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Estimating the survival probability of nuclear-transfer embryos before embryo transfer by a novel biopsy: Oct4 and Sox2 gene expression patterns of a monozygotic twin blastocyst separated at the 2-cell stage of nuclear-transfer embryos

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Abstract: The survival probability of mouse blastocysts developed from nuclear transfer (NT) was estimated prior to embryo transfer using a novel biopsy method. NT embryos were separated into two at the 2-cell stage to produce monozygotic twin blastocysts from a single NT oocyte. We first examined efficient culture methods to produce monozygotic twin blastocysts. Then one of the two blastocysts was used for gene expression analysis, and the other was transferred to a recipient mouse. Co-culture of monozygotic twin blastomeres with fertilized embryos or culture in a small well *in vitro* improved the developmental potential to the blastocyst stage and increased the blastocyst cell number. Although 21.9% of twin somatic cell NT embryos had the same expression levels of Oct4 and Sox2 genes as fertilized embryos, no fetuses were obtained after transfer to recipients.

Key words: Nuclear transfer, Zygotic twin, Diagnosis, Oct4, Sox2

Introduction

Many cloned animals of various species have been produced [1] since the first successful cloning of a sheep in 1997 [2], but the full-term developmental ability of cloned embryos remains very low [3–5]. Abnormalities of epigenetic modifications [6, 7], gene expression [8], and chromosome segregation [9] in preimplantation embryos, and hormonal conditions in recipients in postimplantation stages are possible reasons for the low developmental ability. Attempts to enhance the developmental rate of mouse-cloned embryos have included treatment of cloned embryos with trichostatin A (TSA)-supplemented medium [3, 4], Xist knockdown or knockout by RNA interference [10], and the treatment of cloned embryos with latrunculin A instead of cytochalasin B during activation [11].

It is important to select good cloned embryos before embryo transfer, especially in farm animals, as foster mothers often die after embryo transfer of cloned embryos due to abnormal development of the embryo. The best method of selecting good embryos before embryo transfer, however, has not been established. Although the potential for normal development of fertilized embryos can be easily determined based on visualization or developmental speed, these methods are not effective for cloned embryos [12]. Live cell imaging might be useful for selecting good embryos, but this requires injection of a fluorescent protein coupled with the targeted proteins into the embryo.

In the present study, a unique biopsy method was used to select embryos before embryo transfer. Cloned embryos were separated at the 2-cell stage, and allowed to develop together to the blastocyst stage. One blastocyst was then used for assay and the other blastocyst was used for embryo transfer. This method should result in completely zygotic twin embryos derived from a single nuclear transferred oocyte, without the need for dye injection.

We used Oct4 and Sox2 genes expression patterns

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for diagnosis, because the Oct4 gene was shown to be one of the key genes for the normal differentiation of the inner cell mass in blastocysts [13] and Sox2 is important for maintaining the pluripotent lineage of early mouse embryos [14]. The results of our previous studies [15–18] also suggested that both genes are key factors in the normal development of NT embryos.

The final objective of the present study was to establish a new method of diagnosing cloned embryos before embryo transfer. We first established an efficient method of culturing twin blastomeres to the blastocyst stage *in vitro,* and then examined the gene expression patterns of Oct4 and Sox2 in one blastomere of the twin, and transferred the other embryo to a foster mother to examine whether the cloning efficiency was improved by the determination of specific gene expressions.

Materials and Methods

All experiments and protocols were performed in strict accordance with the Guiding Principles for the Care and Use of Research Animals adopted by Kinki University Committee on Animal Research and Biothics. All chemicals were purchased from Sigma Aldrich Chemical Co., unless otherwise stated.

Collection of fertilized embryos

BDF1 (C57BL/6×DBA) female mice were superovulated with 5 IU pregnant mare serum gonadotrophin and human chorionic gonadotrophin (hCG), respectively given 48 h apart. After hCG injection, the BDF1 female mice were mated with ICR males to collect zygotes 20 h after hCG injection. Zygotes were cultured in KSOM until the 2-cell stage or blastocyst stage. These *in vivo*-fertilized *in vitro*-cultured embryos were used as a control.

Nuclear transfer

Nuclear transfer was performed as described previously [4, 15–18]. Briefly, BDF1 females mice were superovulated as described above and metaphase II oocytes were collected 14 to 15 h after hCG injection. Enucleated oocytes were cultured in KSOM at 37 °C for 1 h in 5% CO_2 in air before NT. Cumulus cells that were obtained at oocyte collection were maintained in M2 medium in 1.5-ml Eppendorf tubes at room temperature until NT. Before NT, collected cumulus cells were centrifuged and mixed with polyvinylpyrrolidone-supplemented M2 medium. After NT with a piezo electric-actuated micromanipulator, the oocytes were transferred to M2 medium for 10 to 15 min at room temperature and cultured in KSOM supplemented with 100 nM TSA for 2 h, and then activated in Ca^{2+} free KSOM supplemented with 100 nM TSA, 10 mM $SrCl_2$, and 5 *μ*g/ml cytochalasin B for 6 h [4, 15–18]. Activated embryos with a pseudopronucleus were cultured in KSOM at 37 °C in 5% CO_2 in air until the 2-cell stage.

Removal of the zona pellucida

The zona pellucida (ZP) of somatic cell nuclear transfer embryos at the 2-cell stage was removed by one of two methods: chemical removal or mechanical removal. At 34 h after hCG injection, 2-cell stage embryos developed from NT were transferred to M2 medium and rinsed. For chemical removal, the embryos were transferred to pH 2.5 acidic Tyrode's medium for ~30 s (AT group). When the ZP had completely disappeared, the embryos were rinsed with M2 medium and cultured. For mechanical removal, the ZP was removed using a piezo electric actuated micromanipulator (Piezo group). The ZP of 2-cell stage embryos was perforated horizontally and the 2-cell embryo extruded. The length of the perforation was half the circumference of ZP [19, 20]. These ZP-free 2-cell embryos were transferred to KSOM and cultured at 37 °C under 5% CO_2 in air until use. Removed ZP were maintained in M2 medium at 4 °C for Experiment 3 (described below).

Separation of blastomeres at the 2-cell stage

ZP-free 2-cell embryos were separated to obtain individual blastomeres by pipetting in M2 medium [20, 21]. Separated blastomeres were cultured *in vitro* as described below.

Culture of twin embryos in vitro

Separated blastomeres were cultured by one of four methods, as described below. In each culture system, the embryos were cultured at 37 °C under 5% CO_2 in air. Blastomeres were transferred to KSOM supplemented with 1:200 stock solution of essential and non-essential amino acid solutions (*Invitrogen*) and 3.5 mg/ml glucose at 64 h after activation, and further cultured until reaching the blastocyst stage at 116 h after hCG injection.

1) A pair of blastomeres was cultured individually in a 1-*μ*l KSOM droplet.

2) A pair of blastomeres was co-cultured with fertilized embryos. Two ZP-free blastomeres were cultured in the same droplet with three *in vivo*-fertilized embryos having a ZP in 3 *μ*l KSOM. The fertilized embryos were easily distinguished from the NT embryos based on the presence or absence of the ZP. Embryos cultured without fertilized embryos were regarded as a control group.

3) Separated blastomeres were reinserted into foreign ZP to examine whether the ZP affects *in vitro* culture.

The re-inserted embryos were cultured individually. Zona-free embryos were used as a control group.

4) Separated twin embryos were cultured individually in very small wells made in the same culture medium droplet of 10*μ*l by pricking with a sterilized needle (WID method). The diameter, depth, and distances between the wells were ~100 *μ*m, ~80 *μ*m, and ~100 *μ*m, respectively. Embryos in the control group were cultured without wells.

Blastocyst cell number

The cell numbers of the inner cell mass (ICM) and trophectoderm (TE) in blastocysts were counted after immunosurgery at 140 h after hCG injection, according to the precedures described in previous reports [22, 23]. Briefly, blastocysts were incubated in M2 medium containing 10% rabbit anti-mouse serum for 30 min, then in M2 medium containing 10% fetal bovine serum for 5 min, and finally in M2 medium containing 10% guinea pig serum, 10 *μ*g/ml propidium iodide, and 10 *μ*g/ml Hoechst 33342 for 30 min at 37 °C. The treated embryos were then examined under a fluorescence microscope to count the cell number in the ICM (stained light blue) and TE (stained pink).

Reverse transcription

At 135 h after hCG injection, one of the twin blastocysts derived from *in vivo*-fertilized and NT embryos were treated with a Cells-to-cDNA^{TM} II kit (Life Technologies) and according to the procedure described in previous reports [15–17]. Briefly, one of the twin embryos was washed twice with phosphate-buffered saline, transferred to 10 *μ*l of cell lysis II buffer, and incubated for 15 min at 75 °C. Then 1 *μ*l DNase I was added to digest the genomic DNA in the samples by incubation of 15 min at 37 °C. Finally, DNase was incubated for 12 min at 75 °C. At this point, the cell lysate was used for reverse transcription and real-time polymerase chain reaction (PCR).

To perform the annealing reaction, 4 *μ*l dNTP mixture, 2 *μ*l random decamers, and 1 *μ*l luciferase RNA were mixed per sample, and then added to 9 *μ*l cell lysate. These samples were incubated for 5 min at 75 °C to anneal. Then, 2 *μ*l RT buffer, 1 *μ*l MMLV RTase, and 1 *μ*l RNase inhibitor were added to an annealed sample, and the mixture was incubated for 60 min at 42 °C, then incubated again for 5 min at 95 °C for reverse transcription.

Quantitative real-time PCR

Quantification of three transcripts was performed by real-time quantitative PCR. PCR was performed using the ABI 7000 PRISM system (Applied Biosystems) according to the procedure desribed in previous reports [15–18]. Sequences and Gene Bank accession numbers of the primer and the probe sets used for amplification of the target genes (Oct4 and Sox2) were the same as those reported previously [15–18]. A 20-*μ*l PCR reaction mixture containing 10 *μ*l Gene Expression Master Mix (Applied Biosystems), 7.85 *μ*l dH_2O, 0.1 *μ*l forward primer, 0.1 *μ*l reverse primer, 0.1 *μ*l probe, and 2 *μ*l cDNA was prepared for each transcript and sample. Thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s, and a combined annealing/extension stage, 60 °C for 1 min.

The comparative C_T method was used for relative quantification of target gene expression levels (ABI Prism Sequence Detection System, Applied Biosystems). The quantification was normalized to the external control, luciferase. Within the log-linear phase region of the amplification curve, the difference between each cycle was equivalent to a doubling of the amplified PCR product. The ΔC_T value was determined by subtracting the luciferase C_T value for each sample from the target gene C_T value of the sample. The $\Delta\Delta C_T$ calculation used the highest or *in vivo*-fertilized embryo sample ΔC_T value as an arbitrary constant which was subtracted from all the other ΔC_T sample values. Fold-changes in the relative mRNA expression of the target gene were determined using the formula $2^{-\Delta\Delta CT}$.

Embryo transfer

ICR strain female mice were mated with vasectomized ICR strain male mice, and female mice observed with a vaginal plug in the morning were regarded as day 0.5 pseudopregnant mice. One of the twin blastocysts was diagnosed, and five *in vivo*-fertilized embryos were co-transferred into oviducts of day 1.0 pseudopregnant mice. Recipient females were sacrified at 18.5 or 12.5 days post conception to collect live fetuses and placentas for examination.

Statistical analysis

The chi-square test and Student's *t*-test were used to analyze the developmental ability data and blastocyst cell number data, respectively. A p-value less than 0.05 was considered significant.

Results

The *in vitro* developmental ability and the cell numbers of blastocysts are shown in Table 1.

1) The proportion of blastocysts in which both blastomeres developed (BL-BL) was quite low in each group

Table 1. Developmental ability of *in vitro* twin nuclear transfer embryos and the cell number at the blastocyst stage

Experimental group*	Zona removal method	Embryo culture method	No.of embryos cultured	No.of embryos developed to blastocyst			Cell number of blastocyst	
				BL-BL	BL-D	D-D	ICM	TE
1)	acid tyrode	single culture	73	3 (4)	16 (22)	54 (74)	6.3 ± 1.6	21.2 ± 8.3
	peizo	single culture	77	8 (10)	13 (17)	56 (73)	4.9 ± 1.9	28.0 ± 9.0
2)	piezo	coculture	89	18 (20)	10 (11)	61 (69)	7.3 ± 4.7	34.4 ± 10.7a
		not-cocultured (control)	81	12 (15)	13 (16)	56 (69)	6.9 ± 2.4	26.9 ± 7.0b
3)	piezo	foreign zona insertion	65	9 (14)	12 (18)	44 (68)	12.0 ± 7.8	25.9 ± 11.2
		zona free (control)	62	6 (10)	7 (11)	49 (79)	10.5 ± 6.0	28.2 ± 7.9
4)	piezo	WID	89	17 (19)a	13 (15)	59 (66)	6.8 ± 2.1	25.1 ± 6.1
		control	85	7 (8)b	14 (16)	64 (75)	8.5 ± 1.9	25.2 ± 4.8

*Corresponding to Materials and Methods. a-b: significantly different ($p<0.05$). BL-BL: both blastomeres developed to blastocysts. BL-D: one blastomere developed to blastocyst. D-D: no blastomere developed to blastocyst.

Table 2. The expression levels of Oct4 and Sox2 in the separated NT embryos

No. of embryos examined		No. of embryos graded as		
		Normal	Abnormal*	
			Down	Up
82	Oct4 only	15	20	3
	Sox2 only	15	14	9
	Both Oct4 and Sox2	18	25	1

*without ~5-fold ranges compared with fertilized embryos.

Table 3. Developmental ability in vivo of nuclear transfer embryos after embyo transfer

	No. of embryso transferred	No. of recipients	Dissection age	No. of fetuses or young
Intact	53	2	Day 12.5	6 (11.3)
twin	41	25		0
Intact	60	3	Full-term	3 (5)
twin	41	21		0

(AT group 4%, Piezo group 10%), and most of the blastomere pairs were degenerated (D-D) (66–75%). Although the difference was not statistically significant, the Piezo group had 20% to 30% higher TE cell and total cell numbers (TE; 21.2 vs 28.0; total; 27.5 vs 32.9).

2) The developmental rate of BL-BL was slightly higher than that of the non-cocultured group (20% vs 15%) but the difference was not significant. The TE cell and total cell numbers of blastocysts cultured with fertilized embryos were significantly higher than those of controls (TE; 34.4 vs 26.9, total; 41.7 vs 32.2). The ICM cell number was the same.

3) The proportion of blastocysts in which both blastomeres developed was 14% in the group of blastomeres cultured in a foreign ZP, and 10% in the control group. Blastomeres reinserted into a foreign ZP had slightly higher developmental ability than ZP-free blastomeres, but the difference was not significant. Both the ICM and TE cell numbers of the groups were similar.

4) The proportion of blastocysts in which both blastomeres developed was low, but significantly higher using the WID method (19%) than that of the control group (4–8%) . Blastocyst cell numbers were the same in both groups.

In our preliminary experiments, the differences in the gene expression levels of Oct4 and Sox2 among individual blastocysts from an *in vivo*-fertilized embryo varied but were within ~5-fold range of the median value. The relative abundance of Oct4 was 0.19–5.23 (average:1.29, median: 1.0, n=39), and that of Sox2 was 0.19–4.95 (average: 1.19, median: 0.9, n=39). Based on this, 18 out of 82 (22%) of analyzed NT blastocysts were considered to be in the normal ranges of both genes, 15 out of 82 (18.2%) had only normal Oct 4 gene levels, and 15 out of 82 (18.2%) had only normal Sox2 gene levels (Table 2). When blastocysts considered to have normal levels of both genes were transferred to recipient mice, no fetus or neonatal mice were obtained at day 12.5 or day 18.5 post pseudopregnancy (Table 3).

Discussion

One strategy to overcome the poor developmental ability of cloned embryos is to modify their epigenetic status [3, 4, 16]. Another strategy is to select a “good embryo”

before embryo transfer. For example, it seems effective to select good embryos before embryo transfer as determined by live-cell imaging. Abnormal chromosomal segregation is observed in more than 90% of cloned mouse embryos by live-cell imaging [9], allowing good embryos to be selected before embryo transfer. In this method, however, a fluorescence protein coupled with targeted proteins must be introduced for observation by the live-cell imaging system, and this could be difficult to perform with farm animals.

Here, we estimated the survival probability of NT mouse blastocysts before embryo transfer using a novel biopsy method that did not use foreign proteins. NT embryos were separated into two at the 2-cell stage to produce monozygotic twin blastocysts from a single NT. Although cloned embryos have the same genetic content as the donor cells, the donor nucleus is damaged and modified to varying degrees by the reprogramming process of enucleated oocytes. Thus, in this study, zygotic twin embryos were produced from a single NT oocyte at the 2-cell stage.

In the present study, we first investigated methods for efficiently producing twin blastocysts. In mammals, monozygotic twin fetuses/young have been produced in bovine [24], sheep [25], goats [26], horses [27], pigs [28], rhesus monkeys [29], and mice [30] by splitting embryos into two during cleavage stage. In the mouse, monozygotic twin fetuses have been produced by splitting 2-cell stage embryos, as performed in the present study [19, 20, 31]. Almost all of those studies used fertilized embryos. Wang et al. [20] produced monozygotic twin fetuses from NT, but it was a nuclear exchange at the 2-cell stage. The birth rate of twin fetuses from in *in vivo*-fertilized embryos was 40% [31], and that of twin fetuses from nuclear exchange at the 2-cell stage was 20% [19].

In the present study, the *in vitro* developmental ability of monozygotic twins derived from NT was very low under all lf the culture conditions examined. On the other hand, intact NT embryos cultured *in vitro* developed to the blastocyst stage at rates ranging from 30% to 50%. Embryos might suffer some damage by the separation processs at the 2-cell stage. Because monozygotic twins were produced at the 2-cell stage, substances crucial for zygotic genome activation might be affected. It was reported in a previous paper [32], that single embryo culture resulted in poor developmental ability (Table 1). It has also been suggested that autocrine and/or paracrine system of embryos do not work in the single embryo cultures [33]. We assume that co-culturing with fertilized embryos supported the developmental ability, compared to single culture of NT embryos, for the same reason.

The cell polarity is influenced by cell-cell interactions [34, 35]. Blastomeres of ZP-free embryos are not tightly packed by the ZP, and thus interactions among blastomeres are thought to be weak with inferior differential ability compared with intact embryos. In ZP-free embryos, blastomeres tend to be planar, not three-dimensional. We observed the morphology of ZP-free embryos at the 4-cell stage, and found that 53% of reinserted embryos and 50% of WID embryos appeared three-dimensional in contrast to 32% to 40% of control embryos [unpublished observation]. Suzuki et al. [36] reported that embryos with abnormal spatial arrangements have lower developmental ability. Removing the ZP may weaken the interactions between blastomeres, leading to a lower ICM number at the blastocyst stage. Blastomeres reinserted into foreign ZP, however, did not exhibit improved developmental ability. There are several possible reasons for this. First, it took a long time to manipulate blastomeres into a foreign ZP outside of the incubator. As described above, the 2-cell stage is under EGA in the mouse, and thus, *in vitro* manipulation such as separation and insertion at the 2-cell stage might affect their subsequent development. Second, reinserted blastomeres are compressed during aspiration by the pipette during reinsertion. A previous study demonstrated that applying pressure to an embryo induces morphologic changes and apoptosis [37]. Thus, additional technical problems must be resolved.

Although significant improvement was not observed in all the culture systems examined, embryos co-cultured with fertilized embryos or cultured in the WID group showed slightly higher developmental abilities to the blastocyst stage in the BL-BL and BL-D groups.

In the present study, gene expression levels of Oct4 and Sox2 were analyzed to select embryos, according to the methods described by Li et al. [15–17]. Oct4 gene expression is essential for development [38]. The twin blastocysts derived from NT had lower Oct4 expression levels, which might be why these embryos failed to implant. In fact, embryos with lower developmental potential also exhibited lower gene expression levels.

The Sox2 gene has an important role in maintaining pluripotency, and embryos with deficits in the Sox2 gene fail to develop to the blastocyst stage [39]. Sox2 gene expression in some embryos derived from NT, however, is higher than that of *in vivo*-fertilized embryos. Pan and Schultz [40] reported that embryos with Sox2 gene overexpression have lower developmental ability, suggesting that higher levels of Sox2 expression lead to disordered regulation of the EGA scheme.

In the present study, we produced monozygotic twin embryos by splitting NT 2-cell embryos and classified the

NT embryo based on gene expression levels: one of the twin blastocysts was used for gene expression analysis, and the other blastocyst was used for embryo transfer. Approximately 20% of the twin blastocysts exhibited almost the same expression level as intact *in vivo*-fertilized embryos, but no fetuses were obtained from such blastocysts. Moreover, Oct4 and Sox2 expression levels largely differed from those of intact embryos. The ICM number of twin blastocysts derived from NT was quite reduced, and not only Oct4 and Sox2, but also other genes essential for fetal development, such as FGF4, Klf4, and c-Myc, might be abnormally expressed or insufficient. It is necessary to improve the methods of culturing twin embryos to increase the cell number at the blastocyst stage. Additionally, expression levels of other genes, especially those cited above, must be analyzed in detail, and compared with those of intact *in vivo*-fertilized embryos and NT embryos.

In conclusion, we successfully produced twin blastocysts from NT embryos by separation at the 2-cell stage. Co-culture with fertilized embryos or culture in WID slightly improved the viability of twin NT embryos to blastocysts, leading to an increase in the cell number. Although the developmental ability of both blastomeres to blastocysts (BL-BL) was still low, zygotic twin embryos from a single NT embryo may be useful for analyzing the reprogramming mechanism and might allow selection of cloned embryos prior to embryo transfer.

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