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Production of somatic cell nuclear transfer embryos using *in vitro*-grown and *in vitro*-matured oocytes in rabbits

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Summary

We examined growing oocytes collected from follicles remaining in superovulated rabbit ovaries, that were grown (*in vitro* growth, IVG) and matured (*in vitro* maturation, IVM) *in vitro*. We produced somatic cell nuclear transfer (SCNT) embryos using the mature oocytes and examined whether these embryos have the ability to develop to the blastocyst stage. In addition, we examined the effects of trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), on the developmental competence of SCNT embryos derived from IVG–IVM oocytes. After growth for 7 days and maturation for 14–16 h *in vitro*, the growing oocytes reached the metaphase II stage (51.4%). After SCNT, these reconstructed embryos reached the blastocyst stage (20%). Furthermore, the rate of development to the blastocyst stage and the number of cells in the blastocysts in SCNT embryos derived from IVG–IVM oocytes were significantly higher for TSA-treated embryos compared with TSA-untreated embryos (40.6 versus 21.4% and 353.1 $\pm$ 59.1 versus 202.5 $\pm$ 54.6, $P < 0.05$). These results indicate that rabbit SCNT embryos using IVG–IVM oocytes have the developmental competence to reach the blastocyst stage.

Keywords: Follicle, *In vitro* growth, Nuclear transfer, Rabbit, Trichostatin A

Introduction

The mammalian ovary includes a large number of oocytes at various growth stages. Nevertheless, most of these oocytes undergo atresia and are not ovulated. If these oocytes could be grown to reach meiotic competence *in vitro*, they would provide a large potential source for protection of endangered species. *In vitro* growth (IVG) of oocytes has been researched and reported in mice (Nayudu & Osborn, 1992; Johnson *et al*., 1995; Cortvrindt *et al*., 1996; Eppig & O'Brien, 1996), pig (Hirao *et al*., 1994; Hashimoto *et al*., 2007b), cattle (Gutierrez *et al*., 2000), and rabbits (Kanaya *et al*., 2007; Sugimoto *et al*., 2009, 2012). In mice (Eppig & Schroeder, 1989) and cattle (Yamamoto et *al*., 1999; Hirao et *al*., 2004), there have been reports of successful production of offspring from mature oocytes after IVG, *in vitro* maturation (IVM), and *in vitro* fertilization. The efficacy of mature oocytes from IVG has previously been shown; however, there are few data on the use of mature oocytes obtained from IVG.

Conversely, somatic cell nuclear transfer (SCNT) can be applied in various fields, such as the reproduction of highly valuable domestic or endangered mammals. Since cloning was first reported in sheep (Wilmut *et al*., 1997), many mammalian species have been cloned successfully using SCNT, including mice (Wakayama *et al*., 1998), cattle (Cibelli *et al*., 1998; Kato *et al*., 1998), pig (Betthauser *et al*., 2000; Polejaeva *et al*., 2000), and rabbits (Chesné *et al*., 2002). Typically, ovulated oocytes have been used as recipients for SCNT. In domestic

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animals such as cattle and pig, IVM oocytes have been used for SCNT. However, there has been only one study reporting the use of IVG–IVM oocytes for SCNT in cattle (Hirao *et al*., 2013).

The present study used rabbits as the experimental animal because they have long been used as experimental models for certain human diseases (Garibaldi & Goad, 1988) and for developing reproductive technologies such as *in vitro* fertilization and intracytoplasmic sperm injection (Hosoi *et al*., 1981; Ogonuki *et al*., 2005). We also reported previously a culture system that is capable of producing blastocyst stage embryos from rabbit IVG–IVM oocytes (Sugimoto *et al*., 2012).

The purpose of this study was to create SCNT embryos using IVG–IVM oocytes as recipients and to investigate the effects of the source of the recipient oocytes on embryonic development *in vitro* using IVG–IVM oocytes. In addition, because trichostatin A (TSA) has been reported to improve the development of cloned embryos in rabbits (Shi *et al*., 2008), we examined the effects of TSA on the development of SCNT embryos using IVG–IVM oocytes as recipients.

Materials and methods

Animals

Animal experiments were approved by the Ethical Committee for Animal Experimentation of Kinki University, Faculty of Biology-oriented Science and Technology. All rabbits for experimentation were obtained from Kitayama Labes Co., Ltd (Nagano, Japan).

Embryo culture medium

The embryos were cultured in CMRL-1066 medium (Gibco) supplemented with 0.146 mg/ml L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 0.55 mg/ml sodium pyruvate (Sigma-Aldrich), 1.861 μl/ml DL-lactate (synthetic and glucose-free; Sigma-Aldrich), 0.063 mg/ml penicillin G potassium salt (Nacalai Tesque, Kyoto, Japan), 5.0 μl/ml gentamicin solution (Sigma-Aldrich), and 20% fetal bovine serum (FBS; HyClone, ThermoFisher Scientific, Waltham, MA, USA) at 38°C with 5% CO_2, 5% O_2, and 90% N_2 under mineral oil for 5 days. Hereafter, this medium is described as m-CMRL.

Collection of oocytes and oocyte–cumulus–granulosa cell complexes

Female New Zealand white rabbits (approximately 3.5 kg in weight and >6 months old) were superovulated by subcutaneous injection of 80 IU pregnant mare serum gonadotropin (Sankyo LifeTech, Kanagawa, Japan) and then intravenously administered 60 IU human chorionic gonadotropin (hCG; Teikoku Zoki, Tokyo, Japan) 72 h later. Mature oocytes were flushed from the oviducts using M2 medium 14 h after hCG injection. Cumulus cells were removed by gently pipetting with 0.6% (w/v) hyaluronidase in M2 medium for 5 min.

Rabbit ovaries were recovered 14 h after hCG injection. Follicles (300–399 μm) in diameter were isolated from the surface of the ovaries. From these follicles, oocyte–cumulus–granulosa cell complexes (OCGCs) were recovered using 26-gauge needles. Only complexes that contained an oocyte of healthy appearance were used for culture.

Growth and maturation of oocytes

Alpha minimum essential medium (11900–024, Invitrogen, Carlsbad, CA, USA) was used for growth. It was supplemented with 0.05% FBS (KPK 22695, HyClone), 3 mg/ml bovine serum albumin (A7638, Sigma-Aldrich), 50 μl/ml ascorbic acid (A4544, Sigma-Aldrich), 10 μl/ml insulin–transferrin–selenium-A (51300–004, Invitrogen), and 10 μl/ml antibiotic/antimycotic solution (anti-BM; A5955, Sigma-Aldrich). The complexes were cultured individually in 50-μl drops of medium under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 7 days. The cultures were totally replenished with fresh medium every day during the growth period. Because granulosa cell-enclosed oocytes showing normal morphology were considered as having survived, only these oocytes were further cultured to maturation. The oocytes were cultured individually in 50-μl droplets of the maturation medium TCM199 (680557, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mg/ml polyvinyl alcohol (average molecular weight 30,000–70,000; P8136–250G, Sigma-Aldrich), 0.2724 mg/ml water-soluble estradiol (E-4389, Sigma-Aldrich), and 10 ng/ml epidermal growth factor under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 14–16 h.

Preparation of donor cells

Ear skin tissue shaved from a transgenic Japanese white rabbit that expressed the marker enhanced green fluorescent protein (EGFP; Hashimoto *et al*., 2007a; Takahashi *et al*., 2007) was washed in Dulbecco's phosphate-buffered saline (PBS; Gibco 21600–010). The cleaned tissue was sliced into small pieces and cultured on a cell culture dish with Dulbecco's modified Eagle's medium (DMEM, Gibco) that contained 10% (v/v) FBS. During the subsequent 5–7 days of culture, fibroblasts grew out around the tissue pieces. These cells were frozen after 2 weeks from the

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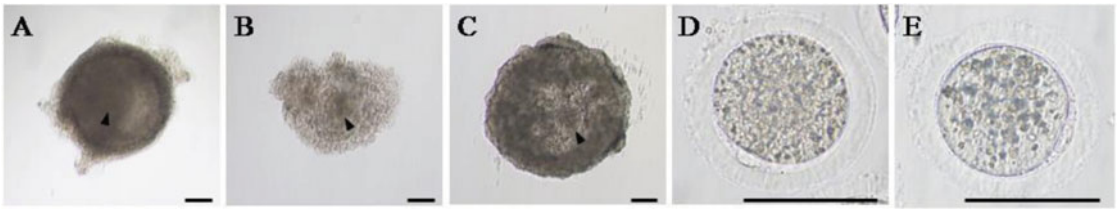

Figure 1 *In vitro* growth and maturation of oocytes from follicles 300–399 μm in diameter. Follicle from rabbit ovary (*A*). Isolated oocyte–cumulus/granulosa cell complex (OCGC) (*B*). Morphology of OCGC on day 7 (*C*). MII stage oocyte after *in vitro* growth and maturation (*D*). Ovulated MII stage oocyte (*E*). Oocytes indicated by arrowheads. Bars = 100 μm.

start of culture. For the preparation of donor cells, thawed fibroblasts from 5–15 passages were cultured to confluence in a 3.5-cm dish and then incubated in DMEM supplemented with 0.5% FBS for 3 days. The cells were harvested with Dulbecco's PBS containing 0.25% (w/v) trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) for 1 min. The suspended cells were washed with DMEM containing 10% FBS and then centrifuged at 350 *g* for 10 min to obtain a cell pellet. The pellet was diluted in DMEM with 10% FBS and maintained at 4°C to prevent aggregation until Electro cell fusion.

Nuclear transfer, fusion, and activation

The oocytes were stained with 5 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B in M2 medium for 10 min. The metaphase plate and first polar body of the oocytes were removed using a micropipette 15–18 μm in outer diameter and visualized by applying ultraviolet (UV) light illumination for 1–2 s. Prior to cell insertion, the enucleated oocytes were allowed to recover in a culture medium for 30 min. A fibroblast was inserted into the perivitelline space of the enucleated oocytes using a micropipette. The cytoplast–fibroblast constructs were then induced to fuse by 2 direct current (DC) pulses of 20 at 2.5 kVcm each in the activation medium (0.3 M mannitol in water that contained 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$). The reconstructed embryos were activated twice by applying the previously described electrical pulses every 30 min and incubated with 2 mM 6-dimethylaminopurine (6-DMAP) in m-CMRL for 2 h. Subsequently, the cloned embryos were washed and cultured in m-CMRL.

Trichostatin A treatment

Immediately following electrical activation, the reconstructed embryos were treated for 2 h with 2 mM 6-DMAP in m-CMRL with 100 nM TSA. Thereafter, the cloned embryos were cultured in m-CMRL with 100 nM TSA for 4 h and then washed and cultured in m-CMRL.

Cell count of blastocysts

The blastocysts were fixed and stained with 4% paraformaldehyde that contained 10 μg/ml Hoechst 33342 for 30 min and then washed twice in PBS that contained 0.1% PVA for 5 min. They were mounted on a glass slide and the cells were counted under a fluorescence microscope using UV light excitation.

Statistical analysis

Fusion, cleavage, development to the blastocyst stage, and number of cells in the blastocysts were evaluated and the data was compared by *t*-test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

In vitro development after SCNT of IVG–IVM oocytes as recipients

Growing oocytes recovered from follicles 300–399 μm in diameter remaining in superovulated ovaries were cultured for growth and maturation *in vitro* (Fig. 1). In total, 121 growing oocytes were obtained from 10 rabbits ($n = 5$) and cultured for growth *in vitro*. Each experiment used two rabbits at a time. After culture for maturation, 57 oocytes reached the metaphase II (MII) stage (Table 1). For the control, 100 ovulated oocytes (MII stage) were obtained from 10 rabbits ($n = 5$; Table 1). With respect to development after SCNT (Fig. 2), the rates of fusion, cleavage and development to the blastocyst stage were not significantly different for SCNT embryos derived from IVG–IVM oocytes compared with those derived from ovulated oocytes (Table 1).

Effect of trichostatin A on SCNT embryo development

To examine the effect of TSA on the development of cloned embryos, the reconstructed oocytes were treated with TSA for 6 h. No distinct increase was observed in the rate of development to the 4-cell

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Table 1 Growth and maturational competence of rabbit oocytes from follicles with diameters of 300–399 μm and preimplantation stage development of SCNT embryos

Oocyte growth and maturation	No. of trials	No. for IVG[a]	No. (%) IVM[b]	Nuclear morphology of oocytes (%)[c]			No. (%) of fused	No. (%) of embryos developed[d]		
				GV/IVM	MI/IVM	MII/IVM No. for nuclear transfer		Cleaved/ fused	Blastocyst/ fused	Blastocyst/ IVG
In vitro	5	121	111 (91.7)	14 (12.6)	40 (36)	57 (51.4)	40 (70.2)	32 (80)	8 (20)	8 (6.6)
In vivo	5	–	–	–	–	100	83 (83)	74 (89.2)	19 (22.9)	–

[a]IVG, *in vitro* growth: growing oocytes were cultured for 7 days.
[b]IVM, *in vitro* maturation: after IVG the oocytes were cultured for 14–16 h.
[c]GV, germinal vesicle; MI, metaphase I; MII, metaphase II.
[d]Blastocysts were examined on day 5 (120 h) of culture after reconstruction.

stage in SCNT embryos derived from both ovulated oocytes and IVG–IVM oocytes in the TSA-treated group. However, compared with the TSA-untreated group, the rate of development to the blastocyst stage and number of cells in the blastocysts were higher in the TSA-treated group for SCNT embryos derived from both ovulated oocytes and IVG–IVM oocytes (Fig. 3; $P < 0.05$).

Discussion

The purpose of this study was to investigate the effects of the source of recipient oocytes on embryonic development *in vitro* using IVG–IVM oocytes. To date, no study has investigated SCNT using IVG–IVM oocytes. To our knowledge, this study is the first one to describe blastocyst formation in rabbit embryos reconstructed using IVG–IVM oocytes.

In SCNT, the blastocyst rate of embryos reconstructed using MI oocytes has been reported to be significantly lower than that of embryos reconstructed using MII oocytes (Miyoshi *et al.*, 2001). In addition, the rate of development to blastocyst of SCNT embryos produced using IVM oocytes was reportedly lower than that of embryos produced using *in vivo* matured oocytes (Wells *et al.*, 1997). Furthermore, it has been reported that the conditions of *in vitro* maturation of recipient oocytes affect the development of SCNT embryos and that maturation of the ooplasm used as a recipient for SCNT is related to the development of SCNT embryos (Holker *et al.*, 2005). The ooplasm contains cytoplasmic factors such as reprogramming factors that affect the health and developmental competency of the oocyte, which can also affect its ability to reprogram and support development following SCNT (Keefer, 2008). In this study, *in vitro* developmental data showed no differences in the cleavage or blastocyst rates between SCNT embryos derived from ovulated oocytes and IVG–IVM oocytes. It can thus be inferred that IVG–IVM oocytes have developmental competency and reprogramming factors following SCNT.

Incomplete nuclear reprogramming and low blastocyst rate are major problems of SCNT. Molecular analysis has elucidated that cloned embryos have abnormal epigenetic modifications such as DNA methylation and histone modifications (Ohgane *et al.*, 2004; Dean *et al.*, 2001). TSA, a histone deacetylase inhibitor (HDACi), has been used to improve this low developmental rate caused by abnormal epigenetic modifications. It has been reported to improve the development of cloned embryos in mice (Kishigami *et al.*, 2006). In rabbits, TSA has been reported to either improve (Shi *et al.*, 2008) or limit the effect on

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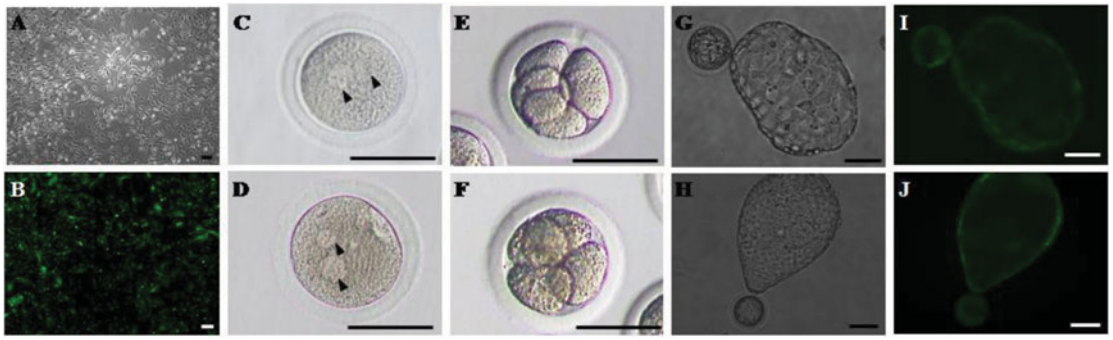

Figure 2 Donor fibroblast and rabbit cloned embryos reconstructed with *in vitro* growth–*in vitro* maturation (IVG–IVM) oocytes or *in vivo* matured oocytes. Fibroblast cell derived from transgenic rabbit expressing enhanced green fluorescent protein (EGFP) under visible light (*A*) and excitation light (489 nm) (*B*). Development of somatic cell nuclear transfer (SCNT) embryos derived from oocytes grown and matured *in vitro* (*C*, *E*, *G*, *I*). Development of SCNT embryos derived from ovulated oocytes (*D*, *F*, *H*, *J*). Pronuclear-like structures (arrowheads) in SCNT embryo after activation (*C*, *D*). Four-cell embryo (*E*, *F*). Blastocyst under visible light (*G*, *H*). Blastocyst under excitation light (489 nm) (*I*, *J*). Bars = 100 μm.

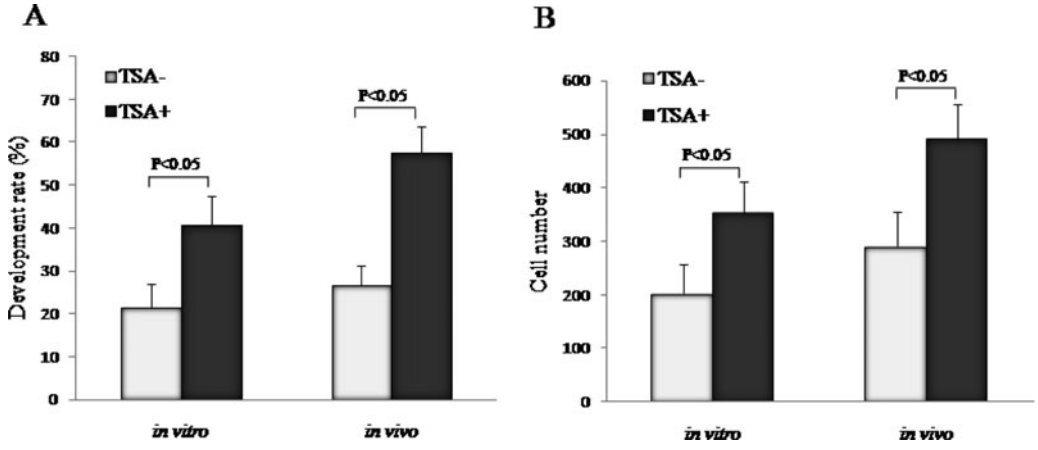

Figure 3 Effect of trichostatin A (TSA) on the development of somatic cell nuclear transfer (SCNT) embryos to blastocyst stage (*A*) and number of cells in blastocysts (*B*). The development rate (%) is shown as the rates to reach blastocyst. The number of cells in the blastocysts (*B*) was determined on day 5 (120 h) of culture after reconstruction. Bars labelled as *in vitro* denote SCNT embryos derived from oocytes grown and matured *in vitro*, whereas those labelled as *in vivo* denote SCNT embryos derived from ovulated oocytes.

the *in vitro* development of SCNT embryos (Meng *et al*., 2009). In the present study, the blastocyst rate of SCNT embryos derived from IVG–IVM oocytes and ovulated oocytes was significantly improved by TSA treatment. In addition, the number of cells in the blastocysts in SCNT embryos derived from IVG–IVM oocytes and ovulated oocytes improved significantly in TSA-treated embryos compared with TSA-untreated embryos. One report demonstrated that TSA had a considerable effect on fibroblasts used as donors. In contrast, another study showed a limited effect of TSA on cumulus cells. The present study showed the effect of TSA on embryonic development using rabbit fibroblasts as donors for SCNT.

In general, IVG–IVM oocytes show poor development after fertilization compared with ovulated oocytes; therefore, IVG in mammals has been studied extensively to achieve complete growth and development (Martins *et al*., 2008; Silva *et al*., 2010; Sugimoto *et al*., 2009, 2012). In the present study, the blastocyst rate after SCNT using IVG–IVM oocytes improved by approximately two-fold by TSA treatment; this effect is similar to that of TSA when SCNT is performed using ovulated oocytes. These results imply an improvement in the efficiency of nuclear reprogramming of donor cells by TSA rather than by an improvement in the quality of IVG–IVM oocytes. We infer that HDACs are effective in improving developmental competence of SCNT embryos derived from both IVG–IVM and ovulated oocytes. Therefore, when improving the developmental competence of SCNT using IVG–IVM oocytes, we concluded that it is possible to use a method similar to that for SCNT using ovulated oocytes.

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In conclusion, this study shows that growing oocytes collected from follicles remaining in superovulated rabbit ovaries have the developmental competence to reach the blastocyst stage after SCNT by performing IVG–IVM. By using the ovarian freezing technique (Donnez *et al*., 2006), IVG–IVM oocytes may become a potential bioresource. In addition, new developments can be anticipated, such as the production of offspring and the establishment of embryonic stem cells from SCNT embryos using IVG–IVM oocytes.

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