Nuclear Transfer in Rats Using an Established Embryonic Cell Line and Cumulus Cells

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Abstract. The present study was performed to establish a rat cell line from blastocysts and to examine the developmental ability of nuclear transfer embryos reconstituted from the established cells and cumulus cells with enucleated mature oocytes. No colonies of embryo-derived cells were observed when morulae were cultured. In contrast, 89% and 40% of the attached embryos formed colonies when blastocysts and hatched blastocystes were cultured, respectively. When the colonies were subcultured in the absence of feeder cells, a cell line with round cell morphology was obtained. This cell morphology was stable up to at least passage 23. The percentage of these embryo-derived cells at G0/G1 was increased when cells were cultured in medium with 0.5% FCS for 48 and 72 h. Ninety-three percent of fresh, non-cultured cumulus cells were at G0/G1. The percentages of reconstructed embryos survival (44–48%), and developed to the 2-cell stage (35%), were significantly higher when the nuclei of cumulus cells were used than when the nuclei of blastocyst-derived cells were used (29% and 3%). The results of the present study indicate that a rat cell line can be established from blastocysts and maintained in vitro for a long period, and that reconstructed embryos obtained by transferring the blastocyst-derived cell nuclei into enucleated oocytes have poorer development potential than those obtained using cumulus cell nuclei. No reconstructed embryos developed beyond the 2-cell stage in vitro.

Key words: Nuclear transfer, Cell line, Cumulus cells, Activation, Rats

Accepted for publication: April 25, 2002
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Nuclear transfer has important implications for not only multiplication of embryos of domestic animals, but also for the elucidation of genomic totipotency or multipotentiality of embryonic and somatic cell nuclei [1–4]. The technique is also useful for the production of transgenic animals [5, 6], and for the generation of gene-targeted animals [7]. Viable lambs were produced from embryos reconstituted with cells of an established embryonic cell line and enucleated oocytes [8, 9]. Recently, cloned animals have been produced from enucleated oocytes receiving nuclei of various somatic cells including cumulus cells in sheep [10], cattle [6, 11, 12], mice [13, 14], goats [15] and pigs [16–18]. However, information on nuclear transfer (NT) in rats is limited. Attempts to produce rats from NT using embryonal blastomeres [19] and various somatic cells including cumulus and fibroblast cells [20–22] have been unsuccessful thus far.

The present study was therefore performed to establish a rat cell line from embryos and to examine the developmental ability of rat NT embryos with embryo-derived cells compared to...
NT embryos with cumulus cells.

Materials and Methods

Preparation of animals
ACI and Wistar rats were placed in polycarbonate cages (25 cm × 40 cm × 20 cm) with wood shavings on the floor in a room at a controlled temperature of 24 ± 2°C and humidity of 65 ± 5% with lights on at 6:00 and off at 18:00. Animals were given bulk type commercial rat feed (High Pure Ace (P); Itochu Co., Ishinomaki, Japan) and tap water ad libitum.

Embryo collection
Sexually mature female ACI rats in proestrus (2–3 months old) were paired with mature males of the same strain. Female rats with sperm in vaginal smears at 10:00 the next morning were considered to be pregnant (day 1). Mated females were sacrificed at 15:00–17:00 on day 5 by cervical dislocation and embryos were collected by flushing the uterine horns with Ca²⁺- and Mg²⁺-free Dulbecco’s PBS (-) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1 mg/ml BSA (fatty acid-free, No. A-6003, Sigma, MO).

Embryo culture
Embryo culture for the formation of colonies was performed as described previously [23] with some modifications. The culture medium was mR1ECM [24–26] supplemented with 0.8 mM NaH₂PO₄ (Wako Pure Chemical Industries Ltd., Osaka), 0.1 mM 2-mercaptoethanol (Wako), nucleosides (0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine, and 0.01 mM thymidine) (all from Sigma), 2000 IU/ml leukemia inhibitory factor (LIF; Chemicon International Inc., CA, USA), 10 ng/ml basic fibroblast growth factor (bFGF; Boehringer Mannheim GmbH, Werk Penzberg, Federal Republic of Germany), and 20% (v/v) FCS (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) instead of polyvinylalcohol. After washing 3 times with medium, morulae, blastocysts and hatched blastocysts (3–5 embryos), collected by uterine flushing, were transferred to the wells of 4-well plates (Nunc) and cultured at 37°C, 5% CO₂ in air until 80% confluent. Culture medium was the same as that for cell culture described previously [23]. Cells were then treated in 0.1 µg/ml colcemid (Wako) for 1 h. After trypsinization in PBS(−) supplemented with 0.25% (w/v) trypsin and 0.5 mM EDTA (T-E) and treatment in 0.075 M KCl solution (Wako) for 20 min, cells were fixed in methanol:glacial acetic acid (3:1) on a glass slide. The chromosome spreads were air dried, stained with Giemsa solution (Merck Ltd., Tokyo) and observed with a microscope (Olympus BX50).

Cell cycle determination of embryo-derived cells and cumulus cells by flow cytometry
Preparation of embryo-derived cells: As described above, after thawing, cells were cultured until confluent. When confluent, the medium was changed for one supplemented with 0.5% FCS, and the culture was continued for 0, 24, 48, 72 or 96 h. At the end of culture, cells were washed once in PBS (-) and then separated in T-E and subjected to flow cytometry.
Preparation of cumulus cells: Immature female
Wistar rats (25–40 days old) were injected intraperitoneally with 10 iu of equine chorionic gonadotrophin (eCG, Teikoku Hormone Mfg. Co., Ltd., Tokyo) followed by an intraperitoneal injection of 10 iu of hCG (Sanyko Kabu Company, Tokyo) 48–52 h later. Eggs were collected as described previously [28]. Briefly, animals were sacrificed by cervical dislocation 20–24 h after administration of hCG. Oocyte-cumulus cell complexes were flushed out of the oviducts with PBS (-) supplemented with 1 mg/ml fatty acid-free BSA (Sigma) and treated with 0.1% (w/v) hyaluronidase (Sigma). Cumulus cells were collected for flow cytometry.

Flow cytometry: To determine the cell cycle stage profiles of blastocyst-derived cells and cumulus cells, flow cytometry was performed. After washing once with PBS (-), cells were fixed in 70% ethanol (Wako) for 30 min on ice. Cells were washed once with PBS (-) and resuspended in 1 ml of 100 U/ml RNase (Stratagene, Austin, Texas) for 30 min to remove RNA. Cells were then washed once with PBS and pelleted. Cell pellets were stained for 30 min at room temperature with 0.005% (w/v) propidium iodide (Sigma) and filtered through a 30-µm mesh before flow cytometry. Cells were analyzed on a FACs Calibur flow cytometer (Becton Dickinson, San Jose, CA) and the percentages of cells within the G0/G1, S and G2/M phases of the cell cycle were determined using the Cell Quest program (Becton Dickinson).

Nuclear transfer by microinjection of cell nuclei into the cytoplasm using a piezo micromanipulator

Recipient oocytes: Immature Wistar rats (25–40 days old) were induced to superovulate as described above. Fifteen hours after hCG injection, animals were sacrificed by cervical dislocation in the morning and cumulus-oocyte complexes (COCs) were collected by flushing the oviducts with fertilization medium consisting of mR1ECM [24–26] containing 110 mM NaCl (Wako) and 4 mg/ml BSA (fatty acid-free, No. A-6003, Sigma, MO) without polyvinylalcohol. COCs were treated with 0.1% (w/v) hyaluronidase (Sigma). Oocytes and cumulus cells were then collected and used as recipient oocytes and donor cells, respectively, in subsequent nuclear transfer.

Enucleation: The oocytes were transferred to 100 µl of PBS(-) supplemented with 7.5 mM D-glucose (Wako), 0.5 mM sodium pyruvate (Wako), 10.0 mM sodium lactate (Sigma), 0.1 mM glutamine (Wako), 0.13 M sucrose (Wako) and 10% FCS (manipulation solution), and the zonae pellucidae were cut with a piezo pulse at intensity 4–10 with speed 2 with a fine glass needle. After cutting, the oocytes were transferred to the fertilization medium. After transfer to manipulation solution supplemented with 5 µg/ml cytochalasin B (Sigma) and 5 µg/ml Hoechst 33342 (Sigma) and culture for 15 min, the oocytes were then transferred to manipulation solution supplemented with 5 µg/ml cytochalasin B. The oocytes were enucleated by removing the first polar body and the metaphase II plate in a small amount of surrounding cytoplasm with a glass pipette. Successful enucleation was confirmed by visualizing the cytoplast and removed cytoplasm under ultraviolet light. After enucleation, the cytoplasts were transferred to fertilization medium and kept in a CO2 incubator until injection of donor cells.

Donor cells: Frozen cells derived from ACI rat blastocysts at passage 4 of culture were thawed and washed once in DMEM supplemented with 10% FCS (v/v). Cells were then transferred to 35-mm dishes (Sumitomo Bakelite Co., Ltd., Tokyo) containing cell culture medium and cultured at 37°C, 5% CO2 in air until confluent. After reaching confluence, cells were cultured in cell culture medium containing 0.5% FCS for 48–72 h, and then treated in T-E to separate individual cells. Cells were transferred to fresh 35-mm dishes and cultured for 30 min. At the end of culture, STO cells attached to the dishes and cells were collected for nuclear transfer.

Recipient cytoplasts and donor cells were transferred to 100 µl of manipulation solution on a microscope stage. The donor cell membrane was broken with a fine glass pipette and the nucleus was separated. Several piezo pulses with intensity 3–4 at speed 2 were used to advance the needle through the zonae pellucidae of enucleated oocytes. After the piece of zonae pellucidae was discarded into the perivitelline, a piezo pulse with intensity 1 at speed 1 was applied to advance the needle through the plasma membrane and then the nucleus was injected into the cytoplasm. Injected oocytes were transferred to fertilization medium and cultured until activation.

Activation and culture: Injected oocytes were washed once in the activation solution, which consisted of 0.3 M mannitol, 0.1 mM CaCl2, 0.1 mM
MgSO₄ (all from Wako), and 1 mg/ml fatty acid-free BSA (Sigma). They were then placed between two wire electrodes (1 mm apart) on the fusion chamber slide with 30 ml of activation solution. A D.C. charge (100 V/mm for 99 µsec) was applied twice to injected oocytes, after which they were transferred to fertilization medium supplemented with 2 mM DMAP and cultured for 2, 3 or 4 h. Three to 21 activated embryos were transferred to 100 µl of mRIECM, cultured for at least 2 days and cleavage was examined.

**Statistical analysis**

The proportions of surviving reconstructed embryos with pronuclei after activation and cleavage shown in Table 2 were subjected to chi-square test.

**Results**

**Establishment of cell lines**

After 2 days of culture, attachment of embryos to feeder cells was observed in all blastocysts and hatched blastocysts, but only in 17% of morulae (Fig. 1a). No colonies of attached embryo-derived cells were observed when morulae were cultured, whereas 89% and 40% of the attached embryos formed colonies in cultures of blastocysts and hatched blastocysts, respectively (Table 1). Round cell morphology in the first passage (Fig. 1b) was stable up to at least passage 23. The number of chromosomes in cells of the fourth passage was 42 (2n).

**Cell cycle of embryo-derived cells and cumulus cells**

The percentages of embryo-derived cells at G0/G1 were 60.6%, 64.0%, 78.7% and 82.3% when cells were cultured in medium with 0.5% FCS for 0, 24, 48 and 72 h, respectively. However, most cells were dead after 96 h of culture and the cell cycle could not be determined. In contrast, 93% of fresh, non-cultured cumulus cells were at G0/G1.

**Nuclear transfer and development of reconstructed embryos in vitro**

When the nuclei of blastocyst-derived cells were injected into cytoplasts, 29% (36/123) of reconstructed embryos survived. Pronucleus formation was observed in 78% (28/36) of the surviving embryos. Three percent (1/36) of reconstructed embryos developed to the 2-cell stage (Table 2). When the nuclei of cumulus cells (Wistar) were injected into cytoplasts, 44–48% of reconstructed embryos survived. The percentages of reconstructed embryos with pronucleus formation that then developed to the 2-cell stage were 75% (15/20) and 35% (7/20), respectively, after reconstructed embryos were activated by electrical stimulation followed by DMAP treatment for 2 h. The percentages of reconstructed embryos with pronucleus formation that then developed to 2-cell stage were 87–88% and 23–14%, respectively, after reconstructed embryos were activated by electrical stimulation followed by DMAP treatment for 3–4 h. The percentages of reconstructed embryos surviving and developing to the 2-cell stage were significantly higher when the nuclei of cumulus cells were used than when those of
blastocyst-derived cells were used. None of the reconstructed embryos developed beyond the 2-cell stage (Table 2, Fig. 2).

**Discussion**

We previously reported establishing a cell line derived from porcine blastocysts *in vitro* and that NT embryos reconstructed from this cell line and enucleated oocytes developed to blastocysts *in vitro* [23]. Campbell *et al.* [8] reported that viable lambs could be produced from NT embryos with cells from an established cell line. Since a cell line can be cultured for a long time *in vitro* and is useful for genetic manipulation *in vitro*, the cell line derived from rat embryos with normal karyotype (2n) in the present study might also be useful for the production of cloned or knockout rats for research purposes.

It has been reported that the cell cycle stage of donor cells is an important factor for the development of nuclear transfer embryos [23]. Production of animals by the nuclear transfer of differentiated cells has been achieved mainly by transferring nuclei in G0 or G1 phase to untreated recipients [6, 8, 10, 11, 14, 15]. In the present study, the population of embryo-derived cell line cells in G0/G1 phase was 60.6% before culture, and this increased to about 80% after culture for 48–72 h in low FCS solution, and that of cumulus cells in G0/G1 phase without culture was 93%. These results are in agreement with previous reports that about 40–60% of embryonic cell line cells in cattle are in G0/G1 phase at any time [29]. It has been reported that percentages of cumulus cells after ovulation were 76-90% or more than 90% in G0/G1 phase of the cell cycle in the mouse [14, 30, 31].

Activation of recipient cytoplasts by electrical or a combination of electrical and chemical stimuli is a critical element of the nuclear transfer procedure [32]. Combined electrical and 6-DMAP treatments were used in nuclear transfer in cattle [6] and
rabbits [32, 33]. Prolonged exposure to 6-DMAP has been shown to result in chromosomal abnormalities in cattle [34, 35]. Our parthenogenetic experiments in rats indicated that a combination of two electrical pulses with 4 h of 6-DMAP treatment significantly improved blastocyst development rates [36]. The results of the present study showed that a combination of two electrical pulses with 2–3 h of 6-DMAP treatment significantly improved cleavage rates of rat NT embryos, demonstrating that electrical stimulation followed by 6-DMAP treatment is also an effective activation procedure in rat nuclear transfer.

Cumulus cells surrounding oocytes have been used to produce cloned mice [14] and cattle [11]. Although a study in porcine NT indicated that there was no difference in developmental ability of NT embryos receiving cumulus cells and fibroblast cells [37], it has been suggested that developmental ability of NT embryos with somatic cells could be different according to cell type in mammals [29, 31, 38, 39]. A recent study in the mouse indicated that more NT embryos receiving follicular epithelial cells developed into blastocysts in vitro and into fetuses in vivo than those receiving cumulus cells [31]. The present study showed that more NT embryos receiving the nuclei of cumulus cells survived and developed to the 2-cell stage than those receiving the nuclei of blastocyst-derived cells. However, none of the NT embryos developed beyond the 2-cell stage in vitro, which might have been due to the failure of normal reprogramming of injected donor nuclei rather than the culture system since both oocytes fertilized in vitro and those activated artificially developed to blastocysts with reasonable percentages in our culture system [28, 36].

Acknowledgments

This work was supported by grants from the Program for Promotion of Basic Research Activities for Innovative Biosciences and the “Research for the Future” Program, the Japan Society for the Promotion of Science (JSPS-RFTF97L00904).

References


