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Effect of culture medium type on canine adipose-derived mesenchymal stem cells and developmental competence of interspecies cloned embryos

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ABSTRACT

Canine adipose-derived mesenchymal stem cells (ASCs) are promising as donor cells for somatic cell nuclear transfer (SCNT). It has been suggested that different cell cultures possess different capacities to support pre-implantation development of SCNT embryos. The aim of this study is to investigate whether two culture medium (RCMEP, Dulbecco's modified Eagle's medium [DMEM]) affect gene expression of ASCs, subsequent development of interspecies SCNT (iSCNT) and gene expression of cloned embryos. The RCMEP-cultured cells contained significantly greater amounts of *SOX2*, *NANOG*, *OCT4*, *DNMT1*, and MeCP2 than DMEM-cultured cells ($P < 0.05$). In iSCNT, the use of DMEM medium for culturing cells resulted in similar development to the blastocyst stage than those derived from RCMEP cultured cells (4.5% and 3.2%, respectively; $P > 0.05$). The expression of all transcripts except for DNMT1 in cloned blastocysts from RCMEP cultured cells followed those of cloned blastocysts derived from DMEM cultured cells. The alteration of gene expression in ASCs by culture medium was not manifested in the iSCNT embryos derived from these cells. Although the culture medium can induce changes of gene expression by ASCs, such alterations in donor cells did not affect the developmental competence or gene expression patterns of iSCNT embryos.

1. Introduction

In efforts to improve the efficiency of somatic cell nuclear transfer (SCNT) in mammals, many studies focused on donor cells have been performed. It is critically important for development of reconstructed embryos that the cell cycle states of the donor cell and the enucleated recipient oocyte are coordinated. In SCNT studies with many species, using donor cells that were synchronized into a quiescent (G0/G1) stage improved cloned blastocyst formation and cloned offspring birth rates [1–4]. Methods such as serum starvation during cell culture or roscovitine are often used to achieve cell-cycle synchronization [5,6].

The type of nuclear donor cell, characterized by its tissue origin and extent of differentiation, is among the key factors affecting the efficiency of SCNT, but cell selection and treatment are controversial areas. In mice, greater numbers

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of offspring were produced through nuclear transfer with embryonic stem cells compared with somatic cells [7]. However, *in vitro* development of mouse embryos cloned using hematopoietic stem cells was inefficient and production of cloned pups was no better than with clones made using other somatic cells such as cumulus, Sertoli, and fibroblast cells [8]. In canine SCNT, cloned offspring have been derived using donor cells of several types, which affected the efficacy of cloning [5,9–11].

The final factor affecting the SCNT procedure is the complete reprogramming potential of donor cells. Successful reprogramming of donor cells can be influenced by *in vitro* culture conditions, including passage number, serum concentration, cell density, and chemical treatment [12–15]. Recent studies in mice have shown that treatment of donor cells with chemicals such as trichostatin A changed epigenetic methylation patterns and improved the quality of cloned blastocysts through induced hyperacetylation in mice [16,17], cattle [18], and pigs [19] SCNT.

To perform this experiment, it is necessary to prepare matured oocyte and donor cell derived from dogs. However, it is difficult to obtain many high-quality recipient canine oocytes because of a limited number of *in vivo* mature oocytes [20] and still low *in vitro* maturation (IVM) rate [21–23]. Therefore, interspecies SCNT (iSCNT) is utilized to analyze gene expression patterns of donor cells and cloned embryos derived from donor cells with different culture medium. The iSCNT technique has been used widely for evaluating the developmental competence of donor cells, investigating development mechanism of the reconstructed embryos, and preserving the endangered animals [24,25].

Thus, the purpose of the present study was to (1) compare gene expression of canine ASCs grown in two culture medium, (2) analyze *in vitro* development of iSCNT embryos derived from ASCs cultured in two different media, and (3) investigate expression patterns of genes related to stemness, reprogramming, and pre-implantation development in iSCNT embryos.

2. Materials and methods

2.1. Donor cell culture and preparations

Canine ASCs were prepared as described previously [11]. In brief, cells were isolated from subcutaneous fat tissue collected from the abdomen of a healthy beagle dog under a protocol approved by Seoul National University. Cryopreserved cells at passage 0 were thawed and cultured in two different medium: RCMEP (ASCs culture medium; Keratinocyte-SFM (Invitrogen)-based medium containing 0.2 mmol/L ascorbic acid, 0.09 mmol/L calcium, 5 ng/mL rat EGF, and 5% fetal bovine serum; RNL Bio Ltd., Seoul, Korea) [26] and Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum. Cell adhesion was examined under a microscope 24 hours later and cells were washed in PBS. The respective media were replaced daily. The ASCs at passage 3 were used as iSCNT donor cells. When the ASCs reached 80% to 90% confluence, they were subpassaged with each medium. Donor cells were synchronized to the G0 phase of the cell cycle by contact inhibition. Before SCNT, cells were disaggregated by 0.25% trypsin EDTA treatment for 3 minutes.

2.2. Flow cytometry analysis

The cell fraction at passage 3 was cultured in RCMEP and DMEM until confluence. After trypsinization, detached cells were suspended in PBS at a concentration of 1×10^6 cells/1 mL. Cells were stained using specific antibodies: CD29 (1:100, BD Pharmigen, San Jose, CA), CD44 (1:100, Serotec, Oxford, UK), and CD90 (1:100, Serotec) were fluorescein isothiocyanate conjugated. CD31 (1:100, BD Pharmigen), CD 34 (1:100, BD Pharmigen), and CD 45 (1:100, BD Pharmigen) antibodies were phycoerythrin conjugated. CD29 is a rat specific and CD105 mouse antibody. CD34, CD44, and CD90 are canine specific; others are specific to human. The degree of cell surface markers was examined by FACS Calibur (BD Biosciences) using CELL Quest software.

2.3. Oocyte collection and IVM

Bovine ovaries were collected from a local abattoir and transported within 2 hours in 0.9% (w/v) NaCl solution at 35 °C. Cumulus oocyte complexes were retrieved from antral follicles 2 to 8 mm in diameter by aspiration with an 18-ga needle attached to a 10 mL syringe. The cumulus oocyte complexes with evenly granulated cytoplasm and comprising more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen), supplemented with 10% fetal bovine serum, 2 mmol/L $NaHCO_3$, and 1% penicillin-streptomycin (v/v). For IVM of bovine oocytes, 30 to 40 cumulus oocyte complexes were cultured in TCM-199 supplemented with 1 μg/mL follicle stimulating hormone (Antrin, Teikoku, Japan), 10 ng/mL epidermal growth factor, 0.57 mmol/L cysteine, 0.91 mmol/L sodium pyruvate, 1% (v/v) Pen-Strep (Invitrogen), at 39 °C in a humidified atmosphere of 5% CO_2 in air for 20 hours.

2.4. Interspecies somatic cell nuclear transfer

We performed iSCNT as described [27]. Briefly, *in vitro*-matured bovine oocytes were denuded of cumulus cells in Hepes-buffered TCM-199 supplemented with 0.1% (v/v) hyaluronidase. Oocytes with extruded polar bodies were selected and exposed to cytochalasin B (5 μg/mL) and Hoechst 33342 (5 μg/mL). Metaphase chromosomes were removed under ultraviolet light with a fine-needle pipette. Enucleated oocytes were randomly divided into two groups for testing canine ASCs cultured in RCMEP and DMEM. Single donor cells cultured in each medium were transferred to an enucleated metaphase II oocyte. A couplet of donor cell–cytoplast complexes was induced to fuse using two pulses of direct current, 35 to 40 V/cm for 15 μs each using an Electro-Cell fusion apparatus (NEPA GENE Co., Chiba, Japan). Chemical activation was performed by incubating the reconstructed embryos in modified synthetic oviductal fluid containing 5 μmol/L ionomycin. Cloned iSCNT embryos were transferred into 40-μL microdrops of modified synthetic oviductal fluid covered with mineral oil and cultured in an atmosphere of 39 °C, 5% CO_2,