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Evaluation of the Quality of Porcine Somatic Cell Nuclear Transfer Embryo by Gene Transcription Profiles

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Abstract. This study aimed to evaluate the quality of porcine somatic cell nuclear transfer (SCNT) embryos by examining its gene transcription patterns. Embryos were produced by SCNT, intracytoplasmic sperm injection (ICSI) or under different conditions, and transcripts of genes for fibroblast growth factor receptor (*FGFr*) 2IIIc, *FGFr72IIIb*, X inactive-specific transcript (*Xist*), interleukin 6 (*IL6*), *IL6* receptor (*IL6r*) α and c-kit ligand, were detected by real-time RT-PCR. The percentages of embryos in which these transcripts were detected were similar in SCNT and ICSI embryos. On the other hand, the transcriptional levels of the *FGFr72IIIb* and *IL6r α* genes were 0.5 times less and 2 times more, respectively, in SCNT blastocysts than those of ICSI blastocysts ($p < 0.05$). When nuclear transfer was performed before or after activation of oocytes, embryos in the latter case showed significantly lower frequencies of having *FGFr72IIIb* (74% vs. 90%) and *Xist* (3% vs. 33%) transcripts compared to the former case embryos ($p < 0.05$). When two lines of nuclear donor cells with different developmental potencies were used, the transcriptional profiles in the reconstructed embryos did not show any significant differences. Our study suggests that expression profiles of *FGFr72IIIb*, *IL6r α* , and *Xist* can be used as markers for the diagnosis of the developmental potency of porcine nuclear transfer embryos.

Key words: Nuclear transfer, Gene transcription, Embryo, Pig, Real-time RT-PCR

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In recent years, the significance of transgenic pigs has been widely recognized for biomedical uses such as organ supply for xenotransplantation, production of hybrid bioartificial organs, models for human diseases and mass production of biologically active compounds. One of the effective technologies for production of transgenic pigs, somatic cell nuclear transfer (SCNT) has been the center of attention instead of the conventional pronuclear DNA injection method [1–3]. With current technology, however, the rate of SCNT

embryos progressing to live young is hovering between 1 and 3% or less [4]. The low birth rate of cloned pigs is the result of fetal death in early development, stillbirth and neonatal death immediately after birth [5]. These abnormalities are thought to arise from failure in genetic reprogramming in the early stages after transplantation of embryos [6]. In other words, normal embryonic development cannot be achieved until the donor DNA is successfully reprogrammed after nuclear transfer. Factors influencing reprogramming of transferred nuclei are wide-ranging from the type and cell cycle stage

of donor cells to nuclear transfer protocol, oocyte activation method, and culture of nuclear-transferred embryos before they are transferred to a recipient gilt. Analyzing the influence of these factors is essential for more efficient production of cloned pigs.

Factors influencing the quality of SCNT embryos should be properly examined after actual transplant to recipient gilts, because abnormalities in development of SCNT embryos often occur after implantation in the uterus [5]. However, such an approach is impractical in domestic animals including pigs and cattle, as they have long gestation periods and a substantial amount of work and cost is required to maintain them until their embryos are evaluated. This limitation is being addressed by molecular embryologic techniques [7–9].

Daniels *et al.* [7] compared the transcription patterns of interleukin 6 (*IL6*), fibroblast growth factor 4 (*FGF4*), *FGF* receptor-2 (*FGFr2*) and other genes in SCNT and *in vitro* fertilized (IVF) embryos to evaluate their viability. The transcripts of these genes [8] as well as X inactive-specific transcript (*Xist*) [9] have been suggested as potential markers for examining the influence of factors such as the donor cell type and the timing of oocyte activation on embryonic development. As reported in those studies, if genes important for early embryonic development can be used as markers to assess developmental potency and viability of SCNT embryos, it would provide useful information for improvement of cloned pig production.

This study aimed to determine if transcriptional analysis of these marker genes is useful for evaluating the quality of porcine SCNT embryos. Real-time RT-PCR was used to examine the transcription profiles in porcine embryos reconstructed by various methods. The gene transcriptional patterns were compared 1) between SCNT and intracytoplasmic sperm injection (ICSI) embryos, 2) among SCNT embryos produced by different protocols, and 3) among SCNT embryos using different donor cell lines.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Preparation of recipient oocytes

Ovaries recovered from gilts at a local slaughterhouse were transported to the laboratory in PBS containing 0.1% (w/v) polyvinyl alcohol (PVA), 75 μ g/ml penicillin and 50 μ g/ml streptomycin. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles (diameter 3–6 mm) using a 20 gauge needle attached to a 10 ml disposable syringe. COCs having several layers of compact cumulus cells were selected and cultured in NCSU23 medium [10] supplemented with 0.6 mM cysteine, 10 μ g/ml EGF, 10 IU/ml eCG (Teikoku Hormone MFG, Tokyo, Japan), hCG (Teikoku) and 10% (v/v) porcine follicular fluid. Oocytes were cultured for 22 h with hormones and then for 18–20 h without hormones in a humidified atmosphere of 5% CO₂ in air at 38.5 C.

Preparation of fetal fibroblasts

Primary culture of fetal fibroblasts was established from day 25 female porcine fetuses by routine cell culture technology. Briefly, body tissues were cut into small pieces and dispersed by exposure to 0.25% trypsin-0.02% EDTA (Invitrogen, Carlsbad, CA). Following digestion, the cells were centrifuged to remove debris and cultured in DMEM supplemented with 15% FBS at 37 C in a humidified atmosphere of 5% CO₂ in air.

After 3 to 4 passages, cells were suspended in medium with 10% (v/v) DMSO (Nacalai Tesque, Kyoto, Japan) and stored frozen in liquid nitrogen. For each experiment the cells were thawed, cultured to subconfluency and then serum-starved for 2 days in medium containing 0.5% FBS.

Nuclear transfer

In vitro matured oocytes with expanded cumulus cells were treated with 1 mg/ml hyaluronidase and denuded of cumulus cells by pipetting. Enucleation was performed using the chemically assisted method [11]. Matured oocytes with the first polar body were cultured in NCSU23 medium supplemented with 0.1 μ g/ml demecolcine, 0.05 M sucrose and 4 mg/ml BSA for 0.5–1 h. Treated oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm using a beveled pipette (30 μ m in diameter) in 10 mM HEPES-buffered Tyrode's lactose medium (TL-HP) containing 0.3% (w/v) polyvinylpyrrolidone (PVP), 0.1 μ g/ml demecolcine, 5 μ g/ml

cytochalasin B (CB) and 10% (v/v) FBS. When a protrusion was observed on the surface of oocyte, it was removed along with the polar body. Enucleation was confirmed by staining the cytoplasts with 5 $\mu\text{g}/\text{ml}$ bisbenzimidazole (Hoechst 33342).

Fetal fibroblasts synchronized by serum starvation for 48 h were used as nuclear donors. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. Donor cell-oocyte complexes were placed in 0.28 M mannitol (Nacalai Tesque) solution containing 150 μM MgSO_4 and 0.01% (w/v) PVA and held between 2 electrode needles. Membrane fusion was induced with a somatic hybridizer (SSH-1, Shimadzu, Kyoto, Japan) by applying a single direct current (DC) pulse (200 V/mm, 10 μsec) and a pre- and post-pulse alternating current (AC) field of 5 V, 1 MHz for 5 sec, respectively [12].

Pre-activation nuclear transfer protocol (Pre-Act NT): Reconstructed embryos were cultured in NCSU23 supplemented with 4 mg/ml BSA for 1–1.5 h before being electrically activated. Activation of reconstructed embryos was induced by a DC pulse (150 V/mm, 100 μsec). The activation solution consisted of 0.28 M mannitol, 50 μM CaCl_2 , 100 μM MgSO_4 and 0.01% PVA. The activated embryos were treated with 5 $\mu\text{g}/\text{ml}$ CB for 3 h to suppress extrusion of the pseudo-second polar body.

Post-Activation nuclear transfer protocol (Post-Act NT): The enucleated oocytes were electrically activated as described above. After activation the oocytes were maintained in NCSU23 for 2–5 h and used for nuclear transfer as described above. The reconstructed embryos were cultured without CB treatment after nuclear transfer.

Intra-cytoplasmic sperm injection (ICSI)

Frozen boar semen prepared by the pellet method in BF5 solution [13] was used to prepare sperm for ICSI. Frozen sperm were prepared by the pellet method in BF5 solution from the artificial insemination semen. The frozen sperm were thawed in BTS solution and sonicated to remove sperm tails (28 kHz, 30 sec). The sperm heads were microinjected to the IVM oocytes by a Piezo-actuated micromanipulator. Microfertilization was carried out in TL-HP with 10% FCS, and the oocytes were electrically activated as described above within the 30 min before the sperm injection.

In vitro culture of embryos

SCNT embryos reconstructed with fetal fibroblasts and ICSI embryos were cultured in NCSU23 supplemented with 4 mg/ml BSA under paraffin oil in a plastic Petri dish under an atmosphere of 5% CO_2 in humidified air at 38.5 C. After 96 h, 10% (v/v) FBS was added to the culture medium.

Gene sequences

The DNA sequences of the seven porcine genes analyzed in this study, *β -actin*, *FGFr2IIIc*, *FGFr72IIIb*, *IL6*, *IL6 α* , *Xist*, c-kit ligand (stem cell factor, *SCF*), were obtained from GenBank.

RNA extraction

Cultured embryos were briefly washed in RNase-free water and separately transferred to a microcentrifuge tube filled with 10 μl RNase-free water and 5 U RNase inhibitor. They were immediately snap-frozen in liquid nitrogen and stored at -80°C . RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction as follows. The embryo was thawed in lysis buffer containing 1% mercaptoethanol and homogenized in a QIAshredder column (Qiagen). The lysate was added to glycogen (molecular biology grade, Ambion, TX) (250 $\mu\text{g}/\text{ml}$) as RNA carrier, and applied onto an RNeasy Mini Spin Column (Qiagen) for adsorption of RNA molecules. RNA was eluted with 25 μl RNase-free water containing 2.5 U RNase inhibitor and stored at -80°C .

Real-time RT-PCR

Each RNA sample extracted from the embryos was analyzed by real-time RT-PCR. Reverse transcription of total RNA and PCR amplification was performed using the QuantiTect Probe RT-PCR kit (Qiagen) and TaqMan Probe (Applied Biosystems, CA, USA). Each probe was labeled with a reporter dye FAM (6-carboxyfluorescein) at the 5' end, and with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine) at the 3' end. Primers and probes were designed based on the sequences obtained from GenBank. By real-time RT-PCR using total RNA from the porcine ovary, the most suitable annealing temperature (T_a) was determined for each sequence between 58 and 62 C. The primer and probe sequences and T_a for each gene are listed in Table 1. Each RT-PCR

Table 1. Primer and TaqMan probe sequences for transcriptional analyses of the target genes in porcine SCNT and ICSI embryos

Gene (GeneBank accession #)	Sequence	Position	T _a
<i>β actin</i> (U07786)	F:CATCACCATCGGCAACGA R:GTTGGCGTAGAGGTCCTTCCT P:CCTTCAACTCGATCATGAAGTGCGACGT	375–392 502–522 467–494	62
<i>FGFr2IIIc</i> (AJ439896)	F:CACGGACAAAGAAATTGAGGTTCT R:TCAACCATGCAGAGTGAAAGGAT P:CCCAATAGAATTACCCGCCAAGCACGTA	12–35 105–127 75–102 (antisense)	58
<i>FGFr2IIIB</i> (AJ439895)	F:AGTTTGTCTGCAAGGTGTACAGTGAT R:CGTATTTGCTGCCGTTCTTTT P:CCCACATCCAGTGGATCAAACACGTG	125–150 185–205 158–183	60
<i>Xist</i> (AJ429140)	F:GAAGCATCAGCCAGCAACAC R:TCATAACCATCACTAGTACCCAAACC P:CCTTTGAGCTTAGGTGAGCAGGATTCCTG	394–413 450–475 420–448	58
<i>IL6</i> (AF309651)	F:AGCTATGAACTCCCTCTCCACAA R:GGCAGTAGCCATCACCAGAAG P:TTCAGTCCAGTCGCCTTCTCCCTGG	42–64 100–120 70–94	60
<i>IL6α</i> (AF147881)	F:TGGTGAGGAAGTTTCAGAACAGTC R:GACGCATAGGGTCACTATGTGG P:AGACTTCCAGGAGCCGTGCCAGTACTC	446–469 561–582 477–503	60
c-kit ligand (L07786)	F:GTAGCAGTAATAGGAAGGCCTCAGA R:AGAGAAGAATGCTGGCAATGC P:ATTGAAGACTCCAGCCTCCAGTGGGC	665–689 727–747 694–719	58

F: Forward Primer, R: Reverse Primer, P: TaqMan Probe, T_a: annealing temperature.

reaction mixture (50 μ l) contained the following: 400 μ M dNTP, 0.4 μ M of each primer, 0.2 μ M of TaqMan Probe, 2.5 mM of MgCl₂, 0.5 volume of 2 $\times$ QuantiTect Probe RT-PCR Master Mix, 10 U of Rnase inhibitor and 0.5 μ l of QuantiTect RT mix. RT-PCR was carried out in the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Thermal cycling conditions were 30 min at 50 C, 50 cycles of 30 sec at 94 C and 60 sec at 58–62 C.

Semi-quantification of gene transcripts was also carried out for blastocysts. Five serial dilutions of ovary-derived total RNA (0.01–100 pg) were used to measure the threshold cycle (C_t, the number of cycles at which PCR products reach a certain amount) of each RNA concentration to obtain the 5-point standard curve for real-time RT-PCR. Transcriptional levels of each gene were quantified using the standard curve. The transcriptional level of β -actin was used as an internal control to determine the relative transcriptional levels of the target genes in each sample.

Experimental design

Exp. 1. Detection of differences in the gene transcription profiles between SCNT and IVF embryos

This experiment aimed to compare the transcription patterns in SCNT and ICSI embryos. After nuclear transfer, 2–4 cell embryos and blastocysts were sampled at 27–29 and 149–175 h, respectively. These SCNT embryos were produced by the Pre-Act method. Embryos were individually analyzed for transcription of the target genes, and the numbers of SCNT and ICSI embryos which had the transcripts were counted. Semi-quantitative transcription analysis was also carried out for the blastocysts.

Exp. 2. Examining differences in the transcription profiles in SCNT embryos reconstructed by two different protocols: comparison between the Pre-Act and Post-Act methods

In this experiment we aimed to compare the transcription patterns in SCNT embryos which received nuclei before (Pre-Act NT) and after oocyte activation (Post-Act NT). The 2–4 cell embryos were collected at approximately 24 h after

Table 2. Gene transcription profiles in porcine SCNT and ICSI embryos

	2–4 cell stage*		Blastocyst stage*	
	ICSI	NT	ICSI	NT
<i>FGFr2IIIc</i>	11/31 (35.5)	8/30 (26.7)	35/35 (100.0)	21/23 (91.3)
<i>FGFr72IIIb</i>	30/31 (96.8)	27/30 (90.0)	35/35 (100.0)	22/23 (95.7)
<i>Xist</i>	12/31 (38.7)	10/30 (33.3)	34/35 (97.1)	22/23 (95.7)
<i>IL6</i>	8/31 (25.8)	4/30 (13.3)	35/35 (100.0)	23/23 (100.0)
<i>IL6α</i>	3/31 (9.7)	7/30 (23.3)	31/35 (88.6)	17/23 (73.9)
c-kit ligand	31/31 (100.0)	30/30 (100.0)	35/35 (100.0)	23/23 (100.0)

* The numbers and percentages (in parentheses) of embryos in which the target transcript was detected are listed for each developmental stage.

nuclear transfer, and the rate of transcription of the target genes was analyzed as described in Exp. 1.

Exp. 3. Changes in the transcription profiles in SCNT embryos reconstructed from different donor cell lines

This experiment used two different donor cell lines to produce SCNT embryos, and their effects on the transcription patterns were analyzed. Two types of fetal fibroblasts, PFF1 and PFF6, have been known to show significantly different blastocyst formation rates when used as nuclear donors. Using these cell lines, SCNT embryos were reconstructed by the Pre-Act method. As described in Exp. 1, the 2–4 cell embryos were collected at approximately 24 h after nuclear transfer, and the rate of transcription was analyzed for each cell line.

Statistics

The significance of the difference in the number of embryos in which transcripts for the gene of interest were detected was determined using the χ^2 test. For the semi-quantitative analysis of transcripts, the Smirnov-Grubbs' test was used for the exception of outliers (significant level 5%). The mean transcriptional level in each group was examined by the t-test (significant level 5%).

Fig. 1. Relative quantification of gene transcripts in porcine SCNT and ICSI blastocysts. * Significant difference ($p < 0.05$) between the transcriptional levels in SCNT and ICSI embryos.

Results

Exp. 1. Difference in the transcriptional profile in SCNT and ICSI embryos

There was no significant difference in the transcriptional profiles of SCNT and ICSI embryos for all the six genes examined (Table 2). On the other hand, as shown in Fig. 1, semi-quantitative analysis of transcripts in blastocysts showed that the transcriptional level of *FGFr72IIIb* in SCNT embryos was significantly lower than that of ICSI embryos ($p < 0.05$). In addition, the transcriptional level of *IL6 α* in SCNT embryos was statistically higher than that in ICSI embryos ($p < 0.05$) (Fig. 1).

Table 3. Transcriptional profiles in SCNT porcine embryos reconstructed by different protocols

	Pre-Act*	Post-Act*
<i>FGFr2IIIc</i>	8/30 (26.7)	6/34 (17.6)
<i>FGFr72IIIb</i>	27/30 (90.0) ^a	25/34 (73.5) ^b
<i>Xist</i>	10/30 (33.3) ^a	1/34 (2.9) ^b
<i>IL6</i>	4/30 (13.3)	9/34 (26.5)
<i>IL6α</i>	7/30 (23.3)	8/34 (23.5)
c-kit ligand	30/30 (100.0)	34/34 (100.0)

^{a, b} $P > 0.05$

* The embryos were reconstructed by either pre-activation nuclear transfer protocol (Pre-Act NT) or post-activation nuclear transfer protocol (Post-Act NT). The numbers and percentages (in parentheses) of embryos expressing the target transcript are listed for each method. The data for the Pre-Act embryos were obtained in the experiment shown in Table 2.

Table 4. Transcriptional profiles in SCNT porcine embryos produced by different donor cell lines

	PFF6*	PFF1*
<i>FGFr2IIIc</i>	8/30 (26.7)	9/30 (30.0)
<i>FGFr72IIIb</i>	27/30 (90.0)	24/30 (80.0)
<i>Xist</i>	10/30 (33.3)	11/30 (36.7)
<i>IL6</i>	4/30 (13.3)	2/30 (6.7)
<i>IL6α</i>	7/30 (23.3)	4/30 (13.3)
c-kit ligand	30/30 (100.0)	29/30 (96.7)

* The numbers and percentages (in parentheses) of embryos in which the target transcript was detected are listed. The embryos were reconstructed either from PFF1 or PFF6 as nuclear donors. The data for the PFF6-derived embryos were obtained in the experiment shown in Table 2.

Exp. 2. Difference in the transcription profiles in SCNT embryos produced by different methods

As shown in Table 3, the numbers of embryos that had transcripts for *FGFr72IIIb* and *Xist* were significantly fewer in the embryos reconstructed by Post-Act NT than those in the embryos produced by Pre-Act NT ($p < 0.05$). There was no difference in the transcription profiles of *FGFr2IIIc*, *IL6*, *IL6 α* and c-kit ligand between the two methods.

Exp. 3. Difference in the transcription profiles when using two different donor cell lines

There were no significant changes in the rates of embryos in which transcripts of the six genes were detected, when using the two different donor cell lines (Table 4).

Discussion

In this study, we selected the *FGF* receptor family, *Xist*, *IL6*, *IL6 α* and c-kit ligand genes as candidate genetic markers for evaluating the developmental potency of SCNT porcine embryos. The *FGF* receptor family [14, 15], *IL6* and *IL6 α* [16, 17] are strongly expressed at the blastocyst stage and are known to play critical roles in post-blastocyst development and implantation. *Xist* is

required for X inactivation [18] and is also expressed strongly in the blastocyst stage [19–21]. The transcription level of *Xist* in female embryos was substantially higher than that of male embryos [19, 21]. It has been reported that in cattle these genes are useful markers for assessing the developmental potency of nuclear transfer embryos at the blastocyst stages [7–9]. The c-kit ligand gene is known to have an important function in early embryonic development, especially in the blastocyst stage [17, 22, 23]. All of these genes start to express at the 2 cell stage [7, 17, 19, 22], that is before genome activation in porcine embryos [24, 25].

When we compared embryos derived from SCNT and ICSI, there was no difference in the transcription profiles of these marker genes both at the early cleavage and blastocyst stages. In contrast, differences between SCNT and ICSI embryos were observed in the transcriptional levels of *FGFr72IIIb* and *IL6 α* . *FGF* [15, 26, 27] and *IL6* [28, 29] have significant functions in blastocyst development, hatching and implantation. Therefore, the differences in the expression levels of these genes may reflect differences in post-blastocyst developmental potency. Also in bovine nuclear transfer and IVF embryos, *Xist* [9], heat shock protein (*Hsp*) and interferon tau (*IF τ*) [30]

showed different expression levels in the blastocyst stage. Together, our data confirms that differences in the developmental potency of SCNT and ICSI embryos may be assessed by the expression levels of the marker genes at the blastocyst stage.

Pre-Act and Post-Act NT were used to compare the transcriptional profiles in embryos derived by the method which had previously cloned young pigs [12] and by the method which does not develop embryos. In our previous study, Pre-Act NT not only showed relatively stable blastocyst formation from NT embryos (10–20%), but also produced live clones [12], suggesting its potential in successfully reprogramming the donor nuclei. On the other hand, most of the NT embryos reconstructed by the Post-Act method ceased development during the early cleavage stages (data not shown). Nuclear transfer was carried out 2 to 5 h after oocyte activation in the Post-Act method. When the nucleus was transferred to an oocyte in which the level of maturation/M-phase promoting factor is dropped to a certain level after activation, premature chromosome condensation and nuclear envelope breakdown often did not occur, thus leading to an extremely low rate of embryo development [31–33]. Our comparative analysis showed that fewer embryos had transcripts for *FGFr72IIIb* and *Xist* in the early cleavage stages when reconstructed by Post-Act NT compared to those derived by the Pre-Act method. Especially the rate of *Xist* transcription was significantly low in the Post-Act embryos indicating expression of *Xist* had not been initiated in most of them.

Knockout of *FGFr2*, a member of the *FGF* receptor family, in mice results in developmental arrest in the blastocyst stage [34]. In addition, transcriptional defect of *Xist* is lethal in the early developmental stages [35–37]. These data suggest that the transcription profile of the embryos reconstructed by the Post-Act method in our study was a characteristic presentation of developmentally defective embryos. Our study provides a basis for the use of these gene expressions as indexes to evaluate the developmental potency of NT embryos reconstructed by an inappropriate method.

The type of donor cells is one of the most important factors that determine the developmental potency in NT embryos [38]. Two types of porcine fetal fibroblasts, PFF1 and 6, examined in this study were derived from 2

littermate fetuses of the same sex and used after the same number of passages in culture. Their growth rate and cell-cycle synchrony were also similar (data not shown). Our preliminary study, however, showed that the rates of blastocyst formation of NT embryos derived from these cell are remarkably different: 12% in PFF6 and 4% in PFF1 (data not shown). Our third experiment aimed to compare the cell lines appropriate and inappropriate as nuclear donors.

Unexpectedly, the two donor cell lines with different potencies did not cause a change in the transcription profiles of NT embryos. One possible reason for this observation is that our study examined the transcriptional profile only in the early cleavage stages. When bovine embryos reconstructed from granulosa cell nuclei and IVF embryos were compared [8], transcription of *FGFr2* was lower in the NT blastocysts, although no difference could be seen in the earlier developmental stages.

The total amount of RNA extracted from a single embryo does not yield a sufficient amount of product after RT-PCR, resulting in difficulty in analysis. In contrast, real-time PCR is highly specific and sensitive, using a fluorescent probe that increases its fluorescent intensity with the copy number of PCR product, and thus it is more suitable for this study. Quantitative analyses of gene transcripts from a single embryo by real-time RT-PCR have been used to evaluate the qualities of human and murine oocytes [39–43].

In conclusion, our data suggest the possible use of real-time RT-PCR for examining the transcription profiles of *FGFr72IIIb*, *IL6ra*, and *Xist* in a single embryo, which in turn can be interpreted for assessment of embryonic quality. We expect that further research with a greater variety and number of genes will provide a stronger basis for evaluating the viability of cloned embryos.

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