in response to miR-101-2 overexpression. β -actin was used as a loading control. Data are shown as mean $\pm$ SD from 3 experimental replicates (* $P < 0.05$).

miR-101-2 Affects the Expression of *ING3* and Related Genes in miR-101-BFF

First, we tested whether miR-101-2 was successfully overexpressed after transfection and found that miR-101-2 expression was greatly upregulated (about 15-fold) in miR-101-BFF compared with that in 513-B1-BFF and BFF ($P < 0.01$; Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15072>).

The *ING3* activates TP53-transactivated promoters and modulates TP53-mediated transcription and apoptosis. Against this background, we tested a series of apoptosis-related genes to reveal whether these regulators are involved in the apoptosis inhibition of miR-101-2. We checked the mRNA levels of some regulators by qRT-PCR, including *ING3*, *TP53*, *CDKN1A*, *BAX*, and *BCL2L1*. The results showed that the levels of *ING3*, *TP53*, *CDKN1A*, and *BAX* decreased significantly in miR-101-BFF ($P < 0.05$) compared with that in 513-B1-BFF, whereas *BCL2L1* level showed an increase in miR-101-BFF ($P < 0.05$) compared with that in 513-B1-BFF (Figure 2). These findings indicate that miR-101-2 can affect the expression of some apoptosis-related genes.

Effects of miR-101-2 Overexpression on Apoptosis, Cell Cycle, and Proliferation

The nuclear donor cell is a crucial factor affecting SCNT embryo quality and the efficiency of SCNT, and many efforts have been made to improve them by improving donor-cell status (Chen et al., 2015; Mizutani

et al., 2015). Furthermore, miR-101 can regulate cell proliferation, the cell cycle, and apoptosis in different cell types (Wang et al., 2014b; Zhou et al., 2015; Qian et al., 2016). Therefore, we detected the apoptosis, cell cycle, and proliferation in donor cells after miR-101-2 overexpression.

The clone cells miR-101-BFF and 513-B1-BFF were first examined by flow cytometry to test whether miR-101-2 could affect apoptosis and cell cycle distribution of BFF. The miR-101-BFF had lower percentages of apoptotic and dead cells than in 513-B1-BFF (Figure 3A). These findings indicate that miR-101-2 could inhibit apoptosis in BFF.

Recently, many studies have focused on the association of miR-101 with apoptosis and cell proliferation through interaction with its target genes. The miR-101 inhibits the apoptosis of trophoblast HTR-8/SVneo cells by targeting endoplasmic reticulum protein 44 during preeclampsia (Zou et al., 2014). By contrast, the overexpression of human *ING3*, a putative homolog of the *Caenorhabditis elegans* *ING3* gene, promotes apoptosis (Nagashima et al., 2003; Wang et al., 2007). In the present study, we found that miR-101-2 down-regulated *ING3* and inhibited the apoptosis of bovine SCNT donor cells. The miR-101-2 was inversely correlated with *ING3* in apoptosis regulation. It has also been suggested that *ING3* overexpression increases the apoptotic rate of RKO cells (WT *TP53*) but not RKO-E6 cells (inactivated *TP53*), and it was further confirmed that *ING3* activates *TP53*-transactivated promoters, including the promoters of *CDKN1A* and *BAX* (Nagashima et al., 2003; Jafarnejad and Li, 2012). The *ING3*-mediated apoptosis occurs in a *TP53*-dependent/independent manner or via the inhibition of *CASP8* or *FAS* activation (Garkavtsev et al., 1998; Wang et al., 2007; Coles and Jones, 2009). In our study, the mRNA levels of *TP53*, *CDKN1A*, and *BAX* were decreased significantly following miR-101-2 overexpression. Accordingly, we infer that miR-101-2 regulation of apoptosis by targeting *ING3* in BFF may occur in a *TP53*-dependent manner.

The flow cytometry results demonstrated that the percentage of cells in the G₁ phase decreased and the percentage of cells in the S phase increased in the clone cells miR-101-BFF compared with that in the clone cells 513-B1-BFF. This indicates that miR-101-2 may be involved in the G₁-to-S phase transition. These findings show that the G₁-to-S phase transition of BFF can be expedited by miR-101-2 (Figure 3B). To investigate the possible function of miR-101-2 in BFF, we examined its effect on cell proliferation. The CCK8 assay results showed that the overexpression of miR-101-2 resulted in the promotion of the growth of BFF compared with that of the vector control (Figure 3C).

Figure 2. The mRNA expression of *ING3* and apoptosis-related genes in 513-B1-bovine fetal fibroblasts (BFF) and miR-101-BFF. *ING3*, *TP53*, *CDKN1A*, *BAX*, and *BCL2L1* mRNA expression was assessed by real-time PCR in 513-B1-BFF (BFF transfected with empty vectors) and miR-101-BFF (BFF with miR-101-2 overexpression). Data are shown as mean $\pm$ SEM from 3 experimental replicates (* $P < 0.05$, ** $P < 0.01$).

Figure 3. Effect of miR-101-2 overexpression on cell status. (A) Flow cytometry results show the effect of miR-101-2 overexpression on cell status. The x-axis represents cell status. Different letters (a,b) indicate $P < 0.05$. (B) Flow cytometry results show the effect of miR-101-2 overexpression on cell-cycle distribution. The x-axis represents the cell cycle stage. G₁ = first gap; S = synthesis; G₂/M = second gap/mitotic phase. Different letters (a,b) indicate $P < 0.05$. (C) CCK8 assays were performed to investigate the effect of miR-101-2 on the proliferation of bovine fetal fibroblasts (BFF) at the indicated time points. Data are shown as mean $\pm$ SEM from 3 experimental replicates (* $P < 0.05$, ** $P < 0.01$).

The miR-101 could inhibit cell proliferation by targeting certain genes (*RAC1*, *FOS*, *MITF*, and *SOCS2*; Konno et al., 2014; Wang et al., 2014a; Zheng et al., 2015). However, we found that miR-101-2 promoted the proliferation of bovine SCNT donor cells, which is probably because it downregulates *ING3*. *ING3* is one member of the well-conserved family of ING proteins, and the overexpression of *ING3* in RKO cells resulted in a decreased proportion of cells in S phase and an increased proportion in G₀/G₁ phase, suppressing cell growth (Nagashima et al., 2003). We found that the downregulation of *ING3* via miR-101-2 overexpression resulted in a decreased proportion of cells in G₁ phase and an increase in proportion in S phase in BFF. These findings showed that miR-101-2 could accelerate the proliferation of BFF.

Characterization of miR-101-2 Expression in IVF and SCNT Embryos

The qRT-PCR results showed that the expression of miR-101-2 in 513-B1-NT group was lower than that in IVF group at the 2-cell and 8-cell stage ($P < 0.05$; Figure 4), whereas it reached a similar level in the IVF group when using miR-101-BFF as donor cells ($P > 0.05$). However, miR-101-2 expression levels at the blastocyst stage showed no significant differences ($P > 0.05$). Wang et al. (2017b) indicated using donor cells with miR-148a overexpression could upregulate its expression in cloned embryo, and similar results were found in another study, with miR-449b being selected (Wang et al., 2017a). These results are in line with our findings.

Effects of miR-101-2 Overexpression on the Quality of Cloned Blastocysts

Primarily, we analyzed the differences in developmental rate among 3 groups. The 2-cell rate and 8-cell rate between the miR-101-BFF NT (miR-101-NT) group and the 513-B1-BFF NT (513-B1-NT) group showed no significant difference (78.42 ± 2.07 vs. 75.76 ± 0.61 and 51.66 ± 1.98 vs. 47.80 ± 1.28 , respectively, $P > 0.05$), which were both lower than that of IVF embryos ($P < 0.05$). The blastocyst rate has been widely used as an index of blastocyst quality in studies on SCNT (Liu et al., 2018). Day-7 blastocyst rate, however, was higher in the miR-101-NT group than in the 513-B1-NT group (39.08 ± 1.08 vs. 32.90 ± 2.10 , $P < 0.05$), attaining a rate comparable to that of IVF embryos, and Day-8 blastocyst rate showed similar tendency in miR-101-NT, 513-B1-NT, and IVF group (44.68 ± 1.35 vs. 37.93 ± 1.97 vs. 47.01 ± 1.43 , Table 1).

Figure 4. The expression pattern of miR-101-2 in in vitro fertilized (IVF) and somatic cell nuclear transfer (SCNT) embryos. Relative miR-101-2 expression level of genes in 2-cell, 8-cell, and d-7 blastocysts from IVF, 513-B1-NT [SCNT embryos produced by using bovine fetal fibroblasts (BFF) transfected with empty vectors as donor cells], or miR-101-NT groups (SCNT embryos produced by using BFF with miR-101-2 overexpression as donor cells). Different letters (a,b) indicate $P < 0.05$. The results from 2-cell IVF samples are set as 1. Data are shown as mean $\pm$ SEM from 3 experimental replicates.

We also performed immunostaining of blastocysts for the trophectoderm marker CDX2 and the DNA marker DAPI to compare the numbers of different cell types among the 3 groups (Figures 5A and 5B). Total cell number for individual 513-B1-NT blastocysts was less than that for IVF blastocysts (86.4 vs. 102.5, $P < 0.05$), but this figure rose significantly when miR-101-BFF was used as donor cells (100.8 vs. 86.4, $P < 0.05$). The ICM/TE ratio was also significantly higher in the miR-101-NT group than in the 513-B1-NT counterpart (28.8 vs. 23.9%, $P < 0.05$), at a level comparable to that of the IVF group, whereas ICM cell number between these groups did not show significant difference (Figure 5B).

To clarify why a higher ICM/TE ratio was found in the miR-101-NT group, qRT-PCR was performed to reveal the expression levels of 4 development-related genes, namely, *POU5F1*, *NANOG*, *SOX2*, and *CDX2*; among these, *POU5F1* and *SOX2* were upregulated in the miR-101-NT group (Figure 6).

The *POU5F1* gene (also known as *OCT4*), which encodes a factor that maintains ICM fate, plays a key role in embryonic development and stem cell pluripotency. It has been reported that *POU5F1* transcript levels were decreased in clones compared with those in IVF embryos at the blastocyst stage (Beyhan et al., 2007). Similar results were obtained in our study. The *SOX2* is also involved in maintaining pluripotency. In ICM cells, *SOX2* collaborating with *POU5F1* and *NANOG* acts as a circuit to form the pluripotent regulatory network (Chen et al., 2008; Kim et al., 2008). The observed rise in the expression of *SOX2* and *POU5F1*, but not in *NANOG* or *CDX2*, in the miR-101-NT group is consistent with previous studies indicating the relationship between the expression of related genes and blastocyst quality (Su et al., 2011; Chen et al., 2015).

Serum starvation treatment is commonly used in SCNT, but it might result in DNA damage and subsequent apoptosis. Therefore, apoptosis was considered as a criterion for the evaluation of blastocyst quality (Hardy, 1997). Here, results indicated the apoptotic rate in 513-B1-NT embryos was significantly higher than that in IVF embryos. The apoptotic rate was lower in miR-101-NT blastocysts than in the 513-B1-NT group ($P < 0.05$), at levels comparable to those in IVF embryos (Figures 5C and 5D). Furthermore, the expression of *ING3* and several genes associated with apoptosis was assessed by qRT-PCR (Figure 6). The *BCL2L1* was upregulated in miR-101-NT blastocysts compared with that in the IVF group and the 513-B1-NT group ($P < 0.05$). The *ING3* expression was higher in 513-B1-NT than in the IVF blastocysts ($P < 0.01$), but it decreased significantly in miR-101-NT blastocysts compared with the 513-B1-NT group ($P < 0.01$). The expression of *BAX* was lower in miR-101-NT blastocysts than in both the IVF and the 513-B1-NT group ($P < 0.05$).

Table 1. Effect of micro RNA miR-101-2 on the development of cloned bovine embryos in vitro¹

Group ²	No. of reconstructed	No. of cleaved embryos (%)	No. of 8-cell (%) per 2-cell	No. of blastocysts on 7 d (%) per 2-cell	No. of blastocysts on 8 d (%) per 2-cell
IVF	211	178 (84.38 $\pm$ 1.16) ^a	101 (56.66 $\pm$ 0.89) ^a	77 (42.80 $\pm$ 1.78) ^a	83 (47.01 $\pm$ 1.43) ^a
513-B1-NT	165	125 (75.76 $\pm$ 0.61) ^b	60 (47.80 $\pm$ 1.28) ^b	42 (32.90 $\pm$ 2.10) ^b	47 (37.93 $\pm$ 1.97) ^b
miR-101-NT	193	151 (78.42 $\pm$ 2.07) ^b	78 (51.66 $\pm$ 1.98) ^b	60 (39.08 $\pm$ 1.08) ^a	67 (44.68 $\pm$ 1.35) ^a

^{a,b}Values with different superscripts within columns differ significantly ($P < 0.05$).

¹Three replicates were performed. The data are shown as mean $\pm$ SEM.

²IVF = in vitro fertilization; 513-B1-NT = somatic cell nuclear transfer (SCNT) embryos produced by using bovine fetal fibroblasts (BFF) transfected with empty vectors as donor cells; miR-101-NT = SCNT embryos produced by using BFF with miR-101-2 overexpression as donor cells.

The suppression of *ING3* was associated with fewer apoptotic germ cells in *C. elegans*. Also, the embryonic death rate was decreased in the *ING3* mutant (Luo et al., 2009). Here, *ING3* expression in SCNT blastocysts was suppressed when using miR-101-BFF as donor cells. The *BCL2* family proteins influence programmed cell death or apoptosis, among which *BCL2L1* is anti-apoptotic, whereas *BAX* is proapoptotic (Martinou and Youle, 2011). We measured the transcript abundance of *BAX* and *BCL2L1* among the 3 groups and found that *BAX* was downregulated and *BCL2L1* was upregulated in the miR-101-NT group compared with their levels in the 513-B1-BFF and IVF counterparts. These results are consistent with previous studies indicating the

relationship between the expression of related genes and apoptosis (Su et al., 2011). Taking these findings together, the changes of expression of *ING3*, *BAX*, and *BCL2L1* might contribute to the decrease in apoptotic rate in miR-101-NT blastocysts.

Collectively, these results demonstrated that the quality of cloned blastocysts from miR-101-BFF was better than for those derived from 513-B1-BFF, which was similar to the quality of IVF blastocysts. Poor embryo quality has been a major problem contributing to pregnancy failure in SCNT (Choi et al., 2013). Great effort has been placed into enhancing developmental competence of SCNT embryos, and they found that those cloned blastocysts with better quality (lower

Figure 5. Overexpression miR-101-2 enhance the early development of somatic cell nuclear transfer (SCNT) embryos. (A) Immunostaining of CDX2 (red) and 4,6-diamidino-2-phenylindole (DAPI; blue) in blastocysts from in vitro fertilized (IVF), 513-B1-NT (SCNT embryos produced by using bovine fetal fibroblasts (BFF) transfected with empty vectors as donor cells), and miR-101-NT (SCNT embryos produced by using BFF with miR-101-2 overexpression as donor cells) groups. Scale bar = 50 μ m. (B) Histogram shows the total cell number, inner cell mass (ICM) cell number, and ICM:trophectoderm (TE) ratio of each blastocyst from IVF, 513-B1-NT, and miR-101-NT groups (n = 34, 28, and 31, respectively). Different letters (a,b) indicate $P < 0.05$. (C) Apoptotic cells (green) in the nuclei (blue) of blastocysts in IVF, 513-B1-NT, and miR-101-NT. Scale bar = 50 μ m. (D) The apoptotic rate in blastocysts from IVF, 513-B1-NT, and miR-101-NT groups (n = 32, 23, and 28, respectively). Different letters (a,b) indicate $P < 0.05$. Data are shown as mean $\pm$ SEM from 3 experimental replicates. TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Figure 6. Relative expression of development-related genes and apoptosis-related genes. Relative expression level of genes in d-7 blastocysts from in vitro fertilization (IVF). 513-B1-NT = somatic cell nuclear transfer (SCNT) embryos produced by using bovine fetal fibroblasts (BFF) transfected with empty vectors as donor cells; miR-101-NT = SCNT embryos produced by using BFF with miR-101-2 overexpression as donor cells. Three experimental replicates were performed. Data are shown as mean $\pm$ SEM from 3 experimental replicates (* $P < 0.05$, ** $P < 0.01$).

apoptotic rate, more total cell number, and so on) tend to be more able to result in development in vivo (higher pregnancy rate, birth rate, and so on) after performing embryo transfer (Su et al., 2015; Liu et al., 2018). However, whether miR-101-2 would improve the in vivo development of bovine SCNT embryos needs further investigation.

CONCLUSIONS

Our results indicate that miR-101-2 can reduce cell apoptosis and expedite the cell cycle. More importantly, it can also enhance the development rate and reduce the apoptotic rate in SCNT embryos and subsequently enhance the early development of bovine SCNT embryos.

ACKNOWLEDGMENTS

We thank our laboratory colleagues for their help in this study. This work was supported by the National Science and Technology Major Project (no. 2016ZX08007-002; Yangling, China).

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