Analysis of Differentially Expressed Genes in Cloned Bovine Placenta

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ABSTRACT

Placenta is the main nutrition source for the fetus during pregnancy. Thus, it has a pivotal function in the pregnant process. Many functions of the placenta have been elucidated. An abnormal placenta is associated with a high rate of pregnancy failure in somatic cloned bovine. Differentially expressed genes (DEGs) were examined in a comparison between normal and cloned bovine placenta using annealing control primer (ACP)-based GeneFishing PCR. Using 120 ACPs, nearly 80 genes were identified and the fragments of 42 DEGs were sequenced. 38 of these genes were known genes and four were unknown.

To determine the DEGs result, six target clones expressing on one-side of a normal and a clone placenta were selected. Through an analysis of the target genes using the real-time PCR, the expressing pattern was found to be somewhat different from the DEGs. Additionally, several genes appeared with the same expression pattern. Taken together, this suggests that the target genes would be essential for research into what influences the placental formative mechanisms during fetal development.

(Key words : Placenta, Differentially expressed genes, Annealing control primer)

INTRODUCTION

The successful production of viable offspring by cloning with somatic cell nuclear transfer (SCNT) has been achieved in ungulate, including sheep (Wilmut *et al.*, 1997), mice (Wakayama *et al.*, 1998, 1999), cattle (Kato *et al.*, 1998, 2000; Wells *et al.*, 1999), goat (Baguisi *et al.*, 1999; Keefer *et al.*, 2002; Zou *et al.*, 2001), pig (Boquest *et al.*, 2002; Polejaeva *et al.*, 2000), rabbit (Chesne *et al.*, 2002), and cat (Shin *et al.*, 2002). The most important challenge facing somatic cell NT technology is the high rate of pregnancy failure occurring throughout the entire gestation period, from embryo transfer to parturition (Heyman *et al.*, 2002; Hill *et al.*, 2000, 2001; Sousa *et al.*, 2001; Wells *et al.*, 1998). Tamada and Kikyo (2004) reported that the successful rate of obtaining viable offspring from cloning is currently less than 5%.

In bovine, a poorly developed placenta with very few placentomes and little vascularization has frequently been described in early pregnancy (Hill *et al.*, 2000). Later in pregnancy, somatic cloning is associated with hydrallantois (i.e., excessive accumulation of allantoic fluid), which is one of the main causes of fetal mortality during the third trimester (Wells *et al.*, 1998). Abnormal placental development has also been reported in the cloning of mice (Eggan *et al.*, 2001; Inoue *et al.*, 2002; Wakayama *et al.*, 1999). The failure of placental formation may account for most instances of first trimester loss in cattle, as fetal development is halted in the absence of the placenta. The absence of vascularization of the chorioallantoic membranes has been implicated as a possible cause of the failure of placentation (Hill *et al.*, 2001).

Abnormal placental development and function have been reported to be linked to a high proportion of postimplantation fetal losses and may cause inadequate mammary gland development in surrogate recipients and the failure of signaling during the preparation process for parturition (Kato *et al.*, 1998; Wakayama *et al.*, 1999, Wells *et al.*, 1999). Therefore, the production of cloned animal with somatic cells is associated with a number of problems. These matters must be solved fundamentally.

This study was performed to investigate the physiological status of somatic cell clone recipients in Korean native cow and to compare differentially expressed genes between a normal and a cloned placenta.

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The expression patterns of six specific DEGs were analyzed using real-time PCR.

MATERIALS AND METHODS

Source for Nuclear Transferred Somatic Cells and Nuclear Transfer

The somatic cells used in this experiment were derived from bovine fetal fibroblast cells collected at 45 days of pregnancy. Fibroblast cells were separated in a trypsin-EDTA treatment and cultured in DMEM plus 10% FBS in a method previously described in the literature (Yang *et al.*, 2006). For NT, frozen-thawed cells were cultured up to the confluent stage and were utilized after the culture was placed in a serum-deficient medium for 5 days. The methods involved in the production of nuclear embryos are described in detail in the literature (Yang *et al.*, 2006). Briefly, donor cells were combined with enucleated oocytes. Fusion of the oocyte-cell complex was induced by a single DC pulse (25V/150 um for 10 usec) using an Electro Cell Fusion Model LF 101 (BEX, Tokyo, Japan) in Zimmerman's mammalian cell fusion medium. After electric stimulation, the fused oocytes were treated with 10 uM Ca ionophore for 5 min and 2 mM DMAP for 3 h. Embryos were cultured in CR1aa medium for 72 h at 38.5℃ in a 5% CO_2, 5% O_2, and 90% N_2. On day 7, good-quality blastocyst was nonsurgically transferred to surrogate cows.

Generation of Placental Tissues

Bovine placental tissues were obtained under general anesthesia from SCNT-derived Korean Native Cattle (KNC) on day 280 of pregnancy. Normal placental tissues were also obtained via cesarean section on day 280 of gestation. The tissues were completely washed two times in ice-cold phosphate buffered saline (PBS, pH 7.2) and were dissected into small fragments. Fetal cotyledons, maternal caruncles and placental tissues were snap-frozen in Liquid nitrogen (LN_2) and were stored at −80℃.

RNA Isolation

Placental tissues were homogenized and total RNAs were extracted using a TRIZOL RNA isolation kit (Invitrogen, Carlsbad, CA). RNA was precipitated with isopropyl alcohol (Sigma, USA), washed with 75% ethanol in DEPC water and were then redissolved. The RNAs were treated with RNase-free DNase I to remove possible contaminating DNA and stored at −80℃. Finally, the RNA was quantified using a Nano Drop Spectrometer (ND-1000, NanoDrop®, USA).

cDNA Synthesis and ACP RT-PCR Analysis

First-strand cDNA synthesis by reverse transcriptase as described by Park and Min (2008) was performed for 1.5h at 42℃ in a final reaction volume of 20 μl containing 3 μg of the purified total RNA, 4 μl of 5X reaction buffer (Promega, Madison, WI, USA), 5 μl of 2 mM dNTP, 2 μl of 10uM cDNA synthesis primer dT-ACP 1 (Gene-Fishing™ DEG kits, Seegene, Korea), and 0.5 μl of RNasin® RNase Inhibitor (40U/uL; Promega).

Second-strand cDNA synthesis and subsequent PCR was conducted in a single tube. Second-strand cDNA synthesis was performed at 50℃ (low stringency) during one cycle of first-stage PCR in a final reaction volume of 49.5 μl containing 3~5 μl of the diluted first-strand cDNA, 5 μl of 10X PCR reaction buffer (Roche Applied Science, Mannheim, Germany), 5 μl of 2 mM dNTP, 1 μl of 10uM dT-ACP 2 and 1 μl of 10uM arbitrary primer (Annealing Control Primer of 120 (GeneFishing™ DEG kits, Seegene, Korea)) preheated to 94℃. The PCR products were subjected to electrophoresis on a 2% agarose gel and stained with 1 mg/ml ethidium bromide.

Cloning and Sequencing

The differentially expressed bands were extracted from the agarose gel using a Geneclean II Kit and a Glassmilk gel extraction Kit (Q-BIOgene, Cambridge, UK). The eluted products were cloned using TOPO TA Cloning Vector (Invitrogen Co., Grand Island, NY). The plasmid DNA was extracted using QIAprep Spin Miniprep Kit (QIAGEN, USA). To verify the insert DNA, the isolated plasmids were sequenced using an automated DNA sequencer (Applied Biosystems, Model 37-30XL, USA). The sequence identity of each product was confirmed by BLAST searches on the combined Genebank/EMBL and expressed sequence tag libraries (dbEST) that were accessed through the National Center for Biotechnology Information homepage (http://www.ncbi. nlm.nih.gov).

Semi-Quantitative RT-PCR

Standard cDNA synthesis by reverse transcription of the total RNA was employed using the Oligo(dT)12~18 primer and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY). The mRNAs of several genes were then detected by RT-PCR with specific primer pairs using reagents supplied with a Taq DNA polymerase kit (TOYOBO, Tokyo, Japan). The PCR products were visualized under ultraviolet light on 2% agarose (Promega, USA) gels in 0.5X TAE buffer containing 1 mg/ml ethidium bromide.

Real-Time PCR

Real-time PCR reactions were performed using a LightCycler device (Roche). The cDNA of the target genes and β-actin were then detected in real time. β-actin mRNA was used as an internal standard. Fast-

Table 1. Sequence similarity and characterization of differentially expressed transcripts

Functional role	Identity	Clone	GenBank Acc. No.	Homology(%)[a]
Cell communication signal transduction	BMP 4	1	XM874429.1	209/209(100%)
	TM4SF	35	XM612305.2	545/546(99%)
	AGTR 1	40	NM174233.2	467/468(99%)
	IGFBP 3	42	AF305712.1	712/713(99%)
	"	49	AF305712.1	715/718(99%)
	"	64	AF305712.1	493/493(100%)
	"	72	M76478.1	271/271(100%)
	"	73	XM869824.1	259/261(99%)
	Colony stimulating factor 1	50	XM876460.1	432/435(99%)
Biological process unknown	Melanoma antigen family D	5	XM590101.2	305/305(100%)
	Cytoplasmic dynein light polypeptied 1	28	AY675078.1	448/449(99%)
	"	30	AY675078.1	348/350(99%)
Energy pathways metabolism	Bifunctional methylenetetra-hydrofolate dehydrogenase	6	XM595711.2	503/503(100%)
	"	18	XM595711.2	512/513(99%)
	Dehydrogenase/reductase	9	XM591168.2	848/852(99%)
	Ketohexokinase	10	XM867407.1	502/504(99%)
	Spermidine synthase	16	XM878596.1	544/544(100%)
	Cytochrome P450	23	NM176644.1	342/345(99%)
	NDST 2	69	NM174777.2	670/675(99%)
Regulation of nucleobase and nucleic acid metabolism	Heterogeneous nuclear ribonucloprotein H1	7	XM877042.1	117/118(99%)
	Brain ribonuclease	19	X59767.1	368/368(100%)
	Fibrillarin	33	XM581057.2	658/665(98%)
	H2A	41	XM870822.1	606/611(99%)
	PPARG	46	NM181024.2	624/625(99%)
Protein metabolism	TFPI	15	XM611595.2	1046/1048(99%)
	CTSZ	21	XM592880.2	620/626(99%)
	Legumain	25	BC111117.1	640/646(99%)
	Cathepsin L	47	BC102312.1	635/635(100%)
Cell growth and/ or maintenance	Collagen type 1	27	BC105184.1	280/285(98%)
	Tubulin a 1	48	XM590059.2	725/727(99%)
	DCN protein	53	BC105175.1	315/316(99%)
	β-tubulin mRNA	57	AY675081.1	884/890(99%)
Immune response	Clusterin	38	NM173902.2	264/266(99%)
	MHC class 1	54	X80934.1	587/604(97%)
Nucleus	Bromodomain-containing proein 4	12	XM875659.1	475/476(99%)
Peptidase activity	Rhomboid-related protein 2	29	XM592863.2	325/327(99%)
Cell motility	Cell adhesion molecule;JCAM	55	XM588961.2	313/314(99%)
Mitochondrial transport	TOM 40 (Haymaker protein)	79	XM587342.2	285/286(99%)
Unclassification	CG12600-PA (LOC532571)	24	XM611687.2	194/198(97%)
	CG13865-PA.3	31	XM598765.2	635/637(99%)
	MCG : 134463 (IMAGE :8041068)	52	BC111337.1	332/332(100%)
	MGC : 127309 (IMAGE : 7949013)	80	BC102354.1	282/284(99%)

[a] The percentages are based on BLAST searches of the GenBank database.
The other numbers in this column show the number of bases (query/subject) that were compared.

Start DNA SYBR green I (Roche, Germany) contains a Taq DNA polymerase enzyme, reaction mix 10X conc. (with reaction buffer, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, and a dNTP mix that includes d-UTP. The fluorescence data were acquired after the extension step during PCR reactions containing SYBR Green 1. Subsequently, PCR products were analyzed by generating a melting curve. The sizes of the PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

Statistical Analysis

Data were analyzed using a Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System, Inc., Cary, NC). The Turkey's Multiple Range Test was used to analyze differences in the mRNA expression assayed by real time PCR. P values of < 0.05 were considered to be statistically significant.

RESULTS

Analysis of DEGs in Cloned Bovine Placenta

To identify genes that were specially expressed at the placenta, mRNA expression profiles of normal and cloned bovine placenta were compared. The analysis generated nearly 80 DEGs, and 42 DEGs were cloned and sequenced. The results were described by the role in process that also had one or more categories (Table 1). Thus, 38 DEGs were known genes, and 4 DEGs were not identified as a function of genes. A Basic Local Alignment Search Tool (BLAST) search revealed genes that were related in terms of cell communication/signal transduction, energy pathway/metabolism, regulation of nucleobase, protein metabolism, cell growth/maintenance, immune response, cell motility, peptidase activity and mitochondrial transport.

Semi-Quantitative RT-PCR

To confirm the results for the ACP RT-PCR analysis, RNAs were subjected to semi-quantitative RT-PCR. The primer sequences, the number of PCR cycles and the annealing temperatures used for the different genes are summarized in Table 2. This led to the selection of genes that were only expressed in the cloned placenta according to the ACP RT-PCR result (Fig. 1). The mRNA expression pattern was then checked using a semi-quantitative RT-PCR method. A slightly different pattern was found compared to the expression obtained in ACP RT-PCR (Fig. 2). This analysis revealed that not all of the target genes followed the ACP RT-PCR results in terms of expression patterns. Two genes (C1 and C4) were only expressed at the cloned placenta in the parturition period. Thus, these genes would have had significant functions in the placenta of the SCNT. However, four genes (C2, C5, C3 and C6) did not show any differences in terms of mRNA expressions.

Real-Time PCR

In addition, the expression patterns of the six selec-

Table 2. Specific primers used for quantification of differentially expressed transcripts

Clone name	Primer	Sequence	Product size (bp)	Annealing temperature (℃)	RT-PCR cycle no.
Collagen	FW	5'-CCCTGACTCTGGAAACAGAC-3'	303	58	27
	RW	5'-TGGTCATGTTCAGTTGGTCA-3'			
Cytoplasmic	FW	5'-GGCCCATATCAAGAAGGACT-3'	469	"	"
dynein I	RW	5'GTTTTTGGATGGATCACTGG-3'			
	FW	5'CCGTGACCTCATTAACTTGG-3'	863	"	"
Fibrillarin	RW	5'CAAAGTGTCCTCCATTACGC-3'			
	FW	5'TTTGCAAGTATAGGGCGTCT-3'	288	"	"
Clusterin	RW	5'GCGTTTCAGAGGAGTTCAGA-3'			
	FW	5'GATCATTGACCTCGTCTTGG-3'	667	"	"
Tubullin α-1	RW	5'GGCTGGGTAAATGGAGAACT-3'			
	FW	5'ACAGTGTTTTCCCACTCAGC-3'	327	"	"
IGFBP-3	RW	5'CCACTCGTCTTCCAGGTTAT-3'			
	FW	5'ATGAGGCTCAGAGCAAGAGA-3'	199	"	23
β-actin	RW	5'ATCTGGGTCATCTTCTCACG-3'			

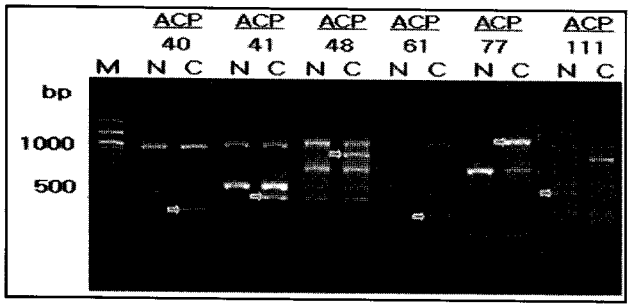

Fig. 1. The results of ACP-based PCR for identification of DEGs from placenta tissues. mRNA from two type of placenta was employed for the synthesis of first-strand cDNA using dT-ACP 1. Using a combination of dT-ACP 2 (reverse primer) and 120 arbitrary ACPs (forward primer), second-strand cDNA sequences were amplified. They were then separated for differentially expressed genes (DEGs) on 1.5 % agarose gels and stained with ethidium bromide for visualization. Bands were excised from the gel for further cloning and sequencing. M: 100 bp ladder marker; N: normal placenta; C: cloned placenta.

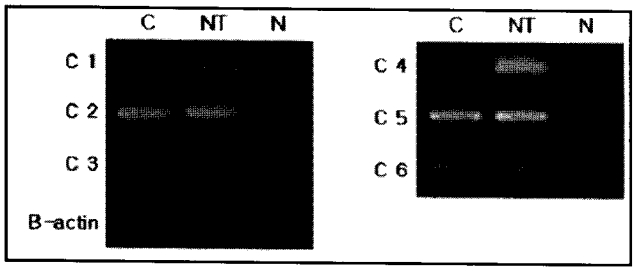

Fig. 2. RT-PCR results for six genes selected. A comparison was made of the expression patterns of the normal and cloned tissues using semi-quantitative RT-PCR. The amplified DNA products were separated on a 2 % agarose gel and stained with ethidium bromide. Collagen (C1), Clusterin (C4) and IGFBP-3 (C6) showed similar expression patterns. Cytoplasmic dynein (C2), Fibrillarin (C3) and Tubullin a-1 (C5) showed similar expression patterns. In addition, Cytoplasmic dynein (C2) and Fibrillarin (C3) showed no significant differences between samples.

ted genes were analyzed using real-time PCR with the specific primer pairs shown in Table 2. Sequence- specific primers are designed to amplify products. To normalize the PCR reaction efficiency, β-actin was used as an internal standard. After normalization with β-actin mRNA levels, the quantitative expression patterns of DEGs were determined (Fig. 3). The results of the real-time PCR and semi-quantitative RT-PCR were identical. This suggests that two genes (collagen type 1 and clusterin) have a same pattern in cloned bovine placenta.

DISCUSSION

As in many mammalian species, the placenta in cattle also produces many proteins, estrogen and progesterone. The production of cloned cattle with somatic cells has been reported in many mammalian thus far. However, the successful rate of obtaining viable offspring from cloning remains at less than 5% (Tamada and Kikyo, 2004). Abnormal placental development and function most likely account for a high proportion of post-implantation fetal losses and may contribute to inadequate mammary gland development in the surrogate recipients and the failure of signaling in preparation for parturition (Eggan *et al.*, 2001; Inoue *et al.*, 2002; Matsuzaki and Shiga, 2002; Wakayama and Yanagimachi, 1998).

In the present study, differentially expressed genes were detected between normal and cloned bovine placentas. In the results, 42 DEGs were cloned and sequenced, 38 DEGs of which were known and 4 DEGs of which were unknown genes. 6 DEGs were identified, and the specific expression was confirmed by RT-PCR (Fig. 2) and real-time quantitative PCR (Fig. 3). Unexpectedly, the ACP RT-PCR results differed slightly from the RT-PCR and real-time PCR results. Thus, the ACP RT-PCR results were not consistent. Sequence results revealed that all DEGs showed a significantly higher sequence similarity (90~100%) with the coding regions of known genes. Thus, genes that are differentially expressed in cloned tissue candidate for regulation of nucleobase (Fibrillarin), cell growth and maintenance (Collagen, Tubullin a-1), immune response (Clusterin), cell communication (IGFBP-3), biological processes unknown (Cytoplasmic dynein I) were found. As shown in Fig. 3, the six selected genes were more commonly detected in cloned placenta than in control placenta. This suggests that these genes have a pivotal role in cloned bovine placenta.

The major collagen of skin, tendon, and bone is the same protein containing 2 alpha-1 polypeptide chains and 1 alpha-2 chain (Lazarides and Lukens, 1971). The fetus contains collagen of a distinctive structure. The genes for types I, II, and III collagens, the interstitial collagens, exhibit an unusual and characteristic structure of a large number of relatively small exons (54 and 108 bp) at evolutionarily conserved positions along the length of the triple helical gly-X-Y portion (Boedtker *et al.*, 1983). Cytoplasmic dynein 1 plays a unique and important role in the initial events of bipolar spindle formation, while any later roles in mitosis may be redundant. Dyenin is a group of microtubule-activated ATPases that serve to convert chemical energy into mechanical energy. They have been divided into the two large subgroups of axonemal and cytoplasmic dyneins (Vaisberg *et al.*, 1993).

Fibrillarin is a component of a nucleolar small nuclear ribonucleoprotein particle thought to participate in the first step of the processing of preribosomal RNA. Aris and Blobel (1991) isolated a cDNA clone encoding human fibrillarin by screening a hepatoma. Okano and

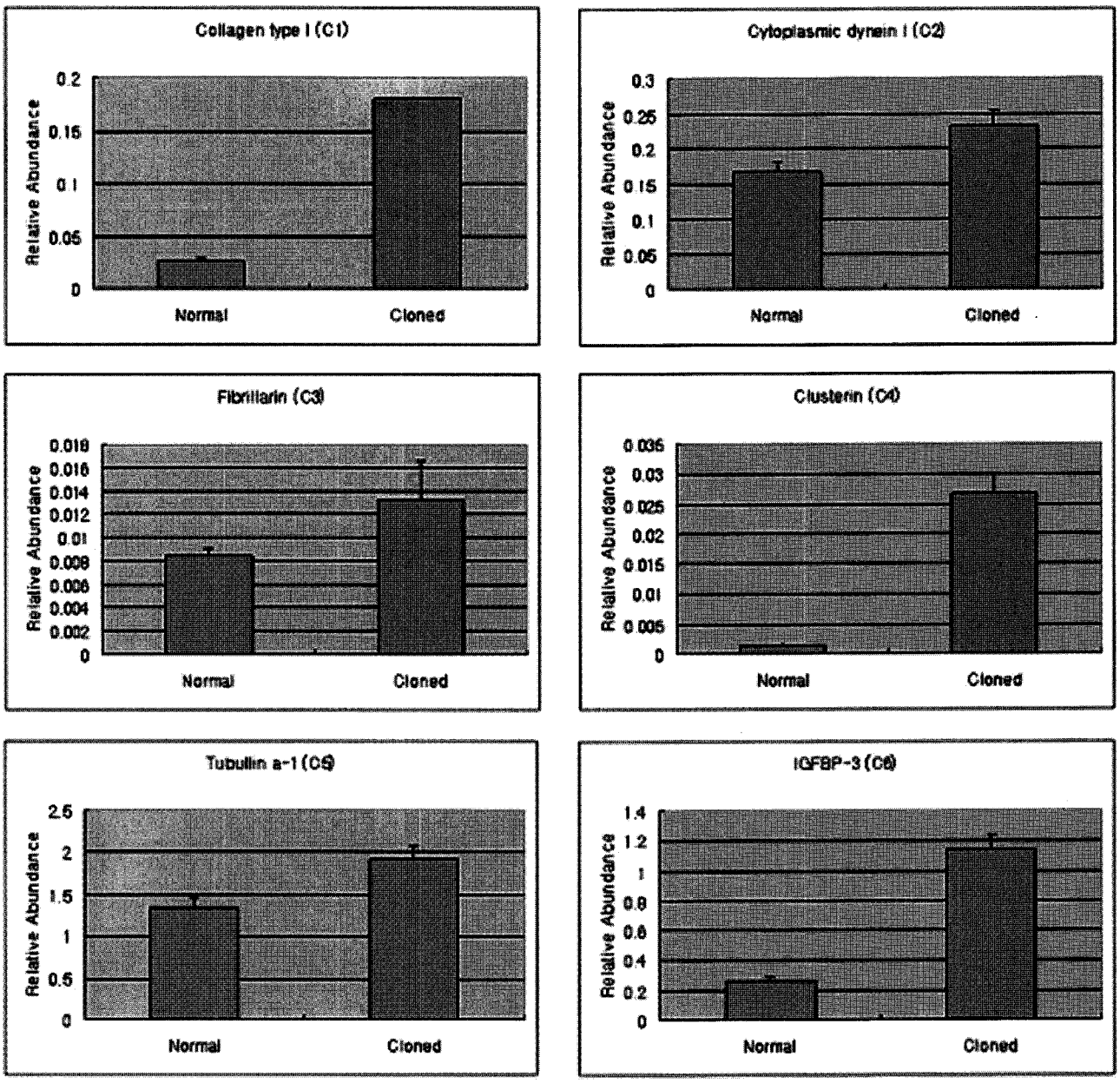

Fig. 3. Relative expression levels of DEGs derived from the placenta by Real-time PCR. Total RNAs from placenta tissues were reverse-transcribed and subjected to real-time quantitative PCR using specific primers (Table 2). All PCRs were conducted in triplicate and normalized for β-actin mRNA expression as an internal standard. Each of these relative values was divided by the value of the calibrator. The relative expression level is presented as an n-fold expression difference compared to the calibrator. Data are shown as means +SE (bars) of triplicate determinations.

Medsger (1990) reported that serum antibodies to the U3 small nuclear ribonucleoprotein were highly specific to systemic sclerosis, were found more frequently in blacks, and were associated with skeletal muscle disease and primary pulmonary arterial hypertension. According to Lewis and Cowan (1990), the alpha-tubulin gene family consists of 15 to 20 dispersed genes, many of which are processed pseudogenes. IGF binding protein plays roles in regulating cell proliferation and apoptosis. The several roles of IGFBP-3 are its function as the major carrying protein for IGF-1 and IGF-2 in the circulation, and its action as a modulator of IGF bioactivity and as a direct growth inhibitor in the extravascular tissue compartment, where it is expressed in a highly regulated manner. Fraser *et al.* (2000) found that IGFBP-3 mRNA is expressed in the endothelium of the human corpus luteum and that the message levels change during luteal development and rescue by human

chorionic gonadotropin. The signal was strong during the early luteal phase, but showed a significant reduction during the mid- and late luteal phases. The level of IGFBP 3 mRNA was higher in the cloned groups than in the control groups. However, Chavatte-Palmer *et al.* (2002) reported a decreased plasma IGF-1 level in nuclear-transferred cloned calves. In contrast, Garry *et al.* (1996) did not find any differences in the plasma level of IGF-1, cortisol, glucagons, and growth hormone between cloned and control groups. Matsuzaki and Shiga (2002) reported that the plasma IGF binding protein level in cloned calves had a greater relative abundance compared with controls. Young *et al.* (2001) suggested parallel increases in the binding capacity of plasma IGFBP-2 and in the expression of the IGFBP-2 level gene in the liver of large offspring fetuses. Thus, these study suggest that an insufficient prepartum rise in the plasma cortisol of cloned calves failed to initiate the switch to an adult mode of the IGF system during late gestation. Thus, the IGF system plays crucial roles in normal embryogenic development and fetal growth.

Clusterin is the major secreted product of Sertoli cells and is thought to play a critical role in spermatogenesis. This protein consists of two 40-kD chains, alpha (34kD) and beta (47kD), covalently joined by disulfide bonds. Fink *et al.* (1993) established a complete physical map of clusterin genes, which spans nearly 20 Kb. Zhang *et al.* (2005) concluded that elevated clusterin levels in human cancers might promote oncogenic transformation and tumor progression by interfering with BAX proapoptotic activities.

As in ungulates of all sorts, the initial period of gestation in cow is characterized by the presence of a large yolk sac. The early nutrition of the embryo is histotrophic, and the early circulation is choriovitelline. Between placentomes, in the intercotyledonary zones, the chorion is avillous. Its adhesion to the endometrium is affected, at least in the cow, by the brush border of the trophoblast. The presence of abundant interdigitating microvilli is a key point, as this relationship usually occurs only between maternal and fetal cells. As in all cases, they form a significant source of histotrophic nutrition (Ramsey, 2006). Especially, IGFBP was reported parallel increase in the binding capacity and in the liver of the large offspring fetuses (Young *et al.*, 2001). Thus, transcriptional regulation may have caused the difference in the plasma IGFBP-3 level between cloned and control calves. Taken together, these results suggest that IGF related-protein is a common condition of large fetuses derived from cloning or *in vitro* embryo transfer, and IGF is one of the important factors responsible for the overweight of cloned calves. In conclusion, the target genes cloned in the present study likely have an influence on the placental formative mechanisms and on fetal development. The genes identified here will provide insight into the mechanisms of placentation.

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