Differential Influences in Sizes and Cell Cycle Stages of Donor Blastomeres on the Development of Cloned Rabbit Embryos

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ABSTRACT : Experiments were conducted to evaluate the effect of blastomere diameters and cell cycle stages on the subsequent development of nuclear transplant rabbit embryos (NT-embryos) using nuclei derived from the 16- or 32-cell stage embryos. All blastomeres and NT-embryos were cultured individually in modified Ham's F-10 medium supplemented with 10% rabbit serum (RS) at 38°C and 5% CO_2 in air. The diameter of blastomeres from 16-cell stage embryos was found twice of those from 32-cell stage (51 vs 27 μm). Significant differences were observed in cleavage rates (≥3 divisions) in the isolated single blastomeres (54 vs 48 for 16-cell; 28 vs 14 for 32-cell, $p<0.05$), but the fusion rates of oocytes with transferred nuclei were similar between small and large single blastomeres derived from either 16-cell or 32-cell stage embryos. When 16-cell stage blastomeres were used as nuclear donors, cleavage rates (≥3 divisions) of the NT-embryos were greater in the small nuclear donors than in the large donors (73 vs 55%, $p<0.05$). On the contrary, significantly higher cleavage (43 vs 6%, $p<0.05$) and developmental rates (14 vs 0%, $p<0.05$) were observed in the large blastomere nuclear donor group of the 32-cell stage embryos. When the cell cycle stages were controlled by a microtubule polymerization inhibitor (Demicolcine, DEM) or the combined treatment of DEM and Aphidicolin (APH), a DNA polymerase inhibitor, fusion rates were 88-96% for the 16-cell donor group (without DEM treatment), which were greater than the 32-cell donor group (54-58%). Cleavage rates were also greater in the transplants derived from G_1 nuclear donor group (93-95%) than those from the DEM and APH combined treatment (73%) for the 16-cell donor group ($p<0.05$). No significant difference was detected in the morula/blastocyst rates in either donor cell stage ($p>0.05$). In conclusion, it appeared that no difference in the developmental competence between large and small isolated blastomeres was observed. When smaller 16-cell stage blastomeres were used as nuclear donor, the cleavage rate or development of NT-embryos was improved and was compromised when 32-cell stage blastomeres were used. Therefore, control nuclear stage of the donor cell at G_1 phase in preactivated nuclear recipients seemed to be beneficial for the cleavage rate of the reconstructed embryo in the 16-cell transplant, but not for subsequent morula or blastocyst development. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 1 : 15-22)***

Key Words : Nuclear Transfer, Cell Cycle Stage, Blastomere, Cloned Embryo, Rabbit

INTRODUCTION

Nuclear cloning is a powerful tool for large-scale production of identical multiplets containing the same genetic materials. After conceptually proposed by Han's Spemann, a German developmental biologist, in 1938, this technology was demonstrated by Briggs and King (1952) in *Xenopus* oocytes for the first time. Solter et al. (1980) then applied this technology to mammalian species and compiled data including nuclear transplant mice, rabbits, pigs, sheep, goats, cows (Wolf et al., 1999) and Rhesus monkey (Meng et al., 1997) were generated thereafter. More recently, a milestone breakthrough with the birth of Dolly the sheep was achieved by Wilmut et al. (1997) using adult mammary gland cells as the donor nucleus. Cloned goats (Baguisi et al., 1999), cattle (Cibelli et al., 1998; Wells et al., 1999; Zakhartchenko et al., 1999; Kubota et al., 2000), and pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000) were also produced by this method. Although a variety of approaches explored, the major obstacle of this technology has been its low efficiency in generating offspring ranging from 0.3% (1/277) in sheep (Campbell et al., 1996; Wilmut et al., 1997), and 1.8-10% in cattle (Cibelli et al., 1998; Wells et al., 1998, 1999; Kubota et al., 2000), to 2-2.8% in mice (Wakayama et al., 1998). Cell cycle stages appear to play a major role in this process. Potential problems in nuclear modification or reprogramming might also exist in all species (Stice and Robl, 1988; Collas and Robl, 1991a; Prather et al., 1999; Robl, 1999), which results in early embryonic loss or late term mortality. In many cases, these problems included hydrallantois, placental edema and/or cardiopulmonary abnormalities (Hill et al., 1999).

Many other factors, such as size of nuclear donor cells, can also contribute to the failure or low efficiency of this technology. Wakayama et al. (1998) suggested that small mouse cumulus cells seemed to be a better source of karyoplasts for producing cloned mice. Tao et al. (1999) demonstrated that small fetal fibroblast cells (diameter <15 μm) support in vitro development better than the large

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cells (>20 μm) in nuclear reconstructed pig embryos. Kubota et al. (2000) reported a similar result in cattle and concluded that larger cells may be in G_2-phase of the cell cycle, which is not the favorable stage for proper DNA reprogramming. However, a smaller cell might undergo apoptosis, which presumably would not be a good candidate for a nuclear donor cell, either. More studies are required to elucidate these phenomena. Furthermore, there is no, or only a very short, G_1-phase of cell cycle in early embryos which usually leads to rapid cleavages without substantial growing (Hinegardner et al., 1964; Laskey et al., 1977; Newport and Kirschner, 1982; Collas et al., 1992a). Previous results showed that kits were derived by nuclear transplant embryos using 8-32-cell stage blastomeres from many labs (Shen et al., 1998; Yang et al., 1990a,b). However, no live young have been reported using differentiated or somatic cells from adult rabbits. More information is required for understanding the physiologic aspects of different nuclear donor cells before the efficiency of animal cloning can be further improved.

Although the nature of embryonic blastomere is different from differentiated somatic cells, the principle or mechanism of nuclear reprogramming after NT is somewhat similar. Therefore, the objectives of this study were to evaluate the effect of size and cell cycle stages of nuclear donor blastomeres on the developmental competence of cloned rabbit embryos.

MATERIALS AND METHODS

Animals, chemicals, and oocyte or embryo collection

Five- to 8-month-old sexually mature New Zealand White rabbits were used throughout this study. Does were superovulated by subcutaneous injection of FSH (0.17 mg/12 h, Sigma F-8001) for 3 consecutive days and intravenous injection of hCG (100 IU) following the last dose of FSH injection based on previous protocol (Carney and Foote, 1990; Foote and Simkin, 1993; Cheng et al., 1988). Oocytes were recovered either by surgery or flushing from the oviducts of animals sacrificed at 14-15 h post-hCG treatment. Two stages of donor cells were obtained by isolating the embryo recovered from 40-41 h (8-cell) and 50-51 h (16-cell) postcoitus, respectively. Ham's F-10-based medium supplemented with 0.4% BSA and 1% rabbit serum (RS) was used for flushing the oviducts and uterine horns. For determination of size of blastomeres, morphologically normal embryos were treated with 0.5% pronase (in 0.88% saline, Sigma P-6911) for 10-15 min, then washed, and pipetted with a small bore glass pipette (diameter: 50-100 μm) to remove zona pellucida as described by Cheng et al. (1989). Most chemicals or reagents were purchased from Sigma Chemical unless otherwise stated.

Classification of nuclear donor cells

Sizes of blastomeres were measured using an image analysis system (Qwin 500, Leica). Average blastomere diameter was calculated and the donor blastomeres were classified into small (<51 μm for 16-cell, <27 μm for 32-cell) and large cells (≥51 μm for 16-cell, ≥27 for 32-cell). Distribution of blastomere diameter is plotted in Figure 1.

Synchronization of cell cycle between donor cells and recipient oocytes

In vitro developmental competence of individual donor blastomeres was first compared between the G_1 and S-phase of the cell cycle stages of donor blastomeres. Three different regimes for cell cycle control were performed as following:

(1) G_1/-DEM (Treatment 1, without chemical treatment): Blastomeres from 8- or 16-cell stage embryos were isolated and in vitro cultured till first division. The divided daughter blastomeres were considered as 16- or 32-cell stage blastomere, respectively, and were immediately inserted to the perivitelline space of enucleated recipient oocytes. (2) G_1/+DEM (Treatment 2, Demicolcine treatment): Demicolcine (0.5% for 10 h, Sigma D-7385), a microtubule inhibitor, was used to control the cell cycle at the presumptive metaphase stage as previous described (Collas et al., 1992a). (3) S/+DEM+APH (Treatment 3, a combined treatment): Aphidicolin (APH, 0.1 μg/ml for 6 h, Sigma A-0781), a DNA polymerase inhibitor, was used to synchronize the cell cycle stage at the G_1/S boundary following DEM treatment. Similarly, all the treated blastomeres were inserted into recipient oocytes immediately after removal of inhibitors.

Oocyte activation and nuclear transfer

Metaphase II (MII) oocytes were enucleated at 16-18 h after ovulation followed by activation treatment based on previous protocol (Yang et al., 1990a; Shen et al., 1997). For activation, enucleated oocytes were equilibrated in mannitol solution for 5 min in a fusion chamber (P/N 450, BTX Inc.) then 6 consecutive electrical pulses (2.5 kv/cm, 60 μs; BTX ECM200, San Diego) were applied with 30 min intervals to activate oocytes. Blastomere insertion was performed 9-10 h after activation treatment and the reconstructed oocytes were subjected to 1-2 more electrical pulses for nuclear fusion (Ozil and Modlinski, 1986; Fissore and Robl, 1993; Shen et al., 1997). Enucleation efficiency of the oocyte was examined under an Olympus fluorescent microscope using Hoechst 33342 staining (10 μg/ml, 10-15 min).

In vitro culture and embryo transfer

Embryos, individual blastomeres, and reconstructed oocytes, were all cultured in Ham's F-10 supplemented with 1.5% BSA and 10%RS for various periods of time.

Developmental competence was evaluated either in vitro or in vivo. Rates of blastocyst formation were examined at the end of culture. Selected pronuclear or 2-4-cell stage transplants were transferred to the oviducts of pseudopregnant or bred recipients as described previously (Cheng et al., 1988; Ju et al., 1991, 2000).

Statistical analysis

Data were analyzed using Chi-square test in the Statistical Analysis System (SAS, 1989).

RESULTS

Experiment 1. *In vitro* development of single blastomeres derived from 16- and 32-cell stage embryos

To evaluate the developmental potential, different sizes of blastomeres were isolated from 16- and 32-cell embryos and cultured individually. This process can serve as a confirmation of the viability of the donor nuclei prior to transplantation. Although blastomeres derived from both 16- or 32-cell embryos were able to cleave further, large cells derived from both stage embryos showed significantly higher developmental rates ($p<0.05$) than the small cells (Tables 1 and 2). Distribution of blastomere diameters is also presented in Figure 1 (a and b). Mean diameters for the 16- and the 32-cell blastomeres are 51 and 27 μm, respectively.

Experiment 2. The effect of size of blastomeres on developmental competence of reconstructed embryos

Large and small blastomeres from either 16- or 32-cell embryos were transferred into the recipient oocytes. The reconstructed embryos were cultured in Ham's F-10 medium supplemented with BSA and 10% RS to evaluate the efficiency of manipulation. In the 16-cell blastomere transfer group, although a slightly higher fusion rate was obtained in the large blastomere transplant group (78 vs 56%, $p<0.05$), no significant difference was observed in activation rate and morula or blastocyst development regardless of the size of blastomere used (18 vs 18%, Table 3). However, cleavage (43 vs 6%, $p<0.05$) and morula or blastocyst rates were significantly higher (14 vs 0%, $p<0.05$) in the large blastomere group when 32-cell stage blastomeres were used as donor nuclei (Table 4).

Table 1. *In vitro* development of large and small blastomeres isolated from 16-cell stage rabbit embryos

Diameter of blastomeres	No. of embryos/does	No. of blastomeres	Cleavage rates (%)[2]
Large (59.71±1.1)[1]	21/4	95	51 (54)[a]
Small (43.5±1.1)	21/4	69	33 (48)[b]
Total (51.1±1.5)	21/4	164	84 (51)

[1] Mean±S.D.

[2] >3 divisions.

[a,b] Values in the same column without common superscripts differ ($p<0.05$).

Table 2. *In vitro* development of large and small blastomeres isolated from 32-cell stage rabbit embryos

Diameter of blastomeres	No. of embryos/does	No. of blastomere	Cleavage rates (%)[2]
Large (29.2±0.3)[1]	16/4	285	80 (28)[a]
Small (22.6±0.2)	16/4	163	23 (14)[b]
Total (26.9±0.2)	16/4	448	103 (23)

[1] Mean±S.D.

[2] >3 divisions.

[a,b] Values in the same column without common superscripts differ ($p<0.05$).

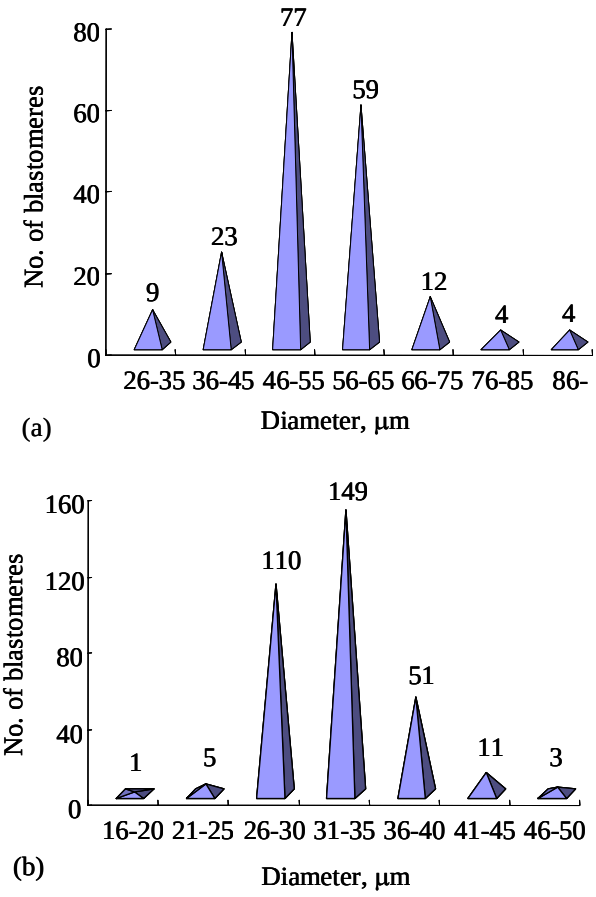

Figure 1. Distribution of the diameter of blastomeres derived from (a) 16-cell stage and (b) 32-cell stage rabbit embryos.

Experiment 3. The effect of cell cycle synchronization between donor nuclei and recipient oocytes

Viability of blastomeres from both 16- and 32-cell stages embryos after Demicolcine treatment was examined (Figures 2a and 2b). Cleavage rates after treatment with different concentrations of this chemical (0.1, 0.5, 1.0, and 2.0 μg/ml) were recorded at 0, 30, 60, and 90 min after removal. Of these, cleavage rate was highest in the 0.5 μg/ml Demicolcine treatment group (86%) for the 16-cell blastomere (Figure 2a), which was significantly higher

Table 3. The efficiency of nuclear transfer procedure using different size of donor cells isolated from 16-cell stage rabbit embryos

Donor cells[1]	No oocytes	No. NT-embryos	Fusion rates[2] (%)	Activation rates[3] (%)	Cleavage rates (%)	Morula/blastocyst (%)
Large	54	27	21 (78)[a]	11 (52)	6 (55)[a]	2 (18)
Small	67	32	18 (56)[b]	11 (61)	8 (73)[b]	2 (18)
Total	121	59	39 (66)	22 (56)	14 (64)	4 (18)

[1] Diameter of donor cells: large>27μm, small<27μm.
[2] Fusion parameters: 2.5kv/cm, 60μsec, 2 pulses.
[3] NT-embryos that cleaved were classified as activated oocytes.
[a,b] Values in the same column without common superscripts differ (p<0.05).

Table 4. The efficiency of nuclear transfer procedure using different size of donor cells isolated from 32-cell stage rabbit embryos

Donor cells[1]	No oocytes	No embryos produced	Fusion rates[2] (%)	Activation rates[3] (%)	Cleavage rates (%)	Morula/blastocyst (%)
Large	56	37	33 (89)	21 (64)	9 (43)a	3 (14)[a]
Small	61	33	26 (79)	16 (62)	1 (6)b	0 (0)[b]
Total	117	70	59 (84)	37 (63)	10 (27)	3 (8)

[1] Diameter of donor cells: large>27 μm, small<27 μm.
[2] Fusion parameters: 2.5 kv/cm, 60 μsec, 2 pulses.
[3] NT-embryos that cleaved were classified as activated oocytes.
[a,b] Values in the same column without common superscripts differ (p<0.05).

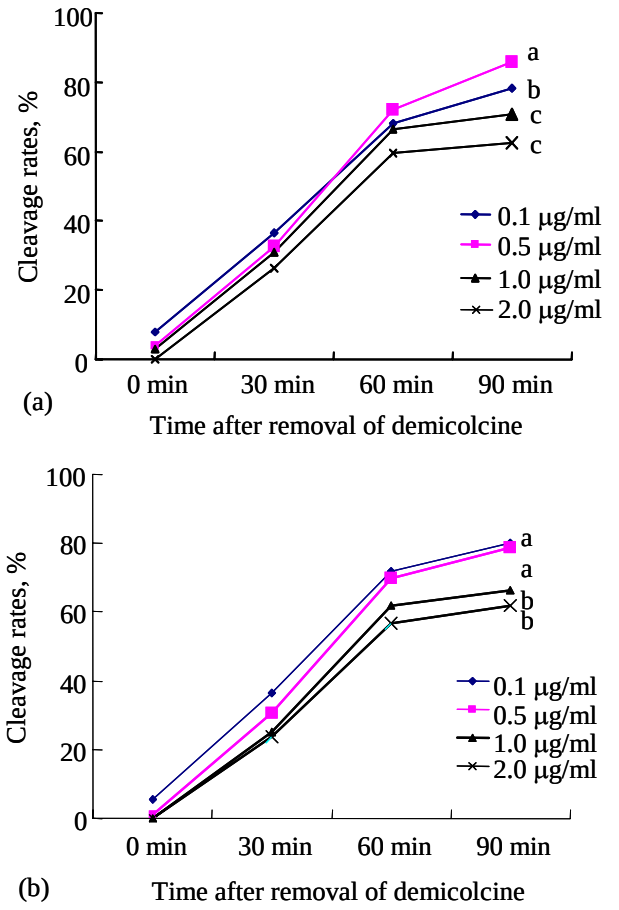

Figure 2. The effect of Demicolcine concentrations on the development of rabbit blastomeres isolated from (a) 16-cell stage and (b) 32-cell stage rabbit embryos. Different characters (a, b, c) for each line represent significant differences (p<0.05).

than other groups. Similarly, for the 32-cell blastomere, although no significant difference was found between 0.1 μg/ml (80%) and 0.5 μg/ml (79%), significantly lower cleavage rates were found in the 1.0 and 2.0 μg/ml groups at 90 min after removal of the chemicals (p<0.05; Figure 2b)

In vitro development under different cell cycle control regimes : No significant differences in rates of fusion, cleavage, and morula or blastocyst formation was observed among treatments within the same embryonic stage (p>0.05). However, the fusion and cleavage rates were significantly higher (p<0.05) in the 16-cell than the 32-cell blastomere transplant group (p<0.05) regardless of the cell cycle stage was in G_1 or S phases (Table 5). Similar low morula or blastocyst development was observed in both embryonic stages of donor blastomeres in this study.

In vivo development of reconstructed embryos : *In vivo* development of the reconstructed embryos was examined in the DEM-treated blastomeres (synchronized at G_1 phase) in both 16- and 32-cell transplants. Fusion rates of 16-cell and 32-cell blastomere transplants appeared to be slightly higher in the G_1/-DEM (83 and 88%) than the DEM-treated group (G_1/+DEM; 78 and 71%) for both the 16- and 32-cell groups.

Although our standard embryo transfer procedure using superovulated embryos had resulted in 70% pregnant rate (Ju et al., 2000), no pregnancy was obtained by transferring the presumptive G_1 blastomere (w/ or w/o DEM treatment) derived from either 16 or 32-cell stage embryos in this study (Table 6).

DISCUSSION

Distribution and development of single blastomeres

The size of blastomeres derived from 16- and 32-cell stage embryos were carefully measured in Experiment 1. Distribution of blastomere diameters for each group fit into

Table 5. *In vitro* development of nuclear transplant embryos derived from cell cycle controlled donor nuclei isolated from 16- and 32-cell stage rabbit embryos

Embryonic stage of donor cells	Treatment groups	No. of oocytes	No. of embryos produced	Fusion rates,[4] %	Cleavage rates5 (>3 divisions), %	Morula and blastocyst, %
16-cell	G_1/-Dem[1]	135	67	89.6 (60/67)[a]	94.9 (37)[a]	10.2 (4)
	G_1/+Dem[2]	56	30	80.0 (24/30)[a]	92.9 (13)[a]	7.1 (1)
	S/+Dem+Aph[3]	62	27	77.8 (21/27)[a]	72.7 (8)[b]	18.1 (2)
32-cell	G_1/-Dem	62	19	57.9 (11/19)[b]	66.7 (94)	16.7 (1)
	G_1/+Dem	68	46	54.3 (25/46)[b]	75.0 (9)	8.3 (1)
	S/+Dem+Aph	48	29	55.2 (16/29)[b]	77.7 (7)	11.1 (1)

[1] G_1/-Dem: Cell cycle stage was presumed at G_1 phase without incubation with Demicolcine.
[2] G_1/+Dem: Cell cycle stage was controlled at G_1 phase by incubation with Demicolcine for 10-12 h.
[3] S/+Dem+Aph: Cell cycle stage was controlled at S phase by incubation with Aphidicolin (Aph) for 6h following 0.5 μg/ml Demicolcine (Dem) for 10-12 h.
[4] Electrofusion parameters: 2.5 kv/cm pulse strength and 60 μs pulse duration for 1 (for 16-cell stage) or 2 (for 32-cell stage) pulses.
[5] Defined by Hoechst 33342 staining.
[a,b] Values between 16- and 32-cell stage or among different cell cycle stages without common superscripts differ ($p<0.05$).

Table 6. *In vivo* development of nuclear transplant rabbit embryos derived from donor blastomeres synchronized at G_1 phase of cell cycle.

Embryonic stages	Treatment	No. of oocytes	No. of embryos produced[1]	Fusion rate, %	No. of embryos transferred	No. of offspring[2,3]
16-cell	G_1/-Dem	42	29	82.8 (24/29)	24	0/12
	G_1/+Dem	50	37	78.4 (29/37)	27	0/0
32-cell	G_1/-Dem	27	16	87.5 (14/16)	14	0/7
	G_1/+Dem	30	17	70.6 (12/17)	11	0/0

[1] NT-embryos were produced by microinjection of donor blastomeres (black Rex) into the enucleated and activated oocytes (New Zealand White).
[2] Recipients were bred by the buck of the same species before embryo transferred.
[3] Control group: standard embryo transfer procedure resulted in 7 out of 10 pregnancy (70%) and 41 kits were born (data not shown).

a normal curve with a mean diameter of 51.1±1.5 μm for 16-cell and 26.9±0.2 μm for 32-cell stage blastomeres (Figure 1a and 1b). These information were useful for preparing the blastomere insertion pipette for rabbit nuclear transfer.

In vitro developmental potential of individual blastomeres was also examined in which a significantly higher cleavage rate (>3 divisions) was observed from large cells of both 16-cell (51 vs 33%, $p<0.05$, Table 1) and 32-cell blastomeres (28 vs. 14, $p<0.05$, Table 2). The different cleavage rates may be a reflection of the cell cycle or viability of individual blastomeres when used as nuclear donor cells. A larger cell indicates that the cell may be in S/G_2 stage in other cell types, but no direct evidence can be applied to embryonic cells.

Effects of stages and sizes of donor blastomeres

Prather et al. (1999) and Tao et al. (2000) reported that 70% of pig mammary cells and fibroblasts of 15 μm in diameter were found being in G_0/G_1 phase, whereas cells with diameters of 20 μm were more likely to be in the S-phase. The effect of blastomere diameters on activation rates and morula/blastocyst development of the transplants derived from 16- or 32-cell stage blastomeres, respectively, were shown in Table 3 and 4. Large cells seemed to exhibit slightly better fusion rates than small cells which is, possibly, due to a larger contact surface between the donor cell and recipient membrane (Zimmermann and Vienken, 1982; Smith and Wilmut, 1998; Collas and Robl, 1991; Stice and Keefer, 1993). This view was further supported by the data in Table 5 where 16-cell blastomeres had much higher ($p<0.05$) fusion and cleavage rates than those in 32-cell stage. It is also possible that the differences in ultrastructures of these partially differentiated blastomeres contribute to the different fusion rates, efficiency of the blastomere transplantation, and hence the development of the transplant embryos. Polarization of blastomeres evaluated by the distribution of microvilli on the vitelline membrane does not occur until the 32-cell stage in rabbit embryos (Koyama et al., 1994). When the embryo becomes compacted, polarized microvilli are found on the outer surface of the blastomere located in the outer layer of the embryos. None or shorter microvillus protrusions are observed in the contact surface of the blastomere in which adhesion molecules, such as uvomorulin or E cadherin, may be expressed locally at this stage (Fleming et al., 1986; Levy et al., 1986; Softon et al., 1992; Clyton et al., 1999). Similarly, membrane morphology, such as rough or smooth, of the donor cells can also affect development of the NT-embryo. Smooth donor cells contribute to better development than rough surface cells after transplantation (Tao et al., 1999). More studies on the blastomere

characteristics are required to interpret their differential contribution of fusion rates and developmental competence.

Similar morula or blastocyst rates were observed in the 16-cell stage donor group. However, no morula or blastocyst development was observed in the small nuclear donor group in the 32-cell stage blastomeres indicating large donor cells might be a better nuclear source for embryo cloning in this stage. Besides, a relatively small cell may be a sign of apoptosis. On the contrary, Prather et al. (1999) reported that small fibroblasts (15 μm) supported *in vitro* development better than large cells (20 μm). One of the major differences between somatic cell cycles and blastomeres is a short growth phase (G-phase), or none at all in the early embryonic blastomeres. (Ito et al., 1981; Collas et al., 1992a). This difference would explain at least part of the controversial result in this study.

The effect of cell cycle synchronization of donor blastomeres

Another factor influencing transplantation efficiency is the cell cycle of donor nucleus (Campbell, 1999; Campbell et al., 1996). We used a colcemid or colchicine analog, demicolcine, to synchronize the cell cycle stage at metaphase. Effects of different levels, ranging from 0.1 to 2.0 μg/ml, on blastomere viability were first examined using cleavage rate after removal of the chemical as a criterion. Concentrations with better blastomere cleavage rates after removal of the inhibitor were found between 0.1 and 0.5 μg/ml for both 16- and 32-cell blastomeres (Figures 2a and 2b), and the latter concentration was chosen for the treatment in this study.

Two different models of nuclear transfer protocol are currently proposed based on synchronization of the cell cycle stage (Campbell et al., 1996; 1999; Stice et al., 2000). One is the G-phase (G_0 or G_1) recipient theory in which a G_0- or G_1-phase donor nucleus is required the metaphase II (MII) recipient oocyte. The MII oocyte is characterized by a high concentration of maturation promoting factor (MPF), which can cause premature chromatin condensation (PCC) and breakdown of the nuclear envelope (NEBD). When a G-phase nucleus is transferred, development of the reconstructed embryo is normal without any appearance of DNA damage (Campbell, 1999). However, when an S-phase nucleus is transferred, the active DNA replication would result in pulverized chromosomes and extensive DNA damage would occur, in turn, result in abnormal or retarded development of the transplant. The other model is use of preactivated oocytes as universal recipients by which more broad spectrum of donor cells can be transferred regardless of their cell cycle stages (Campbell et al., 1996). In this study, we tested this hypothesis using donor nuclei with two presumptive cell cycle stages (G_1 and S) derived from both 16- and 32-cell stage blastomeres (Table 5). Controversially, cleavage rate (>3 divisions) following the G_1 donor cell transplant was significantly higher than in the S phases donor group ($p<0.05$) when from a 16-cell stage blastomere donor, however, this was not found in the 32-cell donor group. Although no significant difference was found in morula or blastocyst rate ($p>0.05$), it seemed slightly higher in the G1/-DEM or S/+DEM+APH nuclear donor group for both 16- and 32-cell blastomeres suggesting unnecessary of controlling the cell cycle stage of donor cells when universal recipients were used.

CONCLUSION

The size of nuclear donor blastomeres or cells may contribute to efficiency of embryo cloning not only because of their physiologic status, but also their morphologic appearance, such as polarization and structure of cell membrane, which may affect the fusion rate, and in turn, the developmental potential. In this study we demonstrated the effect of size in 16-cell and 32-cell blastomeres as donor cells on the development of cloned rabbit embryos. Cell cycle synchronization, however, may not be beneficial in terms of morula or blastocyst development of reconstructed rabbit embryos under this protocol.

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