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Derivation and characterization of putative embryonic stem cells from cloned rabbit embryos

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ABSTRACT

The present study aimed to establish embryonic stem (ES) cell lines, i.e., ntES cells, using rabbit blastocyst stage embryos cloned by somatic cell nuclear transfer. First, we investigated the development of cloned rabbit embryos reconstructed with normal fibroblasts and fibroblasts transfected with enhanced green fluorescence protein (eGFP). Blastocyst rates were 27.4% and 23.9%, respectively, for the embryos reconstructed with normal fibroblasts and fibroblasts transfected with eGFP compared with that from the parthenogenetic group (43.1%). One ntES cell line was established from embryos reconstructed with eGFP-transfected fibroblasts (1 of 17, 5.9%), and three ntES cell lines were derived from those with normal fibroblasts (3 of 17, 17.6%). All the ntES cell lines retained alkaline phosphatase activity and expressed ES cell–specific markers SSEA-4, Oct-4, TRA-1-60, and TRA-1-81. The pluripotency was further confirmed by reverse transcription–polymerase chain reaction analyses of *Oct-4*, *Nanog*, and *Sox-2* expressions in ntES cell lines. The differentiation capacity of ntES cells was also examined *in vitro* and *in vivo*, by which these ntES cell lines were able to differentiate into all three germ layers through embryoid bodies and teratomas. In conclusion, it is apparent that the efficiency of ntES cells derived using eGFP-transfected donor cells is lower than that with nontransfected, normal fibroblasts donor cells. Similar to those from parthenogenetic embryos, all ntES cell lines derived from cloned rabbit embryos are able to express pluripotency markers and retain their capability to differentiate into various cell lineages both *in vitro* and *in vivo*.

1. Introduction

Rabbits (*Oryctolagus cuniculus*) are classical laboratory animals with many advantages over rodents and other species and have been used for studying human diseases including hypertension [1,2], myocardial infarction [3–5],

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bone and cartilage disorders [6,7], arteriosclerosis [8–10], diabetes [11–13], and so forth. With the progress in molecular technology, they have been genetically modified using most recently developed tools including TALEN [14] and CRISPR-Cas9 [15,16] to model several neuronal disorders [17].

Embryonic stem (ES) cells are derived from the inner cells mass of blastocysts, which possess the ability to differentiate into all the cell lineages of animal body. These cells can be potentially used to study and treat various degenerative diseases [18,19]. Disadvantage of embryo-derived ES cells including those from fertilized embryos (f-ES cells) and parthenotes (p-ES cells) is that they are genetically divergent from the donor or the recipient. After transplantation, various degrees of immune response from the recipient are prominent in rejecting the transplanted ES cells [20–23]. One solution to the immune rejection would be to generate isogenic ES cells from the patient [20]. The genetically reprogrammed somatic cells, also known as induced pluripotent stem (iPS) cells, exhibit functional similarities to ES cells, and they have been successfully produced by many laboratories in several species including humans [24–26]. However, more in-depth study on iPS cell biology is indispensable before they can meet the demands for future clinical applications. Moreover, iPS cells differ from ES cells in many ways, such as DNA methylation [27], epigenetic status [28], gene expression profile [29], and response to induced neural differentiation [30]. An ideal alternative to circumvent the immune rejection problem would be to generate patient-specific ES cell lines by somatic cell nuclear transfer (SCNT) [20].

In addition, study on rabbit SCNT has been reported and early-stage embryonic cells from preimplantation embryos were used as donor cells that can support the full-term development of the cloned pubs [31]. Live births of rabbit clones were reported using different types of somatic donor cells including cumulus cells [32–34], fibroblasts [35], and mesenchymal stem cells [17]. However, how to increase rabbit ES (rES) cell cloning efficiencies have not been systemically studied. In our previous study, we found that young oocytes retrieved from preovulatory follicles (follicular oocytes) were better in quality with a better efficiency to support the development of cloned rabbits [36,37]. To continue the study, we further attempt to establish rabbit ES cells from SCNT embryos using follicular oocytes and protocols that we used to derive f-ES and p-ES cells [38,39].

In the present study, we have first successfully generated high-quality cloned blastocysts using donor cells from rabbit ear fibroblasts to establish ntES cell lines, whose morphology, pluripotency, and differentiation potentials are comparable with those of f-ES and p-ES cells.

2. Materials and methods

2.1. Reagents and animals

The care and use of animals for embryo recovery complied with the guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University, Taiwan, ROC (IACUC Permit NO. 96–72). Chemicals and reagents used were mainly purchased from Sigma–Aldrich Co., Ltd., unless otherwise mentioned. The severe combined immunodeficiency (SCID) mice were purchased from BioLasco Taiwan Co., Ltd., and raised in accordance with the IACUC guidelines of National Chung Hsing University. When the sacrifice of animals was essential, all efforts were made to minimize suffering animals.

2.2. Generation and culture of rES cell lines form SCNT embryos

Approximately 12 hours after hCG injection, recipient oocytes were recovered from both the preovulatory follicular oocytes and the ovulated oocytes using flushing medium supplemented with 3.36 g/L $NaHCO_3$ (S5761), 10% fetal bovine serum (FBS; Gibco 26140–079), 20 mM HEPES (H3375), and 1% antimycotics (Gibco 15240). Cumulus cells from *in vivo*–matured rabbit oocytes were removed by repeated pipetting in 0.1% (vol/vol) hyaluronidase in Dulbecco's phosphate-buffered solution (DPBS).

For enucleation, oocytes were transferred to a droplet of HEPES-TCM 199 containing 5 μg/mL cytochalasin B and 10% FBS under inverted microscope (Nikon) equipped with micromanipulation system (Narishige). Oocytes were first hold with holding pipette and the zona pellucida was cut nearby the position of the first polar body (PB) by a fine glass needle. The cytoplasm adjacent to the first PB was then squeezed out, along with the first PB, from the cut with the same glass needle. Success of enucleation was checked by Bisbenzimide (Hoechst 33342) staining for 5 minutes and observed using an inverted microscope equipped with epifluorescence. For nuclear transplantation, donor cells are trypsinized (0.05% trypsin) and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS for 1 hour before transfer. Single donor cells were inserted into the perivitelline space of the enucleated oocytes by micromanipulators. Electro cell fusion (3.2 kv/cm, 20 μs, and three pulses) was applied to fuse the donor cell with the cytoplasm of the reconstructed embryos. Successfully fused embryos were then incubated in the activation medium containing 6-dimethylaminopurine (2 mM) and cycloheximide (5 μg/mL) for 1 hour followed by culturing in the Menezo's B2 medium (Laboratoire CCD, Paris, France) supplemented with 2.5% FBS in an incubator (39 °C, 5% CO_2 with humidified air) for 4 days on the basis of our previous protocols [40].

2.3. Derivation and culture of rES cell lines

After 4 days of culture, cloned rabbit blastocysts were plated on the mitomycin C–inactivated mouse embryonic fibroblast monolayers in the ES cell medium. The medium consisted of 81.5% DMEM (D7777), 15% fetal calf serum (26140–079; Gibco), 4 mM L-glutamine (G8540), 0.5% nonessential amino acids, 0.1 mM α-mercaptoethanol (M7522), and 1000 U/mL murine leukemia inhibitory factor (ESG 1107; Chemicon, Temecula, CA, USA). After embryos attaching to the feeders, culture medium was changed every other day [40]. Seven days after culture, the inner cell mass outgrowths were picked and passaged to fresh feeder cells in the ES cell medium.