Journal of Reproduction and Development, Vol. 53, No. 1, 2007

—Full Paper—

Changes in the mRNA Transcripts of Insulin-Like Growth Factor Ligand, Receptors and Binding Proteins in Bovine Blastocysts and Elongated Embryos Derived from Somatic Cell Nuclear Transfer

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Abstract. The objective of this study was to determine changes in the transcription of insulin-like growth factor (IGF)-related genes in blastocyst (BC)- and elongated (EL)-stage embryos produced by nuclear transfer using somatic cells (NT-SC). Bovine BC (day 7)- and EL (day 15)-stage embryos were obtained from NT-SC or *in vivo* production (Vivo). The relative abundance of mRNA was examined by RT- real-time PCR. The transcript of IGF-II was only detected at the EL stage in both the NT-SC and Vivo embryos. The level of transcription of the IGF-I receptor (r) in the NT-SC embryos was decreased at the EL stage and was significantly ($P<0.05$) lower than at the BC stage. In contrast, the IGF-IIr levels did not differ significantly between the NT-SC and Vivo embryos, regardless of the developmental stage. IGF-binding protein (IGFBP)-2 levels were markedly decreased in the NT-SC and Vivo embryos at the EL stage ($P<0.05$). The IGFBP-3 level in Vivo was significantly ($P<0.05$) increased at the EL stage compared with at the BC stage. However, the IGFBP-3 levels in NT-SC embryos were unchanged and lower ($P<0.05$) than in the Vivo embryos at the EL stage. These results suggest that there are differences in the levels and changes in the transcription of IGF-related genes in bovine embryos produced by NT-SC compared with those produced by Vivo.
Key words: Bovine, Nuclear transfer, Embryo, Gene expression, Insulin-like growth factor
(**J. Reprod. Dev. 53:** 77–86, 2007)

A number of cloned animals have been produced in various species by somatic cell nuclear transfer (NT-SC). However, the efficiency of animal production by NT-SC is still very low. In bovine cloning, a high rate of embryonic, fetal, neonatal, and postnatal abnormality has been consistently observed [1–6]. Although the cause of such abnormalities is unknown, it is possible that the phenomenon is correlated with abnormal epigenetic modifications and gene expression in NT-SC embryos. Recently, it was clearly indicated that epigenetic modifications and gene expression patterns are closely related to the rate of success of cloning in mice [7, 8].

Several investigators have reported [9–12] aberrant transcription patterns for genes that have important roles in either preimplantation development and implantation or early

Accepted for publication: June 16, 2006
Published online: October 24, 2006
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postimplantation development in bovine NT-SC embryos. Recently, we found that bovine NT-SC embryos at the blastocyst (BC) stage show deviations in expression patterns with respect to insulin-like growth factor (IGF)-related genes that are important for regulating the growth of the fetus [13]. For Type II IGF receptor (IGF-IIr) and IGF-binding protein (IGFBP)-3, lower incidences of gene transcription and large variations in gene expression levels were observed in NT-SC embryos. Whether the deviations from the normal pattern of gene expression and development observed in the BC embryos persist throughout fetal development or even up to birth has not yet been determined.

The BC stage is the most common point in embryonic development at which systems such as *in vivo* production, *in vitro* fertilization (IVF), and NT-SC are assessed. Elongated (EL)-stage embryos characterize a stage of development immediately preceding the recognition of pregnancy, onset of conceptus apposition or placentation, and formation of the extraembryonic membranes and embryo proper [14]. Both developmental stages are biological landmarks for embryonic development, and assessments of gene expression in these periods are important for clarifying the cause of the abnormalities in NT-SC embryos, fetuses, and offspring.

The objectives of this study were to evaluate the gene expression of NT-SC embryos developed to the EL stage and to clarify the changes in gene transcription from the BC to EL stage. We determined the frequency and relative abundance of gene transcripts of IGF ligand, receptors, and binding proteins in single bovine embryos derived from NT-SC and development *in vivo* using the sensitive real-time PCR method.

Materials and Methods

Generation of NT-SC embryos

Collection and maturation of oocytes: Oocytes obtained from slaughterhouse ovaries were matured *in vitro* using methods described previously [13]. Briefly, cumulus-oocyte complexes (COCs) were aspirated from 2 to 8 mm follicles. Ten bovine COCs were matured in a 100 μl drop of IVMD-101 medium (Research Institute for the Functional Peptides, Yamagata, Japan [15]) at 39 C in a humidified atmosphere containing 5% CO_2 in air for 22 h.

Nuclear transfer and embryo culture: NT-SC and embryo culture were carried out essentially as described previously [13]. Briefly, bovine fibroblast cells for NT-SC were collected from the skin of a 100-day fetus using 0.1% collagenase (Sigma Chemical, St. Louis, MO, USA) and 0.25% trypsin (Invitrogen, Carlsbad, CA, USA). Primary cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with 10% fetal calf serum (FCS; Roche Diagnostics, Tokyo, Japan) for up to four passages. Prior to NT-SC, fibroblast cells at 50 to 70% confluency were cultured for a further 4-5 days in serum-depleted medium (DMEM with 0.5% FCS).

After *in vitro* maturation, COCs were stripped of their cumulus cells by vortexing in 0.1% hyaluronidase (Sigma) for 5 min. The zona pellucidae of the oocytes were cut with a fine glass needle. The oocytes were enucleated by removing the first polar body and metaphase II plate with a small amount of surrounding cytoplasm in medium supplemented with 5 μg/ml of Cytochalasin B (CB; Sigma). A single donor cell was inserted into the perivitelline space of the enucleated oocytes using a glass pipette. The recipient oocyte-cell complexes were pulsed with two direct current electric pulses of 20 V/150 μm for 50 μsec with a 1-sec interval in Zimmermann's fusion medium [16]. The recipient oocytes were fused with donor cells, simultaneously activated by direct current electric pulses, and then treated with 10 μg/ml of Cycloheximide (Sigma) for 5 h. Cell fusion was assessed at 5 h after the electric pulses. Only embryos in which the donor cell had fused successfully with the cytoplast were cultured. Following NT-SC, the embryos were cultured in modified TALP (mTALP) medium [17] with 0.1% BSA (Sigma) at 39 C in 5% CO_2, 5% O_2 and 90% N_2. On Day 1 (NT-SC=day 0), the embryos were transferred to mTALP supplemented with 3% new born calf serum (Invitrogen) and cultured at 39 C in 5% CO_2, 5% O_2 and 90% N_2. On day 7, BC embryos were collected for the isolation of total RNA.

Embryo transfer and recovery of elongated embryos

Estrus synchronization was performed on the recipient cows for embryo transfer (ET) by administration of a CIDR device (EAZI-Breed™, InterAg, Hamilton, NZ) for 7–14 days and injection

Table 1. Details of primers used for RT-PCR

Genes	Primer sequences (5′–3′)[a]	Annealing temperature (C)	Fragment size (bp)	Gene bank accession no.
IGF-II	F-CGTGGCATCGTGGAAGAGTGT R-GGTGACTCTTGGCCTCTCTGA	56	277	X53553
IGF-Ir	F-ATGCTGTTTGAGCTGATGCGCATGTGCTGG R-GCGCTCGTTCTTGCGGCCCCCGTTCAT	56	354	X54980
IGF-IIr	F-CGCCTACAGCGAGAAGGGGTTAGTC R-AGAAAAGCGTGCACGTGCGCTTGTC	60	293	J03527
IGFBP-2	F-ACTGTCACAAGCATGGCCTG R-CCTCCTGCTGCTCATTGTAGA	56	168	AF074854
IGFBP-3	F-ACTTCTCCTCTGAGTCCAAGC R-CGTACTTATCCACACACCAGC	56	210	M76478
GAPDH	F-CCAGAAGACTGTGGATGGCC R-CTGACGCCTGCTTCACCACC	60	248	U85042

[a]Primer orientation: F, forward; R, reverse.

of PGF2α (cloprostenol 0.5 mg, Resipron-C®, Teikoku Hormone, Tokyo, Japan) 1 day before removal of the device. A total of 25 NT-SC embryos at the BC stage were nonsurgically transferred to 5 recipients (5 embryos per recipient) 6–7 days after estrus. On day 15 (NT-SC=day 0), EL conceptuses were recovered nonsurgically by uterine flushing using a Foley catheter (20 French gauge).

Production of embryos in vivo

Embryos produced *in vivo* (Vivo) were obtained from donor cows treated for superovulation and artificial insemination (AI) using methods described previously [13]. Briefly, a total of 20 IU of FSH (Antorin®, Denka Pharmaceutical, Kanagawa, Japan) was given to the donors twice daily in decreasing doses over 3 days. To induce luteolysis, PGF2α was injected on the third day of superovulation. The donors were bred by AI at 12–24 h after the onset of estrus. On day 7 and day 15 of development (AI=day 0), BC and EL embryos were nonsurgically recovered from 3 (day 7) or 4 (day 15) donor cows by uterine flushing using a Foley catheter (20 French gauge).

Determination of the relative abundance of the gene transcripts in bovine embryos

The basic protocol used to determine the relative abundance of the gene transcripts has been described previously [13].

RNA isolation: BC embryos were treated with 0.5% protease (Sigma) in PBS containing 1% polyvinyl pyrrolidone (PVP) for 5 min and were then washed three times in 1% PVP-PBS. Single BC embryos were added to 5 μl of lysis buffer (0.8% Igepal [ICN Biomedical, Aurora, OH, USA], 5 mM DTT [Invitrogen], and 1 U/μl of RNAsin [Promega, Madison, WI, USA]), snap frozen in liquid nitrogen, and stored at –80 C. Recovered EL embryos that had an embryonic disc were transferred to 1% PVP-PBS, and the lengths of the trophoblasts of intact EL embryos were measured under a stereomicroscope. Then, the trophoblasts of each EL embryos was divided into several pieces approximately 3 to 5 mm in width and washed three times in 1% PVP-PBS. Total RNA was isolated from 1 or 2 pieces of trophoblast using a RNeasy® Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions and was stored at –80 C.

Reverse transcription: RNA samples were heated to 80 C for 5 min, transferred directly to ice, and treated with 1 U/μl of DNase I (Invitrogen) for 15 min at room temperature. For inactivation of the DNase I, 1 μl of 25 mM EDTA was added to the samples and heated for 10 min at 65 C. The following were added to the tube: 5 × RT buffer, 0.1 M DTT, Oligo$(dT)_{15}$ primer (0.5 μg/μl; Promega), 10 mM dNTP mix (Invitrogen) and Rnasin. The samples were then heated at 65 C for 5 min. After denaturation, the samples were kept at 37 C for 10 min, 100 U of reverse transcriptase (SuperScript H-RT; Invitrogen) was added, and then the mixture was incubated at 37 C for 60 min and denatured at 99 C for 5 min. The reaction mixture was diluted with DEPC-treated water to obtain a final volume

of 20 μl.

Real-time PCR: PCR was conducted in a Light Cycler (Roche), and products were detected with SYBR Green, which is included in the QuantiTect® SYBR Green PCR Master Mix (Qiagen). For each quantification, 2 μl of the RT product was used. The amplification program was as follows: preincubation for the activation of HotStarTaq DNA polymerase (Qiagen) at 95 C for 15 min, followed by 50 cycles of denaturation at 94 C for 15 sec; annealing of primers at different temperatures (Table 1) for 30 sec; and elongation at 72 C for 30 sec. After the end of the last cycle, a melting curve was generated by beginning fluorescence acquisition at 65 C and taking measurements every 0.2 C until 95 C was reached. Product identity was confirmed using plots of the melting curve and electrophoresis on 1% agarose gel stained with ethidium bromide.

Quantification: A standard curve was generated by amplifying serial dilutions of a known quantity of amplicons. Amplicons consisted of purified PCR products that were electrophoresed, and a specific band for each of the genes examined was extracted from the gel using a QIAquick® Gel Extraction Kit (Qiagen). The purified PCR products were quantified by measuring absorbance at 260 nm and then diluted. Serial ten-fold dilutions for creation of a standard curve were amplified in every real-time PCR run. The standards and cDNA samples were then co-amplified in the same reaction prepared from a master mix. Fluorescence was acquired at each cycle in order to determine the threshold cycle (Ct) or the cycle during the log-linear phase of the reaction at which fluorescence rose above the background for each sample. Final quantification was conducted using the Light Cycler's quantification software, and the results were reported as the relative expression after normalization of the amount of transcript to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same samples.

Statistical analysis

The trophoblastic length of EL embryos was analyzed using the Mann-Whitney's U test. The mRNA expression level data was analyzed using the Kruskal-Wallis test followed by multiple pair-wise comparisons using the Scheffe method. Differences were considered significant at $P<0.05$.

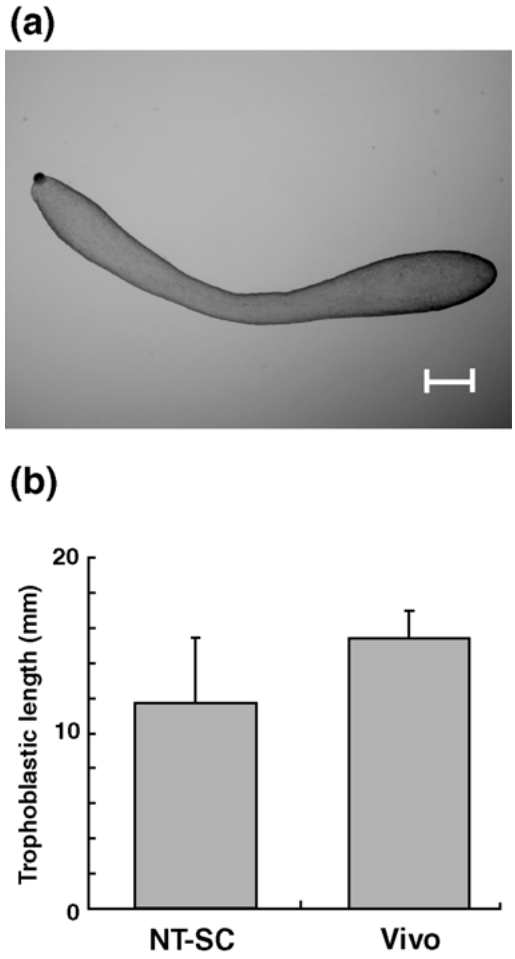

Fig. 1. Representative photograph and physical measurements of a bovine day 15 conceptus. (a) A typical EL embryo obtained by NT-SC. The scale bar represents 1 mm. (b) Trophoblastic length (mean ± SEM) of NT-SC (n=7) and Vivo (n=7) embryos.

Results

Development of day 7 NT-SC embryos and day 15 conceptuses

The BC developmental rate (number of BC/number of 1-cell stage embryos in culture) for NT-SC embryos was 20.9% (29/139). The recovery (number of embryos/number of recipient cows from which embryos were recovered) of BC- and EL-stage embryos was 12/4 (EL) for NT-SC, 34/3 (BC) and 29/4 (EL) for Vivo. The recovery rate (number of embryos recovered/number of embryos transferred) was 48.0% (12/25) for NT-SC.

Table 2. Summary of the RT-PCR results following analysis of the bovine embryos*

Embryo stage	Embryos derived from	No. of embryos with a positive signal / No. of embryos analyzed (%)				
		IGF-II	IGF-Ir	IGF-IIr	IGFBP-2	IGFBP-3
BC	NT-SC	0/14 (0)	14/14 (100)	3/14 (21)	14/14 (100)	7/14 (50)
BC	Vivo	0/14 (0)	14/14 (100)	12/14 (86)	14/14 (100)	13/14 (93)
EL	NT-SC	5/7 (71)	5/7 (71)	7/7 (100)	7/7 (100)	5/7 (71)
EL	Vivo	7/7 (100)	7/7 (100)	7/7 (100)	7/7 (100)	7/7 (100)

* GAPDH signals were detected in all embryos used for RT-PCR analysis.

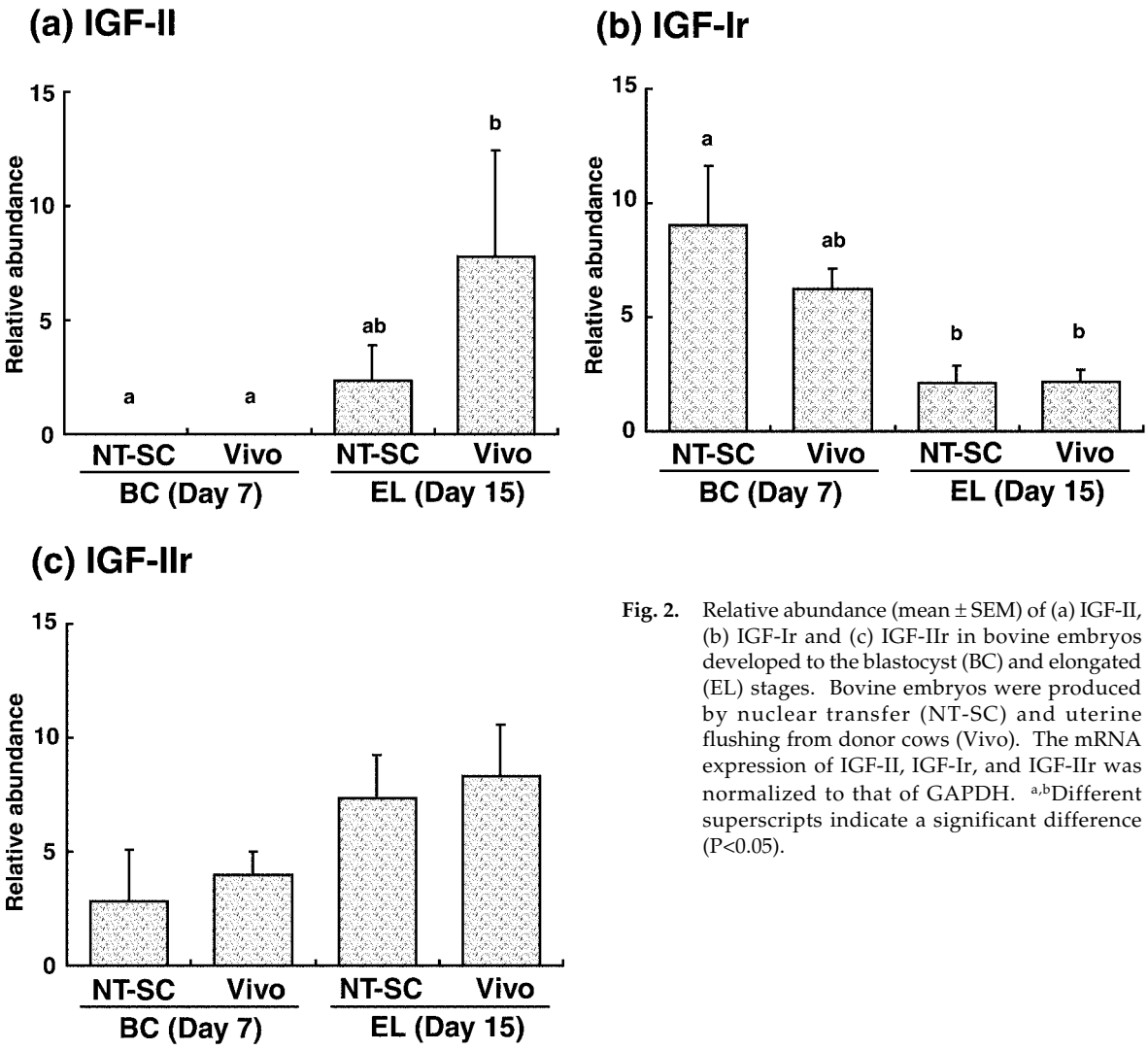

Fig. 2. Relative abundance (mean ± SEM) of (a) IGF-II, (b) IGF-Ir and (c) IGF-IIr in bovine embryos developed to the blastocyst (BC) and elongated (EL) stages. Bovine embryos were produced by nuclear transfer (NT-SC) and uterine flushing from donor cows (Vivo). The mRNA expression of IGF-II, IGF-Ir, and IGF-IIr was normalized to that of GAPDH. [a,b]Different superscripts indicate a significant difference ($P<0.05$).

Fig. 1 shows a typical EL stage embryo and trophoblastic lengths of embryos. Trophoblastic length (range, mean ± SEM) did not differ significantly between NT-SC (4.0–31.0 mm, 11.7 ± 3.7) and Vivo (9.0–20.0 mm, 15.4 ± 1.6).

Frequencies of mRNA transcription in bovine embryos

The transcripts of five genes were analyzed in individual BC- and EL-stage embryos derived from either NT-SC or Vivo. A total of 14 (BC) and 7 (EL) embryos were used in each group to analyze the gene expression. The results are summarized in

Table 2. The transcript of GAPDH was detected in all embryos. The transcript of IGF-II was not detected in BC-stage embryos derived from NT-SC and Vivo, but it was detected at the EL stage in both groups. The transcripts of IGF-Ir and IGFBP-2 were detected with high frequency regardless of the origin and stage of the embryos. The transcripts of IGF-IIr and IGFBP-3 in the BC-stage embryos produced by NT-SC were detected at lower frequencies, 21 and 50%, respectively. However, the transcripts of these genes were consistently detected at the EL stage.

Relative abundances of mRNA transcripts in bovine embryos

The relative abundance of specific transcripts was analyzed using a total of 14 (BC) and 7 (EL) single embryos in each group. The relative abundance of each gene was obtained from the individual embryos, and the mean relative abundance is presented in Figs. 2 and 3. As shown in Fig. 2a, the transcript of IGF-II was detected at only the EL stage. The relative abundance of this transcript was increased significantly ($P<0.05$) in the Vivo embryos. The transcript levels of IGF-Ir were lower at the EL stage than at the BC stage (Fig. 2b). The IGF-Ir level was significantly ($P<0.05$) decreased in the NT-SC embryos at the EL stage. There was no difference in the mean abundances of IGF-IIr transcript between the NT-SC and Vivo embryos (Fig. 2c).

The transcript levels of IGFBP-2 and -3 are shown in Fig. 3. There was no difference in the expression of IGFBP-2 between NT-SC and Vivo on day 7 of development (Fig. 3a). The amount of IGFBP-2 transcript in both groups dropped to its lowest level at the EL stage and was significantly ($P<0.05$) lower than at the BC stage. In contrast, an increase ($P<0.05$) in the level of IGFBP-3 transcription was observed in the Vivo embryos from the BC to EL stage. However, the amount of IGFBP-3 transcript was unchanged in the NT-SC embryos during development from the BC to EL stage, and the relative abundance of IGFBP-3 was significantly ($P<0.05$) lower than that in the Vivo embryos at the EL stage (Fig. 3b).

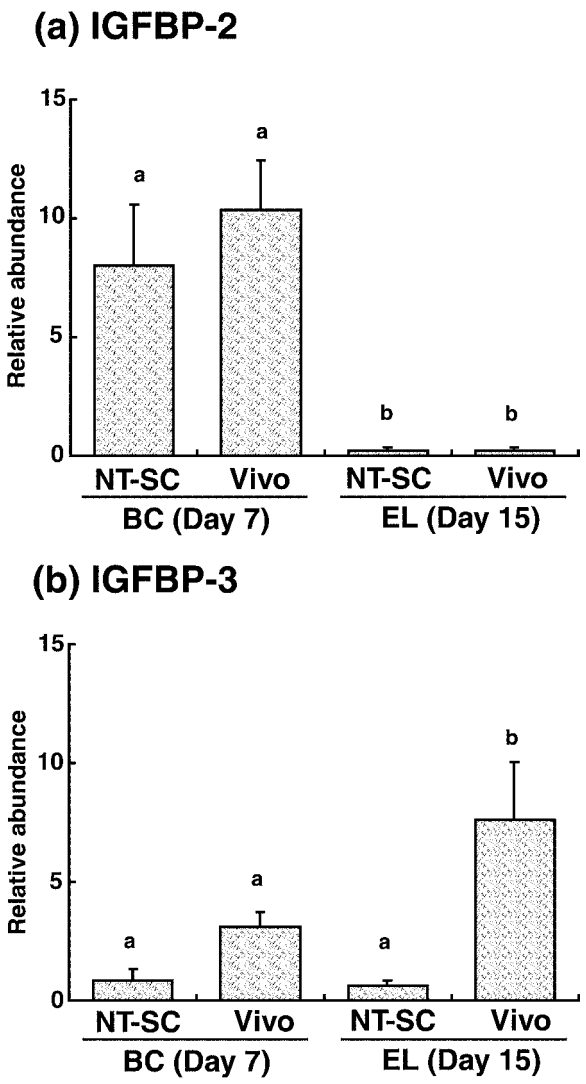

Fig. 3. Relative abundance (mean ± SEM) of (a) IGFBP-2 and (b) IGFBP-3 in bovine embryos developed to the blastocyst (BC) and elongated (EL) stages. Bovine embryos were produced by nuclear transfer (NT-SC) and uterine flushing from donor cows (Vivo). The mRNA expression of IGFBP-2 and IGFBP-3 mRNA was normalized to that of GAPDH. [a,b]Different superscripts indicate a significant difference ($P<0.05$).

Discussion

A number of abnormalities have been reported in cloned animals, including early embryonic loss, abortion, stillbirth, and postnatal death. To clarify the cause of these abnormalities, which occur at various stages of pregnancy, an extensive analysis of gene transcripts using embryos and fetuses at a variety of developmental stages is necessary. Although the expression of various genes in bovine

NT-SC embryos has been analyzed, most studies have attempted to detect the mRNA at the BC stage [9–12]. Therefore, we used BC and EL embryos to evaluate the transcription of IGF-related genes specific to the origin and developmental stage of NT-SC embryos.

Hashizume *et al*. [18] reported that there was no significant difference in the average length of bovine fetuses produced by the AI and NT-SC on day 60 of gestation, but the number of placentomes was about half for NT-SC compared to AI. In the present study, trophoblastic length did not differ significantly between the NT-SC and Vivo embryos on day 15. However, the extent of development and elongation in both groups demonstrated extensive variability similar to that described in a previous report [14]. Although the variation in the development of the conceptus between and within the NT-SC and Vivo embryos does not allow us to draw conclusions about the subsequent establishment of pregnancy beyond day 15 of development, it seems obvious that there were no morphological abnormalities in the NT-SC conceptuses at day 15 of development.

Large offspring syndrome (LOS), which is the phenomenon of increased fetal or birth weight, is well-recognized as an abnormality of NT [1–6]. Phenotypes similar to those described for LOS in cattle and sheep have been found in mice and humans and are related to the altered expression of imprinted genes [19, 20]. Imprinted genes such as IGF-II and IGF-IIr play important roles in regulating embryo development and fetal growth [21–24]. In particular, IGF-IIr expression is strongly related to LOS in the ovine fetus [25]. In the present study, the IGF-II transcript was detected in EL but not BC- stage embryos, which was not consistent with previous reports [14, 26, 27]. An increase in this transcript has been observed as bovine embryos reached the hatched BC stage [24]. Transcripts for IGF ligands in BC- stage embryos may be present at extremely low levels, and so the IGF-II transcript was not detected in our system using individual BC embryos.

Based on our findings, there were no differences in transcript levels for the IGF-IIr gene in embryos from different origins and developmental stages, and this was consistent with previous observations using IVF and Vivo embryos [14, 28]. In contrast to the relative abundance of the IGF-IIr gene, the incidence of NT-SC embryos with the IGF-IIr signal was increased from the BC to EL stage by virtue of transfer to the uterus. Furthermore, the expression of IGF-Ir in the NT-SC embryos was significantly decreased from the BC to EL stage. Lazzari *et al*. [28] reported that although the level of the IGF-Ir transcript at the BC stage was significantly higher in bovine embryos produced from *in vitro* culture systems compared with embryos derived from Vivo, the level was significantly decreased by culture in the sheep oviduct. Maternal IGF ligands and many other components of the IGF system are expressed in the bovine reproductive tract [29, 30], and their levels *in utero* significantly increase from day 15 to 18 of gestation [31]. The IGF receptor transcripts in bovine preimplantation embryos were influenced by IGF ligands [24, 32]. Therefore, changes in the expression of IGF receptors from the BC to EL stage may result in an improvement of the environment for embryo development on ET to the recipient uterus.

The actions of the IGF family are regulated through their association with IGFBPs. IGFBPs also appear to transport IGFs and mediate IGF-independent actions, such as the inhibition or enhancement of growth and induction of apoptosis [33, 34]. To date, six different IGFBPs (IGFBP-1 through -6) have been identified that specifically bind IGF-I and IGF-II. IGFBP-1, IGFBP-3, and IGFBP-4 bind IGF-I and IGF-II with similar affinity, whereas IGFBP-2, IGFBP-5, and IGFBP-6 prefer to bind IGF-II [35]. IGFBPs and their gene transcripts are found in various tissues including parts of the reproductive tract such as the ovarian follicle, oviduct, and uterus [31, 35–37]. Furthermore, bovine early embryos also express mRNAs encoding IGFBPs 2–4 through to the blastocyst stage [37]. In our previous study [13], IGFBP-2 and IGFBP-3 were expressed in bovine blastocyst embryos obtained using various procedures including NT-SC. In the present study, the IGFBP-2 transcript levels in NT-SC and Vivo embryos were significantly decreased in the EL stage compared to the BC stage. This result is consistent with the observation [38] that the expression of IGFBP-2 in bovine NT-SC or Vivo conceptus at day 50 of gestation is localized to the deep stromal tissue of the maternal placentome and does not occur in the fetal villi.

In the present study, a tendency was observed towards lower levels of IGFBP-3 in the NT-SC embryos. Furthermore, the NT-SC embryos at the

EL stage had significantly less transcript for IGFBP-3 than the Vivo embryos. In a previous study [13], the relative abundance of IGFBP-3 in BC embryos obtained by NT-SC was less than that in Vivo embryos but was not different from the levels in IVF embryos. The conditions for culture have been shown to strongly affect transcriptional activity in bovine embryos [11, 39–41]. Therefore, we previously concluded that some environmental factor(s) for embryonic development were responsible for the differences in IGFBP-3 abundance in bovine embryos obtained using various procedures. However, in the present study, the NT-SC embryos developed from the BC to EL stage in the same environment used for producing the Vivo embryos. The results of the present study clearly indicate that the deviation in the amount of the IGFBP-3 transcript in NT-SC embryos is due to the origin of the embryos not the environment during embryonic development. Although the expression of IGFBP-3 in the bovine NT-SC conceptus has been reported to be abnormal at Days 50 to 150 of gestation [38], the present study showed that it was already altered in the day 15 conceptus. It is not clear whether the IGFBP-2 or IGFBP-3 transcript in bovine embryos is related to this specific phenomenon in NT conceptus and offspring. However, as IGFBPs regulate not only the IGF system, but also cell growth and the activities of cytokines in various tissues [42–45], it is quite likely that differences in IGFBP-3 expression are fundamental to the placental abnormalities seen in pregnancies achieved with NT-SC.

In the previous study [13], we demonstrated that there was large variation in the expression of IGF related genes in individual BC embryos, and this type of tendency was strongly observed in NT-SC embryos. Kohda *et al*. [46] reported that there is variation in the gene expression of many genes among mouse clones. In the present study, there was also variation in the relative abundance of individual embryos (data not shown). It is possible that the gene expression level in individual BC embryos is influenced by the ability of inner cell mass (ICM) and trophectoderm (TE). In mouse cloned embryos, abnormal octamer-binding transcription factor-4 expression, such as expression in both ICM and TE cells, and absence of expression in all cells was observed [47]. Although the reason for such abnormality and variation of gene expression in NT-SC embryos is unclear, it may be attributable to initial errors in the reprogramming process after NT.

In conclusion, the results of an analysis of mRNA transcripts at two different stages of development demonstrate that bovine NT-SC embryos show deviations in their expression patterns with respect to IGF-related genes. Although the mean abundance of IGF receptors in NT-SC embryos was not altered, the level of IGFBP-3 transcript was lower in these embryos. Changes in the expression of the IGF family may be responsible for the altered growth characteristics seen in fetuses and offspring originating from bovine embryos obtained using the NT-SC procedure. Further analysis of IGF-related genes in fetuses and placentae developed to the late stages of gestation is necessary to clarify the cause of the abnormalities observed in pregnancies of NT-SC.

Acknowledgments

This research was supported by Research Project for Utilizing Advanced Technologies in the Ministry of Agriculture, Forestry and Fisheries (No. 1602).

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