Timing and Uniformity of Embryonic Gene Activation Affect Subsequent Pre-implantation Development of Cloned Bovine Embryos

Aya KASAMATSU1), Kazuhiro SAEKI1), Tomohiro TAMARI1), Daisaku IWAMOTO1), Atsuhiro TATEMIZO1), Kazuya MATSUMOTO1), Yoshihiko HOSOI1) and Akira IRITANI1)

1)Department of Genetic Engineering, Kinki University, Kinokawa, Wakayama 649-6493, Japan

Abstract. In the present study, we examined the timing of onset, intensity, and mosaicism of embryonic gene expression in bovine nuclear transfer (NT) embryos. The relationship between gene expression and early embryonic development was also examined. To monitor the gene expression of NT embryos, we produced NT embryos with bovine transfected fibroblasts carrying a firefly luciferase gene under the control of a chicken \( \beta \)-actin promoter, an expression system that has previously been shown to be representative of embryonic gene expression in mice. Photon count imaging showed that luciferase luminescence began in NT embryos with fibroblasts 48 hours post fusion (hpf) and reached a plateau at the 4- to 8-cell stage at 60 hpf. Only 4- to 8-cell NT embryos luminescent by 60 hpf developed to the blastocyst stage. At 60 hpf, strongly luminescent embryos developed to the blastocyst stage at a higher rate \( (P<0.05) \) than embryos with weak or absent luminescence. However, embryos with mosaic luminescence developed at a much lower rate \( (P<0.05) \) than those with whole-embryo luminescence, even if the embryos exhibited strong luminescence. Our results indicate that precise and uniform embryonic gene expression at the 4- to 8-cell stage at 60 hpf may be closely related to development of bovine NT embryos to the blastocyst stage.

Key words: Bovine, Cloned embryos, Embryonic gene activation, Pre-implantation development, Timing, Uniformity

Successful generation of cloned cattle by nuclear transfer (NT) with somatic cells has been reported throughout the last decade [1–3]. However, the efficiency of production of cloned cattle is generally low because of first trimester losses of more than 50% during pregnancy after transfer of NT embryos [3, 4]. Even if the offspring survive, they often have increased birth weights, which is referred to as “large offspring syndrome” [5–7]. Recently, it has been reported that aberration of epigenetic modifications such as DNA methylation and histone acetylation occur in bovine NT embryos [8–10]. Additionally, abnormal levels of expression of some developmentally important genes have also been observed at the blastocyst stage in bovine NT embryos [11, 12].

Embryonic gene activation (EGA), defined as the maternal-embryo transition of developmental control, is the first crucial event during mammalian early development because the timing of onset of EGA is concurrent with the developmental block of embryos cultured in vitro [13]. A previous study...
showed that when the firefly luciferase gene under the control of a housekeeping or virus gene promoter is injected into the pronuclei of mouse zygotes, its expression is concomitant with zygotic gene translation at the 2-cell stage and is regulated by the same mechanisms that regulate their EGA [14]. The luciferase assay has previously been used to determine the onset of EGA in mammalian embryos [14–16]. In bovine embryos, EGA mainly occurs at the 4- to 16-cell stages [17, 18]. However, EGA in bovine NT embryos has not been fully examined. Moreover, mosaic gene expression among blastomeres at EGA has been shown in porcine NT embryos [19]. However, the reason why the mosaicism occurred has not been clarified yet.

Herein, we report the timing of onset, intensity, and mosaicism of embryonic gene expression in bovine NT embryos and the effects of the gene expression on the pre-implantation development of NT embryos. Notably, we used stable transfected cells carrying firefly luciferase genes as the donor cells for NT embryos in this study and observed the expression of the luciferase genes as endogenous marker genes in the NT embryos.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Establishment of transfected cell lines

Bovine fibroblasts were obtained from ear skin samples from a 5-month-old, male Japanese black beef calf. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS; BioWest, Paris, France, 10% FBS-DMEM) for four passages at 37°C under 5% CO2 at high humidity. They were then transfected with a plasmid, pIRES2-enhanced green fluorescent protein (EGFP; Clontech, Mountain View, CA, USA), containing a cDNA for improved firefly luciferase gene under the control of the β-actin promoter [15] and a neomycin resistance cassette [pβ-act/luc/IRES/EGFP/neor] using a transfection reagent (GeneJammer; Stratagene, La Jolla, CA, USA). Forty-eight hours after transfection, the culture medium was replaced with DMEM supplemented with 20% (v/v) FBS and 600 µg/ml G-418 (Geneticin; Gibco, Grand Island, NY, USA), and then the cells were cultured in the medium for 15–20 days for selection of neomycin-resistant cells. The surviving cell colonies were further selected by their EGFP fluorescence using a fluorescence microscope (Nikon, Tokyo, Japan). Following several passages of the cells, cell lines expressing continuous EGFP fluorescence were selected. Luminescence of the luciferase (LUC+) in the cells was confirmed in medium containing 500 µM D-luciferin using an imaging photon counter (ARGAS-50; Hamamatsu Photonics, Hamamatsu, Japan). Cells confirmed to have integrated the transgene into their genome by Southern blotting analysis were used for production of NT embryos.

In vitro maturation of oocytes

Bovine ovaries were obtained from a local slaughterhouse and were transported in saline at 20–25°C to the laboratory within six hours. Cumulus-oocyte complexes (COCs) were aspirated from the ovaries, collected, and washed with 25 mM Hepes-buffered TC199 with Hanks' salts (H199-H; Gibco) supplemented with 5% (v/v) FBS and 25 µg/ml gentamicin (FBS-H199-H). The washed COCs were cultured for 21 h in 50 µl of 25 mM Hepes-buffered TC199 with Earle's salts (H199-E; Gibco) supplemented with 5% FBS, 0.5 mM sodium pyruvate, 25 µg/ml gentamicin, 0.02 AU/ml FSH (Antrin; Kawasaki Seiyaku, Tokyo, Japan), and 1 µg/ml estradiol-17β under mineral oil at 39°C in 5% CO2 in air with high humidity (10 COCs/droplet).

Nuclear transfer with transfected fibroblasts

The surrounding cumulus cells were removed by pipetting COCs at 18–21 h post-maturation in FBS-H199-H supplemented with 0.25% (w/v) hyaluronidase. The nuclei of the denuded oocytes were removed as follows. The zona pellucida of an oocyte was slit in the vicinity of the first polar body. The ooplasm was softened by treatment with 5 µg/ml cytochalasin B and was then enucleated by pushing out the MII plate with a little cytoplasm using a fine glass needle. Enucleation was confirmed by visualizing the karyoplast under UV light after staining a small amount of cytoplasm with 20 µg/ml Hoechst 33342. Quiescent cells induced by serum deprivation of the culture
GENE EXPRESSION IN CLONED BOVINE EMBRYOS

medium [3] were inserted into the perivitelline space of the enucleated oocytes. The cell-ooplast couplets were placed in Zimmerman fusion medium [20] at room temperature and electrically fused with two DC pulses of 2.72 kV/cm for 11 µsec each using an Electro-Cell Manipulator (ECM-200; BTX, San Diego, CA, USA). Fused couplets were activated with 5 µM ionomycin for 5 min and then treated with 10 µg/ml cycloheximide in modified synthetic oviduct fluid medium [21] without KH2PO4 (mSOFM) until 6 h post fusion (hpf) at 39 C in 5% CO2 and 5% O2 in air with high humidity. Following activation, the NT embryos were cultured in 50 µl mSOFM (20 to 30 embryos/droplet) covered with mineral oil until determination of their LUC+ activity at 39 C in 5% CO2 and 5% O2 in air with high humidity.

Determination of LUC+ activity in NT embryos

Luciferase activity was detected in NT embryos by determination of the luminescence of the embryos in medium containing 500 µM D-luciferin using an imaging photon counter. The rates of luminescent embryos were recorded for each detection time point. The luminescence was quantitatively determined for 10 min. The luminescence of each blastomere in the embryos was determined after removal of their zona pellucidae with 0.5% (w/v) pronase (Roche Diagnostics, Basel, Switzerland). The mean LUC+ activity of an embryo was calculated from the total photon count accumulated over the 10-min period and was expressed in relative light units (RLUs).

Culture of NT embryos after determination of LUC+ activity

After determination of luminescence, 20–30 cleaved NT embryos were cultured to the blastocyst stage in a droplet of mSOFM at 39 C in 5% CO2 and 5% O2 in air with high humidity because embryo development is more successful with group cultures than with single cultures [22]. In vitro fertilized embryos [21] were added to a culture droplet to adjust the number of embryos to 20–30 if the number of NT embryos in the culture droplet was less than 20. The nuclear transfer blastocysts could be distinguished by the slits in their zona pellucidae.

Experimental design

Experiment 1: This experiment examined the onset of embryonic gene expression in the NT embryos. The profiles of the expression of a luciferase gene in the NT embryos with transfected cells were examined by determining the luminescence from the 1-cell stage (0 hpf) to the blastocyst stage (168 hpf).

Experiment 2: This experiment examined the relationship between the timing of onset of embryonic gene expression in the NT embryos and subsequent development of the embryos. The luminescence in each NT embryo was determined at 60, 72 and 120 hpf. When the luminescence of LUC+ in the NT embryos was detected at 60 hpf, the luminescent embryos were isolated from the experimental pool and were cultured to the blastocyst stage. The luminescence in the remaining embryos was then determined again at 72 hpf, and only luminescent embryos were cultured. The other embryos were cultured separately depending on whether they were luminescent or non-luminescent at 120 hpf. The developmental rates to the blastocyst stage were determined at 168 hpf.

Experiment 3: This experiment examined the relationship between intensity of gene expression in NT embryos and their subsequent development. The luminescence of LUC+ was determined in 4- to 8-cell NT embryos at 60 hpf. The embryos were categorized as being either strong (>10×10^4 RLUs/embryo), intermediate (5 to 10×10^4 RLUs/embryo), weak (<5×10^4 RLUs/embryo), or absent based on the intensity of the luminescence, and then the embryos in each group were cultured separately up to the blastocyst stage.

Experiment 4: This experiment examined the relationship between mosaicism of gene expression among blastomeres in NT embryos and their early development. At 60 hpf, the luminescence of LUC+ was determined in 4- to 8-cell embryos, and then the zona pellucidae of the strongly luminescent embryos were removed. The luminescence of each blastomere was then determined again. The zona-free embryos were categorized by whether all or some of the blastomeres were luminescent (whole-LUC+ and mosaic-LUC+, respectively). These embryos were individually cultured in aggregation drops using a method similar to the WOW system [23] to allow subsequent development to the blastocyst stage.
Statistical analysis

All experiments were repeated at least three times. The data was analyzed with Fisher protected least significant difference (PLSD) tests following ANOVA using the StatView software (StatView version J-4.11; Abacus Concepts, Berkeley, CA, USA). Differences of \( P<0.05 \) were considered to be significant.

Results

Experiment 1: Onset of embryonic gene expression in NT embryos

Five cell lines were obtained after transfection of \( p\beta\text{-act/luc'}/\text{IRES/EGFP}/\text{neo} \) to bovine fibroblasts. Integration of the transgene into the genomes of two of the cell lines (cell lines 1 and 2) was confirmed by Southern blot analysis. In the NT embryos produced from the two cell lines carrying the luciferase gene, luminescence began at 48 hpf (about 20% of the embryos), reached a plateau at 60–80% of the embryos were luminescent, and then began to decrease at 96 hpf until the blastocyst stage at 168 hpf (Fig. 1). Because the characteristics of expression of the luciferase gene were similar in the two cell lines, only the results obtained from cell line 1 are presented below.

Experiment 2: Effect of onset of luminescence on the development of NT embryos to the blastocyst stage

Eighty-eight embryos were examined for luminescence at 60, 72 and 120 hpf, and embryos with visible luminescence at least once were cultured separately to the blastocyst stage. As shown in Table 1, about half of the embryos (48/88, 55%) were luminescent at 60 hpf (at the 4- to 8-cell stage), and 10 (19%) of these embryos developed to the blastocyst stage. None of the remaining embryos that either showed luminescence at a later time or did not show luminescence developed to the blastocyst stage (Table 1).

Experiment 3: Effect of intensity of luminescence at 60 hpf on the development of NT embryos to the blastocyst stage

Fifty-five percent of the NT embryos (137/251) were at the 4- to 8-cell stage at 60 hpf. About one-third of these embryos were strongly luminescent (> 10 \( \times \) 10⁴ RLUs/embryo). A total of 15 embryos developed to the blastocyst stage, and most of these embryos were from the strongly luminescent group (Table 2).

Experiment 4: Effect of pattern of luminescence among blastomeres showing strong luminescence (>10 \( \times \) 10⁴ RLUs/embryo) on the development of NT embryos to the blastocyst stage

Sixty-six percent of the NT embryos (144/219)
developed to the 4- to 8-cell stage. Thirty-eight (26%) of these embryos were categorized as being strongly luminescent at 60 hpf. As shown in Table 3, about two-thirds of the strongly luminescent embryos showed luminescence in all of their blastomeres (Whole-LUC+, Fig. 2A) and the rest showed luminescence in only some of their blastomeres (Mosaic-LUC+, Fig. 2B). Development to the blastocyst stage was significantly greater for the whole-LUC+ NT embryos than for the mosaic-LUC+ NT embryos (P<0.05, Table 3).

**Discussion**

In the present study, we found that embryonic gene expression in the bovine NT embryos took place at 4- to 8-cell stage at 60 hpf. Furthermore, we observed that NT embryos exhibiting an adequate and uniform gene expression at the 4-to 8-cell stage at 60 hpf had higher ability to develop to the blastocyst stage.

The onset of EGA is regulated in embryos by a time-dependent mechanism following fertilization rather than by a particular cell cycle event [14, 24]. Therefore, in the present study, the luminescence of the NT embryos was determined every 12 or 24 hpf. When EGA was examined in bovine NT embryos with stably-transfected cells carrying luciferase genes, none of the embryos were luminescent until 36 hpf, about 20% were luminescent at 48 hpf, and 60–80% were luminescent at 60 hpf (Fig. 1). These results are in agreement with our previous results from observation of transient expression following injection of a luciferase gene into the pronuclei of NT embryos, which showed that luminescence began in bovine NT embryos at 48 hpf and reached the maximum level at 60 hpf [25]. In bovine embryos, embryonic transcription-dependent protein synthesis is first detected during the 4-cell stage at 48 h post-insemination (hpi), and then the number of proteins increases during the 8-cell stage at 60 and 84 hpi [18]. Furthermore, a previous study at our laboratory detected luminescence in fertilized embryos after injection of a luciferase gene, indicating that bovine EGA begins beyond the 4-cell stage 3 days post-insemination [26]. Therefore, EGA in bovine NT embryos may begin 48 to 60 hpf.

---

**Table 2.** Effect of intensity of luminescence at 60 hpf on the development of NT embryos to the blastocyst stage

<table>
<thead>
<tr>
<th>No. of embryos cultured</th>
<th>No. (%) of 4- to 8-cell stage embryos</th>
<th>Intensity (RLU/embryo)</th>
<th>No. (%) of embryos</th>
<th>No. (%) of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>251</td>
<td>137 (55)</td>
<td>Strong (&gt;10 × 10⁴)</td>
<td>48 (35)</td>
<td>14 (29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate (5 to 10 × 10⁴)</td>
<td>22 (16)</td>
<td>1 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weak (&lt;5 × 10⁴)</td>
<td>44 (32)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent (0)</td>
<td>23 (17)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*a, b, c, d Values significantly different (P<0.05).

**Table 3.** Effect of pattern of luminescence among blastomeres showing strong luminescence (>10 × 10⁴ RLU/embryo) on the development of NT embryos to the blastocyst stage

<table>
<thead>
<tr>
<th>Expression pattern of luciferase gene ( % of luminescent blastomeres)</th>
<th>No. (%) of embryos</th>
<th>No. (%) of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole-LUC+ (100%)</td>
<td>24 (63)</td>
<td>13 (54)</td>
</tr>
<tr>
<td>Mosaic-LUC+ (&lt;100%)</td>
<td>14 (37)</td>
<td>2 (14)</td>
</tr>
</tbody>
</table>

*a, b Values significantly different (P<0.05).
during the 4- to 8-cell stages in a similar manner to bovine fertilized embryos.

We showed that only 4- to 8-cell stage NT embryos that became luminescent by 60 hpf developed to the blastocyst stage and that no blastocysts were obtained from embryos that became luminescent at a later time (Table 1). It is well known that embryos subjected to suppression of embryonic transcription using α-amanitin are arrested at 2-cell stage in mouse [27], at 9- to 16-cell stages in cattle [28], and at 8- to 16-cell stage in sheep [29]. Delayed expression of transcription-requiring complex (TRC), a marker of EGA, has also been observed in mouse 2-cell blocked embryos compared with normally developed embryos [30]. Therefore, it appears that the embryonic gene expression of NT embryos must occur at a predetermined time for subsequent development of the embryos.

Our results showed that strongly luminescent embryos at the 4- to 8-cell stage at 60 hpf developed to the blastocyst stage at a higher rate than intermediate and weak luminescent embryos (Table 2). Semiquantitative RT-PCR analysis has shown that the expression levels of developmentally important genes, such as DNMT and Mash2, increase in bovine pre-implantation embryos [11]. The relative expression levels of Xist and Ndn, the imprinted genes correlated with EGA, were significantly higher in in vitro fertilized bovine embryos at the 8-cell stage than in NT embryos [10]. These findings may indicate that the increase in the gene transcripts reflects the developmental ability of the embryos. Therefore, it is likely that developmentally important genes and the luciferase gene were sufficiently expressed in the strongly luminescent NT embryos.

Park et al. [19] reported mosaic expression of EGFP in pig 4- to 8-cell stage NT embryos with somatic cells carrying EGFP under the control of cytomegalovirus promoter. However, the effect of the mosaicism on subsequent development of NT embryos has yet to be clarified. In the present study, mosaic-LUC+ NT embryos developed at a much lower rate than whole-LUC+ NT embryos, even in the embryos that exhibited strong luminescence (Table 3). Our results indicated that NT embryos that fail to express their genes uniformly among blastomeres might fail to develop further.

In conclusion, the present results suggest that precise onset and uniformity of EGA are required for subsequent development of NT embryos. In addition, it may be possible to predict the ability of bovine NT embryos to develop to blastocysts by examining their embryonic gene expression.

Acknowledgements

This study was supported by a Grant-in-Aid for the 21st Century COE Program of the Japan Ministry of Education, Culture, Sports, Science and Technology, a grant from the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technology Excellence Project of the Japan Science and Technology Agency, and a Grant-in-Aid for Scientific Research (N0.18380169) from the Japan Society for the Promotion of Science.

References

1. Wells DN, Misica PM, Tervit HR, Vivanco WH. Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. Reprod Fertil Dev 1998; 10: 369–378.
GENE EXPRESSION IN CLONED BOVINE EMBRYOS

629