Reprod Dev Biol 33(4) : 243-248 (2009)

A Comparative Study of Protein Profiles in Porcine Fetus Fibroblast Cells with Different Confluence States

Rong Xun Han, Hong-Rye Kim, Yunfei Diao, Myung Youn Kim, Chang Sik Park and Dong Il Jin[†]

Division of Animal Science & Resources, Research Center for Transgenic and Cloned Pigs, Chungnam National University, Daejeon 338-708, Korea

ABSTRACT

To examine the differential expression of proteins during the cycling (70～80% confluences) and G0/G1 (full confluences) phases in porcine fetal fibroblast cells, we used a global proteomics approach by 2-D gel electrophoresis (2-DE) and MALDI-TOF-MS. Cycling cell were harvested at approximately 70% to 80% confluent state while cells in G0/G1 phase were recovered after maintenance of a confluent state for 48 hr. Cellular proteins with isoelectric points ranging between 3.0～10.0, were analyzed by 2-DE with 2 replicates of each sample. A total of approximately 700 spots were detected by 2-D gels stained with Coomassie brilliant blue. On comparing the cell samples obtained from the cycling and G0/G1 phases, a total of 13 spots were identified as differentially expressed proteins, of which 8 spots were up-regulated in the cycling cell and 5 were up-regulated in the G0/G1 phase. Differentially expressed proteins included K3 keratin, similar to serine protease 23 precursor, protein disulfide-isomerase A3, microsomal protease ER-60, alpha-actinin-2, and heat-shock protein 90 beta. The identified proteins were grouped on the basis of their basic functions such as molecular binding, catabolic, cell growth, and transcription regulatory proteins. Our results show expression profiles of key proteins in porcine fetal fibroblast cells during different cell cycle status.

(Key words : Porcine fetal fibroblast cells, Cell cycle, 2-D gel electrophoresis, Mass spectrometry)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a technique used for preserving endangered species, cloning animals, and producing transgenic animals (Loi *et al*., 2001; Wilmut *et al*., 2002). SCNT has been successfully used to produce cloned offsprings in a number of mammalian species (Khammanit *et al*., 2008). In SCNT, donor cell cycle stage is an important factor for successfully cloning the animal. Previous studies have carefully selected cells in G1 and G0, and compared cloning efficiency with those cells (Kasinathan *et al*., 2001). Three calves were obtained from cells in G1, while no calves were derived with fetal cells in G0. Other studies, however, showed a 3～7-fold increase in the production of viable offspring with embryos derived from adult quiescent cells (G0) (Wilmut *et al*., 2002). Donor cells at mitotic phase may be considered optimally compatible with metaphase II (MII) oocytes and have resulted in improved rates of blastocyst development using murine embryonic stem (ES) cells, as opposed to interphase nuclei (Zhou *et al*., 2001).

Since the first cloned mammal was produced using SCNT, have been inconsistencies over the importance of the donor cell cycle stage (Wilmut *et al*., 1997). Several studies have shown that when G0/G1 phase nuclei are transferred into MII oocytes, the development of reconstituted embryos is improved because redundant replication of DNA is avoided and the genome can readily be reprogrammed in cells in the G0/G1 phase (Campbell, 1999; Rideout *et al*., 2001; Yu *et al*., 2003). The cell cycle stages of cultured cells can be synchronized by serum starvation, contact inhibition, or chemical treatments (Cho *et al*., 2005). To synchronize cultured cells in the G2/M phase of the cell cycle, the microtubule inhibitor, colchicine, has been used to increase the proportion of G2/M cells in pig mammary fibroblasts cells (Boquest *et al*., 1999).

Currently, two-dimensional gel electrophoresis (2-DE) in combination with MALDI-TOF mass spectrometry (MALDI-TOF-MS) is a frequently applied technique that isolates complex cellular matter or biological fluid proteomes (Görg *et al*., 2000). This technique gives informa-

* This work was supported by a grant No. R11-2002-100-03001-0 from the ERC program of the Korean Science & Engineering Foundation and by a grant #20070401034031 from the BioOrgan Program, Rural Development Administration, Korea.

[†] Corresponding author : Phone: +82-42-821-5876, E-mail: dijin@cnu.ac.kr

tion about the posttranslational modifications and expression level of gene products in normal cells with parameters resulting from the influence of environmental factors. Recent studies have investigated the proteomics of the mouse MII oocyte protein expression pattern (Ma *et al.*, 2008), and the extracellular signal-regulated kinase pathway proteins during the different cell cycle stages have been compared (Roberts *et al.*, 2006). Li *et al.* (2003) used two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to identify the cyclin D1/E increased expression in the human umbilical vein endothelial cells exposed to tumor conditioned medium during cell cycle progression. However, to the best of our knowledge, a direct comparison of porcine fetal fibroblast cell proteins between the cycling cell and the G0/G1 phases has not been reported. This study was designed to identify differential protein expression patterns between the cycling cell and the G0/G1 phase of porcine fetal fibroblast cells using a proteomics approach based on 2-DE and MALDI-TOF MS.

MATERIALS AND METHODS

Cells and Cell Culture

A porcine fetus was obtained from a pregnant sow at day 35 after insemination, and the tissue was cut into small pieces with fine scissors. The tissue was washed 3 times and incubated for 10 min at 37℃ in phosphate buffered solution (PBS) containing 0.05% trypsin and 0.5 mM EDTA. This suspension was then centrifuged at 500 ×g for 10 min. The cell pellet was resuspended in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) medium supplemented with 75 μg/ml penicillin G, 50 μg/ml streptomycin, 5% (v/v) fetal bovine serum (FBS), and 5% (v/v) fetal calf serum (FCS) (Gibco, 26-010-074) and cultured at 38.5℃. Before nuclear transfer, cells were washed twice in PBS and treated with 0.25% trypsin and 0.5 mM EDTA for 2 minutes in a 38.5℃ incubator for single cell isolation. Cells in the cycling cell were harvested at approximately 70% to 80% confluent state, while cells in the G0/G1 phase were recovered after maintenance of a confluent state for 48 hr (Kasinathan *et al.*, 2001).

Cell Lysate Preparation

Cell lysates were prepared from porcine fetal fibroblast cells in the cycling and G0/G1 phases, by scraping the cells from culture dishes, followed by centrifugation (500 ×g, 4℃ for 10 min) and solubilization of the pellets in a buffer solution containing urea (8 M), CHAPS (4%), DTT (70 mM), and resolytes 3～10 (2%). This mixture was centrifuged at 12,000 rpm, 4℃, for 15 min. The protein concentration was determined using the Bradford method, and stored in 1-mg aliquots at −70℃ until later use.

2-D Gel Electrophoresis

Precast 18 cm IPG strips with pH in the 3～10 range were obtained from Amersham Biosciences. For isoelectric focusing (IEF), 2 mg of each protein sample was mixed with modified rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2.5% DTT, 10% isopropanol, 5% glycerol, 2% v/v IPG buffer; pH 3～10) to obtain a total volume of 450 μl (Görg *et al.*, 2000). A mixture of samples was loaded onto an IPG strip (pH 3～10; 180×3×0.5 mm). The strip was allowed to rehydrate overnight in a swelling tray. Following rehydration, the first dimension IEF, was performed using an Amersham Pharmacia Multiphor II IEF unit. Automatic IEF was carried out at 1.5×10^5 Vh. The initial applied voltage was 100 V, and it was gradually increased to a final voltage of 8,000 V. After the first dimension IEF, an IPG gel strip was placed in an equilibration solution (6 M urea, 2% SDS, 50% v/v glycerol, 2.5% acrylamide, 1.5 M Tris-HCl; pH 8.8) containing 5 mM TBP, for 20 min with gentle shaking. The second dimensional separation was performed on 8～16% linear gradient SDS polyacrylamide gels. The gels were placed in 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, the gels were stained using colloidal Coomassie brilliant blue (CBB) G-250. The gels were fixed for 1 hr in a fixation solution (30% v/v methanol, 10% v/v acetic acid) and stained with colloidal CBB G-250 for 24 hr followed by destaining with 1% acetic acid. These gels were then analyzed using Melanie III software (Swiss Institute for Bioinformatics, Geneva, Switzerland). These calculations were applied to the percent-volume parameter, representative of protein expression. Variations in abundance were calculated as the ratio of average values (% vol), between the 2 samples. The process for the validation of variant proteins was carried out by human operators.

Sample Preparation for MALDI-TOF Mass Spectrometry Analysis

In-gel digestion of proteins was performed as previously described (Shevchenko *et al.*, 1996), with some modifications. For CBB-stained proteins, the gel slab was destained by using 120 μl of wash solution (50% v/v acetonitrile, 25 mM NH_4HCO_3; pH 7.8). The gel pieces were then dehydrated with 50 μl of acetonitrile and dried for 30 min in a vacuum centrifuge. The dried gel pieces were rehydrated with 5 μl of trypsin solution (0.0012 μg/μl trypsin in 25 mM NH_4HCO_3; pH 7.8)

and digested at 37℃ overnight. After complete 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% (trifluoroacetic acid) TFA. The extracted peptides were analyzed using MALDI-TOF. 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 the extracted peptide solution from each gel spot piece and the same volume of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% v/v TFA, and 50% v/v acetonitrile), were loaded onto a 96-well MALDI sample plate and crystallized. For each sample, an average of 500 spectra was obtained, and the scans were performed twice. The spectra were automatically calibrated upon acquisition, using an external three-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). The spectra were saved as peak table files to search against an online non-redundant protein sequence database on the internet (SWISS-PROT and/or NCBI [2009/11/03, Data Bank]).

RESULTS

Analysis of Pig Fibroblast Cells Using 2-DE

Differential protein expressions in the porcine fetal fibroblast cells of the cycling and G0/G1 phase were evaluated using 2-DE. The cell samples were obtained from a porcine fetus that was obtained from a pregnant sow at day 35 after insemination. The 2DE images obtained from each 1-mg protein sample extracted from the cycling and G0/G1 phase cells are shown in Fig. 1. In 2-DE, a large number of proteins were separated across the entire pI and MW ranges of the gel. After staining with CBB, approximately 600~700 protein spots per gel were detected using the Image Master 5.0 software. The protein spots that showed differential expression in the cycling and G0/G1 phases are shown in Fig. 2. A total of 13 spots were identified as differentially expressed proteins, of which 8 spots were up-regulated proteins in cycling cell and 5 spots were down-regulated proteins in cycling cell of porcine fetal fibroblast cells.

Identification of Differentially Expressed Proteins

To identify the proteins in the differentially expressed spots, the unprocessed spectra of samples after tryptic digestion and MALDI-TOF MS, were evaluated using the DataExplorer™ software; this resulted in the capture of monoisotopic peaks (Fig. 3). The tryptic peptide masses were used to identify protein candidates using the web-based searching software ProFound (http://129.85.19.192/profoundbin/WebProFound.exe). Thirteen spots were identified as known proteins via SWISS-PROT and NCBI databases. The search results were

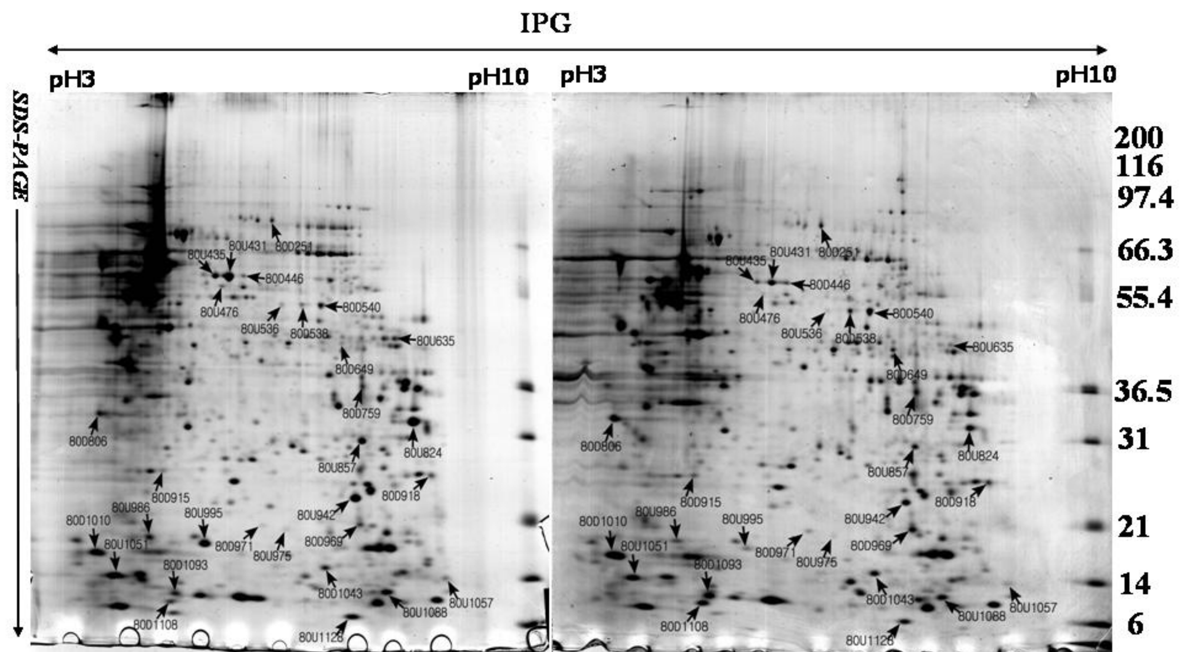

Fig. 1. 2-D PAGE protein separation of porcine fetal fibroblast cell proteins in the cycling and G0/G1 phases, as visualized with CBB staining. The first dimension IEF was carried out in 18 cm 3~10 IPG strips, and the second dimension was performed in an 8~16% gradient gel. A mean of spots was enumerated with ImageMaster 5.0 software.

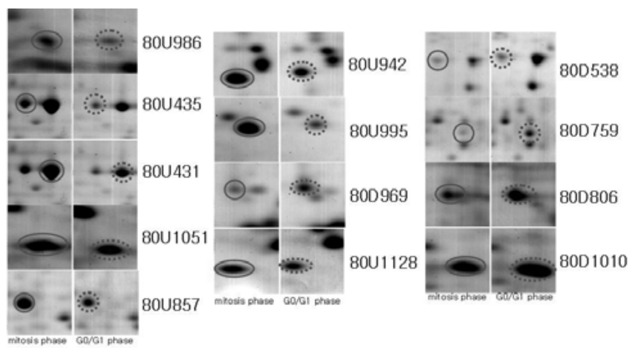

Fig. 2. Differentially expressed protein spots in the cycling and G0/G1 phases of porcine fetal fibroblast cells. The black dotted line represents down-regulation and a solid line represents up-regulation. 80D: down-regulated spots during cycling phase, 80U: up-regulated spots during cycling phase.

evaluated on the basis of accepted standards that take into account the number of peptides matched to the candidate protein, coverage of a candidate protein's sequence by matching peptides, and agreement of the experimental and theoretical pI and M_r with derived values.

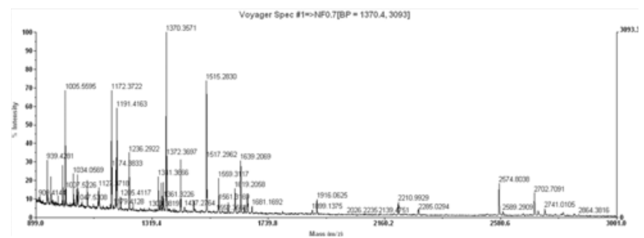

Fig. 3. MALDI-TOF-MS spectra obtained for 80U435 spots. Database searching allowed the identification of protein disulfide-isomerase A3, as seen in Table 1.

The identified proteins are presented in Table 1. On comparing the cell samples obtained from the cycling and G0/G1 phases, we identified 13 spots as differentially expressed proteins, of which 8 spots were up-regulated proteins in+ the cycling cell samples (e.g., similar to serine protease 23 precursor, protein disulfide- isomerase A3, and microsomal protease ER-60) and 5 spots were up-regulated proteins in the G0/G1 phase cell samples (e.g., alpha 2 actinin, heat-shock protein 90

Table 1. Proteins differentially expressed in the cycling and the G0/G1 phases in fetal fibroblast cells, as identified by MALDI-TOF

Spot ID	Est'd Z	Accession No.	Protein information and sequence analyses tools (T)	%	pI	kDa	Protein function
Up-regulation spot in							
80D538	1.15	gi|47155480	Alpha-actinin-2	17	5.8	68.47	Actin binding
80D759	1.15	gi|12082134	Heat-shock protein 90 beta	16	5	82.37	ATP binding
80D806	1.14	gi|1332717	Acylamino acid-releasing enzyme	12	5.3	82.52	Transcription regulation
80D969	1.24	gi|13561970	Follicle stimulating hormone beta subunit	56	5.6	15.85	Hormone activity
80D1010	1.88	gi|28461187	Myosin, light chain 6, alkali, smooth muscle and non-muscle	49	4.6	17.13	Regulatory light chain of myosin
Up-regulation spot in							
80U431	1.65	gi|1583929	Microsomal protease ER-60	15	5.9	57.14	Isomerase
80U435	2.16	gi|729433	Protein disulfide-isomerase A3	27	6.2	57.41	Isomerase
80U857	1.09	gi|1305529	Mx protein homolog	12	6	76.12	Antiviral defense
80U942	2.23	gi|729207	Alpha-crystallin B chain	59	6.8	20.09	Response to heat
80U995	1.27	gi|57102468	Similar to serine protease 23 precursor	33	9.8	43	Proteolysis
80U986	1.32	gi|425551	K3 keratin	15	7.8	64.61	Epithelial cell differentiation
80U1051	1.7	gi|47716872	Galectin-1	44	5.1	15.01	Galactoside binding
80U1128	1.21	gi|51701919	Ubiquitin	41	6.6	8.55	Transcription regulation

[a] The Z score is the distance to the population mean in units of standard deviation. It also corresponds to the percentile of the search in the random match population. Conceptually, this "95th percentile" is different from "95% confidence," in that the search is a correct identification. (The following is a list of Z scores and its corresponding percentiles in an estimated random match population: [(Z: percentile) 1.282: 90.0, 1.645: 95.0, 2.326: 99.0, 3.090: 99.9].)

beta, and follicle stimulating hormone beta subunit). In conformity with the annotation from the UniProt Knowledgebase (Swiss-Prot/TrEMBL) and the Gene Ontology Database, the identified proteins were grouped on the basis of their basic functions such as molecular, catabolic, cell growth, and transcription regulatory proteins.

DISCUSSION

In this study, the expression patterns of porcine fetal fibroblast cell proteins were compared between the cycling and G0/G1 phases using proteomics analysis. Through extensive analysis with MALDI-TOF, several proteins differentially expressed in the porcine fetal fibroblast cell were identified. The expression levels of 13 spots on 2-DE gel differed significantly between the fibroblast cell samples from the cycling and G0/G1 phases. Human fibroblast cells are known to contain 170 protein isoforms or fragments (Bumke *et al*., 2003). Boraldi *et al*. (2003) reported proteomics analysis of the normal human dermal fibroblasts cells. However, differential protein expressions among the porcine fetal fibroblast cells in the different growing phases have not been reported.

In the current study, one spot (80D538) that was up-regulated during the G0/G1 phase in porcine fetal fibroblast cells was identified as alpha-actinin-2. Alpha-actinin is an actin-binding protein with multiple roles in different cell types. In nonmuscle cells, the cytoskeletal isoform is found along with the microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane (Youssoufian *et al*., 1990). Mills *et al*. (2001) observed that murine actinin-2 and actinin-3 are differentially expressed, spatially and temporally, during embryonic development, and in contrast to humans, alpha-actinin-2 expression did not completely overlap alpha-actinin-3 in the postnatal skeletal muscle, suggesting an independent function. Meanwhile, one spot that was up-regulated in the cycling cell in the porcine fetal fibroblast cells (80U986) was identified as K3 keratin. K3 keratin belongs to a large family of approximately 30 distinct peptides that combine to form the intermediate filaments (10-nm fibers) present in the cytoskeleton in epithelial cells. Type I cytokeratins are acidic and range from a molecular weight of 40 to 56.5 kD, whereas type II cytokeratins are neutral or basic and have molecular weights of 53 to 67 kD [Moll *et al*., 1982]. Specific pairs of heterotypic keratin chains are coexpressed during the various stages of epithelial differentiation. The gene for cytokeratin 3 (*KRT3*), a member of the type II subfamily, is expressed together with *KRT12* during "corneal-type" differentiation (Schermer *et al*., 1986). Future work should further examine the relationship between these proteins and the different porcine fetal fibroblast cell cycle stages.

Proteomics analysis has allowed us to visualize protein expression patterns and profiles in the porcine fetal fibroblast cell during the different cell cycle stages. The results of the current study present composite profiles of key cell proteins, the expression of which is affected by the cell cycle stages; the results also reveal that several functional class proteins are up- and down-regulated in the different cell cycle stages of porcine fetal fibroblast cells. The differential expression of these proteins may have a useful for implication in SCNT efficiency.

REFERENCES

1. Boquest AC, Day BN, Prather RS (1999): Flow cytometric cell cycle analysis of cultured porcine fetal fibroblast cells. Biol Reprod 60:1013-1019.
2. Boraldi F, Bini L, Liberatori S, Armini A, Pallini V, Tiozzo R, Ronchetti IP, Quaglino D (2003): Normal human dermal fibroblasts: proteomic analysis of cell layer and culture medium. Electrophoresis 24(7-8):1292-1310.
3. Bumke MA, Neri D, Elia G (2003): Modulation of gene expression by extracellular pH variations in human fibroblasts: a transcriptomic and proteomic study. Proteomics 3(5):675-688.
4. Campbell KH (1999): Nuclear equivalence, nuclear transfer and the cell cycle. Cloning 1:3-15.
5. Cho SR, Ock SA, Yoo JG, Mohana kumar B, Choe SY, Rho GJ (2005): Effect of confluent, roscovitine treatment and serum starvation on the cell-cycle synchronization of bovine foetal fibroblasts. Reprod Domest Anim 40:171-176.
6. 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.
7. Kasinathan P, Knott JG, Wang Z, Jerry DJ, Robl JM (2001): Production of calves from G1 fibroblasts. Nature Biotechnol 19:1176-1178.
8. Li A, Li H, Jin G, Xiu R (2003): A proteomic study on cell cycle progression of endothelium exposed to tumor conditioned medium and the possible role of cyclin D1/E. Clin Hemorheol Microcirc 29(3-4):383-390.
9. Loi P, Ptak G, Barboni B, Fulka, Jr J, Cappai P, Clinton M (2001): Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat Biotechnol 19:962-964.
10. Ma M, Guo X, Wang F, Zhao C, Liu Z, Shi Z, Wa-

ng Y, Zhang P, Zhang K, Wang N, Lin M, Zhou Z, Liu J, Li Q, Wang L, Huo R, Sha J, Zhou Q (2008): Protein expression profile of the mouse metaphase-II oocyte. J Proteome Res 7:4821-4830.

11. Mills M, Yang N, Weinberger R, Vander Woude DL, Beggs AH, Easteal S, North K (2001): Differential expression of the actin-binding proteins, alpha-actinin-2 and -3, in different species: implications for the evolution of functional redundancy. Hum Mol Genet 10:1335-1346.
12. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R (1982): The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31:11-24.
13. Rideout WM 3rd, Eggan K, Jaenisch R (2001): Nuclear cloning and epigenetic reprogramming of the genome. Science 293:1093-1098.
14. Roberts EC, Hammond K, Traish AM, Resing KA, Ahn NG (2006): Identification of G2/M targets for the MAP kinase pathway by functional proteomics. Proteomics 6:4541-4553.
15. Schermer A, Galvin S, Sun TT (1986): Differentiation-related expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49-62.
16. Wilmut I, Beaujean N, de Sousa PA, Dinnyes A, King TJ, Paterson LA, Wells DN, Young LE (2002): Somatic cell nuclear transfer. Nature 419:583-586.
17. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997): Viable offspring derived from fetal and adult mammalian cells. Nature 385:810-813.
18. Youssoufian H, McAfee M, Kwiatkowski DJ (1990): Cloning and chromosomal localization of the human cytoskeletal alpha-actinin gene reveals linkage to the beta-spectrin gene. Am J Hum Genet 47 (1):62-72.
19. Yu YS, Sun XS, Jiang HN, Han Y, Zhao CB, Tan JH (2003): Studies of the cell cycle of *in vitro* cultured skin fibroblasts in goats: work in progress. Theriogenology 59:1277-1289.
20. Zhou Q, Jouneau A, Brochard V, Adenot P, Renard JP (2001): Developmental potential of mouse embryos reconstructed frommetaphase embryonic stem cell nuclei. Biol Reprod 65:412-419.

(Received: 30 November 2009 / Accepted: 15 December 2009)