

# Beneficial Effect of Young Oocytes for Rabbit Somatic Cell Nuclear Transfer

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## Abstract

This study was designed to examine the effect of the age of rabbit oocytes on the developmental potential of cloned embryos. The metaphase II oocytes used for nuclear transfer (NT) were collected at 10, 12, 14, and 16 h post-hCG injection (hpi). The total number of oocytes collected per donor (21.4–23.7) at 12 to 16 hpi was similar, but significantly higher than that collected at 10 hpi (16.2). Additionally, a significant improvement in blastocyst development was achieved with embryos generated by electrically mediated cell fusion (56.0%), compared to those from nuclear injection (13.1 %) (Experiment 1). Markedly higher blastocyst development (45.8–54.5%) was also achieved with oocytes collected at 10–12 hpi than from those collected 14–16 hpi (8.3–14.3%) (Experiment 2). In Experiment 3, the blastocyst rates of NT embryos derived from oocytes harvested 12 hpi (39.2–42.8 %) were significantly higher than from those collected at 16 hpi (6.8–8.4 %) ( $p < 0.05$ ), regardless of the donor cell age. Kinase activity assays showed variable changes of activity in rabbit oocytes over the period of 10–16 hpi; however, there was no correlation with preimplantational development (blastocyst rate vs. MPF,  $R = 0.326$ ; blastocyst rate vs. MAPK,  $R = -0.131$ ). Embryo transfer of NT embryos utilizing 12 hpi oocytes resulted in one full-term but stillborn, and one live cloned rabbit; thus, an efficiency of 1.7 % ( $n = 117$ ) (Experiment 4). These results demonstrated that NT utilizing relatively young rabbit oocytes, harvested at 10–12 h after hCG injection, was beneficial for the development of NT embryos.

## Introduction

**R**ABBITS ARE AN EXCELLENT MODEL for the study of human reproductive (Ogonuki et al., 2005), cardiovascular (Fan and Watanabe, 2003), regenerative biology (Chesne et al., 2002), and diseases (Fan and Watanabe, 2003), because of the similarity of their biochemical and physiological processes to those of humans. In the 1980–1990s rabbits were produced by introducing embryonic cells from preimplantational embryos into enucleated oocytes by nuclear transfer (NT) (Collas and Robl, 1990, 1991; Collas et al., 1993; Stice and Robl, 1988; Yang et al., 1992). When a differentiated somatic cell was used as a nuclear donor for NT, however, it was found that the rabbit was a relatively more difficult species to clone, in comparison to other domestic animals (e.g., cattle and sheep). This may be caused by the rapid cell cycle of its

preimplantational embryo (Chesne et al., 2002), and some other unknown mechanisms of reprogramming the somatic genome within a recipient oocyte (Cervera and Garcia-Ximenez, 2004; Dinnyes et al., 2001; Mitalipov et al., 1999; Tsunoda and Kato, 2004; Yin et al., 2000, 2002). Thus far, only three reported cases worldwide demonstrated success in somatic cell NT of the rabbit. Chesne et al. (2002) first established that live clones were generated from NT with freshly prepared adult rabbit cumulus cells. Later, rabbit clones were produced from cultured fetal (Yang et al., 2007) and adult (Li et al., 2006) fibroblasts. In other cases, high fetal losses and abortion of rabbit clones were observed during the periods of implantation and pregnancy (Inoue et al., 2002; Yin et al., 2002).

The success of somatic cell NT depends upon a series of nuclear remodeling and reprogramming events (Campbell

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et al., 2007; Wilmut et al., 1997). Nuclear remodeling is related to the oocyte's cytoplasmic levels of maturation promoting factors (MPF) and mitogen-activated protein kinase (MAPK), while nuclear reprogramming depends on molecular events, such as methylation, acetylation, etc. The age of a recipient oocyte plays an important role in successful nuclear remodeling and subsequent reprogramming, as well as in the creation of live clones in cattle and mice (Du et al., 1995; Liu et al., 2007). It was believed that higher levels of MPF and MAPK were present in young oocytes, compared to aged ones (Lee and Campbell, 2006; Tian et al., 2002); and that the higher concentration of active MPF was responsible for inducing nuclear remodeling such as nuclear envelope breakdown, premature chromosome condensation (PCC), and other refined chromosomal structural modifications that might be fundamental to molecular reprogramming (Lee and Campbell, 2006; Campbell et al., 2007). However, there were also reports showing that MPF and MAPK activity was a nondirect reprogramming regulator (Tani et al., 2003), and that PCC was not essential for nuclear reprogramming in cattle (Sung et al., 2007). To date, there were no detailed profiles of MPF and MAPK concentrations in rabbit oocytes at and during postovulation. Cervera and Garcia-Ximenez (2003) found that the oocyte age and nuclear donor cell type affected the *in vitro* development efficiency of rabbit NT embryos. Inoue et al. (2002) reported an improved postimplantational development of cloned rabbit embryos using recipient oocytes collected at 13–14 h post-hCG injection (hpi), and after activation with inositol 1,4,5-trisphosphate (IP3). However, neither full-term development nor a live clone was generated from these relatively young recipient oocytes. In fact, the first live clones were produced in 2002 by Renard's group, using oocytes collected at 16 hpi (Challah-Jacques et al., 2003; Chesne et al., 2002).

In this study we designed a series of experiments to determine the relative levels of MPF and MAPK in ovulated rabbit oocytes collected at different times following hCG injection. The efficiency (*in vitro* and *in vivo* developmental potentials) of somatic cell NT with recipient oocytes of different age groups was directly compared. As a consequence, we demonstrated that NT utilizing rabbit oocytes collected at different times revealed differential development of NT embryos *in vitro*, and confirmed the beneficial effect of using relatively young oocytes for NT.

## Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's phosphate-buffered saline (D-PBS; 15240-013, Gibco, Grand Island, NY) containing 0.1% polyvinyl alcohol (PVA; P-8136) was used for flushing oocytes from oviducts (PBS-PVP). The basic washing medium for donor cells was Dulbecco's Minimum Eagle's medium (DMEM; 31600, Gibco). Medium 199 (M199) with Earle's salts, L-glutamine, 2.2 g/L sodium bicarbonate, and 25 mM HEPES (Gibco, 12340-014) was supplemented with 10% fetal bovine serum (SH0070.03, Hyclone, Logan, UT), and used as the standard manipulation medium. Oocyte and embryo cultures were maintained in at 38.5°C in 5% CO<sub>2</sub> and humidified air.

## Animal maintenance and hormone administration

Sexually mature (6–12 month old) New Zealand White (NZW) and Dutch Belted (DB) rabbits were maintained under light cycles appropriate for the experimental design. For oocyte and nuclear donors, NZW rabbits, maintained under a 16 h light:8 h dark cycle, were superovulated with a modified procedure (Dinnyes et al., 2001) consisting of two 3 mg, two 4 mg, and two 5 mg injections of FSH (Folltropin-V, Bioniche Animal Health Canada, Belleville, Ontario, Canada) at intervals of 12 h, followed by 200 IU of human chorionogonadotropin (hCG) (Chorulon, animal use, Intervet Inc, Millsboro, DE). Dutch Belted rabbits served as embryo recipients and were asynchronously induced to ovulate (22-h delay after oocyte donors) by an intramuscular injection of 15 µg per doe of GnRH analogue (Cystorelin, Abbott Laboratories, North Chicago, IL) according to the protocol of Challah-Jacques et al. (2003). Following surgical embryo transfer, recipients were moved into an environment with an 8 h light:16 h dark cycle. Untreated DB rabbits were mated with fertile males to establish pregnancies; after parturition, these mothers served as foster mothers to cloned kits delivered by Caesarian section.

All animal maintenance, care, and use procedures described within were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee. All surgical procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

## Oocyte collection, preparation, and donor cell isolation

According to the experimental design detailed below, at 10–16 hpi, reproductive tracts including ovaries were excised from euthanized NZW donor rabbits by midventral laparotomy and taken into the laboratory for oocyte collection. Cumulus–oocyte complexes (COCs) were either collected from the Graafian follicles of the ovaries (follicular oocytes) or flushed from the oviducts (oviductal oocytes) with 5 mL PBS-PVA per oviduct and collected. Rabbit COCs were treated with 0.5 mg/mL hyaluronidase in PBS for 1 min, and cumulus cells were subsequently removed from the oocytes by careful pipetting with a fine bore glass pipette. The oocytes were examined under light microscopy to discern those with the appearance of a first polar body as metaphase II (MII) oocytes. The MII oocytes from both follicles and oviducts, in the same treatment group, were pooled and maintained for NT.

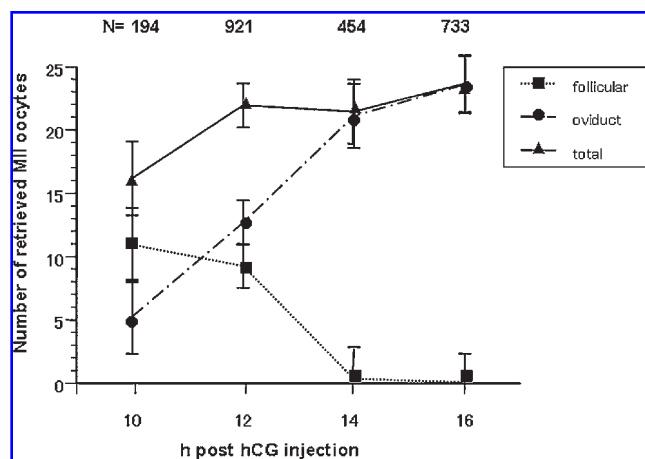
Freshly collected cumulus cells served as the somatic cell nuclear donors in NT, as described by Challah-Jacques et al. (2003). Collected cells were washed in Ca<sup>2+</sup>, Mg<sup>2+</sup> free Dulbecco's PBS (14190-144, Gibco) supplemented with 10% polyvinylpyrrolidone (PVP-40) and centrifuged at 1000 × g. Treatment with PVP helped to wash off membrane damaged cells. The cell pellet was subsequently treated with a 3-min digestion by 0.05% trypsin (103140; ICN, Aurora, OH) and 0.5 mM EDTA (8991; Baker, Phillipsburg, NJ) at 37°C. Trypsinized cumulus cells were subsequently suspended in 10% FBS DMEM and maintained at 37°C prior to NT.

## NT, parthenogenetic activation, and embryo culture

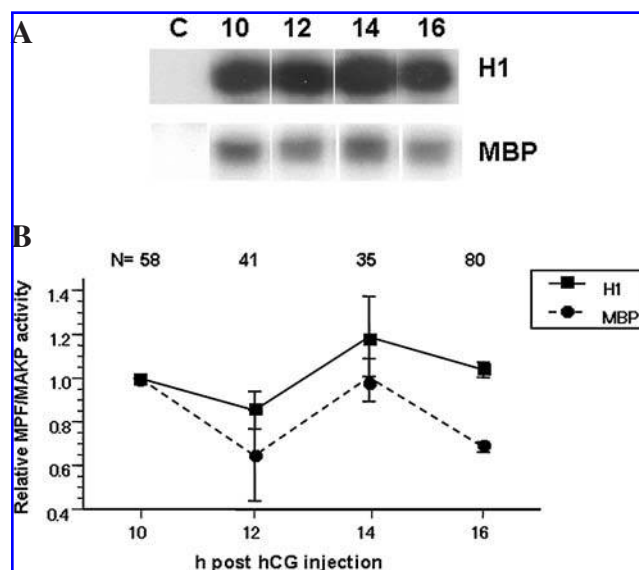
All micromanipulations were carried out using our standard procedure (Du et al., 2006; Kubota et al., 2000). Enu-

cleation was performed by making an incision in the zona pellucida using a glass needle and applying pressure until the polar body, along with some of the surrounding cytoplasm (approximately 1/8 total cytoplasm), was extruded. Successful enucleation was confirmed by fluorescent microscopy of extruded cytoplasm after staining with 10  $\mu\text{g}/\text{mL}$  Hoechst 33342 dye. For the direct nuclear injection method, a cumulus cell was lysed by mechanical pipetting of the cell using an injection pipette, prewashed in 10% high molecular weight PVP (PVP-360), with an inner diameter of 8  $\mu\text{m}$ . The cell, with its partially disrupted cell membrane, was then injected into an oocyte's cytoplasm with the aid of a Piezo-Drill system (Prime Tech, Ibaraki, Japan). The successful injection of a complete nucleus into the oocyte was observed and confirmed under a light microscope with 400 $\times$  magnification. Alternatively, we employed a fusion method, whereby a donor cell with a diameter of approximately 20  $\mu\text{m}$  was selected and transferred into the perivitelline space of an enucleated rabbit oocyte. Donor cell-cytoplasm pairs were then incubated in 0.3 M mannitol supplemented with 0.1 mM  $\text{CaCl}_2$  and 0.1 mM  $\text{MgCl}_2$  for 3 min, and subsequently transferred into an electrical chamber containing the same fusion medium. Cell fusion was induced by applying three direct current pulses of 3.2 kV/cm for a duration of 20  $\mu\text{sec}$ /each by BTX 200 Electro Cell Manipulator (Biotechnologies & Experimental Research Inc., San Diego, CA). Following the completion of DC electric pulses, NT oocytes were incubated for at least 15 min at 38.5°C to facilitate complete cell fusion prior to microscopic examination to determine the fusion rate.

All fused or injected oocytes were cultured in 10% FBS M199 for 1 h before being subjected to activation. Activation of cloned embryos was accomplished by electrical DC stimulation as used for cell fusion, followed by a 1-h incubation in M199 + 10% FBS containing 2.0 mM 6-dimethylaminop-



**FIG. 1.** Rabbit oocyte recovery at different times post-hCG injection. MII oocytes collected from Graafian follicles were defined as follicular oocytes, and those collected from flushed oviducts, as oviductal oocytes. Both follicular and oviductal oocytes were collected at 10 ( $n = 194$ ), 12 ( $n = 921$ ), 14 ( $n = 454$ ), and 16 ( $n = 733$ ) hpi. These values were calculated and classified into three categories: follicular, oviductal, and total average number of oocytes per donor.



**FIG. 2.** Analysis of MPF and MAPK activity in oocytes of different ages. (A) A representative autoradiograph of histone H1 and myelin basic protein (MBP) kinases in the oocytes collected at 10 ( $n = 58$ ), 12 ( $n = 41$ ), 14 ( $n = 35$ ), and 16 ( $n = 80$ ) hpi. (B) Relative activity levels of MPF (shown as H1 activity) and MAPK (shown as MBP activity) for oocytes collected at 12, 14, and 16 hpi were compared to the standardized values of those harvested at 10 hpi.

urine (DMAP, D-2629), 5  $\mu\text{g}/\text{mL}$  cycloheximide (CHX, C-6255).

To evaluate the *in vitro* developmental potential of cloned embryos, following activation the reconstructed oocytes were cultured in 2.5% FBS B2 medium (Laboratories CCD, Paris, France). Cleavage rates were recorded 24 h postculture, and two to four celled embryos were cultured further in the same B2 medium for an additional 4 days to determine their blastocyst development.

#### *In vivo development of rabbit nt embryos*

The cloned embryos, derived from oocytes of different age groups, were transferred into recipients to test their *in vivo* viability. Cloned embryos were cultured for 16 h *in vitro* before being surgically transferred, by midventral laparotomy, into pseudopregnant DB rabbits. Ten to 20 embryos at two- to eight-cell stage were loaded into a 5- $\mu\text{L}$  Drummond microdispenser, and transferred into the oviduct on one side of a recipient. Pregnancy was monitored by palpation and/or ultrasound on day 14–16 postembryo transfer (ET). All pregnancies were allowed to progress to term (days 31–33); some of the deliveries were performed by caesarean section to help ensure live kits.

#### *MPF and MAPK activity*

Kinase activity assays of MPF and MAPK were performed at University of Massachusetts, where reliable procedures were routinely carried out (Fissore et al., 1996; Tian et al., 2002). Five to 10 rabbit oocytes were collected at each of the different time points (10, 12, 14, and 16 hpi) for assays. Oocytes were placed into 0.5-mL centrifuge tubes and frozen

by immediately immersing into liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . At the time of assay, a volume of  $6\text{-}\mu\text{L}$  kinase sample buffer containing  $6.4\text{ mM EDTA}$ ,  $10\text{ mM NaF}$ ,  $100\text{ mM Na}_3\text{VO}_4$  in PBS was added to each tube. MPF and MAPK activities were determined by using H1 (Type III-S, H-5505) and Myelin basic protein (MBP, M-1891) as substrates, respectively, as described previously (Fissore et al., 1996; Tian et al., 2002). Briefly, denuded and lysed rabbit oocytes were incubated with ( $\gamma\text{-}^{32}$ )-ATP for 30 min at  $37^{\circ}\text{C}$ , which allowed active MPF and MAPK in oocytes to phosphorylate the substrate mixtures. The proteins in oocytes were subsequently denatured by heating the reaction mixtures at  $95^{\circ}\text{C}$  for 5 min in sodium dodecyl sulfate (SDS) buffer. The radioactively labeled substrates were electrophoresed onto 15% SDS-polyacrylamide gels; the dried gels were subsequently exposed to Kodak X-ray film. Kinase activity was measured by scanning the autoradiographs with a Kodak Imaging Quantification system. Densitometric values from each treatment group were analyzed by the Quantity One software (BioRad, Hercules, CA). The number of oocytes used in each group for the MPF and MAPK assay was from 5 to 10; thus, the autoradiograph intensity obtained from each age group, for each replicate, was normalized to that of five oocytes per group by calculating their differential intensity. This standardization was achieved by setting the activity of five oocytes collected at 10 hpi as 1.0 unit activity. Therefore, the enzyme activities of other age groups (12, 14, and 16 hpi) were transformed and are given as relative activity values. Four replicates of this assay were performed for each treatment.

### Experiments

**Experiment 1: Comparison of membrane fusion and direct nuclear injection.** The effect of two NT methods (cell fusion vs. nuclear cytoplasmic injection) was compared on the subsequent preimplantational development to blastocyst of NT embryos. The MII oocytes collected from the ovaries or the oviducts at 12 hpi were pooled and randomly assigned to NT with either electrically mediated membrane fusion or Piezo-Drill assisted direct nuclear injection. The NT embryos from both groups were activated with the same activation protocol described above (two electrical pulses combined with an incubation of DMAP and cycloheximide), and embryos were cultured in B2 and 2.5% FBS for 5 days.

**Experiment 2: The effect of oocyte age on the preimplantational development of cloned rabbit embryos.** In Experiment 1 we observed a better development via electrical mediated cell membrane fusion. Therefore, in this experiment, the oocytes were harvested at varying time points (10, 12, 14, and 16 hpi), and were all reconstituted via electrical-mediated cell fusion. Cumulus cells from the same time points were used as nuclear donors. NT embryos were then activated with a combined electrical and chemical activation procedure as described for Experiment 1, and subsequently cultured *in vitro* for 5 days to examine their preimplantational developmental potential.

**Experiment 3: The effect of oocyte age versus donor cell age on the preimplantational development of cloned rabbit embryos.** In order to determine whether the beneficial effect

of young oocytes on NT in Experiment 2 was reinforced by the use of young cumulus donor cells, a  $2 \times 2$  factorial experiment was performed, in which the oocytes were recovered either at 12 hpi (young oocytes) or at 16 hpi (aged oocytes). They were then fused with cumulus nuclear donors harvested from 12 or 16 hpi cumulus-oocyte complexes.

**Experiment 4: Full-term developmental potential of NT embryos reconstructed with oocytes of different ages.** Because the number of oocytes collected per donor at 10 hpi was lower than at other time points, and *in vitro* development was comparable between NT embryos derived from the oocytes at 10 and 12 hpi, only NT embryos reconstructed from the oocytes of 12, 14, and 16 hpi were transferred into recipient rabbits to examine their term developmental potential. In some cases, when sufficient numbers of transferable NT embryos were available from 12 hpi oocytes, we were able to perform separate ET with embryos derived from follicular and oviductal oocytes.

### Statistical analysis

The data on MPF and MAPK activities were subjected to an arc sin transformation. The transformed data were then analyzed by ANOVA (General Linear Model, SPSS 11.0, Chicago, IL) (Snedecor and Cochran, 1980) For the analysis of preimplantational development *in vitro*, the proportions of embryos that reached cleavage (the two- to eight-cell stage), and blastocyst were transformed by an arcsine transformation, and analyzed by Student's *t*-test. A *p* value of less than 0.05 is considered as statistically significant.

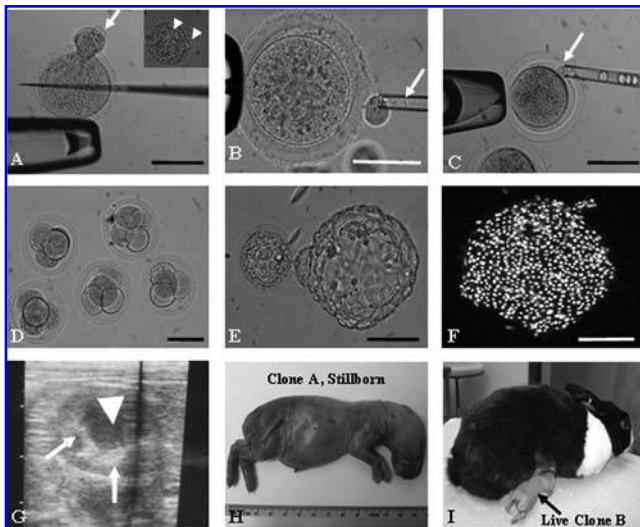
## Results

### Rabbit oocyte collection

A total of 102 rabbits at 6–12 months of age were assigned to four groups for timed oocyte collections following hCG injection (hpi) according to the experimental design: Group A, 10 hpi ( $n = 12$ ); B, 12 hpi ( $n = 42$ ); C, 14 hpi ( $n = 20$ ) and D, 16 hpi ( $n = 28$ ). As shown in Figure 1, the average number of oocytes collected at 10 hpi was 16.2 per doe, significantly lower than that at 12 hpi (21.9 per doe) ( $p < 0.05$ ). From 12 to 16 hpi, the average number per donor remained similar (21.4–23.7 per doe) ( $p > 0.05$ , Fig. 1). Follicular oocytes collected from Graafian follicles comprised 67.3% of the total number of oocytes collected at 10 hpi ( $n = 194$ ), subsequently, at 12 hpi their percentage decreased to 42.2% ( $n = 921$ ), and they further diminished significantly to 1.5% at 14 hpi, ( $n = 454$ ), and finally, by 16 hpi had declined to only 0.3% ( $n = 733$ ) ( $p < 0.05$ , Fig. 1). The percentage of follicular oocytes that showed a first polar body in the 10, 12, 14, and 16 hpi groups was: 95.6% ( $n = 131$ ), 98.4% ( $n = 375$ ), 87.5% ( $n = 8$ ), and 100% ( $n = 2$ ), respectively. Among oocytes collected from the oviducts, the percentage presenting a first polar body at 10, 12, 14, and 16 hpi were: 98.4% ( $n = 63$ ), 99.5% ( $n = 546$ ), 99.1% ( $n = 446$ ) and 99.7% ( $n = 731$ ), respectively.

### MPF/MAPK assay

The relative activity of MPF in the oocytes declined from 10 hpi to 12 hpi (1.0 vs. 0.9); however, it increased to 1.16 at 14 hpi, and was 1.15 at 16 hpi (Fig. 2A and B). The dynam-



**FIG. 3.** Cloned rabbits generated by nuclear transfer (NT) from adult somatic cells. (A) Enucleation of matured rabbit oocyte by pressing out the first polar body (indicated by arrow) along with a portion of cytoplasm through an incision in zona pellucida made by a microneedle. The metaphase plate and polar body chromosomes (near arrow heads) were visualized in the extruded cytoplasm by fluorescent microscopy after staining with 10  $\mu\text{g}/\text{mL}$  Hoechst 33342 dye (top right window). Only enucleated oocytes, whose MII chromosomes were confirmed to be contained in the cytoplasm removed, were used for NT. (B) During NT, partially lysed cumulus cells containing nucleus (arrow) ready to inject into cytoplasm of enucleated oocyte by direct nuclear injection, or (C) an intact cumulus cell transferred into the perivitelline space of an enucleated oocyte (arrow) prior to membrane fusion. (D) Cloned rabbit embryos developed to four-celled stage 16 h after culture *in vitro*, and (E) continued development into hatched blastocyst stage after 5 days of culture, containing 596 stained nuclei (F). Pregnancy was determined by ultrasound on day 14 after embryo transfer (ET); (G) fetus body indicated by arrows, and yolk sac cavity by arrowhead. (H) The first full-term rabbit clone (Clone A) was naturally delivered as a stillborn 33 d after ET, and (I) the second clone (arrow, Clone B) was delivered alive, by C-section, 31 days after ET. Bar = 100  $\mu\text{m}$ .

ics of MAPK activity in these oocytes was found to follow a similar pattern from 10 to 16 hpi. Nevertheless, the most aged oocytes (16 hpi) contained significantly lower MAPK activity than the youngest ones, collected 10 hpi and 14 hpi ( $p < 0.05$ ), but levels similar to those found in the less aged oocytes collected at 12 hpi (Fig. 2B).

#### Experiment 1: Comparison of Piezo-Drill injection versus electrical cell fusion

All rabbit oocytes were enucleated by pressing the first polar body, along with approximately 1/8 of the volume of their cytoplasm, out through an incision made with a microneedle through the zona pellucida (Fig. 3A). The donor cells were transferred directly into oocytes by Piezo-Drill assisted nuclear injection (Fig. 3B), or placed into the perivitelline space followed by electrically mediated fusion (Fig. 3C). After NT and further culture, cloned embryos sequentially developed into four-celled (Fig. 3D), morulae, and blastocyst stage *in vitro* (Fig. 3E and F). The NT results (Table 1) illustrate that the percentage of donor nuclei successfully introduced into the cytoplasm of oocytes was greater with direct Piezo-Drill-assisted nuclear injection than via cell fusion ( $p < 0.05$ ). Electrically mediated fusion of NT embryos, however, resulted in significantly higher embryo development rates to morulae (58.2 vs. 17.7%) and blastocysts (56.0 vs. 13.1%) ( $p < 0.05$ ). Overall blastocyst development, based on the total number of oocytes suitable for NT, was also higher in the fusion group (39.3 vs. 12.9%,  $p < 0.05$ ). Moreover, the efficiency of the preimplantational development of cloned NT embryos from membrane fusion was as good as that of the parthenogenetic activation controls (56.0 vs. 49.9%,  $p > 0.05$ ). The total number of cells in the NT blastocyst was no different between those from direct injection ( $345 \pm 35$ ,  $n = 8$ ) and fusion methods ( $386 \pm 12$ ,  $n = 15$ ) ( $p > 0.05$ ).

#### Experiment 2: The effect of oocyte age on the preimplantational development of cloned rabbit embryos

Based on the improved NT efficiency achieved by the membrane fusion method in Experiment 1, oocytes collected at all time points hpi were subjected to the fusion based NT protocol for determining the effect of oocyte age on *in vitro* development of cloned embryos. The fusion rate was similar among all age groups (10, 12, 14, and 16 hpi, 61.5–75.7%).

TABLE 1. EFFECT OF SOMATIC NUCLEAR TRANSFER PROCEDURES ON THE DEVELOPMENT OF CLONED RABBIT EMBRYOS *IN VITRO*

Treatment	Donor cell type	No. of replications	No. oocytes	% Oocyte Fused/survived	% Embryos developed to*			
					Cleavage	Four-cell	Morulae	D5 BLs
Fusion	Cumulus	4	55	68.6 $\pm$ 6.5 <sup>a</sup>	70 $\pm$ 11.8 <sup>ab</sup>	60.5 $\pm$ 13.0 <sup>ab</sup>	58.2 $\pm$ 7.7 <sup>a</sup>	56.0 $\pm$ 9.9 <sup>a</sup>
Injection	Cumulus	4	71	97.1 $\pm$ 1.8 <sup>b</sup>	37.5 $\pm$ 9.7 <sup>a</sup>	24.8 $\pm$ 6.3 <sup>a</sup>	17.7 $\pm$ 7.5 <sup>b</sup>	13.1 $\pm$ 9.1 <sup>b</sup>
PA	N/A	4	51	N/A	75.3 $\pm$ 3.8 <sup>b</sup>	68.4 $\pm$ 5.8 <sup>b</sup>	54.4 $\pm$ 10.5 <sup>a</sup>	49.9 $\pm$ 6.8 <sup>a</sup>

<sup>a,b</sup>Values with different superscripts within columns differ,  $p < 0.05$ . Cumulus cells derived from cumulus–oocyte complexes of adult rabbits at 6–12 month age. In the injection group, the oocytes surviving after Piezo-Drill nuclear injection were calculated and cultured further *in vitro*. In fusion group, fused NT embryos were cultured for development *in vitro*. Cleavage, the sum of two- and four-celled after a 16-h culture *in vitro*; four-cell, four-cell embryos after a 16- culture; BL, blastocyst; D5, day 5; Fusion, NT with membrane mediated fusion; Injection, NT using donor nuclear cytoplasmic injection, PA, parthenogenetic activation as control. N/A, not applicable.

\*The percentage of embryonic development was calculated based upon the number of fused oocytes used for NT.

TABLE 2. EFFECT OF OOCYTE AGE ON THE DEVELOPMENT OF CLONED RABBIT EMBRYOS *IN VITRO*

Treatment	Total No. of oocytes	No. of replications	Fused oocytes (%)	Percentage of embryos developed to*			
				Cleaved	Four-cell	Morula	D5 BLs
10 h	72	4	61.5 ± 7.0 <sup>a</sup>	75.0 ± 16.5 <sup>a</sup>	66.7 ± 16.8 <sup>a</sup>	54.2 ± 16.0 <sup>a</sup>	45.8 ± 18.3 <sup>a,b</sup>
12 h	144	4	71.7 ± 4.8 <sup>a</sup>	66.7 ± 13.8 <sup>a</sup>	60.6 ± 16.0 <sup>a</sup>	57.6 ± 13.1 <sup>a</sup>	54.5 ± 14.8 <sup>a</sup>
14 h	62	4	75.7 ± 11.9 <sup>a</sup>	48.2 ± 12.6 <sup>a</sup>	30.4 ± 7.2 <sup>a,b</sup>	19.6 ± 7.4 <sup>b</sup>	14.3 ± 7.8 <sup>b,c</sup>
16 h	128	4	65.2 ± 3.5 <sup>a</sup>	41.7 ± 3.9 <sup>a</sup>	25.0 ± 2.3 <sup>b</sup>	18.3 ± 18.8 <sup>b</sup>	8.3 ± 1.6 <sup>c</sup>

<sup>a,b,c</sup>Values with different superscripts within columns differ,  $p < 0.05$ . Cumulus cells derived from cumulus–oocyte complexes of adult rabbits at 6–12 month age. Fused NT embryos were arranged for development *in vitro*. Cleavage, the sum of two- and four-celled after a 16-h culture *in vitro*; four-cell, four-cell embryos after a 16-h culture; BL, blastocyst; D5, day 5.

\*The percentage of embryonic development was calculated based upon the number of fused oocytes used for NT.

Between the oocytes collected at 10 and 12 hpi, we found no statistically significant difference in cleavage, development to morulae (54.2 vs. 57.6%), or to blastocysts (45.8 vs. 54.5%) ( $p > 0.05$ ) (Table 2). However, a significant decrease in blastocyst development was observed in oocytes collected at 14 (14.3%) and 16 hpi (8.3%), compared to those at 12 hpi (54.5%,  $p < 0.05$ ). In the correlation comparison between *in vitro* development of cloned embryos and the relative activity of MPF and MAPK, the R indices were  $-0.131$  (blastocyst rate vs. MAPK), and  $0.326$  (blastocyst rate vs. MPF); these indicate that the NT efficiency does not appear to have a linear correlation with MPF/MAPK activity.

### Experiment 3: The effect of oocyte age versus donor cell age on development of cloned rabbit embryos *in vitro*

As indicated in the Table 3, the blastocyst rates from NT embryos derived from oocytes 12 hpi (39.2–42.8%), with six replicates, were significantly higher than those from oocytes 16 hpi (6.8–8.4%) regardless of the donor cell age ( $p < 0.05$ ). Among oocytes of the same age group, we observed no significant difference in blastocyst development when using donor cumulus cells from different ages of COCs (12 vs. 16 hpi) (Table 3).

### Experiment 4: Full-term development of NT embryos from different aged oocytes

The full-term developmental potential of cloned embryos was compared to determine the effect of oocyte age on the

efficiency of generating live rabbit clones. Because of the significantly lower number of oocytes harvested at 10 h hpi, and due to the lack of statistically significant differences in development between those collected at 10 and 12 hpi, oocytes acquired at 12, 14, and 16 hpi only were used in this NT trial. The resultant NT embryos were transferred into 22 h delayed asynchronous recipients as detailed above. In the nuclear injection group, 140 NT embryos constructed from 12 hpi oocytes were transferred into six recipients; one pregnancy was revealed by palpation on day 14 (1/6, 16.7%), but there was no term development. As indicated in Table 4, five recipients (5/12, 41.6%), two recipients (2/12, 16.7%), and two recipients (3/12, 25.0%) from the 12, 14, and 16 hpi oocyte groups, respectively, were identified as pregnant on days 14–16 following embryo transfer (ET) (Fig. 3G). In the 12 hpi oocyte group, one full-term, but stillborn (Fig. 3H) clone (Clone A, weighing 76 g) was delivered naturally on day 33 post-ET. Anatomical and morphological evaluation revealed the deceased kit to have no apparent deformities, and no observable defects in major organs. One live clone (Clone B, weighing 57 g) (Fig. 3I) was born by Cesarean section on day 31 post-ET (Table 4). Interestingly, the deceased Clone A was generated from ovulated oocytes, while the live Clone B was from follicular oocytes, although both were collected 12 hpi. The overall efficiency of rabbit cloning using oocytes at 12 hpi was 1.7% ( $n = 117$ , Table 4). In contrast, neither full-term fetuses nor live offspring were delivered from recipients receiving NT embryos generated from oocytes 14 and 16 hpi.

TABLE 3. EFFECT OF BOTH OOCYTE AND NUCLEAR DONOR AGE ON THE DEVELOPMENT OF CLONED RABBIT EMBRYOS *IN VITRO*

Treatment					Percentage of embryos developed to*			
Oocyte age (h)	Donor cell age (h)	Total No. of oocytes	No. of replications	Fused oocytes (%)	Cleaved	Four-cell	Morula	D5 BLs
12	12	125	6	70.3 ± 6.3 <sup>a</sup>	73.8 ± 4.5 <sup>a,b</sup>	58.5 ± 3.1 <sup>a</sup>	50.1 ± 12.4 <sup>a</sup>	42.8 ± 7.2 <sup>a</sup>
16	12	132	6	66.7 ± 3.1 <sup>a</sup>	44.7 ± 4.2 <sup>c</sup>	25.9 ± 4.8 <sup>b</sup>	10.9 ± 2.5 <sup>b</sup>	6.8 ± 2.5 <sup>b</sup>
12	16	95	6	65.9 ± 6.9 <sup>a</sup>	85.3 ± 11.1 <sup>b</sup>	69.5 ± 7.5 <sup>a</sup>	49.4 ± 2.1 <sup>a</sup>	39.2 ± 3.1 <sup>a</sup>
16	16	144	6	69.2 ± 7.3 <sup>a</sup>	55.6 ± 8.9 <sup>a,c</sup>	35.3 ± 7.1 <sup>b</sup>	11.6 ± 3.2 <sup>b</sup>	8.4 ± 2.3 <sup>b</sup>

<sup>a,b,c</sup>Values with different superscripts within columns differ,  $p < 0.05$ . Cumulus cells derived from cumulus–oocyte complexes collected at either 12 or 16 h after hCG injection (hpi) from adult rabbits at 6–12 month age. Fused NT embryos are prepared for development *in vitro*. Cleavage, the sum of two- and four-celled after a 16-h culture *in vitro*; four-cell, four-cell embryos after a 16-h culture; BL, blastocyst; D5, day 5.

\*The percentage of embryonic development was calculated based upon the number of fused oocytes used for NT.

TABLE 4. EFFICIENCY OF RABBIT SOMATIC CELL NT WITH RECIPIENT OOCYTES AT DIFFERENT AGES

Oocyte age (h)	Donor cell type	NT procedure	No. cloned embryos	No. of recipients	No (%) of pregnant	No (%) of clones <sup>b</sup>	% Clones/embryos transferred
12	Cumulus	Fusion	117	12	5 (41.6)	2 (16.7)	1.7
14	Cumulus	Fusion	161	12	2 (16.7)	0 (0)	0
16	Cumulus	Fusion	228	12	3 (25.0)	0 (0)	0

<sup>a</sup>Oocytes were collected from superovulated ova donors at 12, 14, and 16 h posthuman chorionic gonadotropin (hCG) injection (hpi).

<sup>b</sup>Clone A (stillborn, weighted 76 g), delivered naturally on day 33 post-ET, was generated using an oocyte flushed from the oviduct of a donor rabbit 12 hpi. Clone B (live, weighted 57 g), born by Cesarean section on day 31 post-ET, was cloned with an oocyte from the Graafian follicle of the rabbit ovary collected 12 hpi.

## Discussion

The rabbit represents a unique species that provides an excellent model from which to extrapolate to humans. Due to the unavailability of rabbit stem cells capable of germ line transmission, NT in combination with gene targeting provides a valuable technology to produce gene targeted transgenic rabbits. Rabbit was the pioneering species that was used to define embryonic NT and reprogramming in mammals. In the 1980s, rabbit NT using embryonic cells (blastomeres and/or inner cell mass cells) was shown successful. However, it has been very challenging when highly differentiated somatic cells were used as nuclear donors. It was until 2002 when the first success in rabbit somatic cell NT was reported by Renard's group (Chesne et al., 2002). In that report, careful consideration was taken for both the rapid kinetics of the cell cycle of rabbit embryos and the narrow window of time available for their implantation following transfer to recipients. Later, Li et al. (2006) used adult fibroblast cells, and Yang et al. (2007) used fetal fibroblasts and cumulus cells, and both reports produced live clones. Overall, rabbit NT efficiency using somatic cells remains very low, and the mechanism(s) responsible for the low NT success rates has yet to be revealed.

The important role of the age of the recipient oocyte in competent NT reprogramming was reported in cattle (Du et al., 1995), and mice (Liu et al., 2007). Yet, the effect of recipient oocyte age on rabbit NT efficiency has not been clearly demonstrated. Among published studies, rabbit oocytes used for NT were either collected at 13–14 hpi (Li et al., 2006; Dinnyes et al., 2001; Inoue et al., 2002; Mitalipov et al., 1999), or 15–16 hpi (Chesne et al., 2002; Yang et al., 2007). Cervera and Garcia-Ximenez (2003) demonstrated that oocytes collected at 13 hpi supported higher fusion rates and improved morulae and blastocyst development when compared to those of aged oocytes (17 hpi); however, only *in vitro* developmental data were included in that report. Our study demonstrated the beneficial effect of utilizing a young recipient oocyte in rabbit, both *in vitro* and *in vivo* to achieve successful reprogramming of the highly differentiated somatic nuclear genome. In an earlier *in vitro* study, we separated the oocytes collected from oviducts versus ovaries. The results showed that most rabbit oocytes (95.6–98.4%) from Graafian follicles were mature at MII at 10–12 hpi. We observed the trends that oocytes from ovarian follicles supported better embryo development (Du et al., unpublished) than those from oviducts. Thus, for *in vitro* experiments, both follicular

and oviducal MII oocytes (with polar body), within the same age group (primarily 10 and 12 hpi), were pooled for NT in order to eliminate the variable of oocyte origin (follicular vs. oviducal), and focus on age. For *in vivo* experiments (e.g., ET), the embryos derived from follicular oocytes were separated from those derived from oviducal oocytes whenever possible (i.e., 10–24 transferable embryos/recipient). Interestingly, the live clone was generated using an oocyte collected 12 hpi from an ovarian follicle, while the stillborn clone was from an NT embryo constructed with an oocyte collected 12 hpi by flushing the oviduct. Certainly it is still premature to conclude that oocytes from ovarian follicles would support better *in vivo* development after NT in rabbits. Future studies will be imperative to closely examine the effects of oocytes collected from oviducts versus follicles.

Our data demonstrated that the age of rabbit oocyte was profoundly more significant than that of the donor cell in the reprogramming of the somatic genome. Obviously, there was a close relationship between the oocyte and cumulus cells in the COCs, but the aging effects observed in our study were mostly due to factors affecting the oocyte's cytoplasm, and therefore, its reprogramming ability. We do not attribute these age effects to the donor cell's DNA itself, which is transferred into the oocyte cytoplasm. Meanwhile, our success in using young oocytes at 12 hpi implied that only a very narrow window of time, estimated as 2–4 h, is optimal for NT manipulation, and/or there exists a stringent limit to the plasticity of rabbit oocyte cytoplasm in which biochemical and physiological reprogramming of an introduced nucleus can take place.

In early rabbit NT studies using embryonic donor cells (e.g., from blastomeres or ICMs), aged oocytes were used as recipients because young oocytes were found difficult to be activated (Adenot et al., 1997; Yang et al., 1992). In those studies, the oocytes were activated by either electrical stimulation (Stice and Robl, 1988), or with the protein synthesis inhibitor CHX (Yang et al., 1992), or kinase phosphorylation inhibitor DMAP (Adenot et al., 1997). Meanwhile, a single activation stimulation might not be sufficient to activate a rabbit oocyte, particularly on a freshly ovulated, young oocyte (Escriva and Garcia-Ximenez 2000). Recently, activation of rabbit somatic NT embryos was performed by double DC pulses of 1.2 kV/cm for 20  $\mu$ sec after electrical fusion (Shi et al., 2008), but only 22.6% NT embryos developed to blastocysts (Shi et al., 2008). The combination of electric and chemical stimulation was a critical step in the pursuit of more effective NT in rabbits, as reported previously (Chesne

et al., 2002; Dinnyes et al., 2001). Activation using a combination of  $IP_3$  with DMAP was reported to promote an effective blastocyst development of both parthenogenetically activated oocytes (50.0%) and NT embryos (29.6%) (Mitalipov et al., 1999). Dinnyes et al. (2001) demonstrated a combined electrical pulse and 2-h DMAP treatment could significantly improved blastocyst development of activated oocytes (29.0%), and resulted in blastocysts with cell numbers comparable to those of fertilized embryos. Yang et al. (2007) reported 34% blastocysts developed from NT embryos activated by combined electrical pulse, 1.9 mM DMAP and cytochalasin B, as well as a live clone. The beneficial effect of CHX in combination with DMAP was found in blastocyst development of cattle NT embryos when compared with a cycloheximide only activation regime (Du and Yang, unpublished data). Chesne et al. (2002) reported a 47% blastocyst development from rabbit NT embryos with a combination of electrical pulse, CHX and DMAP. Li et al. (2006) used the similar activation procedures to clone rabbits from cultured adult cultured fibroblast cells. In our study, electrical stimulation combined with CHX and DMAP was used in parthenogenetic activation and in NT, and was sufficient to activate young age oocytes (10–12 hpi). A substantial blastocyst development of up to 56.0% was achieved in NT that was similar to the rate in the parthenogenetically activated group (49.9%). In future studies, a comprehensive and detailed comparison of various activation procedures is necessary in order to ascertain the best activation system for rabbit NT embryos.

Nuclear reprogramming is a complicated process, involving not only cellular nuclear-cytoplasm interactions (Campbell et al., 1996), but also epigenetic modifications (Yang et al., 2007) and molecular differentiation (Latham, 2004; Yang et al., 2007). In our study, the overall MPF activity was not dramatically altered in the oocytes collected from 10 to 16 hpi, but MAPK activity was shown to decrease by 16 hpi. We did not find correlation between the MPF and MAPK activity, and the NT efficiency at different oocyte age, suggesting that MPF and MAPK activity did not significantly influence developmental potentials of rabbit embryos cloned from oocytes of various ages. In cattle, MPF was shown not to be a key regulatory factor for reprogramming (Tani et al., 2003); however, it was possible that MPF acted as a promoter, or at least an initiator, of reprogramming, because a somatic nucleus was introduced into a cytoplasm containing high concentrations of MPF (Liu and Yang 1999; Liu et al., 1998). The molecular reprogramming factors, or at least the reprogramming initiation molecules, certainly reside in the cytoplasm of the matured oocyte, and their function may cease after activation (Du et al., 2002). However, the exact levels of MPF and MAPK activities after NT micromanipulation were not compared when the donor nucleus was exposed to the ooplasm in our study.

In addition, our results were different from the findings of Chesne et al. (2002), where live clones were produced using oocytes collected at 16 hpi. In our study, two full-term rabbit clones were both generated from oocytes collected 12 hpi. The reasons for these discrepancies might be due to technical differences in the methods used for NT, or to the relative skill of different technicians in performing these exacting techniques. We considered that our NT micromanipulation skills might be inferior to those of the French group;

however, this does not negate the oocyte age effect that was clearly demonstrated here, because all our NTs were carried out by the same technician. A direct comparison of oocyte age on cloning efficiency was not performed in that study (Chesne et al., 2002). Other factors contributing to the discrepancies between these studies may include differences in animal breeds and different superovulation protocols. In the French study, matured INRA-1077 or F1 (1077 X GD22) does were used for experiments; superovulation was performed with five injections, whereas in our study, we used New Zealand White rabbits as donors and Dutch Belted as recipients, and employed the six injection regime of FSH treatment.

In the present study, we also examined the effects of different NT method. Somatic cell NT, in various animal species, is accomplished by a donor nucleus being introduced into the oocyte's cytoplasm either by direct nuclear injection (Inoue et al., 2002; Onishi et al., 2000; Wakayama et al., 1998), or via electrically mediated membrane fusion (Bethausser et al., 2000; Chesne et al., 2002; Du et al., 2002; Kato et al., 1998; Kubota et al., 2000; Wilmut et al., 1997). Nevertheless, the relative efficiency of these two NT approaches has not been directly compared in rabbits (Inoue et al., 2002; Yin et al., 2002). We demonstrated that NT by electrically mediated membrane fusion was more effective than was nuclear injection for cloned rabbit embryonic development. The reason for this differential development has yet to be determined. In fact, direct nuclear injection was very effective in mouse somatic cell NT, in which chemical activation with strontium (10 mM), omitting an electrical stimulation, was routinely performed (Wakayama et al., 1998; Wakayama et al., 2001; Wakayama et al., 2006). In rabbit, improved postimplantational development of NT embryos was shown on days 16–20 after embryo transfer by the activation of  $IP_3$  by Ogura's group (Inoue et al., 2002), but without producing a live clone or term development. In our study, NT embryos derived from both approaches were treated with the same regime of combined electrical and chemical activation. Nevertheless, the transfer of 140 NT embryos derived from nuclear injection did not result in any full-term development. We hypothesize that the electrical pulse used for activation may have an adverse impact on nuclear-injected NT embryos in rabbits. It will be most advantageous to test and develop chemical activation protocols that, in particular, promote the term development of injection-derived embryos. Other possibilities are that (1) the 8- $\mu$ m inner diameter Piezo needle used to inject the 15–20- $\mu$ m rabbit cell may damage cell nucleus during injection; (2) the injection procedure could accidentally activate the oocytes, thus impairing further development. These assumptions need to be confirmed.

In conclusion, our study demonstrated an age-dependent factor in rabbit oocytes used for NT. A higher preimplantational development was achieved following NT using oocytes harvested at 10–12 hpi. Our success, although modest, of preferentially generating full-term/live clones from oocytes collected 12 hpi lends support to the hypothesis that young oocytes possess a more effective spectrum of reprogramming factors, and thus, are more capable of redirecting a differentiated genome to express developmental totipotency.



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## Author Disclosure Statement

The authors declare that no competing financial interests exist.

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