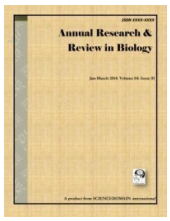

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In vitro Development of Chimeric Bovine Embryos Created by Combining a Single Blastomere of SCNT Embryos with an IVF Embryo

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KS, MK and ARAH designed the study. Authors CT, DI and TN performed the experiments. Authors KS, DI and MK analyzed the data. Authors KS and DI wrote the paper. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

In this study, we produced chimeric embryos from a single blastomere of somatic cell nuclear transfer (SCNT) bovine embryo and *in-vitro* fertilized (IVF) bovine embryo, and examined the rate of blastocyst development and the contribution of the SCNT blastomere to the blastocyst in order to improve cloning efficiency. We produced SCNT embryos from bovine fibroblasts carrying a luciferase gene under the control of the β-actin promoter. At 96 hours post fusion, chimeric embryos were produced by transferring a single blastomere of a 16-cell SCNT embryo to the perivitelline space of an IVF embryo. At 4 days after production of the chimeric embryos, half of the embryos reached the blastocyst stage, which is the same as that of IVF embryos. Furthermore, luciferase activity in blastocysts from the chimeric embryos was detected in both the inner cell mass and

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trophectoderm. These results indicate that single blastomeres of SCNT embryos contributed to both the inner cell mass and trophectoderm in blastocysts from chimeric embryos.

Keywords: Somatic cell nuclear transfer; chimera; bovine embryo; cell aggregation; early development.

1. INTRODUCTION

Animal cloning by somatic cell nuclear transfer (SCNT) has many applications for agriculture and biomedicine and is also a valuable tool for basic research and the conservation of threatened species. Since the birth of Dolly [1], cloned offspring have been produced in several species [2]. However, the efficiency of SCNT remains low due to high rates of embryonic, perinatal and neonatal loss [3,4]. Somatic cell nuclear transfer has also been associated with extraembryonic abnormalities such as placentomegaly and reduction of placentome number [5,6].

Chimeric embryos created by combining an SCNT embryo with an SCNT or in-vitro fertilized (IVF) embryo have been reported to improve the efficiency of blastocyst and full-term development in mice and rabbits [7,8]. Chimeric bovine embryos created by combining an IVF embryo with a parthenogenetic or SCNT embryo have been also developed to term [9,10]. Early studies using aggregates of two developmentally asynchronous mouse embryos showed that early dividing blastomeres have a greater tendency to contribute to the inner cell mass than do later dividing ones [11-13].

In this study, we examined the developmental competence of chimeric bovine embryos created by combining an IVF embryo at the 8-cell stage and a single blastomere of an SCNT embryo at the 16-cell stage. The SCNT embryo was produced from a fibroblast carrying a luciferase gene so that the fate of the SCNT cell could be traced. Cells derived from the SCNT blastomere were localized in a blastocyst obtained from the chimeric embryos.

2. MATERIALS AND METHODS

2.1 Ethics

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of Kinki University (#KAAT-21-001).

2.2 Oocytes Collection and In-Vitro Maturation

Bovine oocytes were matured as described previously [14]. Briefly, bovine ovaries were obtained from a local slaughterhouse and were transported in saline at 20-25℃. Cumulus-oocyte complexes (COCs) were collected from the ovaries, and then they were washed with 25 mM Hepes-buffered TCM199 with Hanks' salts (199H: Gibco, Invitrogen Life Technologies, Tokyo, Japan) supplemented with 5 % (v/v) FBS and 25 μl/ml gentamicin (FBS-199H). The washed COCs were cultured for 18-21 h in 50 μl of 25 mM Hepes-buffered TCM199 with Earle's salts (199E: Gibco) supplemented with 5% FBS, 0.5 mM sodium pyruvate, 25 μg/ml gentamicin, 0.02 AU/ml FSH (Antrin: Kyoritsu Pharmaceutical, Tokyo, Japan) and 1 μg/ml estradiol-17β covered with paraffin oil at 39℃ in 5% CO_2 in air in high humidity (10 COCs/droplet).

2.3 Preparation of Donor Cells for Nuclear Transfer

We used bovine transfected fibroblasts carrying an improved *firefly luciferase (luc$^+$)* gene under the control of a *chicken β--actin* promoter as a nuclear donor [15]. The promoter was bound to the upstream of the luciferase gene and internal ribosome entry site (IRES) sequence and an enhanced green fluorescent protein (EGFP) gene were added to the downstream of the luciferase gene, followed by a neomycin resistance cassette [p*β-act/luc$^+$/IRES/EGFP/(neo^r)*]. The p*β-act/luc$^+$/IRES/EGFP/(neo^r)* was transfected into the fibroblasts using a transfection reagent (GeneJammer: Stratagene, La Jolla, CA, USA). Forty-eight hours after transfection, the culture medium was replaced with Dulbecco's Modified Eagle's Medium (DMEM: Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20% fetal bovine serum (FBS: BioWest, Paris, France) and 600 μg/ml G-418 (Geneticin: Invitrogen Corp, Carlsbad, CA, USA), and then the cells were cultured in the medium for 15-20 days for selection of neomycin-resistant cells. The surviving cell colonies were further selected by their EGFP

fluorescence under UV light to obtain stably transfected cells.

Quiescent cells induced by serum starvation (0.4% FBS-DMEM) for 7 days were used for nuclear transfer. Only EGFP-positive cells were used for SCNT.

2.4 Production of SCNT Embryos

Somatic cell nuclear transfer was carried out as described previously [16]. The surrounding cumulus cells were removed by pipetting COCs at 18-21 h post-maturation in FBS-199H containing 0.25% (w/v) hyaluronidase. The zonae pellucidae of oocytes with the first polar body were slit with a fine glass needle. A small volume of the cytoplasm and the polar body was moved by the pressing the oocyte with the needle. Enucleation was confirmed by visualizing the karyoplast under UV light after staining a small amount of cytoplasm with 20 µg/ml Hoechst 33342. Donor cells that were synchronized at the G0 or G1 phase were inserted into the perivitelline space of the enucleated oocytes. These couplets were held by two needle electrodes [17] and were electrically fused by two direct current pulses of 2.72 kV/cm for 11 µsec each using an Electro Cell Manipulator (ECM-200; BTX, San Diego, CA, USA) in Zimmerman fusion medium [18]. Fused couplets were activated with 5 µM ionomycin for 5 min and then treated with 10 µg/ml cycloheximide in modified synthetic oviduct fluid medium [mSOFM: 14] without KH_2PO_4 until 6 hours post fusion (hpf) at 39℃ in 5% CO_2, 5% O_2 and 90% N_2 with high humidity. Following activation, the SCNT embryos were cultured in 50 µl CR1aa supplemented with 5% FBS (5% FBS-CR1aa) covered with paraffin oil until luminescence was detected at 39ºC in 5% CO_2, 5% O_2 and 90% N_2 with high humidity (20-30 embryos/droplet).

2.4 *In vitro* Fertilization

In-vitro fertilization was carried out as described previously [14]. Briefly, frozen-thawed spermatozoa were washed with a discontinuous gradient Percoll solution [Amersham Biosciences, Uppsala, Sweden; [19]. Matured COCs that had been cultured for 21 h in the maturation medium were inseminated with Percoll washed spermatozoa in a defined medium [20] modified by excluding glucose and supplemented with heparin (yielding final concentrations of 2 x 10^6 sperm and 10 µg heparin/ml). Oocytes and spermatozoa were co-incubated for 18 h under 5% CO_2 in air at 39℃ with high humidity (10 COCs/100 µl). Following fertilization, the surrounding cumulus cells were removed from the oocytes by gently pipetting. The oocytes were cultured under the same conditions as the SCNT embryos.

2.5 Determination of Luciferase Activity in SCNT Embryos

Luciferase activity was detected in SCNT embryos as described previously [16]. Briefly, SCNT embryos that had developed to the16-cell stage at 96 hpf were selected under a stereomicroscope (x60). The luminescence of the embryos was then detected in a medium containing 500 µM D-luciferin using an imaging photon counter (ARGAS 50: Hamamatsu Photonics, Shizuoka, Japan). The luciferase activity of luminescent embryos was recorded. The mean LUC^+ activity of each embryo was calculated from the total photon count accumulated in the 10-min period and was displayed in relative light units (RLUs).

2.6 Production of Chimera Embryos by Microinjecting a Single Blastomere of a SCNT Embryos into an IVF Embryos

The zonae pellucidae of the SCNT embryos with luminescence were removed after determination of luciferase activity. The SCNT embryos were washed in 5% FBS/0.1% PVA-199H, placed in PBS (-) for 5 min and then segregated by gently pipetting. The segregated blastomeres were kept in 5% FBS/CR1aa at 39℃ in 5% CO_2, 5% O_2 and 90% N_2 with high humidity until production of chimeric embryos. At 48 hpi, IVF embryos that had developed to the 8-cell stage were selected under a stereomicroscope (x60) and then the zonae pellucidae of the embryos was slit using a fine glass needle. Single blastomeres from SCNT embryos at the 16-cell stage were transferred in 199H supplemented with 0.1% (w/v) polyvinyl alcohol and 100 µg/ml Phytohemagglutinin (PHA) and inserted into the perivitelline space of the IVF embryos at the 8-cell stage. Chimeric embryos that showed luminescence were cultured in 50 µl 5% FBS-CR1aa covered with paraffin oil until day 4 after production of chimeric embryos at 39℃ in 5% CO_2, 5% O_2 and 90% N_2 with high humidity.

2.7 Statistical Analysis

All data were analyzed with chi-square test with Fischer's exact probability test. Differences of $p<0.05$ were considered to be significant.

3. RESULTS AND DISCUSSION

In the first experiment, 16 chimeric embryos were produced by microinjecting single blastomere of SCNT embryos into IVF embryos at the 8-cell stage (Fig. 1). Four days later, production of chimeric embryos, the blastocyst rate of chimeric embryos was higher than that of SCNT embryos and was the same as that of IVF embryos (Table 1).

In the next experiment, we examined the luciferase activity of the different types of embryos. Luciferase activity was detected in both the inner cell mass and trophectoderm in all blastocysts of SCNT and chimeric embryos, but not in blastocysts of IVF embryos (Table 1 and Fig. 2). Cells derived from the single blastomere were localized in both the inner cell mass and the trophectoderm in all chimeric embryos. At the 32-cell stage, differentiation of the inner cell mass and trophectoderm during the generation of blastocyst tends to depend on where the blastomeres are located in the embryo [21]. Further studies are needed to determine how the location of the microinjected SCNT blastomere in a chimeric embryo (i.e., an inner or outer location) affects in-vitro development and the distribution of cells derived from the SCNT blastomere. In this study, we did not examine the in-vivo development of the chimeric embryos. Although microinjection of a single IVF blastomere into SCNT embryos was shown to improve the potential to develop to full-term, most tissues were found to have originated from IVF-derived cells [9]. Further studies are needed to determine the ability of the chimeric embryos to full-term and the contribution of a single SCNT blastomere to the germ line of the offspring.

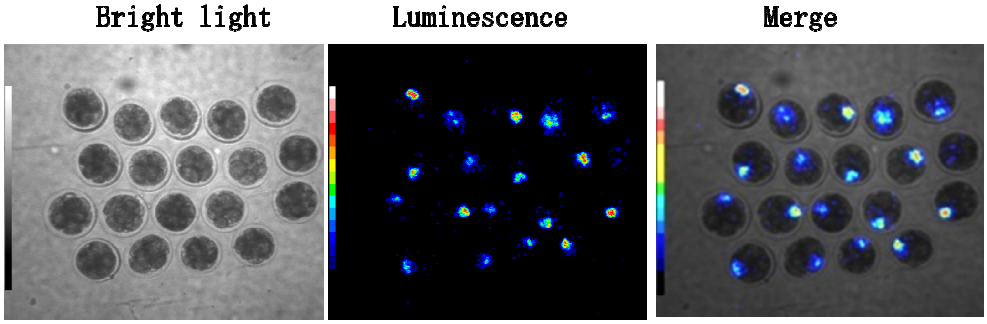

Fig. 1. Luminescent SCNT-derived cells in chimeric embryos

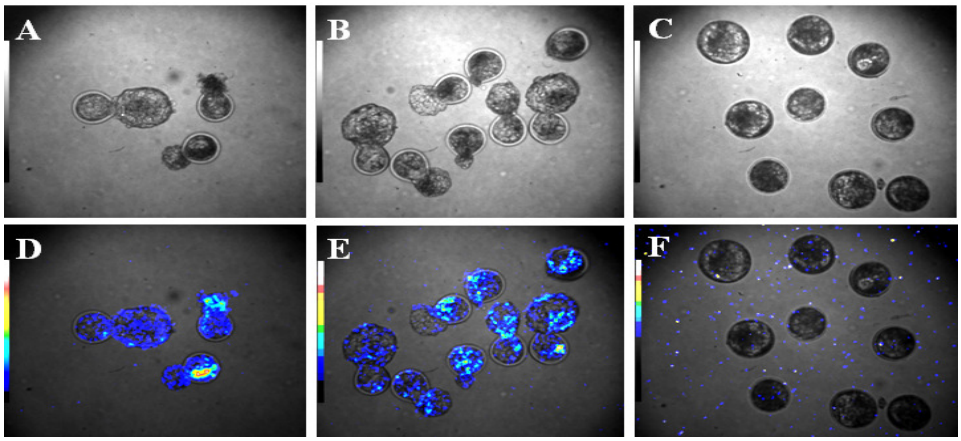

Fig. 2. Luminescence image of SCNT, chimeric and IVF blastocysts

Upper panel: Bright light image of SCNT (A), chimeric (B) and IVF (C) blastocysts. Lower panel: Luminescence image of SCNT (D), chimeric (E) and IVF (F) blastocysts. Luciferase activity in all SCNT and chimeric blastocysts was detected

Table 1. Early development and localization of luminescent activity in chimeric embryos created by combining a single blastomere of an SCNT embryo with an IVF embryo

Group	Developmental stage		No. of embryos cultured	No. (%) of blastocysts	No. (%[1]) of luminescence positive
	SCNT	IVF			
SCNT	8- or 16-cell		18	3(17)[a]	3(100)[a]
Chimeric	16-cell	8-cell	16	8(50)[b]	8(100)[a]
IVF		8-cell	25	13(52)[b]	0(0)[b]

[1]*Percentages with respect to the number of blastocysts obtained,* [a-b] *Superscripts in the each column denote significant differences (p<0.05)*

4. CONCLUSION

In conclusion, our results show that all chimeric embryos produced by microinjection of a single blastomere of SCNT embryos into an IVF embryo can develop to the blastocyst stage, and that cells derived from a single SCNT blastomere contribute to both the inner cell mass and trophectoderm in the all chimeric blastocysts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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