Cloned Placenta of Korean Native Calves Died Suddenly at Two Months after Birth Displays Differential Protein Expression

Kim, Hong Rye, Jae Ku Kang, Hye Ran Lee, Jong Taek Yoon[1], Hwan Hoo Seong[2], Jin Kwan Jung[2], Chang Sik Park and Dong Il Jin[†]

Research Center for Transgenic Cloned Pigs, Chungnam National University

ABSTRACT

Cloned calves derived from somatic cell nuclear transfer (SCNT) have been frequently lost by sudden death at 1 to 3 month following healthy birth. To address whether placental anomalies are responsible for the sudden death of cloned calves, we compared protein patterns of 2 placentae derived from SCNT of Korean Native calves died suddenly at two months after birth and those of 2 normal placentae obtained from AI fetuses. Placental proteins were separated using 2-Dimensional gel electrophoresis.

Approximately 800 spots were detected in placental 2-D gel stained with coomassie-blue. Then, image analysis of Malanie III (Swiss Institute for Bioinformatics) was performed to detect variations in protein spots between normal and SCNT placentae. In the comparison of normal and SCNT samples, 8 spots were identified to be up-regulated proteins and 24 spots to be down-regulated proteins in SCNT placentae, among which proteins were high mobility group protein HMG1, apolipoprotein A-1 precursor, bactenecin 1, tropomyosin beta chain, H^+-transporting ATPase, carbonic anhydrase II, peroxiredoxin 2, tyrosine-rich acidic matrix protein, serum albumin precursor and cathepsin D. These results suggested that the sudden death of cloned calves might be related to abnormal protein expression in placenta.

(Key words : Cloned calves, Placenta, 2-D gel electrophoresis, Mass spectrometry)

INTRODUCTION

Over the past few years, since the production of a cloned lamb form Somatic cells (Wilmut et al., 1997), a variety of species of animals have been cloned (Kato et al., 1997; Wakayama et al., 1998; Baguisi et al., 1999; Wells et al., 1999; Polejaeva et al., 2000; Shin et al., 2002). However, the efficiency of somatic cell nuclear transfer in animals has been exceedingly low with <5% of survival (Wilmut et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Cibelli et al., 2002). Also, the clones that develop to term possess myriad of disorders including obesity, large offspring syndrome, organ defects, respiratory failure, diabetes, and arthritis that might be the causes of sudden death (Young et al., 1998; Hill et al., 2000; McCreath et al., 2000; Ono et al., 2001; Dyer et al., 2002; Tamashiro et al., 2002). In bovine, SCNT is currently an unproductive process, often resulting in embryonic and fatal death as well as high occurrence of abnormalities (Chavatte-Palmer et al., 2002; Hill et al., 2002). These heavy fetal losses and abnormalities have been suggested to link with abnormal development of placentea (Hill et al., 1999).

Abnormal epigenetic reprogramming of the donor nuclei, resulting in aberrant expression of genes needed for proper development and in improper genomic imprinting, has been implicated as a cause for then abnormalities observed clones (Reik et al., 2003; Han, et al., 2003). Reprogramming error on the nucleus of donor cell during SCNT process before their transfer into recipients might due to the abnormal phenotype of cloned embryo that could affect the later development (Tanaka et al., 2001). Chung et al. (2003) reported the evidence that abnormal gene expression patterns such as insufficient imprinting

* This work was supported by grants No R11-2002-100-03001-0 the ERC program of the Korean Science & Engineering Foundation and by a grant from BioGreen 21 Program, Rural Development Administration, Korea.

[1] Genetic Engineering Institute, Hankyong National University.

[2] National Livestock Research Institute, RDA.

† Corresponding author : Dong Il Jin, Research Center for Transgenic Cloned Pigs, Chungnam National University, TEL : +82-42-821-5876; FAX: +82-42-822-6712, E-mail: dijin@cnu.ac.kr

or methylation of genes in reconstituted embryos with SCNT. Also, the low developmental potential of SCNT embryos was related tounsuitable remodeling of chromatin on specific genes by histon acetylation (Cezar et al., 2003; McGraw et al., 2003). These results in abnormal DNA methylation and histon acetylation in cloned embryos showed only fractional evidences that resulted from incomplete reprogramming on the genome of SCNT embryos. Global screening of gene expression is needed to identify the specific genes or gene products causing developmental failure by incomplete reprogramming following SCNT.

Currently, Two-dimensionalgel electrophoresis in combination of MALDI-TOF mass spectrometry is a commonly used technique to separate the complex cellular proteome (Görg et al., 2000). It gives information in a high-throughput mode about the state of the gene products, post-translational modification and expression levels as well as changes in the normal cells with parameters resulting from the effect of external factors or various physiological disorders. By now, these proteomics approaches have not been used to analyze cloned placenta in animals.

In this experiment we have analyzed differential protein patterns between placentae derived from SCNT of Korean Native calves died suddenly at two months old and normal placentae obtained by afterbirth of KNC fetuses fertilized by artificial insemination (AI). And global proteomics approach was exploited by using 2-D gel electrophoresis and mass spectrometry to identify placenta proteins associated with SCNT cloned.

MATERIALS AND METHODS

Placental Samples

We analyzed protein patterns of 2 placentae derived from SCNT of Korean Native calves died suddenly at two months after birth and 2 normal placentae obtained by afterbirth of AI-derived fetuses. The two SCNT-derived sampleswere collected in March 2003 in Hankyong National University. And normal placentae were obtained from Hankyong National University in March 2003. Placental samples were stored in liquid nitrogen just after collection until apply.

Extraction of Solubilized Proteins from Placenta

For 2-D PAGE, soluble proteins were extracted as formerly described (Joubert-Caron et al., 2000), with some modifications by the authors. Briefly, lysis buffer A (containing 1% SDS, 1 mM PMSF, protease inhibitor cocktail (Roche), 100 mM Tris-HCl, pH 7.0) for pH 4~7 was applied to Korean Native cattle placenta with equal volume to tissue weight (ex. 100 μl buffer / 100 mg wet weight) (Joubert-Caron et al., 2000), and samples were suspended with sonication for 15sec, and then chilled in ice. Lysis buffer B (7 M urea, 2 M thiourea, 4% CHAPS, 0.1 M DTT, 1 mM PMSF, protease inhibitor 40 mM Tris-HCl, pH 7.0) was applied to the suspended samples. The samples were shaken gently for 1hr at room temperature with gentle shaking. The solubilized protein extracts were quantified by Bradford pro-tein assay (Bio-Rad) and then final protein samples were stored at -70℃.

2-D Gel Electrophoresis

Precast 18cm IPG strips with pH 4~7 range were obtain Amersham Biosciences. One mg of preparative protein samples used for isoelectric focusing (IEF). The samples was mixed with rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 0.4% DTT, 2% v/v IPG buffer pH 4~7) to total volume of 450 μl (Görg et al., 2000). A mixture of samples was loaded onto an IPG strips (pH 4~7; 180 × 3 × 0.5 mm). The strip was allowed to rehydrate overnight in swelling tray. After rehydration, first dimension, IEF, was performed using an Amersham Pharmacia Multiphor II IEF unit. Automatic isoelectric focusing was carried out for with 1.5 $\times 10^5$ Vh. Voltage started at 100 V and gradually increased to a final voltage of 8000 V. After the first dimensional IEF, IPG gel strip were placed in an equilibration solution (6 M urea, 2% SDS, 50% v/v glycerol, 2.5% acrylamide, 1.5M Tris-HCl, pH 8.8) containing 5 mM TBP for 20min with gentle shaking. The second dimensional separation was performed on 8~16% linear gradient SDS polyacrylamide gels. The gels were placed into an ISO-DALT system (Hoefer Scientific Instruments, San Francisco, CA, USA). The gels (200×250 ×1.0 mm) were run overnight at 10~15 mA per gel until the bromophenol blue marker dye had disappeared at the bottom of the gel.

Staining and Image Analysis of 2-D-Gels

After 2-DE, gels for Mass Spectrometry were stained with method using colloidal coomassie brilliant blue (CBB) G-250. The gels were fixed for 1 hr in fixation solution (30 % v/v methanol, 10% v/v acetic acid) and stained the gel with colloidal CBB G250 for 24hr followed by being destained with 1% acetic acid. The gels were analyzed by Melanie III software (Swiss Institute for Bioinformatics, Geneva, Switzerland). These calculations were applied to the percent volume (%vol) parameter representative of the protein expression. Variations in abundance were calculated as the ratio of average values (%vol) for a group between the two classes. The process for validation of variant proteins was carried out by human operators.

Sample Preparation for MALDI-TOF Mass Spectrometry Analysis

In-gel digestion was performed mainly as previously described (Shevchenko et al., 1996) with some modifications. For coomassie-stained proteins, the gel slab was destained by using 120 μl wash solution (50% v/v ace-

tonitrile, 25 mM NH_4HCO_3, pH 7.8). Then the gel pieces were dehydrated with 50 µl of acetonitrile and dried of for 30 min with a vacuum centrifuge. The dried gel pieces were rehydrated with 5 µl of trypsin solution (trypsin at a concentration of 0.0012 µg/µl in 25 mM NH_4HCO_3, pH 7.8). If needed, additional ammonium bicarbonate buffer was added to completely cover the gel pieces. The digestion was performed at 37℃ overnight. After completion of the digestion, the supernatant was transferred to another Eppendorf tube. To extract residual peptides, the gel pieces were sonicated for 20 min at 30℃ in a solution of 50% acetonitrile/0.5% TFA. Extracted peptides were used for MALDI-TOF analysis. After extracted peptides, mass spectrometric analysis of peptide mass fingerprinting (PMF) was performed using a Voyager-DE STR MALDI-TOF-MS (PerSeptive Biosystems, Framingham, MA, USA). Approximately 1 µl of extracted peptide solution from each gel spot piece and the same volume of matrix solution (10 mg/ ml α-ciano-4-hydroxycinnamic acid, 0.1% v/v TFA, and 50% v/v acetonitrile) were loaded onto a MALDI sample plate (96 well) and crystallized. For each sample, the average of 500 spectra was obtained and scans were performed twice. Spectra were calibrated upon acquisition automatically using an external 3-point calibration. Peak assignment was performed manually using DataExplorer™ software that is part of the Voyager-DE STR MALDI-TOF-MS software package (PerSeptive Biosystems, Framingham, MA, USA) and spectra were saved as peak table files (*.pkt) to search against non-redundant protein sequence database on the internet (SWISS-PROT and/or NCBInr (2004/05/01, Data Bank).

RESULTS AND DISCUSSION

Analysis of Placenta Proteomes by 2-DE

Differential protein expressions in the placentae were evaluated using 2-DE analysis of total protein extracts. To show molecular-level difference in protein that might relate to the functional differences between SCNT and normal placentae, the protein patterns of 2 placentae of cloned Korean Native calves survived for two months after birth and 2 normal placentae obtained by after birth of AI fetuses were compared. For comparison, extract samples were separated in the same conditions. The 2-DE patterns obtained after separation of each 1mg protein sample from control placenta and SCNT placenta were shown in Fig. 1. The MALANIE III software (Swiss Institute for Bioinformatics, Geneva, Switzerland), following the staining with Comassie blue, detected approximately 800~900 protein spots per gel. Image analysis of the two series of Coomassie blue-stained gels permitted us to detail the spots as well as their level of expression (% vol: area * OD*100). Indicated spots in Fig. 1 (A) represent up-regulation, while Fig. 1 (B) represent down-regulation (A-: SCNT specific spots, b-: SCNT down-regulation spots c-: SCNT up-regulation spots, d-: normal specific spots). The expression levels of some variants were statistically analyzed and represented by the mane of % volume histograms (Fig. 2). The magnification of discrepancy protein spots in the normal and SCNT placentae were shown in Fig. 3. In the comparison of the series of gels obtained fromnormal and SCNT samples, at least 36 protein spots were identified to be different in intensity, of which 9 spots were up-regulated proteins in SCNT placenta while 27 spots were down-regulated proteins.

Identification of Differentially Expressed Spots

To probe proteome variation of two samples were excised from gels and identified after tryptic digestion by MALDI-TOF MS. Unprocessed spectra of samples were processed by DataExplorer™ software and resulted to lists of monoisotopic peaks (Fig. 4). The tryptic peptide masses were used to look for protein candidates in web-based searching software, ProFound (http://129.85.19.192/ profound _bin/WebProFound.exe). Thirty-two spots were identified as the known proteins in SWISS-PROT and NCBInr databases. The search results were evaluated on the basis of

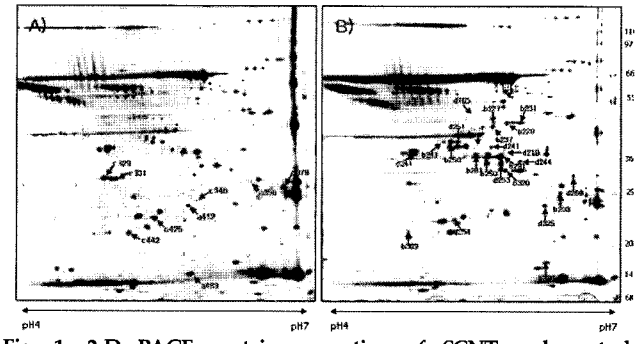

Fig. 1. 2-D PAGE protein separation of SCNT and control placenta as visualized by CBB staining. First dimension was 18Cm 4~7 linear IPG, second dimension was an 8~16% gradient gel. Separation of 1mg of extract. By this way, a mean of 800 spots were enumerated with MELANIE 3 software. Comparison of proteomes of SCNT and control placenta. The spot represents expression variation ((A) SCNT, (B) Control). A Dotted line represents down -regulation and solid line represents up-regulation.

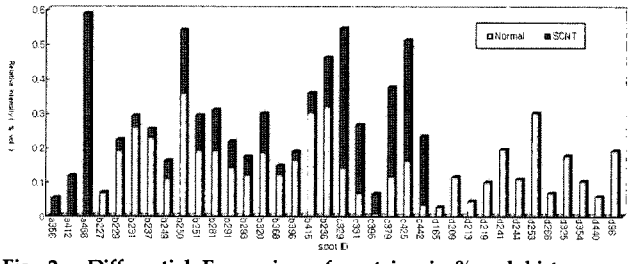

Fig. 2. Differential Expression of proteins is % vol histograms. a: SCNT specific spots, b: SCNT down-regulation spots, c: SCNT up-regulation spots, d: Normal specific spots.

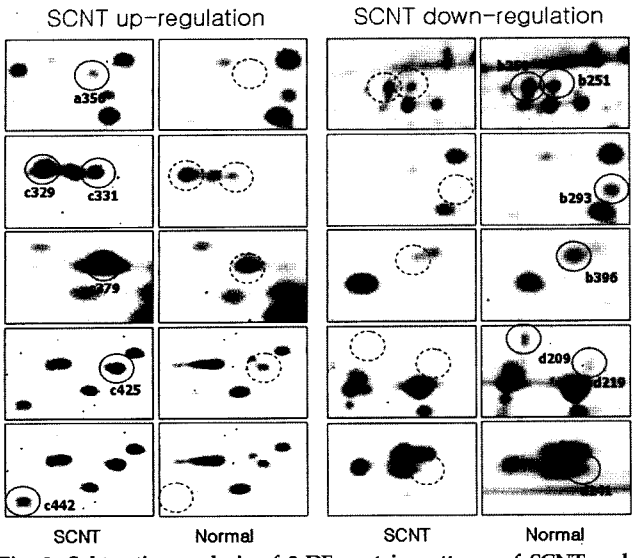

Fig. 3. Subtractive analysis of 2-DE protein patterns of SCNT and control. A black Dotted line represents down-regulation and solid line represents up-regulation.

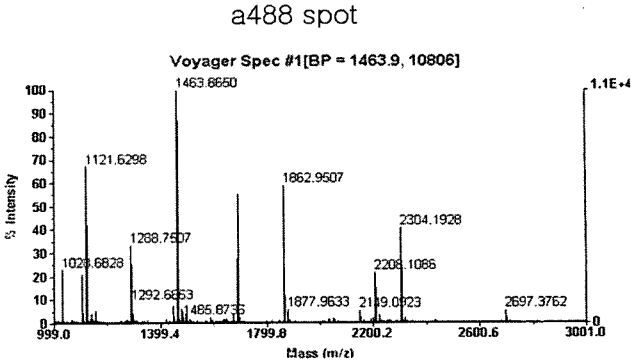

Fig. 4 MALDI-TOF-MS spectra of a488 spots. Database searching allowed the identification of bovine bactenecin 1, as reported in Table 1.

accepted standard that take account into the number of peptides matched to the candidate protein, the difference in the number of matched peptides between the candidate protein and the next best fit, the coverage of the candidate protein's sequence by the matching peptides and agreement of the experimental and theoretical pI and M_r with values (Jensen et al., 1999). Proteins identified and the supporting identification data were represented in Table 1. The spots that correspond to the identified proteins in Table 1 were indicated in Fig. 1.

Characteristics of Identified Proteins

We analyzed a proteomics study in which we compare SCNT and normal placentae. The expression levels of 36 spots differed significantly when placenta protein of normal and SCNT were compared. The threshold, at least 2 times

Table 1. Differentially expressed proteins identified by MALDI-TOF

spot ID	Est'd Z [a)]	accession No	Protein Information	%	pI	kDa
a 356	1.81	P10103	BOVIN HIGH MOBILITY GROUP PROTEIN HMG1 (HMG-1)	29	5.6	25.1
a 412	2.4	P15497	BOVIN APOLIPOPROTEIN A-I PRECURSOR (APO-AI)	48	5.7	30.26
a 488	2.37	P22226	BOVIN CYCLIC DODECAPEPTIDE PRECURSOR (BACTENECIN 1)	55	8.1	18.01
b 227	2.36	P02769	BOVIN SERUM ALBUMIN PRECURSOR	20	5.8	71.76
b 229	2.4	P02769	BOVIN SERUM ALBUMIN PRECURSOR	25	5.8	71.76
b 231	2.37	P02769	BOVIN SERUM ALBUMIN PRECURSOR	37	5.8	71.76
b 237	2.39	P02769	BOVIN SERUM ALBUMIN PRECURSOR	31	5.8	71.76
b 249	2.35	P02769	BOVIN SERUM ALBUMIN PRECURSOR	18	5.8	71.76
b 250	2.4	P02769	BOVIN SERUM ALBUMIN PRECURSOR	29	5.8	71.76
b 251	2.38	P02769	BOVIN SERUM ALBUMIN PRECURSOR	31	5.8	71.76
b 281	2.41	P02769	BOVIN SERUM ALBUMIN PRECURSOR	23	5.8	71.76
b 291	2.28	P02769	BOVIN SERUM ALBUMIN PRECURSOR	23	5.8	71.76
b 293	2.42	P02769	BOVIN SERUM ALBUMIN PRECURSOR	28	5.8	71.76
b 320	2.37	P02769	BOVIN SERUM ALBUMIN PRECURSOR	27	5.8	71.76
b 368	2.36	P02769	BOVIN SERUM ALBUMIN PRECURSOR	15	5.8	71.76
b 396	2.3	P02769	BOVIN SERUM ALBUMIN PRECURSOR	15	5.8	71.76
b 415	2.33	P02769	BOVIN SERUM ALBUMIN PRECURSOR	22	5.8	71.76
b 236	2.36	P02769	BOVIN SERUM ALBUMIN PRECURSOR	31	5.8	71.76
c 329	1.97	P02561	HORSE TROPOMYOSIN BETA CHAIN, PLATELET	31	4.6	28.52
c 331	1.8	S18887	H+-transporting ATPase (EC 3.6.1.35) catalytic chain, renal - pig (fragment)	45	6.2	41.81
c 379	2.4	P00921	BOVIN Carbonic anhydrase II (Carbonate dehydratase II) (CA-II)	53	7.9	28.96
c 425	2.28	Q9BGI3	BOVIN Peroxiredoxin 2	45	5.4	22.28
c 442	1.37	P19427	BOVIN DERMATOPONTIN (TYROSINE RICH ACIDIC MATRIX PROTEIN) (TRAMP) (22 KD EXTRACELLULAR MATRIX PROTEIN) (DERMATAN SULFATE PROTEOGLYCAN-ASSOCIATED PROTEIN 22K)	30	5	22.68
d 209	2.4	P02769	BOVIN SERUM ALBUMIN PRECURSOR	25	5.8	71.76
d 213	2.36	P02769	BOVIN SERUM ALBUMIN PRECURSOR	21	5.8	71.76
d 219	2.36	P02769	BOVIN SERUM ALBUMIN PRECURSOR	16	5.8	71.76
d 241	2.11	AAB26186.1	cathepsin D {EC 3.4.23.5} [cattle, Peptide Partial, 346 aa]	28	5.4	37.69
d 244	2.38	P02769	BOVIN SERUM ALBUMIN PRECURSOR	35	5.8	71.76
d 253	2.41	P02769	BOVIN SERUM ALBUMIN PRECURSOR	32	5.8	71.76
d 266	2.37	P02769	BOVIN SERUM ALBUMIN PRECURSOR	24	5.8	71.76
d 325	2.27	P02769	BOVIN SERUM ALBUMIN PRECURSOR	22	5.8	71.76
d 96	2.36	P02769	BOVIN SERUM ALBUMIN PRECURSOR	37	5.8	71.76

up- or down-regulation, was chosen arbitrarily to exclude proteins that differ in integrated intensity due to small variations occurring randomly during the experimental setup. In the comparison of normal and SCNT samples, a total of 36 protein spots were expressed differentially, of which 8 spots were up-regulated proteins such as high mobility group protein HMG1, apolipoprotein A-1 precursor, bactenecin 1, tropomyosin beta chain, H^+- transporting ATPase, Carbonic anhydrase II, Peroxiredoxin 2, tyrosine rich acidic matrix protein and 24 spots were down-regulated proteins such as serum albumin precursors, cathepsin DAnd 4 spots could not be identified (Table 1). Proteins related with, structural component of chromatin, cholesterol transport, immune system, reversible hydratation of carbon dioxide, signal transduction, signaling cascades of growth factors, regulates actin filament stability, transport and protein degradation are differentially expressed between normal and SCNT placentae.

As listed and summarized in Table 1 and Fig. 1, several proteins could be identified as differentially expressed in SCNT placenta. Many of them were identified as serum albumin precursors that may result from the aberrant angiogenesis processes in SCNT placenta. Failure of reprogramming in developmentally important genes must have affected these differential gene expressions in SCNT placenta. In conclusion, abnormal gene expressions in SCNT placenta may be related with the sudden death of cloned calves.

REFERENCES

1. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y (1999): Production of goats by somatic cell nuclear transfer. Nat Biotechnol 17:456-461.
2. Cezar GG, Bartolomei MS, Forsberg EJ, First NL, Bishop MD, Eilertsen KJ (2003): Nome-wide Epigenetic alterations in cloned bovine fetuses. Biol Reprod 68:1009-14.
3. Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP (2002): Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 66:1596-1603.
4. Chung YG, Ratnam S, Chaillet JR, Latham KE (2003): Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. Biol Reprod 69: 146-53.
5. Cibelli JB, Campbell KH, Seidel GE, West MD, Lanza RP (2002): The health profile of cloned animals. Nat Biotechnol 20:13-14.
6. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C (1998): Ponce de Leon FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280:1256-1258.
7. Dyer O (2002): Dolly's arthritis dents faith in cloning. BMJ 324:67.
8. Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000): The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21:1037-1053.
9. Han YM, Kang YK, Koo DB, Lee KK (2003): Nuclear reprogramming of cloned embryos produced *in vitro*. Theriogenology 59:33-44.
10. Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, ThompsonJA, Winger QA, Westhusin ME (2000): Evidence for placental abnormalityas the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 63:1787-1794.
11. Hill JR, Edwards JF, Sawyer N, Blackwell C, Cibelli JB (2001): Placental anomalies in a viable cloned calf. Cloning 3:83-88.
12. Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller MW, Thompson JA, Looney CR, Westhusin ME, Robl JM, Stice S (1999): Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). Theriogenology 51:1451-1465.
13. Hill JR, Schlafer DH, Fisher PJ, Davies CJ (2002): Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. Biol Reprod 67:55-63.
14. Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME (2000): Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol Reprod 62:1135-1140.
15. Jensen ON, Wilm M, Shevchenko A, Mann M (1999): Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Methods Mol Biol 112:513-530.
16. Joubert-Caron R, Le Car JP, Montandon F, Poirier F, Pontet M, Imam N, Feuillard J, Bladier D, Rossier J, Caron M (2000): Protein analysis by mass spectrometry and sequence database searching: a proteomic approach to identify human lymphoblastoid cell line proteins. Electrophoresis 21:2566-2575.
17. McCreath KJ, Howcroft J, Campbell KH, Colman A, SchniekeAE, Kind AJ (2000): Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066-1069.
18. McGraw S, Robert C, Massicotte L, Sirard MA (2003): Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. Biol Reprod 68:383-9.
19. Ono Y, Shimozawa N, Ito M, Kono T (2001): Cloned mice

from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol Reprod 64:44-50.
20. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407:86-90.
21. Reik W, Santos F, Dean W (2003): Mammalian epigenomics: reprogramming thegenome for development and therapy. Theriogenology 59:21-32.
22. Tamashiro KL, Wakayama T, Akutsu H, Yamazaki Y, Lachey JL, Wortman MD, Seeley RJ, D'Alessio DA, Woods SC, Yanagimachi R, Sakai RR (2002) Cloned mice have an obese phenotype not transmitted to their offspring. Nat Med 8:262-267.
23. Tanaka S, Oda M, Toyoshima Y, Wakayama T, Tanaka M, Yoshida N, Hattori N, Ohgane J, Yanagimachi R, Shiota K (2001): Placentomegaly in cloned mouse conception caused by expansion of the spongiotrophoblast layer. Biol Reprod 65:1813-1821.
24. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R (1998): Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369-374.
25. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997): Viable offspring derived from fetal and adult mammalian cells. Nature 385:810-813.
26. Young LE, Sinclair KD, Wilmut I (1998): Large offspring syndrome in cattle and sheep. Rev Reprod 3:155-163.
(Received: 30 December 2004 / Accepted: 28 February 2005)