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Effect of primary culture medium type for culture of canine fibroblasts on production of cloned dogs

Geon A Kim [a], Hyun Ju Oh [a], Min Jung Kim [a], Young Kwang Jo [a], Jin Choi [a], Jin Wook Kim [b], Tae Hee Lee [c], Byeong Chun Lee [a,*]

[a] Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, Korea
[b] College of Veterinary Medicine, Chungbuk National University, Cheongju, Korea
[c] College of Natural and Mathematical Sciences, University of Maryland Baltimore County, Baltimore, Maryland, USA

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ABSTRACT

Fibroblasts are common source of donor cells for SCNT. It is suggested that donor cells' microenvironment, including the primary culture, affects development of reconstructed embryos. To prove this, canine embryos were cloned with fibroblasts that were cultured in two different primary media (RCMEp vs. Dulbecco's modified Eagle's medium [DMEM]) and *in vivo* developments were compared with relative amount of stemness, reprogramming, apoptosis gene transcripts, and telomerase activity. Donor cells cultured in RCMEp contained a significantly higher amount of *SOX2*, *NANOG*, *DPPA2*, *REXO*1, *HDAC*, *DNMT1*, *MECP2* and telomerase activity than those cultured in DMEM ($P < 0.05$). *In vivo* developmental potential of cloned embryos with donor cells cultured in RCMEp had a higher birth rate than that of embryos derived from DMEM ($P < 0.05$). The culture medium can induce changes in gene expression of donor cells and telomerase activity, and these alterations can also affect *in vivo* developmental competence of the cloned embryos.

1. Introduction

Various treatment protocols for preparation of donor cells have been developed to improve SCNT efficiency in mammals [1,2]. In donor cells, intrinsic factors such as differentiation status and modifying culture conditions can affect nucleus reprogramming efficiency of cloned embryos [3,4]. Cell culture condition can induce changes of gene expression patterns of nuclear donor cells [5,6]. The culture medium used for donor cells affects the development of cloned bovine embryos [7,8]. It also affects the preimplantation development of horse nuclear transfer embryos [9]. However in canines, little attention has been focused on effects of the primary culture medium on donor cells for dog cloning.

Global epigenetic reprogramming of donor cells has been reported as a major event that occurs after SCNT, regulating the success of cloning [10–12]. Previous studies reported that differentiated somatic cells in a highly methylated state appear to be incompletely reprogrammed in cloned embryos [13,14]. Highly methylated somatic donor cells are used to generate cloned embryos, in turn, have been shown to be abnormally hypermethylated [15,16]. Cell characteristics including morphology, function such as overexpression of cytokines, gene expression patterns, resistance to apoptosis, and telomere length can all be changed by *in vitro* culture (IVC) conditions [17]. It is also reported that gene expression level of pluripotency genes in donor cells is linked with cloning efficiency [18,19]. The change of methylation or acetylation status induced by extract or chemical treatments of oocytes also resulted in pluripotency gene expression changes [20,21].

For the present study, we used two culture media, one is Dulbecco's modified Eagle's medium (DMEM) with 10%

* Corresponding author. Tel.: +82 2 880 1269; fax: +82 2 873 1269.
E-mail address: bclee@snu.ac.kr (B.C. Lee).

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fetal bovine serum (FBS) used as the standard medium for culture of fibroblasts, another is RCMEp that is used as establishing for human mesenchymal stem cells. Therefore, the objective of this study is to investigate whether the primary culture medium for canine somatic cells can alter expression of pluripotent state, reprogramming state, apoptosis and the effect on telomerase activity in canine adult fibroblast donor cells; consecutively, altered donor cell will affect canine cloned embryos development.

2. Materials and methods

2.1. Somatic cell culture and preparation

Primary cultures of fibroblasts were established as described in our previous studies with minor modification [22]. In brief, abdominal full-thickness skin was excised from 7- to 9-year-old male beagles under general anesthesia. Immediately after collection, the skin fragments were placed in PBS (Invitrogen, Carlsbad, CA, USA) on ice and transported to the laboratory. After all the fat tissue and blood vessels were removed from the skin with scissors, skin tissues were trimmed to 0.5 cm × 0.5 cm fragments and finely minced into small pieces (~1 mm × 1 mm) which were evenly distributed onto the bottom of a tissue culture dish for culture. Two culture media were used: DMEM (high glucose; Invitrogen) supplemented with 10% FBS (Invitrogen), penicillin–streptomycin (Invitrogen); and RCMEp (K-stem cell bio media for adipose-derived stem cells culture; K-stem cell Ltd, Seoul, Korea). RCMEp is DMEM (Invitrogen)-based medium containing 0.2 mmol/L of ascorbic acid, 10 ng/mL of fibroblast growth factor (FGF), 1% nonessential amino acid (Invitrogen), and 10% FBS. All samples were cultured in 60-mm^2 tissue culture dishes at 39 °C in air containing 5% CO_2, and fresh medium was added every 3 to 4 days till confluence. The number of days required to achieve confluence of the primary culture was recorded for each sample (for only primary confluence). After retrieving the cells from each primary culture medium, cells were cultured and passaged at confluence in each primary culture medium. Cells at passage 0 were harvested by incubation for 2.5 minutes at 39 °C with 0.25% trypsin in 0.01% EDTA and collected by centrifugation; then, they were cryopreserved. Before SCNT, cells at passages 2 to 4 were disaggregated by treatment with trypsin-EDTA. For measuring the size of donor cells, an automated cell counter (Countess, Invitrogen) was used. Diluted donor cells were suspended in PBS (Invitrogen) containing 0.1% FBS (Invitrogen). Suspension solution including donor cells was mixed with the 0.4% trypan blue (Invitrogen) of the same volume. After mixing gently by pipetting, 10 μL of the sample mixture was transferred to the chamber ports on one side of the Countess cell-counting chamber slide. The slide was inserted completely into the instrument, and the mean viable cell size was automatically recorded.

2.2. Canine oocyte collection, somatic cell nuclear transfer, and embryo transfer

For recovery of *in vivo*–matured oocytes, the plasma progesterone concentration of candidate oocyte donor bitches was monitored by an IMMULITE 1000 (Siemens Healthcare Diagnostics Inc., Flanders, NJ, USA). Elevation of plasma progesterone concentration in candidate oocyte donor bitches to 4 ng/mL or higher was considered as the ovulation day [23,24]. Three days after ovulation, *in vivo*–matured oocytes were collected by flushing oviducts using HEPES-buffered tissue culture medium-199 (Invitrogen) supplemented with 10% (v:v) BSA (Invitrogen) and 2-mM $NaHCO_3$. Nuclear transfer was performed as described earlier [22]. After collection, *in vivo*–matured oocytes were denuded of cumulus cells in tissue culture medium-199 supplemented with 0.1% (v:v) hyaluronidase, and oocytes with extruded first polar bodies were selected and exposed to cytochalasin B (5 μg/mL) and Hoechst 33342 (5 μg/mL). The metaphase chromosomes were removed under ultraviolet light by aspiration with a fine needle pipette. Single donor fibroblast cells that had been cultured in each medium were transferred into the perivitelline space of oocytes. Each donor cell–cytoplast couplet was induced to fuse two pulses of DC 72 V for 15 μs, using an Electro-Cell Fusion apparatus (NEPA GENE Co., Chiba, Japan) in 0.26-M mannitol solution containing 0.1-mM $MgSO_4$, 0.5-mM HEPES, and 0.05% (wt/vol) BSA [1,22,25]. Chemical activation was performed by incubating reconstructed embryos in modified synthetic oviductal fluid medium containing 10-μM calcium ionophore for 4 minutes at 38.5 °C. Cloned embryos were transferred into 40 μL of modified synthetic oviductal fluid with 1.9-mM 6-dimethylaminopurine for 4 hours in an atmosphere of 39 °C, 5% CO_2, 5% O_2, and 90% N_2.

Reconstructed cloned embryos were transferred into oviducts of synchronized canine recipients. Under general anesthesia, embryos were inserted into the ampullary portion of the oviduct using a 3.5-Fr Tom-Cat catheter (Sherwood, St. Louis, MO, USA).

2.3. Determination of relative abundance of genes in donor cells by quantitative PCR

Cultured cells at passages 2 to 4 were disaggregated by treatment with trypsin-EDTA. After trypsin-EDTA treatment, donor cells were collected and washed in sterile PBS. Total RNA was extracted by using the easy-spin (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Kyunggi, Korea) from donor cells cultured in each of the two primary culture media. To synthesize complementary DNA of each sample, reverse transcription was carried out at 50 °C for 50 minutes using random hexamer and SuperScript III Reverse Transcriptase (Invitrogen) in a 20-μL reaction volume. Real-time quantitative polymerase chain reaction (PCR) was done according to the Takara Bio Inc. (Shiga, Japan) guidelines. A total PCR reaction volume of 20 μL was made by adding 2 μL of complementary DNA, 1-μL forward primer, 1-μL reverse primer, 8-μL SYBR Premix Ex Taq, 0.4 μL of ROX Reference (Takara Bio Inc.), and 7.6 μL of Nuclease-free water (Ambion Inc., Austin, TX, USA). The reaction was carried out using a 7300 Real-Time PCR Cycler System (Applied Biosystems, Foster City, CA, USA). The thermal profile for reverse transcription quantitative PCR was 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 20 seconds, and 72 °C for 40 seconds.