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Research Paper

Treatment donor cells with UNC0638 modify the abnormal histone H3K9 dimethylation and gene expression in cloned goat embryos

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ABSTRACT

Epigenetic modifications are considered crucial to the reprogramming of somatic cell nuclear transfer (SCNT) embryos and subsequent *in vitro* development. The abnormal histone H3K9 dimethylation (H3K9me2) status has recently been reported in SCNT embryos. The present study was designed to evaluate the effect of treatment donor cells with UNC0638, a specific inhibitor of H3K9 methyltransferase G9A, on *in vitro* development, H3K9me2 levels and gene expression in goat SCNT embryos. Different concentration of UNC0638 was applied to treat goat fetal fibroblasts (GFFs), and the treated GFFs were used as donor cells for SCNT. The results showed that 0.2 μM UNC0638 decreased significantly the level of H3K9me2 in treated GFFs ($P < 0.05$), and did not influence the cell viability ($P > 0.05$). Compared with intracytoplasmic sperm injection (ICSI) embryos, SCNT embryos derived from untreated GFFs (C-SCNT) presented higher level of H3K9me2 ($P < 0.05$). Although the *in vitro* developmental rate was not improved, the abnormal H3K9me2 level was corrected in SCNT embryos from UNC0638-treated GFFs (T-SCNT) compared with C-SCNT embryos. Furthermore, UNC0638 treatment could promote the mRNA expression of key developmental genes Nanog and Oct4 ($P < 0.05$), but did not affect the imprinted genes H19 and IGF2R expression in goat SCNT embryos ($P > 0.05$). In conclusion, these results indicated that treatment donor cells with UNC0638 may have beneficial effects in terms of modifying the abnormal H3K9me2 status and gene expression in cloned goat embryos.

1. Introduction

Many small molecular inhibitors have been applied to improve development of SCNT embryos by specifically modifying the DNA methylation, histone methylation or histone acetylation status. For example, 5-aza-2′-deoxycytidine and trichostatin A could inhibit DNA methylation and histone deacetylation, respectively (Ding et al., 2008; Tsuji et al., 2009). Inhibiting histone deacetylation could improve development efficiency and blastocyst quality of cloned embryos (Dai et al., 2010; Miyoshi et al., 2010; Su et al., 2011). Correcting the abnormal epigenetic modifications can improve the development of SCNT embryos.

The abnormal H3K9me2 status has been observed in cloned embryos of mouse (Wang et al., 2007), cattle (Santos et al., 2003), sheep (Fu et al., 2012) and pig (Huang et al., 2016a). These studies reported that cloned embryos showed higher level of H3K9me2 than IVF embryos at the same development stage. The aberrant reprogramming of histone methylation might be a barrier of the somatic nucleus reprogramming in cloned embryos (Matoba et al., 2014). In addition, H3K9me2 is associated with gene silencing, including inactivation of Oct4 during differentiation of embryonic stem cells or embryogenesis (Kimura et al., 2004). The Oct4 expression was incompletely activated in blastocyst of SCNT embryos (Bortvin et al., 2003). However, the characteristics of H3K9me2 pattern in cloned goat embryos have not been studied.

BIX01294 has been identified as an inhibitor of histone-lysine methyltransferase G9A, and it can selectively reduces H3K9me2 levels (Kubicek et al., 2007). BIX01294 has been successfully used to enhance the generation of induced Pluripotent Stem cells (Shi et al., 2008). It has been reported that treatment with BIX-01294 enhanced the developmental competence of porcine cloned embryos through improving epigenetic reprogramming and gene expression (Huang et al., 2016a). Although treatment with BIX-01294 can not improve the *in vitro* developmental rate of cloned embryos in sheep and mouse, BIX-01294 could correct abnormal H3K9me2 modification (Fu et al., 2012; Huang et al., 2016b). Recently, UNC0638 was identified as another G9A

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inhibitor to reduce H3K9me2 level, and it has been shown to be less toxic than BIX01294 (Vedadi et al., 2011; Fu et al., 2014). In the present study, the effects of treatment donor cells with UNC0638 on *in vitro* development, H3K9me2 levels and gene expression in cloned goat embryos were investigated.

2. Materials and methods

2.1. Ethics statements

The entire experimental procedure was designed under the consideration of animal welfares and approved by the Animal Care and Use Committee of the College of Veterinary Medicine, Northwest A & F University.

2.2. Nuclear donor cells preparation and UNC0638 treatment

Nuclear donor cells were derived from a female Saanen Dairy goat fetus which was 40 days old of pregnancy. The small skin piece was cut and washed three times by phosphate-buffered saline (PBS), and then was minced into 1 mm^3 pieces and spread into 60 mm dishes. Then the tissues were cultured in DMEM (Gibco, Grand Island, USA) medium composed of 10% fetal bovine serum (FBS, Gibco), 100 mg/mL streptomycin and 100 IU/mL penicillin. After 1–2 weeks, goat fetal fibroblasts (GFFs) were amplified up to 90% confluence and then were passaged. Based on previous report (Vedadi et al., 2011), UNC0638 was dissolved into various concentrations (0.1, 0.2, 0.5, 1 μM) with dimethyl sulphoxide (DMSO) and then GFFs were treated 48 h for cell viability assays, immunostaining and cell cycle analysis.

2.3. Cell viability assay

Cell viability was tested using Cell Counting Kit (CCK-8) (Beyotime, Jiangsu, China) as described in a previous study (Wu et al., 2013). Briefly, GFFs were subcultured into 96-well plates at the same concentrations and treatment with different concentrations of UNC0638. After treatment for 48 h in incubator, 10 μL CCK-8 solutions were added into each well. The plate was kept in incubator for 2 h and then was read using a spectrophotometer (Epoch Biotek, America). The absorbance value (Optical density, OD) was tested at 450 nm wave length. The experiment was repeated 3 times. The percentage of cells viability (%) = (OD treatment group/OD control group) × 100.

2.4. Immunostaining and quantification of fluorescence intensity

Immunofluorescence staining was performed as described in our previous study (Su et al., 2011). Both cells and embryos were fixed for 30 min in 4% paraformaldehyde, and then permeabilized for 30 min in 0.1% Triton X-100 in PBS. All following steps were performed at room temperature unless mentioned. Immune Staining Wash Buffer was used for washing the cells and embryos after every step. The samples were blocked in the Immune Staining Blocking Solution (Beyotime, P0102) for 12 h at 4 °C. The samples were stained with H3K9me2 antibodies (Beyotime, AH438) overnight at 4 °C, which was diluted 1:500 in Immune Staining Primary Antibody Dilution Buffer (Beyotime, P0103). After extensive washing, the samples were incubated in Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (Beyotime, A0423; Diluted 1:500). Finally, the samples were stained briefly with 4, 6-diamidino-2-phenylindole (DAPI) (Beyotime, C1005) for 3 min. Embryos were putted in a drop of Antifade Mounting Medium (Beyotime, P0126) on glass slides and covered with glasses. Both cells and embryos were observed under Nikon eclipse Ti-S microscope equipped with a Nikon DS-Ri1 digital camera (Nikon, Tokyo, Japan). All images were captured using the same settings and without any adjustment of constant or brightness. The mean fluorescence intensity of H3K9me2 levels was measured using Image-Pro Plus (Version 6.0; Media Cybernetics) as reported previously (Su et al., 2012). The fluorescence intensity is expressed relative to that of 2-cell ICSI embryos or 0 μM UNC0638 treated cells (set as 100%). The experiment was repeated three times and samples without primary antibody were used as a negative control.

2.5. Cell cycle analysis

Cell cycle analysis was performed by flow cytometry as described in a previous study (Hayes et al., 2005). GFFs treated with 0, 0.2 or 0.5 μM UNC0638, respectively, were incubated for 48 h and then washed with PBS 3 times, then trypsinized, centrifugalized and fixed in 70% precooling ethanol at 4 °C overnight. GFFs were collected and suspended in PBS supplemented with 0.1 mg/mL RNase. The samples were centrifugalized, dyed with 50 μg/mL propidium iodide (PI) containing RNase-free (Beyotime, C1052, Jiangsu, China) for 30 min at 37 °C in dark. The cell cycle were analyzed by a flow cytometer (Beckman Coulter).

2.6. Oocyte collection and in vitro maturation (IVM)

The collection and *in vitro* maturation (IVM) of oocytes was performed as described in our previous study (Liu et al., 2011). Briefly, ovaries were collected separated from the surrounding tissue, and Cumulus-oocyte complexes (COCs) were achieved from ovaries. COCs were washed and cultured for 22–24 h at 38.5 °C in medium TCM199 supplemented with 10% (v/v) FBS, 1 μg/mL 17-estradiol, and 0.075 IU/mL Human Menopausal Gonadotropin (HMG). After 20 h IVM, COCs were transferred into PBS supplemented with 0.1% hyaluronidase to disperse the cumulus cells. Oocytes with a first polar body and evenly granulated ooplasm were selected for ICSI or SCNT.

2.7. Intracytoplasmic sperm injection (ICSI)

Fresh semen was collected by artificial vagina from bucks. The sperm motility was evaluated, and then 200 μL semen were transferred into the bottom of 10 mL sterile tube containing 3 mL Blacket & Oliphant (BO) solution. After 30 min, floated-up sperms were collected and added into a droplet of 10% polyvinilpirrolidone solution for ICSI. The procedure of ICSI was performed according to a previous study (Jiménez-Macedo et al., 2005). Briefly, the sperm tail was broken by the injection pipette, and then a sperm were aspired and injected into the ooplasm. The first body was placed at the 6 or 12 o'clock position while the preferred position of injection was at the 3 o'clock point and sperm was released at 9 o'clock position. Then the ICSI embryos were activated in 5 umol/L ionomycin for 5 min, and then cultures in 200 μL mSOF medium.

2.8. Nuclear transfer

The nuclear transfer was carried out in accordance with previous study (Liu et al., 2011). Briefly, both the first polar body and metaphase plate were removed and then a round donor cell was injected into the perivitelline space of oocytes. The couplets were fused by electrofusion and incubated for 2–3 h in TCM-199 supplemented with 10% FBS and 7.5 μg/mL cytochalasin B. The reconstructed embryos were activated in 5 μM ionomycin for 5 min and then 2 mM 6-dimethylaminopurine for 4 h. Following activation process, the presumptive embryos were washed extensively and cultured in 200 μL mSOF covered with mineral oil.

2.9. Quantitative real-time PCR (qPCR)

The qPCR was performed on StepOne Plus reaction system (ABI, USA) according to a previous study (Su et al., 2012). The embryos RNA isolation and RT reaction was conducted using SuperScript™ III Cells Direct™ cDNA Synthesis System (Life technologies, USA). The cDNA was used for qPCR to quantify the mRNA levels using SYBR Premix

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