The effects of activation protocols on the development of cloned goat embryos

Malee Apimeteetumrong, Anone Thuangsanthia, Narong Leingcharoen, Viboon Yiengvisavakul, Ayuth Harintharanon, Annop Kunavongkrit, Jureerat Sumretprasong, Xavier Vignon, Mongkol Techakumphu

FULL PAPER *Theriogenology*

The Effect of Activation Protocols on the Development of Cloned Goat Embryos

Malee APIMETEETUMRONG[1,2], Anone THUANGSANTHIA[1], Narong LEINGCHAROEN[1], Viboon YIENGVISAVAKUL[1], Ayuth HARINTHARANON[1], Annop KUNAVONGKRIT[2], Jureerat SUMRETPRASONG[3], Xavier VIGNON[4] and Mongkol TECHAKUMPHU[2]

[1]The Bureau of Biotechnology for Animal Production, Department of Livestock Development, Pathumthani, 12000, [2]Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330, [3]Faculty of Veterinary Medicine, Chiengmai University, Chiengmai, Thailand 50100 and [4]Biologie du Developpement et Reproduction, 78352 Jouy en Josas, France

(Received 20 April 2004/Accepted 27 July 2004)

ABSTRACT. This study was conducted to compare the developmental competence of somatic nuclear transfer (NT) embryos, after either ionomycin or ethanol activation, in locally bred goats. Donor cells were prepared from the ear skin fibroblasts of a female goat. Cells, at passage 3–8, starved by culturing in 0.5% FCS for 4–8 d, were used for NT. Immature oocytes were obtained from FSH-stimulated goats and matured for 22 hr before enucleation and NT. After fusion, the reconstructed embryos were activated with either ionomycin or ethanol followed by culturing in 6-dimethylaminopurine (6-DMAP) and cytochalasin B (CB), for 3 hr. In experiment I, the fuse d NT embryos (n=63, ionomycin and n=68, ethanol treatments, respectively) were cultured in B_2 with a Vero co-culture system and their developmental competence was evaluated through to Day 9. In experiment II, the NT embryos at the 2–4 cell stage on Day 2 derived from each treatment (ionomycin n=46, and ethanol n=37), were transferred into 10 synchronous recipients. There were no significant differences between the NT embryos derived from the ionomycin and ethanol groups, in fusion (86.3% versus 82.9%), cleavage (90.5% versus 82.4%) and for morula/blastocyst development rates (9.5% versus 5.9%). Sixty percent (3/5) of the recipients from ionomycin became pregnant by midterm (2.5 mts) while only 20% (1/5) from ethanol treatment was pregnant by Day 45. The results demonstrate that activation with either ionomycin or ethanol in combination with 6-DMAP-CB treatment does not affect the development of cloned goat embryos.

KEY WORDS: activation, developmental competence, goat, nuclear transfer.

J. Vet. Med. Sci. 66(12): 1529–1534, 2004

Since the cloned sheep named "Dolly", was produced by transferring nuclei from adult cells into enucleated oocytes [27], there have been many studies carried out to produce other cloned mammals such as mice, cattle, goats, pigs and rabbits [1, 5, 19, 25, 26]. The nuclear transfer (NT) technique has been efficiently used for producing transgenic, cloned farm animal offspring. These animals are capable of producing valuable proteins, which could have a marked impact on the pharmaceutical industry [22] such as alpha-1-antitrypsin, fibrinogen, human clotting factor IX and antithrombin III [1], human IgG heavy and light chains linked to beta-casein vector [4], enhanced green fluorescent protein [10] and *neo* gene [32].

Activation is an important step in the NT procedure and ethanol and ionomycin have been widely used as activating agents in several species, such as cattle [15, 20, 21], mice [8], sheep [16], and goats [1, 4, 6, 11, 32]. There are a few reports on the development of goat oocytes after parthenogenetic activation by treatment with ionomycin and ethanol, both followed by exposure to 6-diethylaminopurine (6-DMAP) [17, 18]. Nevertheless, comparison of the development of NT goat embryos with either ionomycin or ethanol, as an activating agent, has not been reported. The objective of this study was to compare the developmental competence *in vitro* and *in vivo* of NT goat embryos, after activation, by using either ionomycin or ethanol, both followed by incubation in 6-DMAP-cytochalasin B (CB).

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma-Aldrich Company (St. Louis, MO) and the media from Gibco Invitrogen Corporation (Grand Island, NY).

Preparation of donor cells: Donor cells were prepared from an ear skin fibroblast cell line taken from a 3-year-old Native female goat by using the protocol previously described for bovines by Vignon *et al.* [25]. The skin biopsies were washed three times in Dulbecco's phosphate-buffered saline (DPBS) and cut into small pieces before being attached to the bottom of 60-mm culture dishes. Two to three m*l* of culture medium was slowly added to the culture dishes and the cell explants were then cultured at 38.5°C in a humidified air atmosphere with 5% CO_2 for 4–5 d until a primary fibroblast monolayer was established. The culture medium was Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin. Primary cells were trypsinized with 0.05% trypsin-EDTA, frozen in 10% dimethyl sulfoxide in FCS and stored in liquid nitrogen. For the experiments, the cells were thawed and cultured for 3–4 d. When the cells had grown to 80–90% confluence, they were starved, by culturing in a medium supplemented with 0.5% FCS for 4–10 d. Before NT, the cells were harvested by trypsinization, resuspended in DMEM, supplemented with 10% FCS and

immediately transferred into the recipient oocytes. Cells cultured between passages 3–8 were used for NT.

Preparation of recipient oocytes: The oocytes were recovered from superovulated goats (Native, Saanen and Native-Saanen crossbred goats). The gonadotropin treatment was performed with a modified program of Reggio *et al.* [22]. The estrous cycle was synchronized with the insertion of a controlled internal drug release device (CIDR-G, 0.3 g progesterone, Eazi Breed, InterAg, New Zealand), which was removed on Day 8 (Day 1 = day of insertion). Ovarian superstimulation was obtained with FSH (Folltropin® -V, Vetrepharm Canada Inc., Ontario, Canada) by giving 8 decreasing doses (i.m.), one injection every 12 hr, starting on Day 4 of CIDR-G insertion (total dose equivalent to 200 mg NIH-FSH-P1). Oocyte collection was performed 24 hr after the final FSH injection [22]. Ovaries were exteriorized and the oocytes were aspirated from follicles with a diameter ≥ 2 mm, by using a 21-gauge needle attached to silicone tubing that was connected to an electric vacuum pump (Cook Veterinary Products, Eight Mile Plains, Australia). Cumulus-oocyte complexes (COCs) having at least 2 layers of cumulus cells were selected and matured in maturation medium for 22–23 hr, at 38.5°C, in a humidified air atmosphere with 5% CO_2. The maturation medium consisted of Medium 199 (M199) supplemented with 10% FCS, 10 μg/m*l* FSH, 10 μg/m*l* pLH, 1 μg/m*l* 17-β estradiol, 10 mM β-mercaptoethanol [23], 0.55 mg/m*l* pyruvate and 50 IU/m*l* penicillin-streptomycin. After maturation, the cumulus cells were removed by culturing the COCs for 3 min in M199-Hepes containing 0.5 mg/m*l* hyaluronidase and pipetting for 1 min. Cumulus cell-free oocytes were then cultured in M199, supplemented with 10% FCS and 0.5 μg/m*l* Hoechst 33342, for 20–30 min at 38.5°C in a humidified air atmosphere with 5% CO_2 before enucleation. Oocyte enucleation was performed in M199-Hepes supplemented 10% FCS, 5 μg/m*l* CB and 0.5 μg/m*l* Hoechst 33342 overlaid with mineral oil on the stage of an inverted microscope equipped with Narishige micromanipulators and epifluorescent illumination. The metaphase plate and the first polar body were removed with a glass enucleation pipette (20 μm outside diameter). Confirmation of successful enucleation was achieved by visualizing the metaphase plate under ultraviolet light.

Somatic cell nuclear transfer and activation: The enucleated oocytes were fused with donor cells by applying 2 DC pulses of 2.0 kV/cm for 50 μsec [6]. Two hours after fusion, the reconstructed embryos were activated, either by exposure to 5 μM ionomycin for 5 min at 38.5°C, in M 199 supplemented with 10% FCS or to 7% ethanol for 5 min at 38.5°C in M 199, supplemented with 10% FCS and followed by culturing in 2 mM 6-DMAP and 5 μg/m*l* CB for 3 hr [6, 24]. Fusion was observed at the end of the activation period. The fused embryos were then cultured in B_2 medium (CCD, Paris, France), supplemented with 7% FCS and Vero cells, for 9 d at 38.5°C in a humidified air atmosphere with 5% CO_2. The cleavage and development of NT embryos were observed on Day 2 and Days 7–9 (Day 0 = day of fusion). At the end of the culture period, the NT embryos were fixed and stained with Hoechst 33342 by the method of Begin *et al.* [2]. The numbers of nuclei were counted under ultraviolet light. The embryos, on Day 9 of culture, which had greater than 16 nuclei but less than 32 nuclei, were classified as morulae [12]. The embryos, on Day 9 of culture, which had greater than 32 nuclei, were classified as blastocysts [12].

Parthenogenetic activation of oocytes: Parthenogenetic activation of oocytes was done as a control group, for the development of NT embryos in the same way as was in the NT group. Denuded matured oocytes were exposed to 0.5 μg/m*l* Hoechst 33342, for 20–30 min at 38.5°C in a humidified air atmosphere with 5% CO_2 and then observed for signs of MII and the first polar body, under ultraviolet light. Electric stimulation was done with the same techniques as described above for NT embryos. Stimulated oocytes were then cultured for 2 hr in M199, supplemented with 10% FCS. After culture, they were activated, with either ionomycin or ethanol, followed by culturing in 6-DMAP plus CB, as described above and then further cultured under the same conditions as the NT embryos. Activated oocytes were observed for cleavage and development into the morula and blastocyst stages. At the end of the culture period, the numbers of nuclei in the parthenogenetic embryos were counted under ultraviolet light.

Preparation of recipient animals and embryo transfer: Recipient goats (Native, Saanen and Mixed breed) were synchronized by the same protocol as used for the donors, but not given FSH. An injection of 175 μg of Estrumate® (Schering-Plough Animal Health, NSW, Australia) was given 36 hr before CIDR-G removal. Estrous was detected by using vasectomized male goats 24–48 hr after CIDR-G removal. On Day 2 (Day 0 = day of fusion), NT embryos at the 2–4 cell stage from each activation treatment were transferred into 10 recipients (6–12 embryos/recipient) on Day 2 of their cycle (Day 0 = estrus). Ultrasonography scanning was performed on all recipients on Days 30, 45 and 60 of gestation for pregnancy examination and fetal development.

Statistical analysis: Fusion, cleavage rates and development into morula and blastocyst stages in both groups of NT embryos, as well as the parthenogenetic embryos were compared by means of Fisher's exact test and SAS software. Pregnancy rates were also compared with Fisher's exact test and SAS software. Differences were considered significant at $P<0.05$.

RESULTS

The effect of activation protocols on developmental potential of NT embryos and parthenogenetic embryos in vitro: The rate of fusion of NT embryos was not significantly different (Table 1) in the ionomycin and ethanol treatment groups (86.3% and 82.9%, respectively). The rate of development to the morula and blastocyst stages of NT embryos showed no significant differences between the ionomycin and ethanol treatment groups (9.5% and 5.9%,

Table 1. Effect of activation protocols on the developmental potential of NT embryos and parthenotes *in vitro*

Activation protocols	Groups	No. of activation /reconstructed	No. of fused (%)	No. of[c] cleaved (%)	No. of[c] (%) Morula/Blastocyst
Ionomycin[a]	NT	73	63 (86.3)	57 (90.5)	6 (9.5)[d]
	Parthenotes	26	–	22 (84.6)	11 (42.3)[e]
Ethanol[b]	NT	82	68 (82.9)	56 (82.4)	4 (5.9)[d]
	Parthenotes	12	–	11 (91.2)	4 (33.3)[e]

NT experiments were replicated 6 times in both activation protocols.
Parthenogenetic activations were replicated twice.
a) Five micromole ionomycin followed by incubation in 2 mM 6-dimethylaminopurine + 5 μg/m*l* cytochalasin B for 3 hr.
b) Seven percent (v/v) ethanol followed by incubation in 2 mM 6-dimethylaminopurine + 5μg/m*l* cytochalasin B for 3 hr.
c) Percentage based on number of embryos fused/cultured.
d, e) Values with different superscripts in the same column differ significantly ($P<0.01$).

Table 2. Effect of activation protocols on the developmental potential of NT embryos *in vivo*

Activation protocols	No. of cultured	No. of (%)[c] 2–4 cell stage	No. of transferred	No. of recipients	No. of pregnant (%) Day 30	Day45	Day 60	No. of kids
Ionomycin[a]	68	56 (82.3)	45	5	3 (60)	3 (60)	3 (60)	1[d]
Ethanol[b]	47	38 (80.9)	37	5	1 (20)	1 (20)	0	0

a) Five micromole ionomycin followed by incubation in 2 mM 6-dimethylaminopurine + 5μg/m*l* cytochalasin B for 3 hr.
b) Seven percent (v/v) ethanol followed by incubation in 2 mM 6-dimethylaminopurine + 5 μg/m*l* cytochalasin B for 3 hr.
c) Percentage based on number of embryos cultured.
d) Mummified fetus.

respectively). There were no significant differences in the cleavage rates of NT embryos and parthenogenetic embryos derived from both activation treatments but the development rate to the morula or blastocyst stages of NT embryos, derived from both treatments, was significantly lower ($P<0.01$) than that of the parthenogenetic embryos (Table 1).

The effect of activation protocols on developmental potential of NT embryos in vivo: A total of 46 NT embryos at the 2–4 cell stage derived from the ionomycin treatment group were transferred into five recipients (Table 2) with each recipient receiving, on average, 9.0 ± 1.0 (mean ± SEM) embryos. A total of 37 NT embryos at the 2–4 cell stage, derived from the ethanol group, were transferred into five recipients (Table 2), with each recipient receiving, on average, 7.4 ± 0.4 (mean ± SEM) embryos. Because of the small number of recipients receiving NT embryos derived from the ionomycin and ethanol treatment groups, there were no significant differences in the rates of pregnancy at Day 30 (60%, 3/5 and 20%, 1/5, respectively). Pregnancy status was observed in all three recipients from the ionomycin group through to Day 60 but it was not observed after Day 45 in the recipient from the ethanol group. One mummified fetus (Crown-Rump Length, CRL = 5 inches) connected with cotyledons (number of cotyledons = 54) was taken out of a recipient in the ionomycin group on Day 145 of gestation.

DISCUSSION

In this study, the NT embryos derived from the two different activation protocols appear to have a similar developmental competence *in vitro*. Fusion rates achieved from both activation protocols (86.3% and 82.9% in the ionomycin and ethanol groups, respectively) were similar to that previously reported in sheep [27] but higher than that reported by Reggio *et al.* [22] in goats (85% versus 63%, respectively), with ionomycin as the activating agent. There were no significant differences in the fusion and cleavage rates between the two different protocols in our study. This suggests that the activation protocols had no effect on the fusion and cleavage rates of NT goat embryos.

The morula/blastocyst rate was low in both treatment groups (in 9.5% ionomycin and 5.9% ethanol groups) under our conditions, and the rate was much lower than that (34.4%) previously reported by Zou *et al.* [31] when used in *in-vivo* matured cytoplasts as recipients and NT embryos cultured *in vivo* for 6 d. In the case of the ionomycin group, the rate was also much lower than that previously reported by Chesne *et al.* [6], who used *in vivo* matured oocytes as recipient cytoplasts. This may be due to the *in vivo* conditions offering a better environment than the *in vitro*. In our study, there was no significant difference in the development rate to the morula/blastocyst stages of parthenogenetic embryos, derived from both activation treatments, which corresponds to a recent report [17]. Our results were differ-

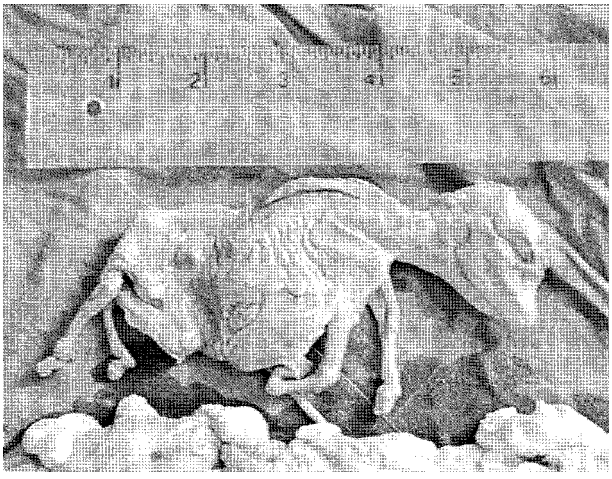

Fig. 1. Mummified fetus, with 5 inch-crown-rump length and 54 cotyledons, was surgically recovered at Day 145 of gestation.

ent from that reported by Loi *et al.* [16] for parthenogenetic sheep embryos. Those authors showed a highly significant difference in the development of the blastocysts derived from ionomycin treatment, after culturing in uteri for 7 d, when compared with ethanol treatment (58.4% versus 19.1%, respectively). Higher development rates were achieved in parthenogenetic embryos when compared to NT embryos in the present study. This may be due to matured oocytes being treated by combined activation, electric stimuli and ionomycin or ethanol as was done in these experiments. It has been demonstrated that the combined treatment with electric and chemical stimulation yields the highest rate of blastocyst formation [9]. In our results, it should be noted that mean cell numbers per blastocyst in both NT and parthenogenetic embryos was lower than 100 (data not shown), which may indicate delayed and/or abnormal development [3].

Ethanol has been used in cattle to effectively induce oocyte activation [20, 21, 30] as well as in rabbits [14]. Presicce and Yang [20] demonstrated that a combined ethanol and cycloheximide (a protein synthesis inhibitor) treatment would induce activation of both young (20 hr) and aging (40 hr) IVM bovine oocytes at very high frequencies (90–100%). In addition, ethanol can induce a single Ca^{2+} rise, which results both from extracellular entry and from mobilization of intracellular stores [16]. On the other hand, ionomycin exclusively mobilizes intracellular calcium stores when used as an activating agent [16]. In the present study, we exposed the reconstructed embryos from each group to each activating agent for the same period, followed by 6-DMAP treatment. Similar development rates *in vitro* were achieved by both activation treatments. Therefore, from our results, either ionomycin or ethanol can activate goat oocytes.

The culture system may also contribute to the low development of embryos. The efficiency of the culture system may be expressed in terms of the percentage of embryos that cleaved and reached more than the 8-cell stage [28]. In addition, the culture of preimplantation mouse embryos in the presence of serum, influences the regulation of growth-related imprinted genes, leading to aberrant growth and behavior [7]. It has been demonstrated that *in vitro* development to the morula-blastocyst stage was higher when NT goat embryos were cultured at low oxygen tension, with a defined medium (36.3%), than in co-culture with Vero cells (27.5%) [6]. The optimal culture system for developing goat embryos needs to be further investigated. In the present study, NT embryos were transferred at early cleavage stages to avoid long-term culture *in vitro*. The avoidance of long-term culture may have alleviated some of the detrimental effects of *in vitro* culture [10]. In sheep and cattle, it was observed that there was an increased incidence of large offspring, after the transfer of embryos cultured in the presence of serum, indicating the possibility of detrimental effects of *in vitro* culture on fetal development [29].

A 60% (3/5) pregnancy rate at Day 30 was achieved after embryo transfer of NT embryos derived from the ionomycin treatment. This pregnancy rate, based on the number of pregnant recipients, per total number of recipients, was slightly higher than that previously reported by others [10, 22]. Similar to the findings of earlier studies [10, 11], prenatal loss after Day 30 ultrasound scan was not observed in the ionomycin treatment group in the present study. A 20% (1/5) pregnancy rate at Day 30 was achieved after embryo transfer of NT embryos, derived from the ethanol treatment, which was lower than that reported (55.5%) by Baguisi *et al.* [1] but those authors lost all thereafter. This is similar to the results of our study, in which recipient received embryos derived from the ethanol group exhibited vesicle formation and were resorbed after Day 45 of gestation. As for the ionomycin group, three of them were found pregnant at term. From the final diagnosis, it was found that two recipients showed signs of pseudopregnancy, with multiple vesicles in their uteri, while the other one carried a mummified fetus with a crown rump length of 5 inches and 54 cotyledons (Fig. 1). The cause of these abnormalities may be due to incomplete reprogrammation of the donor nuclei and consequent low quality cloned embryos, as resulted in a low number of nuclei, under our conditions. Koo *et al.* [13] suggested that early fetal losses or abnormalities might be due to aberrant allocations of NT embryos to the ICM and TE cells during early development. The total cell number, as well as the number of TE cells, of NT embryos, was significantly lower than that of either *in vivo*-derived or IVF embryos in cattle. They also suggested that the NT embryos having a reduced number of TE cells at the preimplantation stage, or incomplete blastocyst formation, might form an abnormal placenta, leading to developmental failures and later fetal loss. In the present study, we transferred NT embryos into recipients as early as possible to avoid undesired factors affecting the developmental potential from *in vitro* culture. The pregnancy could not be maintained to term when transferring NT embryos derived from ethanol

treatment. This result strongly confirms the report of Baguisi *et al.* [1].

In conclusion, our results demonstrate that somatic nuclei, obtained from locally bred goats, could be activated by ionomycin or ethanol treatment in combination with 6-DMAP plus CB treatment. Although no live offspring could be produced, embryonic implantation occurred in the recipients in our study. More experiments need to be performed to confirm the results. Further studies need to investigate the relative contributions of the type of somatic nuclei and the remodeling/reprogramming capabilities of recipient oocytes, as well as appropriate culture systems for the development of NT goat embryos.

ACKNOWLEDGMENTS. This study was financially supported by The Bureau of Biotechnology for Animal Production and Thailand Research Fund (TRF). MA was supported for her PhD by Royal Golden Jubilee Program, TRF. The authors wish to thank Dr. Nucharin Songsasen for critical reading of the manuscript, Dr. Padet Tummaruk for statistic analysis and Dr. Terry Heard for English revision.

REFERENCES

1. Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, 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. and Echelard, Y. 1999. Production of goats by somatic cell nuclear transfer. *Nature Biotech.* **17**: 456–461.
2. Begin, I., Bhatia, B., Baldassarre, H., Dinnyes, A. and Keefer, C.L. 2003. Cryopreservation of goat oocytes and *in vivo* derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. *Theriogenology* **59**: 1839–1850.
3. Booth, P.J., Viuff, D., Tan, S., Holm, P., Greve, T. and Callesen, H. 2003. Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. *Biol. Reprod.* **68**: 922–928.
4. Chen, L.H., Behboodi, E., Regio, B.C., Destrempes, M.M., Green, H.L., Ziomek, C.A., Denniston, R.S., Echlard, Y., Godke, R.A. and Meade, H.M. 2001. Production of transgenic goats from a transfected fibroblast cell line. *Theriogenology* **59**: 259 (abstr.).
5. Chesne, P., Adenot, P.G., Vigliatta, C., Baratte, M., Boulanger, L. and Renard, J.P. 2002. Cloned rabbit produced by nuclear transfer from adult somatic cells. *Nature Biotech.* **20**: 366–369.
6. Chesne, P., Perreau, C., Laloy, E., Poulin, N., Bouttier, A., Cognie, Y., Vignon, X., Heyman, Y. and Mermillod, P. 2002. Somatic nuclear transfer in goats: influence of the culture system on peri-implantation development. *In*: p. 152 (abstr.) The 18th Scientific Meeting of European Transfer Embryo Association, Rolduc, The Netherlands.
7. Fernandez-Gonzalez, R., Jimenez, A., Bilbao, A., Rodriguez de Fonseca, F., Pintado, B. and Gutierrez-Adan, A. 2003. Culture of preimplantation muse embryos with fetal calf serum affects fetal expression of imprinted genes and post-natal growth and behavior. *Theriogenology* **59**: 320 (abstr.).
8. Hardy, K. and Handyside, A.H. 1996. Metabolism and cell allocation during parthenogenetic preimplantation mouse development. *Mol. Reprod. Dev.* **43**: 313–332.
9. Hyun, S., Lee, B., Lee, G., Lee, E., Lim, J., Kang, S. and Hwang, W. 2003. Recruit of porcine oocytes excluded from nuclear transfer program for the production of embryos following parthenogenetic activation. *J. Vet. Med. Sci.* **65**: 51–56.
10. Keefer, C.L., Baldassarre, H., Keyston, R., Wong, B., Bhatia, B., Bilodeau, A.S., Zhou, F.J., Leduc, M., Downey, B.R., Lazaris, A. and Karatzas, C.N. 2001. Generation of dwarf goat (*Capra hircus*) cloned following nuclear transfer with transfected and nontransfected fetal fibroblasts and *in vitro*-matured oocytes. *Biol. Reprod.* **64**: 849–856.
11. Keefer, C.L., Keyston, R., Lazaris, A., Bhatia, B., Begin, I., Bilodeau, A.S., Zhou, F.J., Kafidi, N., Wong, B., Baldassarre, H. and Karazas, C.N. 2002. Production of cloned goats after nuclear transfer using adult somatic cells. *Biol. Reprod.* **66**: 199–203.
12. Koeman, J., Keefer, C.L., Baldassarre, H. and Downey, B.R. 2003. Developmental competence of prepubertal and adult goats oocytes cultured in semi-defined media following laparoscopic recovery. *Theriogenology* **60**: 879–889.
13. Koo, D.B., Kang, Y.K., Choi, Y.H., park, J.S., Kim, H.N., Oh, K.B., Son, D.S., Park, H., Lee, K.K. and Han, Y.M. 2002. Aberrant allocations of inner cell mass and trofectoderm cells in bovine nuclear transfer blastocysts. *Biol. Reprod.* **67**: 487–492.
14. Liu, C.T., Chen, C.H., Cheng, S.P. and Ju, J.C. 2002. Parthenogenesis of rabbit oocytes activated by different stimuli. *Anim. Reprod. Sci.* **70**: 267–276.
15. Liu, L., Ju, J.C. and Yang, X. 1998. Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. *Mol. Reprod. Dev.* **49**: 298–307.
16. Loi, P., Ledda, S., Fulka, J., Cappai, P. and Moor, R.M. 1998. Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. *Biol. Reprod.* **58**: 1177–1187.
17. Ongeri, E.M., Bormann, C.L., Butler, R.E., Melican, D., Gavin, W.G., Echelard, Y., Krisher, R.L. and Behboodi, E. 2001. Development of goat embryos after *in vitro* fertilization and parthenogenetic activation by different methods. *Theriogenology* **55**: 1933–1945.
18. Ongeri, E.M. and Krisher, R.L. 2001. Glucose and pyruvate metabolism of preimplantation goat blastocysts following *in vitro* fertilization and parthenogenetic activation. *Cloning Stem Cells* **3**: 115–123.
19. Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. and Campbell, K.H. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature (Lond.)* **407**: 86–90.
20. Presicce, G.A. and Yang, X. 1994. Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. *Mol. Reprod. Dev.* **37**: 61– 68.
21. Presicce, G.A. and Yang, X. 1994. Parthenogenetic development of bovine oocytes matured *in vitro* for 24 hr and activated by ethanol and cycloheximide. *Mol. Reprod. Dev.* **38**: 380–385.
22. Reggio, B.C., James, A.N., Green, H.L., Gavin, W.G., Behboodi, E., Echelard, Y. and Godke, R.A. 2001. Cloned transgenic offspring resulting from somatic cell nuclear transfer in the goat: oocytes derived from both follicle-stimulating hormone-stimulated and nonstimulated abattoir-derived ovaries. *Biol. Reprod.* **65**: 1528–1533.
23. Songsasen, N. and Apimeteetumrong, M. 2002. Effects of β-mercaptoethanol on formation of pronuclei and developmental

competence of swamp buffalo oocytes. *Anim. Reprod. Sci.* **71**: 193–202.
24. Susko-Parris, J.L., Liebfried-Rutledge, M.L., Northey, D.L., Schutzkus, V. and First, N. 1994. Inhibition of protein kinases after induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. *Dev. Biol*. **166**: 729–739.
25. Vignon, X., Chesne, P., LeBourhis, D., Flechon, J.E., Heyman, Y. and Renard, J.P. 1998. Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. *C.R. Acad. Sci. Sir. III Life Sci*. **321**: 735–745.
26. Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature (Lond.)* **394**: 369–374.
27. Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H.S. 1997. Viable offspring derived from fetal and mammalian cells. *Nature (Lond.)* **385**: 810–813.
28. Yadav, P.S., Saini, A., Kumar, A. and Jain, G.C. 1998. Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Anim. Reprod. Sci.* **51**: 301–306.
29. Young, L.E., Sinclair, K.D. and Wilmut, I. 1998. Large offspring syndrome in cattle and sheep. *Rev. Reprod*. **3**: 155–163.
30. Zakhartchenko, V., Alberio, R., Stojkovic, M., Prelle, K., Schernthaner, W., Stojkovic, P., Wenigerkind, H., Wanke, R., Duchledr, M., Steinborn, R., Mueller, M., Brem, G. and Wolf, E. 1999. Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. *Mol. Reprod. Dev.* **54**: 264–272.
31. Zou, X., Chen, Y., Wang, Y., Luo, J., Zhang, Q., Zhang, X., Yang, Y., Ju, H., Shen, Y., Lao, W., Xu, S. and Du, M. 2001. Production of cloned goats from enucleated oocytes injected with cumulus cell nuclei or fused with cumulus cells. *Cloning* **3**: 31–37.
32. Zou, X., Wang, Y., Cheng, Y., Yang, Y., Ju, H., Tang, H., Shen, Y., Mu, Z., Xu, S. and Du, M. 2002. Generation of cloned goats (*Capra hircus*) from transfected fetal foetal fibroblast cells, the effect of donor cell cycle. *Mol. Reprod. Dev.* **61**: 164–172.