—Original Article—

Improvement of the Developmental Ability of Nuclear Transfer Embryos by Using Blastomeres from *In Vitro* Fertilized Embryos Selected According to the Early Developmental Stage and Cell Division Status as Donor Cells in Cattle

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Abstract. This study was conducted to improve the developmental ability of nuclear transfer (NT) embryos by using blastomeres from *in vitro* fertilized (IVF) embryos with high quality as donor cells. The IVF embryos selected at the 2-cell stage at 24-h postinsemination (hpi) and again at the ≥ 8 -cell stage at 48 hpi (Selected-IVF-embryos) showed the highest blastocyst formation rate among embryos. When blastomeres from the Selected-IVF-embryos (Selected-NT group) or Nonselected-IVF-embryos (Non-selected-NT group) were used as donor cells for NT, the blastocyst formation rate in the Selected-NT group (25.6%) was significantly higher than that in the Non-selected-NT group (13.5%). When blastomeres from the Selected-IVF-embryos at 108 (contained many cells before cell division) and 126 hpi (contained many cells immediately after cell division) were used as donor cells for NT (108- and 126-NT groups, respectively), the 126-NT group showed a significantly higher blastocyst formation rate (32.1%) than the 108-NT group (16.8%). Embryo transfer of blastocysts in the 126-NT group showed that 11 of 23 recipients became pregnant; nine calves were obtained. For the NT embryos reconstructed using *in vivo* derived embryos, 9 of 20 recipients became pregnant; seven calves were obtained. These results indicate that the blastocyst formation rate of NT embryos can be improved by using blastomeres from IVF embryos selected at the early developmental stage, especially immediately after cell division, and that the resultant NT embryos have a high developmental ability to progress to term that is comparable to NT embryos reconstructed using *in vivo* derived embryos.

Key words: Bovine, Embryo, *In vitro* fertilization, Nuclear transfer

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Nuclear transfer (NT) technologies are now widely used by numerous laboratories to produce clone animals from embryonic and somatic cells. However, the overall efficiency of clone animal production is influenced by the different sources of donor cells; early pregnancy losses of cattle established from somatic nuclear transfer (sNT) are commonly above 50% [1, 2], and the frequency of early fetal mortality is about two times higher with sNT than with embryonic nuclear transfer [3]. Therefore, it can be expected that a higher proportion of transferred NT embryos reconstructed from less-differentiated embryonic blastomeres would result in viable offspring compared with sNT. In addition, the production of embryonic cloned calves is permitted in Japan and food products derived from NT cattle can be on the market, thereby increasing commercial acceptance of the technology. Furthermore, the use of blastomeres from the same embryos for nuclear transfer to produce numerous identical bovine offspring is of great value for multiplication of genotypes having a superior economic value and is expected to contribute to their genetic improvement.

In NT technology using embryos at an early developmental stage as donor cells, *in vitro* fertilized (IVF) embryos are often used

because they can be easily produced *in vitro*. However, using IVF embryos as nuclear donor cells for NT has been reported to result in a poor development of the resultant NT embryos up to the blastocyst stage compared with that of NT embryos obtained using *in vivo* derived donor embryos [4, 5]. In addition, the productive efficiency of NT embryos derived from blastomeres of different IVF embryos at the same developmental stage varies considerably [6], indicating that each blastomere of different IVF embryos may have a different developmental ability; this may reflect the fact that the developmental rate of IVF embryos to the blastocyst stage is about 40% and not 100% due to malfunction of many blastomeres resulting in the death of embryos during *in vitro* development [7]. Therefore, the selection of blastomeres with high quality as donor cells will probably influence the development of the resultant NT embryos.

It has been reported that the cell cycle of the donor blastomeres at NT influences the developmental ability of NT embryos [8–12]. Moreover, the development of NT embryos is improved by transplanting the embryonic cells immediately after cell division to the activated recipient oocytes [13, 14]. Thus, the cell cycle and cell division status of blastomeres as donor cells for NT may have effects on the development of the resultant NT embryos. For selection of blastomeres having a high developmental ability, it is important to select IVF embryos with a high developmental rate to the blastocyst stage.

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This study was conducted to establish a reliable selection method for IVF embryos that have a high developmental rate to the blastocyst stage and are suitable as a source of appropriate donor blastomeres for NT and to examine whether transplanting blastomeres from the selected IVF embryos improves the productive efficiency of the resultant NT embryos.

Materials and Methods

In vitro maturation (IVM) and *in vitro* fertilization (IVF) of oocytes

Ovaries of Japanese Black cows were collected from an abattoir and transported to the laboratory in sterile saline solution (0.9% NaCl) at 25 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2–6 mm in diameter and put into Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen, Carlsbad, CA, USA) supplemented with 3% calf serum (CS; Invitrogen). The COCs were washed in the *in vitro* maturation (IVM) medium and then placed into 100- μ l drops of IVM medium under mineral oil (Nacalai Tesque, Kyoto, Japan). The IVM medium was TCM199 (Invitrogen) supplemented with 5% CS, 100 IU/ml penicillin G (Meiji Seika, Tokyo, Japan) and 100 μ g/ml streptomycin (Meiji Seika). The COCs were cultured in an incubator at 38.5 °C in an atmosphere of 5% CO₂ in air. Each drop of the IVM medium contained approximately 20 COCs. After 20–22 h of culture, COCs were transferred into BO medium [15] containing frozen-thawed spermatozoa from one bull that had been treated for capacitation with caffeine (10 mM; Sigma-Aldrich, St. Louis, MO, USA) and heparin (novo-heparin 1000 U/ml) (Mochida Pharmaceutical, Tokyo, Japan) using the methods described by Niwa and Ohgoda [16]. For the experiments described below, the moment of addition of the oocytes into a suspension medium containing spermatozoa was defined as the time of insemination.

Selection of IVF embryos

Following 5 h of insemination, the presumptive zygotes were cultured in 100- μ l drops of *in vitro* culture (IVC) medium, which was composed of CR1aa medium [17] supplemented with 5% CS, under mineral oil in a humidified atmosphere containing 5% CO₂ in air at 38.5 °C. At 24-h postinsemination (hpi), the presumptive zygotes were denuded of cumulus cells by gentle pipetting. The embryos were classified based on their cell number into three groups, the 1-cell, 2-cell and more than 3-cell groups. Each group of classified embryos was transferred into 100- μ l drops of IVC medium (10 to 20 embryos/drop) and cultured separately until their subsequent classification. Each group of embryos was then classified by their cell number again at 48 hpi into two groups, the 2 to 7-cell (<8-cell) and 8-cell or more ($\geq$ 8-cell) groups. Six groups of classified embryos were cultured as a separate group in an identical IVC medium for 8 days after insemination.

*Source of *in vivo* derived embryos*

In vivo embryos were obtained from superovulated Japanese Black cattle (4.6 $\pm$ 1.1 yr olds), and their blastomeres were used as donor cells for NT. Donor cows were superovulated with a decreasing (5, 5; 4, 4; 3, 3 mg) 3-day regimen of follicle-stimulating

hormone (FSH-P; Kawasaki Pharmaceutical, Kanagawa, Japan), and 500 μ g cloprostenol (Estrumate; Schering-Plough Animal Health, Tokyo, Japan) was administered at 48 h after the first FSH-P injection. All donor cows were observed in estrus at 48 h after the cloprostenol treatment and then artificially inseminated (AI) within 12 h with frozen semen. Nonsurgical recovery of the embryos was performed on Day 5 (Day 0 was defined as the day of AI) to collect morula-stage embryos having 16 to 32 blastomeres. These *in vivo* derived embryos were recovered a few hours before nuclear transfer (NT) and kept in DPBS supplemented with 20% CS at 38.5 °C until use.

Method of NT

For recipient oocytes, COCs collected as described above were matured *in vitro* for 20–22 h. The IVM COCs were treated in M2 medium [18] supplemented with 0.5% hyaluronidase (Sigma-Aldrich) for 5 min. Then the cumulus cells were removed from COCs using gentle pipetting. The cumulus-free oocytes were placed in micromanipulation drops of DPBS supplemented with 20% CS and 7.5 μ g/ml cytochalasin B (Sigma-Aldrich) and then enucleated by pushing out a small amount of cytoplasm directly beneath the first polar body. The enucleated oocytes were activated by combined treatments with Ca-ionophore (5 μ M; Sigma-Aldrich) for 5 min, a DC pulse of 0.75 kV/cm for 50 μ sec (LF100, Life Tec, Tokyo, Japan) and cycloheximide (10 μ g/ml; Sigma-Aldrich) for 6 h using the methods reported by Aoyagi *et al.* [19]. For preparation of blastomeres as donor cells, the zonae pellucidae of IVF- and *in vivo* derived embryos were removed by cutting with a glass needle. Blastomeres were isolated by pipetting the zona-free embryos in Ca²⁺- and Mg²⁺-free DPBS. A blastomere was aspirated with an injection pipette and transferred to the perivitelline space of the activated oocytes. The blastomere-oocyte complexes were transferred to Zimmerman cell fusion medium [20] and then placed between the two electrodes of the fusion chamber. Membrane fusion between the enucleated recipient oocyte and blastomere was carried out by exposure to a DC pulse of 0.75 kV/cm for 50 μ sec. One hour after the electric pulse, the fusion rates of the blastomere and recipient oocyte were evaluated using a stereomicroscope (SMZ, Nikon, Tokyo, Japan).

IVC of IVF and NT embryos and their evaluation

All the IVF and NT embryos were cultured in CR1aa medium supplemented with 5% CS for 8 days in a humidified atmosphere containing 5% CO₂ in air at 38.5 °C. The cleavage rates of the NT embryos were assessed at 48 h after fusion, and the developmental rates to the blastocyst stage of IVF and NT embryos after 8 days of IVC were assessed under a stereomicroscope. For counting of cell numbers, blastocysts derived from the IVF and NT embryos were stained with DPBS containing 50 μ g/ml Hoechst 33342 (Sigma-Aldrich). The total cell numbers of the embryos were counted individually under an inverted microscope (Eclipse TE 300, Nikon, Tokyo, Japan) equipped with an epifluorescence (TE-FM, Nikon) and ultraviolet filter block (UV-1A, Nikon).

Embryo transfer

Recipient cows were synchronized by administration of 500 μ g

cloprostenol about 3 days before the onset of desired estrus. Some reconstituted embryos that developed to the blastocyst stage at 7 or 8 day after NT were transferred nonsurgically to recipient cows at 6–8 days after estrus. One or two NT embryos per recipient were nonsurgically introduced into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum. Pregnancies were determined by ultrasonography (SSD-500, Aloka, Tokyo, Japan) 30 and 60 days after embryo transfer.

Experimental design

Experiment 1: The effect of morphological selection (the number of blastomeres) of IVF embryos according to the early developmental stages on their subsequent development to the blastocyst stage was investigated. The IVF embryos were selected according to the number of blastomeres assessed twice at 24 and 48 hpi. Details were explained in the Selection of IVF embryos section above. Six groups of embryos that had been cultured separately for 8 days postinsemination were investigated for their blastocyst formation rates and total cell numbers. The group of IVF embryos showing the highest developmental rate to the blastocyst stage was defined as the Selected-IVF-embryos and used in Experiment 2.

Experiment 2: The effect of using the Selected-IVF-embryos, which were selected as 2-cell stage embryos at 24 hpi, subsequently selected again as ≥ 8 -cell stage embryos at 48 hpi according to the same selection method used in Experiment 1 and then cultured for an additional 3 days (5 days in total), as a source of donor blastomeres on the development of the resultant NT embryos (Selected-NT group) was investigated; that is, the fusion rates of the donor and recipient cells and the cleavage and blastocyst formation rates of the NT embryos were investigated. The results were compared with those of NT embryos obtained using IVF embryos (16–32-cell stage) chosen at random after 5 days of IVC without selection as a source of donor blastomeres for NT (Non-selected-NT group).

Experiment 3: The precise timing of cell division for the Selected-IVF-embryos was investigated. The cell numbers of the Selected-IVF-embryos were evaluated every 6 h from 102–138 hpi during IVC using a fluorescent dye to monitor the cell division status of the embryos. The cell division profile of the IVF embryos at a certain time from 102–138 hpi during IVC was expressed as the ratio of the divided cells; an increased number of cells during each 6-h period of IVC out of the number of cells at the beginning of the period.

Experiment 4: The influence of the cell division stage of donor cells and the timing of NT on the developmental ability of the resultant NT embryos was investigated. When the Selected-IVF-embryos had a high ratio of cell division (containing many cells immediately after cell division), they were assumed to be at the stage “after cell division”. When the embryos had a low ratio of cell division (containing many cells before cell division), they were assumed to be at the stage “before cell division”. Based on the results of Experiment 3, the embryos at 108 hpi and 126 hpi (108 hpi and 126 hpi groups, respectively) were used as donor embryos at the stage “before cell division” and at the stage “after cell division”, respectively, for NT, and rates for the fusion of donor and

recipient cells and cleavage and blastocyst formation were compared between the NT embryos obtained from the Selected-IVF-embryos at the two different stages.

Experiment 5: The influence of the source of donor embryos, IVF or *in vivo* derived embryos, on the developmental ability of the resultant NT embryos was investigated. Based on the results of experiment 4, the Selected-IVF-embryos at 126 hpi (126-NT group) were used as a source of donor blastomeres for NT because they were proven to enhance the developmental ability of the resultant NT embryos to the blastocyst stage. *In vivo* derived embryos (16-cell to 32-cell stages) were used as a source of donor blastomeres for NT (*In vivo*-NT group). Some reconstituted NT embryos that developed to the blastocyst stage at day 7 or 8 day after NT in both groups were transferred to synchronized recipient cows. The NT embryos not used for embryo transfer were stained with fluorescent Hoechst 33342, and then the total cell numbers were counted individually under an inverted microscope equipped with epifluorescence.

Statistical analyses

Differences in fusion rates of donor and recipient cells, cleavage and blastocyst formation rates of embryos and pregnancy and calving rates among experimental groups were analyzed using chi-squared analysis. The average numbers of cells per blastocyst were analyzed using one-way ANOVA. Individual comparisons between means were made using the Student's *t*-test. A value of $P < 0.05$ was considered to be significant.

Results

Experiment 1

A total of 382 embryos were used for selection of IVF embryos according to the number of blastomeres. The number (percentage of the total number) of IVF embryos that had been selected as 1-cell, 2-cell and more than 3-cell stage embryos at 24 hpi were 63 (16.5%), 211 (55.2%) and 108 (28.3%), respectively. The developmental rate to the blastocyst stage of the selected IVF embryos is shown in Table 1. The developmental rate to the blastocyst stage (84.3%) of embryos that had been selected as 2-cell stage embryos at 24 hpi, and then selected again as ≥ 8 -cell stage embryos at 48 hpi (the Selected-IVF group) was significantly higher than those of the other groups after selection (0–39.6%) and that of the Non-selected-IVF group (44.2%) calculated based on the total number of embryos produced without selection ($P < 0.05$). These blastocysts had a significantly higher number of cells (108.6) than those of the embryos from the other groups (42.3–67.3; $P < 0.05$).

Experiment 2

Table 2 shows the fusion rates of donor and recipient cells and the cleavage and blastocyst formation rates of NT embryos from the Non-selected-NT and Selected-NT groups. No differences were observed in the fusion or cleavage rate between the two NT groups. However, the blastocyst formation rate of the NT embryos from the Selected-NT group (25.6%) was significantly higher than that of the Non-selected-NT group (13.5%; $P < 0.05$).

Table 1. Effects of selection of *in vitro* fertilized (IVF) embryos according to the number of blastomeres at 24 and 48 h postinsemination on subsequent *in vitro* development to the blastocyst stage* in cattle

Selection of embryos according to No. of blastomeres**		No. of replicates	No. of embryos	No. of blastocysts (%)	Mean no. of cells per blastocyst (mean $\pm$ SD)
24 h	48 h				
≥ 3	≥ 8	4	71	25 (35.2) ^a	57.4 $\pm$ 20.7 ^a
≥ 3	<8	4	37	9 (24.3) ^a	42.3 $\pm$ 7.1 ^a
2	≥ 8	4	115	97 (84.3) ^b	108.6 $\pm$ 33.3 ^b
2	<8	4	96	38 (39.6) ^a	67.3 $\pm$ 26.9 ^a
1	≥ 8	4	33	0 (0) ^c	—
1	<8	4	30	0 (0) ^c	—
Total		4	382	169 (44.2) ^a	88.2 $\pm$ 28.6 ^a
Non-selected-IVF-embryos					

* All the IVF embryos were cultured for 8 days in total. ** The number of blastomeres in IVF embryos observed at 24 and 48 h postinsemination. ^{a,b,c} Values within the same columns with different superscripts significantly differ ($P < 0.05$).

Table 2. Effects of selection of *in vitro* fertilized embryos as donor embryos for nuclear transfer (NT) on subsequent *in vitro* development of NT embryos to the blastocyst stage in cattle

Source of donor embryos	No. of replicates	No. of recipient oocytes	No. of fused oocytes (%)	No. of cleaved embryos ⁺ (%)	No. of blastocysts ⁺⁺ (%)
Selected-IVF *	4	150	125 (83.3)	105 (84.0)	32 (25.6) ^a
Non-selected-IVF**	4	130	104 (80.0)	87 (83.7)	14 (13.5) ^b

* Selected-IVF: *In vitro* fertilized bovine embryos, which had been selected as 2-cell stage embryos at 24 h post insemination (hpi) and subsequently selected again as ≥ 8 -cell stage embryos at 48 hpi, were cultured for additional 3 days (5 days in total). ** Non-selected-IVF: *In vitro* fertilized bovine embryos (16–32-cell stage) chosen at random after 5 days of *in vitro* culture without selection. ⁺ The cleavage rates of NT embryos were assessed at 48 h after fusion. ⁺⁺ The developmental rates to the blastocyst stage of NT embryos were assessed after 8 days of *in vitro* culture. ^{a,b} Values with different superscripts within the same column significantly differ ($P < 0.05$).

Experiment 3

The total cell numbers of the Selected-IVF-embryos, evaluated every 6 h from 102–138 hpi (36 h) during IVC, are shown in Fig. 1. The average cell number increased from 17.9 to 51.3 cells during the 36 h. The ratios of divided cells were 8.7–28.9% during each 6-h period of IVC. A high ratio of cell division was observed from 120–126 hpi during IVC (28.9%). In contrast, a low ratio of cell division was observed from 102–108 hpi during IVC (8.7%). Thus, it can be suggested that the embryos at 108 hpi (108 hpi group) contain many cells before cell division. In contrast, the embryos at 126 hpi (126 hpi group) contain many cells that divided within 6 h before. Thus, they should contain many cells just after cell division.

Experiment 4

The developmental abilities of the NT embryos produced by using different sources of donor cells at two different cell division stages, the before cell division (108 hpi group) and after cell division (126 hpi group) stages, are shown in Table 3. No differences were observed for the fusion rate of donor and recipient cells and the cleavage rate after NT, but the blastocyst formation rate for the 126 hpi group (32.1%) was significantly higher than that for the 108 hpi group (16.8%, $P < 0.05$).

Fig. 1. Number of cells in *in vitro* fertilized embryos and their rate of cell division according to postinsemination time. The cell number of the Selected-IVF-embryos was evaluated every 6 h from 102–138 hpi during *in vitro* culture (IVC) to evaluate the cell division status of the embryos. The cell division profile of IVF embryos at a certain time from 102–138 hpi during IVC was expressed as the ratio of the divided cells, an increased number of cells during each 6-h period of IVC out of the number of cells at the beginning of the period.

Table 3. Effects of the different cell division stages of the Selected-IVF-embryos* as donor embryos on the developmental ability of the nuclear transfer (NT) embryos in cattle

Source of donor embryos: Selected-IVF-embryos cultured for	No. of replicates	No. of oocytes	No. of fused oocytes (%)	No. of cleaved embryos ⁺ (%)	No. of blastocysts ⁺⁺ (%)
108 h**	6	178	155 (87.1)	123 (79.4)	26 (16.8) ^a
126 h***	6	196	168 (85.7)	136 (81.0)	54 (32.1) ^b

* Selected-IVF-embryos: *In vitro* fertilized bovine embryos, which had been selected as 2-cell stage embryo at 24 h post insemination (hpi) and subsequently selected again as ≥8-cell stage embryos at 48 hpi, were cultured for 108 or 126 h in total. ** The Selected-IVF-embryos at 108 hpi having many cells before cell division. *** The Selected-IVF-embryos at 126 hpi having many cells after cell division. ⁺ The cleavage rates of NT embryos were assessed at 48 h after fusion. ⁺⁺ The developmental rates to the blastocyst stage of NT embryos were assessed after 8 days of *in vitro* culture. ^{a,b} Values with different superscripts within the same column significantly differ (P<0.05).

Table 4. Comparison of developmental ability to the blastocyst stage and to progress to term after embryo transfer of nuclear transfer (NT) embryos produced by using the Selected-IVF-embryos and *in vivo* produced embryos as donor embryos

Source of donor embryos	No. of replicates	No. of recipient oocytes	No. of fused oocytes (%)	No. of cleaved ⁺ embryos (%)	No. of blastocysts ⁺⁺ (%)	Mean no. of cells per blastocyst (mean ± SD)	No. of pregnancies/ No. of transfers (%)	No. of calves/ No. of transfers (%)
Selected-IVF-embryos*	5	118	103 (87.3)	84 (81.6)	42 (40.8)	86.5 ± 16.0	11/23 (47.8)	9/23 (39.1)
<i>In vivo</i> produced embryos**	5	130	106 (81.5)	89 (84.0)	38 (35.8)	83.1 ± 17.3	9/20 (45.0)	7/20 (35.0)

*Selected-IVF-embryos: *In vitro* fertilized bovine embryos, which had been selected as 2-cell stage embryo at 24 h post insemination (hpi) and subsequently selected again as ≥8-cell stage embryos at 48 hpi, were cultured for 126 h in total. ***In vivo* produced embryos: 16-cell to 32-cell stage embryos were collected using a nonsurgical flushing from superovulated cows 5 days after artificial insemination. ⁺ The cleavage rates of NT embryos were assessed at 48 h after fusion. ⁺⁺ The developmental rates to the blastocyst stage of NT embryos were assessed after 8 days of *in vitro* culture.

Experiment 5

The developmental abilities of the NT embryos produced by using the Selected-IVF-embryos at 126 h of IVC or the *in vivo* derived embryos as a source of donor blastomeres for NT are shown in Table 4. No difference was observed in the fusion rate of donor and recipient cells and the cleavage or blastocyst formation rate between the NT embryos derived from both sources of embryos. Also, there was no difference in the average cell number between the blastocysts. NT blastocysts obtained from the Selected IVF-embryos at 126 hpi (126 hpi group) were transferred to 23 recipients (1 blastocyst was transferred to 18 cows, and 2 blastocysts were transferred to 5 cows), and 11 of them became pregnant. Nine recipients carried to full term, but two recipients aborted. NT embryos derived from *in vivo* derived embryos were transferred to 20 recipients (1 blastocyst was transferred to 16 cows, and 2 blastocysts were transferred to 4 cows), and 9 of them became pregnant. Seven recipients carried to full term, but two recipients aborted. Five males and four females were obtained in the 126 hpi-NT group, and four males and three females were obtained in the *In vivo*-NT group. A total of 16 calves were obtained in the 2 groups, and all of them were morphologically normal. The mean birth weights of the nuclear transfer calves of the 126 hpi-NT and *In vivo*-NT groups were 37.9 ± 9.2 kg and 35.7 ± 7.0 kg respectively.

Discussion

Our results demonstrate that the blastocyst formation rate of NT embryos can be improved by using blastomeres from IVF embryos

selected at the early developmental stage and that the resultant NT embryos have a high developmental potential to go to term that is comparable to NT embryos reconstructed using *in vivo* derived embryos. The reason for this improvement must have been the selection of IVF embryos used as donor embryos. To our knowledge, this is the first to report showing that the same pregnancy rates can be obtained after ET of NT embryos produced by using IVF and *in vivo* derived embryos as the source of donor blastomeres.

Many reports have described that the timing of first cleavage after IVF has a major effect on subsequent *in vitro* development of embryos [21–26]. In particular, the developmental rate to the blastocysts stage was significantly higher for embryos that cleaved early after 24–30 h of insemination than those of embryos that cleaved slowly [21–23]. Besides, it has also been reported that the blastocyst formation rates of IVF embryos were different according to the developmental stage after 60 h of insemination; the time at which the blastocyst formation rate was checked was later than that reported above [27]. Thus, we compared the blastocyst formation rates of IVF embryos selected according to the number of blastomeres assessed at 24 h (around the first cleavage) and 48 h (around the third cleavage), and then the embryos were cultured *in vitro* for 8 days in total. It was found that the developmental rate to the blastocyst stage of embryos that had been selected as 2-cell stage embryos at 24 hpi and then selected again as ≥8-cell stage embryos at 48 hpi (the Selected-IVF group) was significantly higher than those of the other groups. Although it was previously reported that the blastocyst formation rates of embryos selected as

2-cell stage embryos at 24 and 30 hpi were 52% [25] and 65.7% [24], respectively, and higher than those of other embryos, only one selection was used in those reports, and the rates seem to be lower than those obtained in the present study. Furthermore, when the blastocyst formation rates were recalculated according to selection only at 24 hpi in the present study, the rate of IVF embryos selected as 2-cell stage embryo that developed to the blastocyst stage was 64.0% and significantly lower than that of the IVF embryos selected twice at 24 and 48 hpi. Thus, it can be concluded that the developmental rate to the blastocyst stage and the quality of the IVF embryos were effectively improved by the two selections of the IVF embryos according to the early developmental stage.

In the present study, a significant decrease of the blastocyst formation rate was observed for the IVF embryos having more than 3 blastomeres at 24 hpi irrespective of number of blastomeres at 48 hpi compared with the Selected-IVF group. Also, IVF embryos having 2 blastomeres at 24 hpi and less than 8 blastomeres at 48 hpi showed a significantly lower blastocyst formation rate compared with the Selected-IVF group. Thus, 2 consecutive selections of IVF embryos would be important to make sure that the selected embryos have the proper timing of cleavages that will result in high developmental ability and the desired quality for NT. In contrast, Ulloa *et al.* [28] reported that the blastocyst formation rates of >8-cell stage embryos at 48 hpi were lower than those of 5-cell to 8-cell stage bovine embryos and that the incidence of chromosomal abnormalities of >8-cell stage embryos at 48 hpi was significantly higher than those of 5-cell to 8-cell stage embryos. In our study, IVF embryos having more than 8 blastomeres at 48 hpi were hardly observed in the embryos selected as 2-cell stage embryos at 24 hpi, but they were observed in the embryos selected as more than 3-cell stage embryos at 24 hpi. Thus, it seemed that IVF embryos having more than 3 blastomeres at 24 hpi corresponded to the embryos having more than 8 blastomeres at 48 hpi in the report by Ulloa *et al.* [28]. As for the IVF embryos with an especially high cleavage speed postinsemination, the developmental rate to the blastocyst stage seemed to be low.

Zakhartchenko *et al.* [6] reported that the developmental rate to the blastocyst stage of NT embryos was different according to the size of blastomeres used as donor cells. Although the blastomeres they obtained were from the same stage embryos, the NT embryos derived from small blastomeres showed a significantly higher blastocyst formation rate than the NT embryos derived from larger blastomeres. They suggested that small blastomeres were superior as donor cells for NT because they have greater developmental ability than larger blastomeres. Although the sizes of the blastomeres were not measured in the present study, it can be also suggested that the developmental potential of NT embryos was improved by using the Selected-IVF-embryos as donor embryos because they were estimated to contain a lot of blastomeres with a greater developmental ability.

Smith *et al.* (1988) reported that cell cycle synchronous transplantation between the donor nucleus and cytoplasm is important for the development of reconstituted mouse NT embryos [29]. Furthermore, it was reported that the cell cycle stage of nuclear donor cells at the time of NT influenced the development of reconstituted NT embryos in cows [9–11], rabbits [8] and mice [12]. On the other

hand, the use of enucleated and activated oocytes as recipient cytoplasts resulted in an increased frequency of development to the blastocyst stage of embryos reconstituted from unsynchronized donor nuclei from totipotent cell types [19, 30–32]. Furthermore, when the synchronized donor cells were transplanted into activated oocytes, the cell stage of the donor blastomeres immediately after division improved the blastocyst formation rate of the NT embryos [12, 13]. Thus, in the present study, we used enucleated and activated IVM oocytes as recipient cytoplasts and estimated the suitable IVF embryos as donor embryos for NT. The present results similarly showed that the blastocyst formation rate of NT embryos was higher using IVF embryos having many cells immediately after cell division as the source of donor cells. Activated recipient cytoplasts are presumed to be at the S-phase with a low level of maturation promoting factor [33]. On the other hand, the G1-phase period of donor embryonic nuclei is very short and immediately shifts to the S-phase of the cell cycle [33, 34]. Therefore, the Selected-IVF-embryos just after cell division were used as the source of donor cells in the present study because many of their blastomeres should be at the S-phase. In the present study, although the cell cycle was not confirmed and the definite stage of the cell cycle of the blastomeres used as donor cells was uncertain, it was suggested that the early cell cycle stage after cell division improved the blastocyst formation rate of the NT embryos based on investigation of the cell division ratio of the donor embryos. Moreover, the donor embryos were selected twice or 3 times at the early cleavage stage in the present study. Therefore, it was thought that the Selected-IVF-embryos were at a similar developmental stage and that their cell division had been well synchronized.

When the Selected-IVF-embryos were used for NT, the blastocyst formation rates and total cell numbers of the NT embryos were similar to those of NT embryos obtained from the *in vivo* derived embryos. Moreover, when the obtained blastocysts were transplanted into recipient cows, the productive efficiency of the clone calves was similar to that of NT embryos derived from *in vivo* derived embryos.

In conclusion, when a blastomere from an IVF embryo was used as a donor cell for NT, the blastocyst formation rate of the NT embryo was improved by transplanting the nucleus from an IVF embryo selected according to the morphological development patterns in the early development stage and just after cell division. The resultant NT embryos have a high developmental ability to progress to term after embryo transfer that is comparable to NT embryos reconstructed using *in vivo* derived embryos.

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