

## Follicular Oocytes Better Support Development in Rabbit Cloning Than Oviductal Oocytes

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### Abstract

This study was conducted to determine the effect of rabbit oocytes collected from ovaries or oviducts on the developmental potential of nuclear transplant embryos. Donor nuclei were obtained from adult skin fibroblasts, cumulus cells, and embryonic blastomeres. Rabbit oocytes were flushed from the oviducts (oviductal oocytes) or aspirated from the ovaries (follicular oocytes) of superovulated does at 10, 11, or 12 h post-hCG injection. The majority of collected oocytes were still attached to the sites of ovulation on the ovaries. We found that follicular oocytes had a significantly higher rate of fusion with nuclear donor cells than oviductal oocytes. There was no difference in the cleavage rate between follicular and oviductal groups, but morula and blastocyst development was significantly higher in the follicular group than in the oviductal group. Two live clones were produced in follicular group using blastomere and cumulus nuclear donors, whereas one live clone was produced in the oviductal group using a cumulus nuclear donor. These results demonstrate that cloned rabbit embryos derived from follicular oocytes have better developmental competence than those derived from oviductal oocytes.

### Introduction

**R**ABBITS PROVIDE an excellent model for extrapolation to humans, as they have similar biochemical and physiological processes. Cloned rabbits have been generated by introducing embryonic cells from preimplantation 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). However, the rabbit is reportedly more difficult to clone with somatic donor cells than other domestic animals, such as cattle and sheep. Rabbit preimplantation embryos have a relatively short cell cycle, and the mechanism responsible for somatic genome reprogramming in recipient oocytes is unknown (Cervera and Garcia-Ximenez, 2004; Dinnyes et al., 2001; Mitalipov et al., 1999; Tsunoda and Kato, 2004; Yin et al., 2000, 2002). To date, only a few cases of successful somatic cell cloning of rabbits have been reported

worldwide. The first live rabbit clones were generated from fresh rabbit cumulus cells (Chesne et al., 2002). Later, rabbit clones were produced using cultured adult fibroblasts (Li et al., 2006) and cumulus cells (Meng et al., 2009) as nuclear donors. More recently, Zakhartchenko et al. (2011) generated live clones from genetically manipulated mesenchymal stem cells.

The success of somatic cell NT is thought to depend upon a series of nuclear remodeling and cytoplasmic reprogramming events. In cattle and mice, the age of a recipient oocyte plays an important role in these events to generate live clones (Du et al., 1995; Liu et al., 2007). Cervera and Garcia-Ximenez (2003) also demonstrated that oocyte age and donor cell type affect cloning efficiency. Young oocytes are known to have higher levels of MPF and MAPK than aged oocytes (Lee and Campbell, 2006; Tian et al., 2002). High MPF activity is responsible for the induction of nuclear envelope breakdown,

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premature chromosome condensation, and other chromosomal modifications fundamental to molecular reprogramming (Campbell et al., 2007; Lee and Campbell, 2006). The first live clone rabbits were produced using oocytes collected at 16 h after human chorionadotropin (hCG) treatment (Challah-Jacques et al., 2003; Chesne et al., 2002). Inoue et al. (2002) then showed that postimplantation development of cloned rabbit embryos is improved by collecting recipient oocytes at 13–14 h post-injection (hpi) of hCG. We have found that embryos cloned using oocytes collected at 12 hpi, but not at later time points, result in a live pup (Du et al., 2009). This suggests that using young oocytes harvested at 10–12 hpi is beneficial for the development of cloned rabbit embryos.

In this study, we compared the capacity of oocytes collected from ovarian follicles (ovulated but attached to the ovulation site or in the follicles, defined as follicular oocytes) or ovulated oocytes (oviductal oocytes) to reprogram rabbit somatic cells. The development of cloned embryos derived from these two types of recipient oocytes was assessed. Our findings show that follicular oocytes improve cloning efficiency and subsequent embryonic development in rabbits.

## Materials and Methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The medium was prepared as described previously (Du et al., 2009). Briefly, 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-PVA) and collecting cumulus–oocyte complexes (COCs) from the follicles of the ovary. Tissue and cell cultures were maintained in Dulbecco's Minimum Eagle's medium (DMEM; 31600, Gibco). Medium 199 (M199), which contains 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 (FBS; SH0070.03, Hyclone, Logan, UT) to serve as the standard manipulation medium. Rabbit oocytes and embryos were cultured in 2.5% FBS B2 medium (Laboratories CCD, Paris, France) at 38.5°C in 5% CO<sub>2</sub> and humidified air.

### Preparation of donor and recipient rabbits

Sexually mature (6- to 18-month-old) New Zealand White (NZW) rabbits and F1 hybrids between NZW and Rex rabbits were maintained under a 12-h light/12-h dark cycle. NZW and F1 rabbits (oocyte donors) were superovulated with hormones, as described by Du et al. (2009). Briefly, we administered two 3-mg, two 4-mg, and two 5-mg injections of Folltropin-V (FSH; Bioniche Animal Health Canada, Belleville, Ontario, K8N 5J2) at 12-h intervals. We then administered 200 IU of hCG (Chorulon, animal use, Intervet Inc, Millsboro, DE). NZW rabbits were induced to asynchronously ovulate with a 22–26-h delay to oocyte donors by mating them with vasectomized males and then administering an intramuscular injection of 15 µg GnRH analogue (Cystorelin, Abbott Laboratories, North Chicago, IL) (Challah-Jacques et al., 2003). Untreated NZW rabbits were mated with fertile males to generate foster mothers for cloned kits.

All animal maintenance, care, and use procedures were reviewed and approved by the Institutional Animal Care

and Use Committee of National Taiwan University and the University of Michigan. All surgical procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

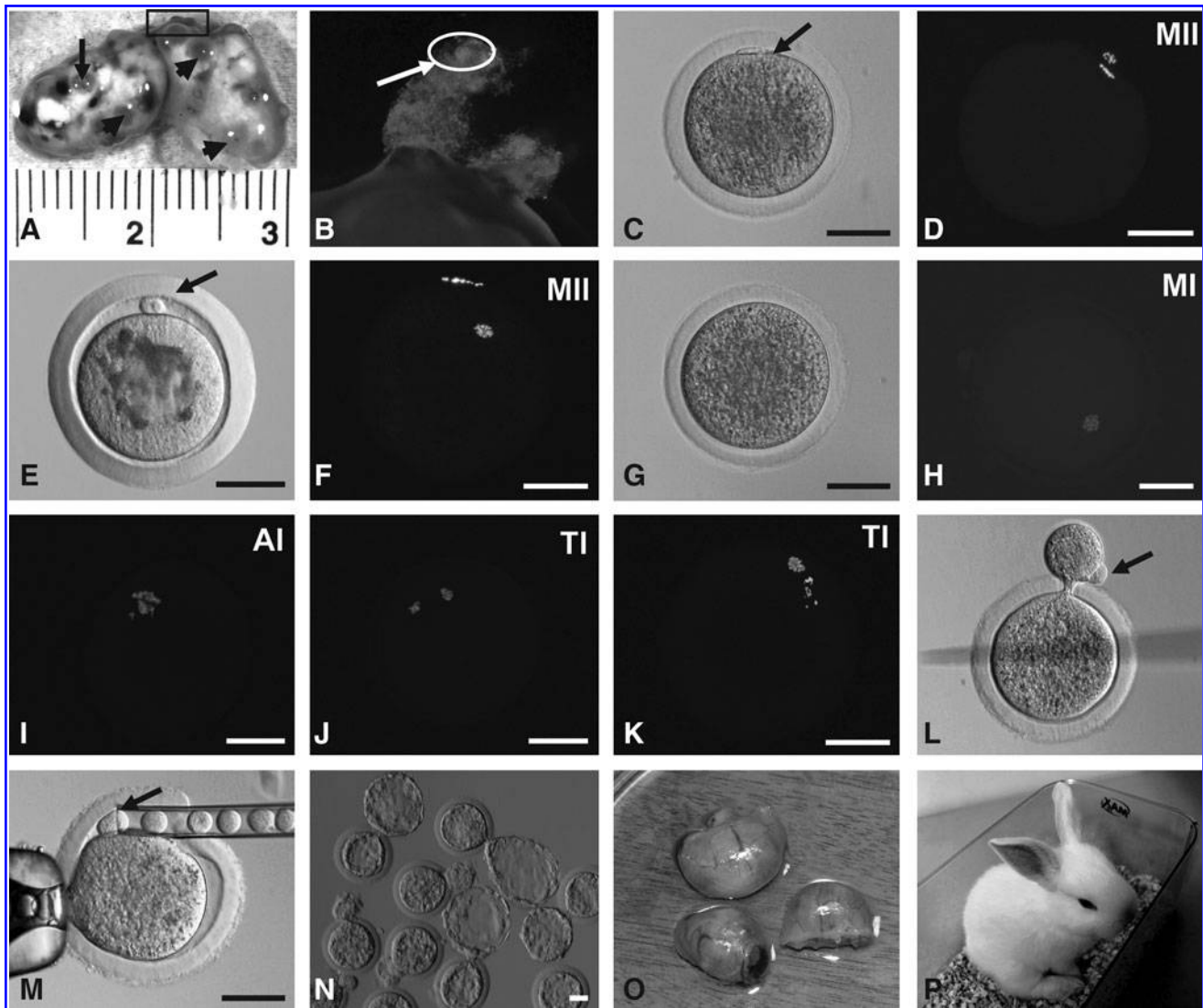
### Collection of oviductal and follicular oocytes

The reproductive tracts including the ovaries were excised from euthanized donor rabbits by midventral laparotomy. Oocytes were then collected from oviducts and ovarian follicles as described below at 10, 11, and 12 hpi. COCs were derived from either the oviducts (oviductal oocytes) or the ovaries (follicular oocytes).

Oviductal oocytes were collected by flushing each oviduct with 5–7 mL PBS-PVA. At the time of collection (10–12 hpi), some oocytes had undergone ovulation but were still at the top of the ovulation site and not in the oviduct (Fig. 1A and B). Therefore, the follicular oocytes included those (1) extracted from the Graafian follicles by rupturing the follicular wall to release COCs (inside, Fig. 1A) as well as those (2) picked up from the ovulation sites (outside, Fig. 1B). Rabbit COCs were treated with 0.5 mg/mL hyaluronidase in PBS for 1 min, and cumulus cells were completely removed from the oocytes by careful pipetting with a fine bore glass pipette. The oocytes were examined by light microscopy to discern the appearance of the first polar body (PB) (Fig. 1C and E). Some of the oocytes from oviducts and ovaries were also then stained with 10 µg/mL Hoechst 33342 for 10 min and examined by fluorescent microscopy to assess maturation status (Fig. 1D and F). Oocytes without the first PB were characterized as being in metaphase I (MI) (Fig. 1H), anaphase I (AI) (Fig. 1I), telophase (TI) (Fig. 1J), or transition from telophase I to metaphase II (TI/MII) (Fig. 1K), respectively. Because most oocytes with the first PB were at MII, the follicular oocytes collected inside and outside of the follicles were pooled for the experiments during NT; however, MII oocytes collected from either follicles or oviducts at different times (10, 11, or 12 hpi) were designated as separate treatment groups. Immature oocytes were cultured in 10% FBS M199 at 38.5°C in 5% CO<sub>2</sub> and humidified air for 3 h. Maturation status was then examined. The MII-stage oocytes derived from the *in vitro* maturation group were not used for NT.

### Preparation of donor cells from rabbit tissues and morulae/early blastocysts

Fibroblasts, cumulus cells, and embryonic cells isolated from morula/early blastocysts derived from NZW rabbits were prepared for NT and subsequent embryo transfer. For collection of fibroblasts, skin notches were taken from the ear of an adult rabbit. Cell culture was performed as described by Du et al. (2002). Briefly, a skin sample with a diameter of 0.5 cm was cut into four to five pieces, washed, placed in a Falcon 35×10-mm culture dish (Becton Dickinson, 3001) containing 10% FBS DMEM, and cultured at 37°C in 5% CO<sub>2</sub> humidified air. Fibroblast monolayers formed around the tissue explants in 5 to 7 days. Fibroblasts were cultured until confluence was reached. During passaging, cultured cells were washed with 1 mL of Dulbecco's PBS and then gently digested with a 3-min incubation at 37°C in 250 µL of 0.05% trypsin and 0.5 mM EDTA (Gibco). Trypsinization was terminated by adding 10% FBS in DMEM and washing. The cell



**FIG. 1.** Generation of cloned rabbits by nuclear transfer (NT) with follicular oocytes. (A) A 2.9-cm-long ovary collected at 11 hpi. The ovulation site is indicated with an arrow and the follicles with arrow heads. (B) High-magnification view of the frame in A. The ovary contained an oocyte-cumulus complex (indicated with an arrow and circled). The oocyte had been ovulated, but was still attached to the ovary. (C) A follicular oocyte with a PB (arrow). Note the small perivitelline space. (D) This oocyte had reached metaphase II (MII), as seen by Hoechst 33342 staining ( $10 \mu\text{g}/\text{mL}$ ). (E) Oviductal oocyte with PB (arrow). Note the large perivitelline space. (F) This oocyte had also matured to MII. (G) Follicular oocyte without the first PB. Such follicular oocytes were in (H) metaphase I (MI), as seen by one cluster of chromosomes; (I) anaphase I (AI), as seen by two clusters adjacent to one another; or (J, K) telophase (TI), as seen by the migration of chromosomes in opposite directions. Oocytes in J and K have different appearances, likely because the oocyte in K is in TI/MII transition. (L) Enucleation of a mature rabbit oocytes. The first PB (indicated with an arrow) along with a portion of cytoplasm was pressed out through an incision, which was made in the zona pellucida using a microneedle. Only enucleated oocytes lacking MII chromosomes, as confirmed by fluorescent microscopy, were used for NT. (M) During NT, an intact donor cell was transferred into the perivitelline space of an enucleated oocyte (arrow) prior to membrane fusion. (N) Cloned rabbit embryos developed into hatching blastocysts after 5 days of culture. (O) Pregnancy was terminated by caesarean section on day 19 after embryo transfer (ET), and fetus bodies were recovered. (P) A 4-week-old rabbit clone (a follicular oocyte reconstructed with a cumulus cell) that was delivered alive, by Caesarean section, 31 days after ET. Bars =  $50 \mu\text{m}$ .

suspension was then centrifuged at 1000–1500 rpm for 5 min, and cells were seeded into new dishes, where they were maintained for an additional 3 to 5 days. Fibroblasts at passage 8 to 10 were used for NT after being serum starved in 0.5% FBS DME for 7 to 9 days. Prior to NT, cells were disassociated by trypsinization, resuspended in 0.5% FBS in DMEM, and allowed to recover for about 1 h at  $37^\circ\text{C}$ .

Embryonic donor cells at the morulae and early blastocyst stage were used for NT as a control for somatic cell NT. Rabbit zygotes were collected from the donors at 18 hpi after super-ovulation and mating with a male. They were then cultured for 72 h to late morulae and early blastocyst stage. After removal of the zona pellucida by treatment with acidic PBS (pH 2.0) and  $5 \text{ mg}/\text{mL}$  pronase E, embryos were incubated for 2 to

3 min in cell disaggregating solution containing 0.25% trypsin. They were then gently pipetted with a fine, fire-polished capillary pipette in M199 with fetal calf serum until individual cells were disaggregated. Isolated embryonic cells had diverse morphology, and only the small, round-shaped, and healthy looking cells were selected for NT.

Freshly collected cumulus cells served as the somatic cell nuclear donors in NT, as described by Challah-Jacques et al. (2003) and Du et al. (2009). Briefly, cumulus cells were washed in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free D-PBS (14190-144, Gibco) supplemented with 10% polyvinylpyrrolidone 40 one time and centrifuged at  $1000\times g$ . The cumulus cell pellet was subsequently treated with trypsin-EDTA for 5 min at  $37^{\circ}\text{C}$ . Trypsinized cumulus cells were then separated into individual cells in 10% FBS DMEM and maintained at  $37^{\circ}\text{C}$  for at least 30 min prior to NT.

#### NT, activation, and embryo culture

The NT micromanipulations were carried out using our standard procedure (Du et al., 2006; Kubota et al., 2000). The steps of enucleation, donor cell transfer, electrical membrane fusion were completed with 2 to 3 h. The number of 100–150 oocytes per batch was used for NT procedure. An incision was made into the zona pellucida using a glass needle, and the PB, along with approximately one-eighth of the surrounding cytoplasm, was extruded by pressing with the glass needle as indicated in Fig. 1L. A donor cell with a diameter of 18–20  $\mu\text{m}$  was selected and transferred into the perivitelline space of an enucleated rabbit oocyte (Fig. 1M). Donor cell-cytoplasm pairs were preincubated in 0.3M mannitol supplemented with 0.1 mM  $\text{CaCl}_2$  and 0.1 mM  $\text{MgCl}_2$  for 2 to 3 min. They were then transferred into an electrical chamber that had wires at a 1-mm distance and contained 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 using a BTX 2001 Electro Cell Manipulator (Biotechnologies & Experimental Research Inc., San Diego, CA). Following the completion of the DC electric pulses, NT oocytes were incubated for 60 to 80 min at  $38.5^{\circ}\text{C}$ . For all experimental groups, NT procedures were finished within 2 to 3 h of oocyte collection.

After the incubation was complete, the fused NT embryos were activated. Activation of cloned embryos was conducted using the same electrical DC setting used for cell fusion and by subsequently incubating embryos for 1 h in M199 containing 10% FBS, 2.0 mM 6-dimethylaminopurine (Sigma, D-2629), and 5  $\mu\text{g}/\text{mL}$  cycloheximide (Sigma, C-6255). After activation, the NT embryos were cultured in 2.5% FBS B2 medium to investigate their *in vitro* developmental potential. Cleavage rates were recorded 16 to 18 h postculture, and two- to four-celled embryos were cultured in the same B2 medium for an additional 4 days to monitor blastocyst development (Fig. 1N).

#### Embryo transfer

The cloned embryos, derived from follicular or oviductal oocytes reconstructed with skin fibroblasts (adult), cumulus cells (adult), or embryonic donor cells (morula/early blastocysts), were transferred into recipients to test their *in vivo* viability. As described by Du et al. (2009), NT embryos were cultured for 16 to 18 h *in vitro* before being surgically trans-

ferred, by midventral laparotomy, into pseudopregnant NZW rabbits. Embryos at the two- to four-cell stage were loaded into a 5- $\mu\text{L}$  Drummond microdispenser and transferred into both oviducts of a recipient. At 16 to 19 days after embryo transfer (ET), pregnancy was monitored by palpation. Some pregnant recipients were terminated on day 19 to examine implantation status and collect the fetus (Fig. 1O). Other pregnancies were allowed to progress to day 31 post-ET, and the live kits were delivered by Caesarean section (Fig. 1P).

## Experiments

### *Experiment 1: Comparison of the preimplantational development of cloned rabbit embryos derived from oviductal or follicular oocytes collected at 12 hpi*

In Experiment 1, the oocytes were harvested at 12 hpi either from oviducts or follicles (inside and outside) on ovaries of donor rabbits. They were then reconstituted with cultured fibroblast cells via electric field-mediated cell fusion. Fibroblasts were starved in 0.5% FBS DMEM for 7 to 9 days prior to NT. NT embryos derived from follicular or oviductal recipient oocytes were then activated with a combined electrical and chemical activation procedure as described above. They were subsequently cultured for 5 days to examine their preimplantational developmental potential.

### *Experiment 2: The effect of follicular oocyte collection time on the preimplantational development of cloned rabbit embryos*

We investigated the *in vitro* development of NT embryos derived from follicular recipient oocytes collected at 10, 11, or 12 hpi. Oocytes were fused with cultured fibroblast donor cells, which were serum starved and treated identically as in Experiment 1.

### *Experiment 3: In vivo and full-term developmental potential of NT embryos reconstructed with follicular versus oviductal oocytes*

Cloned embryos derived from follicular or oviductal oocytes were transferred into different recipients. The number of transferred NT embryos varied from 17 to 30 per recipient. Fibroblasts, freshly prepared cumulus cells, or embryonic cells (morula/early blastocysts) were used to generate the NT embryos, and embryos were transferred into different recipients to examine the implantation or term development on day 31 post-ET.

#### Statistical analysis

Data related to the status of donor ovaries [e.g., ovary size, number of ovulation sites, follicular size, and the number of oocytes collected (follicular vs. oviductal) at different time points (10, 11, and 12 hpi)] were subjected to an arcsin transformation. The transformed data were then analyzed by ANOVA (General Linear Model, SPSS 11.0, Chicago, IL). For the analysis of preimplantational development *in vitro*, the proportions of embryos that reached cleavage (the two- to eight-cell stage), the morulae stage, and the blastocyst stage were subjected to arcsine transformation and analyzed using either Student's *t*-test or ANOVA. A *p*-value  $<0.05$  was considered significant.

## Results

### *Ovarian responses to superovulation at different times after hCG injection*

Seventy-four rabbits at 6 to 12 months of age were superovulated with FSH and underwent oocyte collections at 10 hpi ( $n=20$ ), 11 hpi ( $n=22$ ), or 12 hpi ( $n=32$ ). For each animal, data from each ovary were paired (e.g., volume of ovaries, the number of ovulation sites, and number of follicles). As shown in Table 1, the ovary pair volume (Fig. 1A) ranged from 1.29 to 1.69 cm<sup>3</sup>, and the number of ovulation sites ranged from 26.2 to 32.9 per donor, with no differences in either of these parameters being noted among the three groups. Nevertheless, the average number of follicles on a pair of ovaries decreased from 33.3 per donor at 10 hpi to 16.6–19.0 per donor at 11–12 hpi ( $p<0.05$ ). When the follicles were classified as having a diameter less than 0.8 mm (type A follicles) or ranging from 0.8 to 1.6 mm (type B follicles), 78.2% of follicles in the 10 hpi group were found to be type B follicles, while more follicles in the 11 hpi (90.6%) and 12 hpi (83.9%) groups were type B follicles ( $p<0.05$ ).

### *Oocyte yields and maturation at different times after hCG injection*

Oocytes were collected from oviducts by flushing (oviductal), from ovaries (follicular) by collecting oocytes just ovulated but still attached to ovulation sites (outside, Fig. 1B), or from follicles with a diameter of 0.8 to 1.6 mm. The total number of oocytes collected per donor did not differ among the 10, 11, or 12 hpi groups and ranged from 40.1 to 51.4 (Table 2). However, an analysis of the percentage of oocytes collected from oviducts revealed that only 7.8% of oocytes were collected from oviducts at 10 hpi, with this percentage increasing to 16.6% at 11 hpi and to 34.6% at 12 hpi ( $p<0.05$ ). Conversely, more than 91.2% of oocytes were collected from ovaries at 10 hpi, with this number decreasing to 65.4% at 12 hpi ( $p<0.05$ ). Interestingly, each group yielded a comparable percentage of follicular oocytes, which were collected from outside of ovulated sites (Fig. 1B) (46.6–47.2%) or from inside follicles (52.8–53.4%). Based on the number of ovulation sites on the ovaries and the number of oocytes collected by oviduct flushing, oocyte recovery from oviducts was only 13.0% at 10 hpi, but increased to 24.5% at 11 hpi and 45.7% at 12 hpi ( $p<0.05$ ) (Table 2). These results indicate that not all ovulated oocytes move into the oviducts at the time of oocyte collection (10–12 hpi).

As shown in Table 3, when oocytes moved into oviducts at 10 to 12 hpi, as many as 98.3 to 100% of oviductal oocytes reached MII phase with the appearance of the first PB. The MII chromosome cluster could be clearly seen in these cells using Hoechst 33342 staining and fluorescent microscopy (Fig. 1E and F). In contrast, follicular oocytes had a smaller perivitelline space than oviductal oocytes (Fig. 1C and G vs. Fig. 1E). In all age groups, only 63.8 to 69.9% of follicular oocytes had the first PB and were at MII (Fig. 1C, D) ( $p>0.05$ ). The remaining oocytes did not have the first PB and had matured to varied degrees, with these oocytes being in MI (Fig. 1H), AI (Fig. 1I), TI (Fig. 1J), and TI/MII transition (Fig. 1K). Fluorescent microscopy revealed that 96.3% of follicular oocytes with a PB ( $n=151$ ) and 98.2% of oviductal oocytes with a PB ( $n=404$ ) had matured to MII phase ( $p>0.05$ ) (Fig. 2). However, no oocytes matured to MII phase if they did not extrude the PB, regardless of their source [i.e., follicular ( $n=153$ ) or oviductal ( $n=25$ )] (Fig. 2). Of the follicular oocytes without a PB, 81.3% were in TI, 2.2% in AI, and 16.5% in MI. Of the oviductal oocytes without a PB, 75.7% were in TI and 24.3% in AI.

Finally, a total of 247 immature oocytes collected at 10, 11, or 12 hpi were pooled and cultured for 3 h. Subsequent staining with Hoechst 33342 and examination by fluorescent microscopy revealed that 90.7% of cultured oocytes reached MII.

### *Preimplantation development of cloned embryos derived from oviductal vs. follicular oocytes*

Next, we compared the efficiency of somatic cell NT in oviductal and follicular oocytes collected at 12 hpi. Cultured fibroblasts served as the nuclear donors. The donor cell was transferred directly into the perivitelline space of the enucleated oocyte (Fig. 1M), and electrically mediated fusion was performed. After NT and further culture, cloned embryos sequentially developed into two- to four-celled, morulae, and blastocysts (Fig. 1N). As shown in Table 4, the fusion rate was higher in follicular oocytes than in oviductal oocytes (81.5 vs. 59.1%,  $p<0.05$ ). The percentage of embryos developing into two- to four-cell stage was comparable for follicular and oviductal oocytes. However, the percentage developing into morulae and blastocysts was significantly lower for oviductal oocytes than for follicular oocytes (32.8 vs. 51.6% for morulae; 14.7 vs. 32.9% for blastocysts;  $p<0.05$ ). There was not different in cell number per NT blastocyst between follicular ( $n=8$ ) vs. oviductal ( $n=5$ ) group (176.4 vs. 233.6,  $p>0.05$ ).

TABLE 1. STATUS OF OVARIES COLLECTED FROM DIFFERENT TIMES POST-hCG INJECTION

Treatment (hpi)	No. replicates	Ovary pair volume (cm <sup>3</sup> )	No. ovulation Sites	No. Follicles on ovary pair	% follicles sized with an diameter (mm) ranged at	
					<0.8	0.8–1.6
10	16	1.69±0.18 <sup>a</sup>	30.6±4.4 <sup>a</sup>	33.3±2.8 <sup>a</sup>	24.8±5.2 <sup>a</sup>	78.2±4.5 <sup>a</sup>
11	13	1.28±0.15 <sup>a</sup>	26.2±3.4 <sup>a</sup>	19.0±5.4 <sup>b</sup>	9.4±5.0 <sup>a</sup>	90.6±2.7 <sup>b</sup>
12	11	1.29±0.19 <sup>a</sup>	32.9±4.0 <sup>a</sup>	16.6±2.2 <sup>b</sup>	16.1±3.8 <sup>a</sup>	83.9±6.9 <sup>a,b</sup>

<sup>a,b</sup>Values with different superscripts within columns differ,  $p<0.05$ .

Oocytes were collected from superovulated ova donors at 10, 11, and 12 h post-human chorionic gonadotropin (hCG) injection (hpi). The volume of ovaries, number of ovulations, number of follicles, and diameter of unovulated follicles were examined and accumulated as a pair (two ovaries) per donor.

TABLE 2. OOCYTE COLLECTION FROM THE DONORS AT DIFFERENT TIMES POST-HCG INJECTION

Treatment (hpi)	No. replicates	Total No. oocytes collected	% Collected per animal				No. ovulation sites	% Oocytes collected from oviducts	
			Oviductal	Follicular		Outside			Inside
				Subtotal	Outside				
10	16	51.4 ± 4.7 <sup>a</sup>	7.8 ± 1.9 <sup>a</sup>	91.2 ± 2.0 <sup>a</sup>	47.2 ± 4.9 <sup>a</sup>	52.8 ± 5.0 <sup>a</sup>	30.6 ± 4.4 <sup>a</sup>	13.0 ± 3.4 <sup>a</sup>	
11	13	40.1 ± 4.5 <sup>a</sup>	16.6 ± 2.5 <sup>b</sup>	83.4 ± 2.5 <sup>b</sup>	46.6 ± 5.2 <sup>a</sup>	53.4 ± 5.2 <sup>a</sup>	26.2 ± 3.4 <sup>a</sup>	24.5 ± 3.2 <sup>b</sup>	
12	11	41.7 ± 3.7 <sup>a</sup>	34.6 ± 2.5 <sup>c</sup>	65.4 ± 2.5 <sup>c</sup>	46.9 ± 6.4 <sup>a</sup>	53.1 ± 6.4 <sup>a</sup>	32.9 ± 4.0 <sup>a</sup>	45.7 ± 4.2 <sup>c</sup>	

<sup>a,b,c</sup>Values with different superscripts within columns differ,  $p < 0.05$ .

Oocytes were collected from superovulated ova donors at 10, 11, and 12 h post-human chorionic gonadotropin (hCG) injection (hpi). The number of ovulations, number of oocytes collected either from oviducts, or on ovaries, named as follicular oocytes, ovulated but attached to ovulation sites (outside), or collected within follicles (inside), were examined and accumulated as a pair (two ovaries/oviducts) per donor.

#### Preimplantation development of cloned embryos derived from follicular oocytes collected at different times after hCG injection

Because the use of follicular oocytes clearly yielded better NT efficiency than the use of oviductal oocytes, we next investigated the effect of follicular oocyte age on the *in vitro* development of cloned embryos. For these experiments, follicular oocytes collected at 10, 11, or 12 hpi were subjected to NT using fibroblasts as nuclear donors. As shown in Table 5, the enucleation rate decreased from 80.2% at 10 hpi to 68.2% at 11 hpi and 67.7% at 12 hpi ( $p < 0.05$ ) (Table 5). Nevertheless, the fusion rate was similar among all age groups, ranging from 64.5% to 84.1%. Oocyte age also did not significantly affect cleavage (84.9–91.1%), development into morulae (41.1–48.9%), or development into blastocysts (27.0–34.5%) ( $p > 0.05$ ) (Table 5). There was not different in cell number per NT blastocyst among follicular groups collected at 10 hpi ( $n = 12$ ), 11 hpi ( $n = 9$ ), and 12 hpi ( $n = 14$ ) (214 vs. 154 vs. 220.5,  $p > 0.05$ ).

#### In vivo and full-term developmental potential of cloned embryos reconstructed with oviductal versus follicular oocytes

To understand how oocyte source affects the full-term developmental potential of cloned embryos, we compared the efficiency of generating live rabbit clones using NT embryos derived from follicular or oviductal oocytes. Follicular

and oviductal oocytes were reconstructed with skin fibroblasts (adult), cumulus cells (adult), or embryonic donor cells (blastomeres from morula/early blastocysts). Cloned embryos were then transferred into separate recipients to test their *in vivo* viability. The number of transferred NT embryos ranged from 17 to 30 per recipient. As shown in Table 6, when embryonic donor cells, adult fibroblasts, and freshly prepared cumulus cells were used as nuclear donors, the pregnancy rate varied from 33.3 to 60.0% in the follicular oocyte group and from 0 to 25% in the oviductal oocyte group. In each group receiving fibroblast nuclear donors (i.e., follicular-fibroblast and oviductal-fibroblast group), one pregnancy was terminated on day 19 post-ET to examine implantation. Three conceptuses were collected from the follicular-fibroblast animal (Fig 1O), and two were collected from the oviductal-fibroblast animal. The remaining recipients in these two groups were allowed to complete a full, 31-day gestation. However, no live clones were born, and only implantation sites were observed (four in the follicular-fibroblast group; two in the oviductal-fibroblast group). In contrast to the follicular-fibroblast group, the follicular oocyte-embryonic donor cell group had one live clone birth (2.9% success rate), as did the follicular-cumulus group (1.1% success rate) (Fig. 1P). Similarly, one clone was generated from an oviductal-cumulus group (0.7% success rate) (Table 6).

#### Discussion

Successful nuclear reprogramming by somatic cell NT has been demonstrated by the generation of several species of cloned animals. However, the mechanism underlying reprogramming remains unknown. In mouse studies, cytoplasts derived from MII oocytes and mitotic zygotes could support the development of somatic cell NT embryos (Egli et al., 2007, 2008, 2009; Gao et al., 2002). In contrast, nucleus-free cytoplasts of germinal vesicle oocytes and interphase zygotes are incompetent in remodeling the donor nucleus and supporting embryo development following NT (Gao et al., 2002; Wakayama and Yanagimachi, 2001). Interestingly, selective removal of chromatin and the nuclear membrane from the interphase zygote, which allows most of nuclear material to be retained in the cytoplasm, yields a type of cytoplasm that is capable of supporting cloned embryo development (Greda et al., 2006). The above-mentioned studies show that undefined reprogramming factors located close to the nuclei of germinal vesicle oocytes and fertilized

TABLE 3. MATURATION STATUS OF OOCYTES COLLECTED FROM OVIDUCTS VERSUS OVARIES AT DIFFERENT TIMES POST-HCG INJECTION

Treatment (hpi)	No. replicates	Oocyte source			
		Oviductal		Follicular	
		Total No.	% with Pb	Total No.	% with Pb
10	20	81	98.3 ± 1.2 <sup>a</sup>	814	69.9 ± 6.0 <sup>b</sup>
11	23	214	100.0 ± 0 <sup>a</sup>	745	63.8 ± 5.3 <sup>b</sup>
12	20	301	99.6 ± 0.4 <sup>a</sup>	755	77.9 ± 5.1 <sup>b</sup>

<sup>a,b</sup>Values with different superscripts within rows differ,  $p < 0.05$ .

Oocytes were collected from superovulated ova donors at 10, 11, and 12 h post-human chorionic gonadotropin (hCG) injection (hpi). The oocytes collected from either oviducts or on ovaries (ovulated but attached to ovulation sites and inside follicles), were examined.

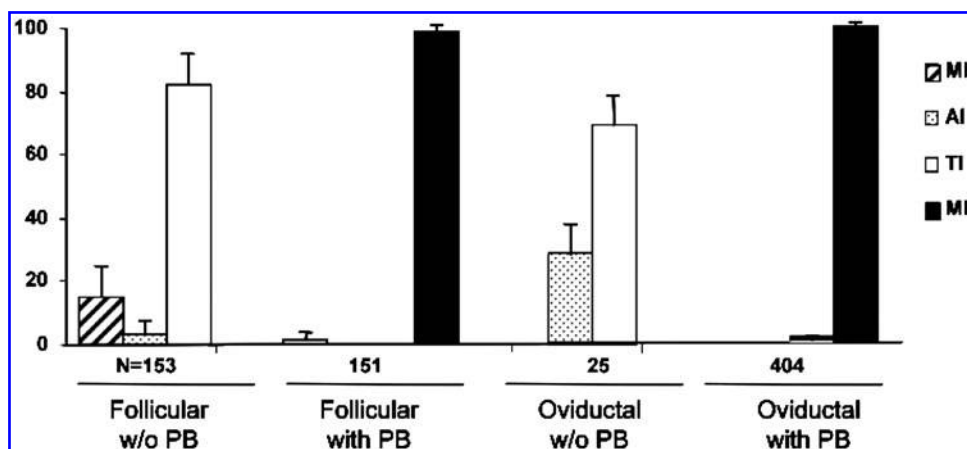


FIG. 2. Maturation status of oocytes collected from oviducts and ovaries. Rabbit oviductal oocytes were collected from oviducts by flushing with D-PBS, while oocytes collected from either outside ovulation sites on ovaries or from 0.8–1.6-mm follicles were pooled as follicular oocytes. Both oviductal and follicular oocytes were examined under a microscope to confirm the appearance of the first PBs. The oocytes were classified into four categories: follicular without

PB (follicular w/o PB,  $n=153$ ), follicular with PB (follicular with PB,  $n=151$ ), oviductal without PB (oviductal w/o PB,  $n=25$ ), and oviductal with PB (oviductal with PB,  $n=404$ ). Oocytes were staining with  $10\ \mu\text{g}/\text{mL}$  Hoechst 33342 and examined by fluorescent microscopy to determine maturation status (MI, AI, TI, and MII) for statistical analysis.

embryos are released and accumulate in metaphase cytoplasts. Among these recipient cytoplasts, those derived from MII oocytes have been shown to be the most potent for reprogramming introduced somatic nuclei.

The important effect of the age of the recipient oocyte on NT efficiency has been reported in cattle (Du et al., 1995), mice (Liu et al., 2007), and rabbits (Du et al., 2009). Rabbit oocytes used for NT have been collected at a wide range of times, from 13 to 14 h to 15 to 16 h after hCG/luteinizing hormone (LH) injection (Dinnyes et al., 2001; Inoue et al., 2002; Li et al., 2006; Mitalipov et al., 1999). We have previously shown that performing NT with young rabbit oocytes harvested at 10 to 12 hpi is more beneficial for the development of NT embryos to blastocysts *in vitro* than using those collected at 14 to 16 hpi (Du et al., 2009). This success in using young oocytes implies that, especially for rabbit NT, a very narrow time window of oocyte age exists, estimated at 2 to 4 h. However, in that study, both follicular and oviductal MII oocytes (with PB), primarily collected at 10 and 12 hpi, were pooled for *in vitro* experiments to eliminate the variable of oocyte origin (follicular vs. oviductal) and to focus on the age of oocytes at collection.

In the present study, we separated the oocytes collected from oviducts and ovaries and compared their ability to support embryo development. We observed that follicular oocytes from ovarian follicles (including those that had just

been ovulated, but were still attached to the ovaries and had not yet entered the oviducts) supported better embryonic development than those flushed from oviducts. The mechanisms underlying the better reprogramming ability of rabbit follicular oocytes compared to oviductal oocytes remain to be addressed. In our previous study, overall MPF activity was not dramatically changed in the oocytes collected from 10 to 16 hpi (Du et al., 2009). Tani et al. (2003) demonstrated that MPF is not a critical regulatory factor for reprogramming in cattle. We assume that MPF, acting as a promoter, can still initiate reprogramming since a somatic nucleus was introduced into oocyte cytoplasm with a high concentration of MPF. Activation of oocytes prior to transfer to a somatic nucleus significantly decreases the development of cloned embryos in cattle (Du et al., 2002) as well as in rabbits (Du et al., data not shown). Reprogramming molecules or initiation factors may be more abundant in the cytoplasm of very young oocytes—in our case follicular oocytes—than in oviductal ones. The reprogramming ability of oocytes appeared to decrease after ovulation. It is also important to notice that the fusion rates dramatically declined when ovulated oocytes entered the oviducts (Table 5) at 12 hpi. The properties of the oocyte membrane may change after the ovulation process, making fusion with the introduced donor cell difficult. Hundreds of macromolecules important for fertilization and early cleavage during embryonic development, such as

TABLE 4. EFFICIENCY OF RABBIT SOMATIC CELL NT WITH RECIPIENT OOCYTES COLLECTED FROM OVIDUCTS VERSUS OVARIES AT 12 H POST-HCG INJECTION

Treatment	No. oocytes	No. replicates	Euncleation (%)	Fusion (%)	% Embryos developed to <sup>1</sup>		
					Cleavage (2–4 celled)	Morulae	Blastocyst
Follicular	658	10	77.8 ± 2.7 <sup>a</sup>	81.5 ± 3.8 <sup>a</sup>	87.9 ± 3.9 <sup>a</sup>	51.6 ± 4.8 <sup>a</sup>	32.9 ± 3.8 <sup>a</sup>
Oviductal	640	10	77.7 ± 3.5 <sup>a</sup>	59.1 ± 6.2 <sup>b</sup>	90.0 ± 3.3 <sup>a</sup>	32.8 ± 4.7 <sup>b</sup>	14.7 ± 2.8 <sup>b</sup>

<sup>1</sup>The percentage of embryonic development was calculated based upon the number of fused oocytes used for NT.

<sup>a,b</sup>Values with different superscripts within columns differ,  $p < 0.05$ .

Oocytes were collected from superovulated ova donors at 12 h post-human chorionadotropin (hCG) injection (hpi). The oocytes collected from either oviducts or on ovaries (ovulated but attached to ovulation sites and inside follicles), were used for NT. Cultured fibroblasts were used as nuclear donors for NT. Fused NT embryos were arranged for development *in vitro*. Cleavage, the sum of two- and four-celled after 16–18 h culture *in vitro* (IVC); morulae, at 72 h IVC; blastocyst at day 5 IVC.

TABLE 5. EFFICIENCY OF RABBIT SOMATIC CELL NT WITH FOLLICULAR RECIPIENT OOCYTES AT DIFFERENT AGES

Treatment (hpi)	No. oocytes	No. replicates	Enucleation (%)	Fusion (%)	% Embryos developed to <sup>1</sup>		
					Cleavage (2–8 celled)	Morulae	Blastocyst
10	271	5	80.2±4.9 <sup>a</sup>	84.1±4.1 <sup>a</sup>	91.1±3.4 <sup>a</sup>	48.9±5.9 <sup>a</sup>	34.5±4.8 <sup>a</sup>
11	288	5	68.2±5.0 <sup>a,b</sup>	64.5±8.9 <sup>a</sup>	89.9±6.2 <sup>a</sup>	48.1±11.3 <sup>a</sup>	27.0±6.2 <sup>a</sup>
12	289	6	67.7±1.5 <sup>b</sup>	70.3±7.1 <sup>a</sup>	84.9±9.3 <sup>a</sup>	41.1±8.3 <sup>a</sup>	29.9±5.1 <sup>a</sup>

<sup>1</sup>The percentage of embryonic development was calculated based upon the number of fused oocytes used for NT.

<sup>a,b</sup>Values with different superscripts within columns differ,  $p < 0.05$ .

Oocytes were collected from superovulated ova donors at 10, 11, and 12 h post-human choriogonadotropin (hCG) injection (hpi). The follicular oocytes collected from ovaries (oocytes, ovulated but attached to ovulation sites and inside follicles), were used for NT. Cultured fibroblasts were used as nuclear donors for NT. Fused NT embryos were arranged for development *in vitro*. Cleavage, the sum of two- and four-celled after 16–18 h culture *in vitro* (IVC); morulae, at 72 h IVC; blastocyst at day 5 IVC.

regulatory molecules, protease inhibitors, growth factors, cytokines, binding proteins, enzymes, and immunoglobulins, have been found in the mammalian oviduct lumen (Bui et al., 2000). In particular, oviduct-specific glycoprotein (OGP), which is found only in the oviduct, has been localized to the zona pellucida, perivitelline space, and plasma membrane of oviductal oocytes and embryos (McCauley et al., 2003). As already mentioned, we speculate that the rabbit oocyte membrane changes as a result of exposure to the oviduct environment. Although these changes may be critical for fertilization (i.e., prevent polyspermy), they may affect the fusion rate following the NT. Jim Robl's group has also reported that the fertilized cytoplasm is much more difficult to fuse with embryonic donor cells than unfertilized oocytes (Stice and Robl, 1988). Reduced fusion may also be attributable to the fact that the perivitelline space is larger in oviductal oocytes than in follicular oocytes, a feature that may make weaken contact between the enucleated oocyte and donor cell.

Here, we found that the ovulation sites on ovaries at 10, 11, and 12 hpi were similar. However, at 10 hpi, most ovulated oocytes did not appear to enter the oviducts, but attached to the ovulation sites on ovaries. When the collection time was prolonged to 11 and 12 hpi, many more oocytes (as many as 45.7%) were in the oviducts, although 30.7% and 38.9% of ovulated oocytes were still attached to the ovulation sites at 11 and 12 hpi, respectively. Most oocytes collected from oviducts were arrested at MII phase. In this study, follicular oocytes included those ovulated but still attached

to the ovulated sites (outside) as well as those from 0.8 to 1.6-mm Graafian follicles (inside). Of the follicular oocytes collected at 10, 11, and 12 hpi, 63.8 to 77.9% possessed the first PB and matured to MII phase. The remaining oocytes that did not extrude the first PB were at different phases of meiosis (MI, AI, TI, and TI/MII transition). The majority of immature oocytes were able to undergo further maturation and became arrested in MII phase after 3 h of culture. We also found that follicular oocytes possessed a similar ability to promote the blastocyst development *in vitro*, regardless of the collection time (i.e., 10, 11, or 12 hpi).

In the present study, we also compared the *in vivo* developmental potential of cloned embryos derived from follicular oocytes and oviductal oocytes. Two clones were generated from follicular oocytes reconstructed with either embryonic or cumulus donor cells, while one clone was born in the oviductal group reconstructed with cumulus cells. When fibroblast cells were used as donor cells, 2.8% (follicular-fibroblast) and 1.4% (oviductal-fibroblast) implantations/fetuses were achieved, but no live clone was generated. This indicates that the cell type and degree of differentiation of the donor genome may play a pivotal role during the reprogramming process in NT embryos. Meng et al. (2009) studied the effect of TSA on development of cloned embryos, also compared the possibility whether the cotransfer of parthenogenetic embryos with NT embryos could increase *in vivo* development potential. A number of 13 live clones were generated with the oocytes collected from Hycote hybrid rabbits in their study. However, it is difficult

TABLE 6. EFFICIENCY OF RABBIT SOMATIC CELL NT WITH RECIPIENT OOCYTES AT DIFFERENT AGES

Oocyte source	Donor cell type	No. cloned embryos	No. recipients	No. (%) pregnant	No. (%) fetuses/implantations	No. (%) of live clones
Follicular	Embryonic mor/EB	34	2	1 (50.0)	0 (0)	1 (2.9)
	Fibroblast <sup>1</sup>	250	9	3 (33.3)	7 (2.8)	0 (0)
	Cumulus	91	5	3 (60.0)	6 (6.6)	1 (1.1)
Oviductal	Embryonic mor/EB	118	6	0 (0)	0 (0)	0 (0)
	Fibroblast <sup>1</sup>	246	8	2 (25.0)	4 (1.6)	0 (0)
	Cumulus	139	8	1 (12.5)	2 (1.4)	1 (0.7)

<sup>1</sup>One pregnant recipient from either follicular or oviductal group was terminated on day 19 post-ET, fetuses were collected. Remaining pregnancies were allowed to progress to day 31 post-ET. The clones were born by Caesarean section on day 31 post-ET.

Oocytes were collected from superovulated ova donors at 10, 11, and 12 h post-human choriogonadotropin (hCG) injection (hpi). The oocytes collected from either oviducts (oviductal) or on ovaries (follicular, either ovulated but attached to ovulation sites or inside follicles), were used for NT. Cultured fibroblasts, freshly prepared cumulus cells and embryonic cells from mor/EB (morulae/early blastocysts) were used as nuclear donors for NT.



to directly compare our study with that of Meng's group. Nuclear reprogramming process is a complicated issue, many experimental variables would result in different outcomes, such as the source of animals (hybrids) for oocyte collection (Wakayama and Yanagimachi, 2001), donor cell types (Du et al., 2002), activation conditions, epigenetic reagents used (i.e. TSA), personal skills of technicians (Du et al., 2009). In our experimental conditions, the results of *in vitro* development for NT embryos have shown the statistical difference between two groups of oocytes (follicular vs. oviductal).

In summary, our study demonstrates that follicular oocytes collected from ovaries possess a higher reprogramming capacity and ability to promote the development of cloned embryos than oviductal oocytes. The higher success rates associated with generating clones from follicular oocytes collected from ovaries (either outside or inside follicles) further support our assumption that younger rabbit oocytes, at MII phase, are effective at reprogramming, allowing them to direct the totipotent development of a donor genome that is introduced via NT.

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### Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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