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Research Article

Transgenic Bovine Nuclear Transfer Embryos from Adult Somatic Cell Lines*

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Abstract: This study was performed to examine whether adult fibroblast cells can be manipulated genetically and whether clonal lines derived from those cells can support embryonic development. A primary adult cell line was established from the ear of an aged cow. Adult cells with a plasmid containing the enhancer green fluorescence protein (EGFP) gene, and non-transfected cells were used for cloning. Green fluorescence expression was observed in 35/49 (71.4%) adult clones. The developmental rates of embryos were significantly lower for cell lines expressing EGFP (11.4%) than for non-transfected cells (20.1%, $P < 0.05$). However, there was no decrease in nuclear transfer (NT) developmental rates (21.5%) when donor nuclei from EGFP transfected cell lines not expressing EGFP but retaining neomycin-resistance gene expression were used as donor nuclei. The NT embryos from transfected and non-transfected cell lines had similar morphology and cell numbers. The results indicated that adult cells can complete clonal propagation including transfection and selection and can be used to produce transgenic NT embryos.

Key Words: Bovine, nuclear transfer, adult fibroblast, green fluorescent protein

Erişkin Vücut Hücresi Hatlarından Elde Edilen Transgenik Sığır Nükleer Transfer Embriyoları

Özet: Bu çalışma olgun hayvandan elde edilen fibroblast hücrelerinin genetik değişikliğe uğratılıp uğratılamıyacağını ve bu hücrelerden geliştirilen hücre hatlarının embriyonik gelişimi destekleyip desteklemiyeceğini araştırmak için yapıldı. Hücre hattı yaşlı bir sığırın kulak dokusundan geliştirildi. Yeşil floresan protein genini (EGFP) taşıyan hücreler ve transfekte edilmemiş olgun hücreler klonlama için kullanıldı. Yeşil floresanın expresyonu olgun hücre klonlarının 35/49 (% 71,4)'ünde tespit edildi. Yeşil floresan proteini exprese eden hücrelerden oluşan embriyoların gelişme oranı (% 11,4) transfekte edilmemiş hücrelerden oluşanlardan (% 20,1, $P < 0,05$) daha düşüktü. Bununla beraber EGFP ile transfekte edilen ve EGFP'yi exprese etmeyen ancak neomisin dirençlilik geninin taşıyan hücrelerin nükleusları kullanıldığında, nükleer transfer embriyolarının gelişme oranında azalma olmadı (% 21,5). Transfekte edilmiş ve transfekte edilmemiş hücrelerden oluşan embriyolar benzer morfoloji ve hücre sayısına sahipti. Bu sonuçlar olgun hücrelerin transfeksiyon ve seleksiyonu içeren klonal çoğalmayı tamamlayabileceğini ve bu hücrelerin transgenik nükleer transfer embriyolarının üretiminde kullanılabileceğini gösterdi.

Anahtar Sözcükler: Sığır, nükleer transfer, olgun fibroblast, yeşil floresan protein

Introduction

Transgenic farm animals have important applications for research as well as for commercial purposes. Genetically modified animals may be successfully produced in a number of species by the injection of the gene into the pronucleus of fertilized oocytes. However, transgene DNA integrates into their genome in less than 5-10% of animals. In addition, transgenic animals generated using pronuclear microinjection are commonly mosaic, an integrated transgene is not present in all cells because the timing of integration is random (1). The birth of calves following nuclear transfer (NT) with cultured transgenic fetal cell lines demonstrates the possibility of producing cloned transgenic cattle (2). More recently, genes were targeted to specific sites in the genome using a homologous recombination in fetal ovine fibroblast cells and live lambs were generated from these cells (3). A key advantage of using NT to produce transgenic and gene-

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targeted livestock is the use of cultured transgenic cells as donor nuclei. Genetically transformed cells can be selected in vitro and cells only with stably integrated transgenes are used as donor cells. All animals created from such cells should be transgenic.

In animal agriculture, additional genetic selective advantages are gained when genetic enhancements can be made in already known superior genotypes. To gain this advantage, adult, genetically enhanced donor cells must be used in the NT procedure. The β galactosidase gene has been widely used as a reporter gene for monitoring gene expression but enhanced green fluorescence protein (EGFP) is a more useful reporter system because it allows the detection of gene expression in living cells (4,5). Recently, several reports have described the use of GFP variants on producing transgenic NT offsprings or embryos in goats (6), pigs (7,8) and cattle (9,10). Therefore, GFP was chosen as a marker gene for this study.

In cattle, sheep, pigs and goats, fetal cells have been used for transgenic livestock animal production because of their rapid growth and potential for many cell divisions before senescence in culture (2,11-13), whereas adult somatic cell cloning has primarily been used to replicate a particular female (14-16) or male (17,18) genotype. To date, there are limited studies using adult transgenic donor cells in the nuclear transfer procedure. Recently, it has been shown that adult somatic cells could remain totipotent for cloning after long-term culture suggested the feasibility of genetic manipulation (19). In a previous study, we demonstrated that genetically modified granulosa cells could be used to produce transgenic NT embryos after long-term culture (9). In this study, we examined whether adult fibroblast cells after transgenic clonal propagation in long-term culture can be used for NT transfer and support embryonic development in vitro.

Materials and Methods

Genetic transformation of cells and establishment of transgenic cell lines

A tissue biopsy was obtained from the ear of a 13-year-old cow. Tissue explants were cultured in Dulbecco's Modified Eagle's medium (DMEM) F-12 (Sigma) supplemented with 10% fetal bovine serum (FBS, Bio Whitaker Inc, Walkersville, MD) and 1% (v:v) penicillin /streptomycin (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin, Sigma) at 37 ºC in a tissue culture plate in a humidified atmosphere of 5% CO_2 and 95% air. Fibroblast cells were transfected with a plasmid containing EGFP gene under the control of the cytomegalovirus (CMV) promoter and neomycin resistant gene, which allows selection using geneticin, under control of SV40 promoter (pEGFP-N1, Clontech Laboratories, Inc. Palo Alto, CA) using a polyamine transfection reagent (GeneJammer, Stratagene, CA) according to the manufacturer's instructions. After transfection the cells were exposed to 600 μg/ml of Geneticin (G418, Sigma) for 20 days and single colonies were isolated in the presence of G418 and expanded. Transfected cell colonies were picked up using round paper membrane colony discs allowing the attechment of cells on the surface of them (Fisher Scientific, Springfield, NJ). The cells were examined for EGFP expression under a fluorescent microscope using a standard fluorescein isothiocyanate (FITC) filter set and marked as positive and negative cell lines. A portion of cells from each EGFP positive and negative cell line was plated for chromosome analysis and DNA isolation. Genomic DNA was isolated from each cell line cultured into 24-well plates using a DNA purification kit (Wizard Genomic DNA kit, Promega purification) and analyzed by PCR for the presence of the transgenes. The chromosomes of cells were counted to examine the ploidy of the transfected and expanded adult fibroblast cell lines by using a standard preparation of metaphase spreads (19). The population doubling (PD) number of clonal lines was estimated using the formula log10 (N/N0) x 3.33 (N: number of cells harvested, N0: number of cells plated).

Recipient cytoplasm preparation and NT

In vitro maturation of bovine immature oocytes and enucleation were performed as described previously (2,10,16). In brief, bovine cumulus-oocyte complexes (COCs) were recovered by the aspiration of small antral follicles on ovaries obtained from a slaughterhouse. Only COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogeneous ooplasm were selected. They were matured in TCM 199 (Gibco Inc, Grand Island, NY) supplemented with 10% FBS, 50 μg/ml sodium pyruvate, 1% v:v penicillin/streptomycin (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin), 1 ng/ml rIGF-1 (Sigma), 0.01 U/ml bLH and 0.01 U/ml bFSH (Sioux Biochem. Sioux Center, IA) in four-well plates overlaid with mineral oil. Maturation was performed at

39 ºC in a humidified 5% CO_2 in air for 16-18 h. After maturation, the cumulus-corona was totally removed by vortexing COCs in TL Hepes medium containing 100 U/ml hyaluronidase (Sigma). Oocytes matured for 16-18 h were enucleated with a 15-µm (internal diameter) glass pipette (Eppendorf, Westburg, NY) by aspirating the first polar body and MII plate in a small volume of surrounding cytoplasm in TL Hepes supplemented with 7.5 µg/ml Cytochalasin B (Sigma). The oocytes were previously stained in TL Hepes containing 2 µg/ml Hoechst 33342 and 7.5 µg/ml Cytochalasin B for 10-15 min. Enucleation was performed under ultraviolet light to ensure the removal of oocyte chromatin.

Two different EGFP positive cell lines, one expressing a high level (AF1-gfp) and the other a low level of EGFP (AF12-gfp), and one EGFP negative cell line (AF47-neo) from transfected adult fibroblast cells, and non-transfected adult fibroblast cells were used as donors. The donor cells were cultured with 10% FBS. A single cell was inserted into the perivitelline space of the enucleated oocyte by using a 15-µm (internal diameter) glass pipette (2). For transfer, the brightest cells in each transgenic cell line were selected under UV light using the FITC filter set. Oocyte-cell complexes were placed in TCM 199 containing 10% FCS at 39 ºC in 5% CO_2 in air until fusion.

Fusion and activation of oocyte-cell couples

Oocyte-cell couples were fused by using a needle-type electrode (7,9) in Zimmermann's fusion medium (20). The single cell-oocyte couple was sandwiched between two wires arranged in a straight line and attached to micromanipulators. A single direct current pulse of 40 V for 20 µs was applied. The activation of NT units was performed as described previously (9,21,22) after modification. In brief, 2 h after fusion, nuclear transfer oocytes were exposed to calcium ionophore (5 µM for 10 min, free acid, Sigma), followed by incubation in TCM 199, supplemented with 10% FBS, 2.5 µg/ml Cytochalasin D (Sigma), 10 µg/ml Cycloheximide (Sigma) for 1 h at 39 ºC in 5% CO_2 in air and in TCM 199 with 10% supplemented FBS and 10 µg/ml Cycloheximide for 5 h at 39 ºC in 5% CO_2, 5% O_2 and 90% N_2.

In Vitro Culture of Reconstructed Embryos

Nuclear transfer oocytes were cultured in 50 µl culture drops of BARC medium (9,22,23) containing bovine serum albumin placed into a 60-mm culture plate overlaid with mineral oil at 39 ºC in 5% CO_2, 5% O_2 and 90% N_2 for 5 days and cleaved NT embryos were transferred in 50 µl culture drops of BARC + BSA medium containing 5% FBS and cultured for an additional 2 days.

Blastocyst staining

Blastocyst stage embryo nuclei were stained on slides in a PBS solution and 10% glycerol containing 100 µg/ml of Hoechst 33342. A drop (~20 µl) of staining solution containing 1-3 embryos was placed in the center of a slide and a cover slip was placed over the drop and the edges sealed. Nuclei were visualized and counted using UV light.

Statistical analysis

Experiments from all different cell lines were repeated three times. Differences among the groups were analyzed by one-way ANOVA. Data were analyzed after arcsin square transformation (SigmaStat, Jandel Scientific).

Results

EGFP expression in fibroblast cells

The day after transfection with the EGFP gene using a polyamine transfection reagent, green fluorescence was observed in approximately 70% of fibroblast cells. Following transfection and selection, 49 colonies from transfected adult cells were picked up and expanded. When examined under UV/FITC filter, 35 (71.4%) of total colonies expressed EGFP. Visual observations suggested that the expression level of EGFP differed among the clonal lines. Sixteen (45.7%) clonal lines expressing EGFP were classified as high expressing clones, and 19 (54.2%) as low expressing clones. At the time of NT, all cells from EGFP transgenic cell lines expressed EGFP. Visually it was observed that each cell line behaved differently in culture, and generally lines expressing EGFP proliferated slower than lines not expressing EGFP. Each clonal line had 1.3-1.8 x 10^6 cells (estimated total of 20.3-20.8 PDs) when they were frozen.

Characterization of donor cells and integration of transgenes

A majority of cells showed a normal chromosomal complement (60 chromosomes including XX chromosomes) in non-transfected adult fibroblasts at passage 4 (94.8%). Adult fibroblasts (at passage 4) were used for transfection and all transgenic adult cell lines were examined for chromosome numbers. A majority of

the cells from each line (78.4-90%) showed a normal chromosome number (60, including XX chromosomes). Chromosome numbers of the lines used for NT are presented in Table 1. The presence of both genes (Neo and EGFP) was examined by PCR in all lines. The cell lines from adult fibroblast expressing EGFP had both genes, but lines not expressing EGFP only had the neo-gene.

EGFP expression and developmental competence of NT embryos

The fusion rate for NT units was determined 2 h after fusion pulse and green fluorescence was observed in all fused NT units when examined under UV light. About 90% of cleaved embryos expressed EGFP after 3 days of culture, and there was no evidence of mosaicism in any expressing embryos. EGFP expression was observed in all blastocysts from both transgenic cell lines. There was a variation visually in EGFP expression between blastocysts from lines AF1-gfp and AF12-gfp. Embryos from AF1-gfp expressed a higher level of EGFP than embryos from AF12-gfp. When the results were compared separately, development rates of NT units from two different EGFP positive lines (11.4% vs. 11.3%) and one EGFP negative line (21.5%) were similar. However, when data were combined there was a significant difference between NT units from positive and negative lines (Table 2, 11.4% vs. 21.5 %, respectively, $P < 0.05$). The development rates of NT units from EGFP negative and non-transfected cells were similar (Table 2, 21.5% vs. 20.1%). Cell numbers and the morphology of blastocysts from all experimental groups were similar.

Discussion

This study showed that adult bovine fibroblast cells can complete transgenic clonal propagation including transfection and culture under antibiotic selection during a long-term culture period and can be used for NT, and support embryonic development in vitro. In agriculture, it would be advantageous to use adult cells from progeny-

Table 1. Chromosome analysis of donor cells.

Cell	Chromosome number			no. of spreads counted
	<60 (%)	60 (%)	>60 (%)	
AF1-gfp[a]	4 (6.6)	49 (81.6)	7 (11.6)	60
AF12-gfp[a]	3 (4.6)	57 (87.6)	4 (6.1)	65
AF47-neo[b]	5 (8.3)	48 (80)	7 (11.6)	60
AF[c]	1 (1.2)	74 (94.8)	3 (3.8)	78

[a] EGFP and neo-positive adult fibroblast

[b] EGFP negative and neo-positive adult fibroblast

[c] Non-transfected adult fibroblast

No significant differences ($p > 0.05$)

Table 2. Development of NT units.

Cell	NT units	NT units fused (%)	NT units cleaved (%)	Blastocyss (%)	Cell no. of blastocyst
AF-gfp[a]	641	368 (57.4)	181 (49.2)d	42 (11.4)d	87.4 ± 5.0
AF-neo[b]	140	79 (56.4)	61 (77.2)e	17 (21.5)e	92.6 ± 10.9
AF[c]	202	104 (51.4)	68 (65.4)e	21 (20.1)e	95.5 ± 0.9

[a] EGFP positive adult fibroblast (both AF1 and AF12 lines)

[b] EGFP negative (only neo-positive)

[c] Non-transfected adult fibroblast

d,e Values within columns with different letters are significantly different ($p < 0.05$)

tested animals for the multiplication of superior genotypes. Although not tested here, it is likely that adult fibroblast cells can serve as a suitable donor for the production of transgenic NT offspring. A previous study showed that transgenic granulosa cells at a late passage number can be used for producing transgenic embryos (9). Adult fibroblasts can be an alternative cell type for producing NT embryos since they can be obtained from either sex.

We used EGFP as a marker to determine gene expression into adult cells and NT embryos derived from these cells. Although GFP is thought to be a non-toxic biological marker, Hanazono et al. (24) reported that high expressing cells (the brightest cells) died within a matter of days after transfection. They hypothesized that this was due to a deleterious effect of the gene product. Hadjantonakis et al. (25) reported a variation of GFP expression on transfected mouse embryonic stem (ES) cells and interpreted this phenomenon as an effect of the gene copy number or the site of integration. They did not, however, report any deleterious effect of GFP on ES cell viability. In the present study, the toxicity levels or copy number may have affected the growth of the transfected fibroblast cells expressing EGFP. Cell lines expressing EGFP proliferated slower than EGFP negative cell lines. However, the expression level of EGFP did not affect the development of NT units or cells. Transgenic mice and bovine embryos expressing the GFP gene have been reported (1,25-27) but a systematic examination for the toxicity of the gene product has not yet been performed. In the present study, variations in the intensity of EGFP expression were observed among clonal cell lines. EGFP expression in blastocyst stage NT embryos also differed between the two EGFP transgenic cell lines. In a recent study (28), clonal lines of transgenic fibroblast cells derived from the same fetus resulted in different embryo development rates when used for NT in pigs. In the present study, there was no significant difference in the developmental rates between embryos from the two EGFP cell lines (expressing low and high levels of EGFP). However, the developmental rates of embryos from EGFP-positive cells were lower than those from EGFP-negative cells. The low embryo development rate observed in this study might be the result of the negative effect of EGFP on donor cells and the development of transgenic embryos.

Generally early passage numbers (3-5 passages) of adult donor cells have been used for nuclear transfer (2,11,12,16,17,29). Although live clones have been obtained from adult somatic cells in sheep (14) and cattle (16-18,30), the production of transgenic animals by cloning has been limited to fetal derived transfected somatic donor cells (2,3,12). Kasinathan et al. (31) found that cells from a 15-year-old animal had a life span of about 18 PDs, and suggested that adult cells are likely to become senescent before selection for transgenic clonal lines would be complete. However, cells from a 13-year-old animal could complete clonal propagation in this study. Two studies (2,31) reported that fetal fibroblast cells had 30 PDs; however, another study (32) indicated that fibroblast cells of the same age had 60 PDs. Therefore, PDs for adult cells might vary among animals or with culture conditions. Although PDs were not examined, two recent studies demonstrated that developmental rates of NT embryos derived from adult cells after long-term culture (10-15 passages) were higher than those of embryos derived from shorter-term culture (five passages) (9,19). In the present study, the percentage of cells with normal chromosome numbers in all experimental groups was similar to those obtained by Kubota et al. (19), who used adult fibroblast cells at passage 10 and 15. Developmental rates to the blastocyst stage for transgenic fibroblast cell derived NT embryos were similar to those obtained by Beyhan et al. (33), who used adult bovine fibroblast and cumulus cells, and those obtained by Shiga et al. (18), who used muscle cells as donor material. Furthermore, NT embryo development rates were similar to transfected granulosa (9) and fetal fibroblast donor cells (2,10). Total cell numbers in blastocyst stage embryos derived from transfected and non-transfected donor cells were higher than those reported by Akagi et al. (34) and similar to those reported by Kasinathan et al. (31). Although the blastocyst development rate from EGFP positive cell lines was lower than that from negative cell lines, when compared with other NT studies, the results obtained from the present study indicate that the transfection and selection of transgenes did not impair the developmental potential of NT embryos.

In conclusion, this study demonstrated that embryonic development is not affected in NT embryos derived from adult fibroblast cells by transfection with a gene of interest and selection with an antibiotic. The lower

developmental rate of embryos produced from cells expressing EGFP may indicate a deleterious effect of EGFP. Therefore, adult cells can complete the transgenic clonal propagation required in gene targeting and so may be used to produce transgenic cloned calves.

References

1. Chan, A., Kukolj, G., Skalka, A., Bremel, R.: Timing of DNA Integration, Transgenic Mosaicism, and Pronuclear Microinjection. Mol. Reprod. 1999; 52: 406-413.
2. Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A., Robl, J.M.: Cloned Transgenic Calves Produced from Non-Quiescent Fetal Fibroblasts. Science 1998; 280: 1256-1258.
3. McCreath, K.J., Howcroft, J., Campbell, K.H.S., Colman, A., Schnieke, A.E., Kind, A.J.: Production of Gene-Targeted Sheep by Nuclear Transfer from Cultured Somatic Cells. Nature 2000; 405: 1066-1069.
4. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C.: Green Fluorescent Protein as a Marker for Gene Expression. Science 1994; 263: 802-805.
5. Prasher, D.C.: Using GFP to see the light. Trends Genet. 1995; 11: 320-323.
6. Keefer, C.L., Baldassarre, H., Keyston, R., Wang, B., Bahatia, B., Bilodeau, A.S., Zhou, J.F., Leduc, M., Downey, B.R., Lazaris, A., Karatzas, C.N.: Generation of Dwarf Goat (*Capra hircus*) Clones Following Nuclear Transfer with Transfected and Nontransfected Fetal Fibroblasts and In Vitro-Matured Oocytes. Biol. Reprod. 2001; 64: 849-856.
7. Miyoshi, K., Taguchi, Y., Sendai, Y., Hoshi, H., Sato, E.: Establishment of a Porcine Cell Line from In Vitro-Produced Blastocysts and Transfer of the Cells into Enucleated Oocytes. Biol. Reprod. 2000; 62: 1640-1646.
8. Koo, D.B., Kang, Y.K., Choi, Y.H., Park, J.S., Kim, H.N., Kim, T., Lee, K.K., Han, Y.M.: Developmental Potential and Transgene Expression of Porcine Nuclear Transfer Embryos Using Somatic Cells. Mol. Reprod. Dev. 2001; 58: 15-21.
9. Arat, S., Rzucidlo, S.J., Gibbons, J., Miyoshi, K., Stice, S.L.: Production of Transgenic Bovine Embryos by Transfer of Transfected Granulosa Cells into Enucleated Oocytes. Mol. Reprod. Dev. 2001; 60: 20-26.
10. Roh, S., Shim, H., Hwang, W., Yoon, J.: In Vitro Development of Green Fluorescent Protein (GFP) Transgenic Bovine Embryos after Nuclear Transfer Using Different Cell Cycles and Passages of Fetal Fibroblasts. Reprod. Fertil. Dev. 2000; 12: 1-6.
11. Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Desrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W., Echelard, Y.: Production of Goats by Somatic Cell Nuclear Transfer. NatBiotech. 1999; 17: 456-461.
12. Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A., Cambell, K.H.S.: Human Factor IX Transgenic Sheep Production by Transfer of Nuclei from Transfected Fetal Fibroblasts. Science 1997; 278: 2130-2133.
13. Kuhholzer, B., Tao, T., Machaty, Z., Hawley, R.J., Greenstein, J.L., Day, B.N.: Production of Transgenic Porcine Blastocysts by Nuclear Transfer. Mol. Reprod. Dev. 2000; 56: 145-148.
14. Arat, S., Gibbons, J., Rzucidlo, S.J., Miyoshi, K., Venable, A., Waltenburg, R., Stice, S.L.: Bovine Cloning Using Adult Donor Cells Treated with Roscovitine. Biol. Reprod. Suppl. 1. 2001; 173 (Abstract).
15. Wilmut, I., Schnieke, A.E., McWhir, J., Kind, K.L., Campbell, K.H.S.: Viable Offspring Derived from Fetal and Adult Mammalian Cells. Nature 1997; 385: 810-813.
16. Wells, D.N., Misica, P.M., Tervit, H.R.: Production of Cloned Calves Following Nuclear Transfer with Cultured Adult Mural Granulosa Cells. Biol. Reprod. 1999; 60: 996-1005.
17. Hill, J.R., Winger, Q.A., Long, C.R., Looney, C.R., Thompson, J.A., Westhusin, M.E.: Development Rates of Male Bovine Nuclear Transfer Embryos Derived from Adult and Fetal Cells. Biol. Reprod. 2000; 62: 1135-1140.
18. Shiga, K., Fujita, T., Hirose, K., Sasae, Y., Nagai, T.: Production of Calves by Transfer of Nuclei from Cultured Somatic Cells Obtained from Japanese Black Bulls. Theriogenology 1999; 52: 527-535.
19. Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Tabara, N., Barber, M., Yang, X.: Six Cloned Calves Produced from Adult Fibroblast Cells After Long-Term Culture. P. Natl. Acad. Sci. USA 2000; 97:990-995.
20. Zimmermann, U., Vienken, J.: Electric Field-Induced Cell-to-Cell Fusion. J. Membrane Biol. 1982; 67: 165-182.
21. Lui, L., Ju, J.C., Yang, X.: Parthenogenetic Development and Protein Patterns of Newly Maturated Bovine Oocytes after Chemical Activation. Mol. Reprod. Dev. 1998; 49: 298-307.
22. Gibbons, J., Arat S., Rzucidlo, S.J., Waltenburg, R., Respess, D.S., Venable, A.M., Stice, S.L.: Enhanced Survivability of Cloned Calves Derived from Roscovitine-Treated Adult Somatic Cells. Biol. Reprod. 2002; 66: 895-900.
23. Powell, A.M., Graninger, P.G., Talbot, N.C., Wells, K.W.: Effects of Fibroblast Source and Tissue-Culture Medium on Success of Bovine Nuclear Transfer with Transfected Cells. Theriogenology 2001; 55: 287 (Abstract).

24. Hanazono, Y., Yu, J-M., Dunbar, C.E., Emmons, R.V.B.: Green Fluorescent Protein Retroviral Vectors: Low Titer and High Recombination Frequency Suggest a Selective Disadvantage. Hum. Gene Therapy 1997; 8: 1313-1319.

25. Hadjantonakis, A-K., Gertsenstein, M., Ikawa, M., Okabe, M., Nagy, A.: Generating Green Fluorescent Mice by Germline Transmission of Green Fluorescent ES Cells. Mechanism Dev. 1998; 76: 79-80.

26. Ikawa, M., Kominami, K., Yoshimura, Y., Tanaka, K., Nishimura, Y., Okabe, M.: Green Fluorescent Protein as a Marker in Transgenic Mice. Dev. Growth Differ. 1995; 37: 455-459.

27. Kato, M., Yamanouchi, K., Ikawa, M., Okabe, M., Naito, K., Tojo, H.: Efficient Selection of Transgenic Mouse Embryos Using EGFP as a Marker Gene. Mol. Reprod. Dev. 1999; 54: 43-48.

28. Kühholzer, B., Hawley, R.J., Lai, L., Kolber-Simonds, D., Prather, R.S.: Clonal Lines of Transgenic Fibroblast Cells Derived from the Same Fetus Result in Different Development When Used for Nuclear Transfer in Pigs. Biol. Reprod. 2001; 64: 1695-1698.

29. Polejaeva, I.A., Chen, S.H., Vaught, T., Page, R., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A., Campbell, K.H.S.: Cloned Pigs Produced by Nuclear Transfer from Adult Somatic Cells. Nature 2000; 407: 86-90.

30. Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H., Tsunoda, Y.: Eight Calves Cloned from Somatic Cells of Single Adult. Science 1998; 282: 2095-2098.

31. Kasinathan, P., Knott, J.G., Moreira, P.N., Burnside, A.S., Jerry, D.J., Robl, J.M.: Effect of Fibroblast Donor Cell Age and Cell Cycle on Development of Bovine Nuclear Transfer Embryos In Vitro. Biol. Reprod. 2001; 64: 1487-1493.

32. Lanza, R.P., Cibelli, J.B., Blackwell, C., Cristofalo, V., Francis, M.K., Baerlocher, G.M., Mak, J., Schertzer, M., Chavez, E.A., Sawyer, N., Lansdorp, P.M., West, M.D. Extension of Cell Life-Span and Telomere Length in Animals Cloned from Senescent Somatic Cells. Science 2000; 288: 665-669.

33. Beyhan, Z., Mitalipova, M., Chang, T., First, N.L.: Developmental Potential of Bovine Nuclear Transfer Embryos Produced Using Different Type of Adult Donor Cells. Theriogenology 2000; 53: 210 (Abstract).

34. Akagi, S., Takahashi, S., Noguchi, T., Hasegawa, K., Shimizu, M., Hosoe, M., Izaike, Y.: Development of Embryos Using Cumulus Cells for Nuclear Transfer. Theriogenology 2000; 53: 208 (Abstract).