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Nuclear-Cytoplasmic Interaction and Development of Goat Embryos Reconstructed by Nuclear Transplantation: Production of Goats by Serially Cloning Embryos[1]

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ABSTRACT

The time of pronuclear formation of in vivo-matured oocytes was examined. Maturation-promoting factor (MPF) activity in enucleated and electrically activated oocytes was checked by assessment of nuclear envelope breakdown (NEBD) of fused blastomeres. The effect of stage of the cell cycle of donor cells and recipient oocytes on DNA synthesis and development of reconstructed embryos was studied. MPF activity declined rapidly to approximately 63.2% at 1 h, to 9.7% at 5 h, and to the level at which NEBD cannot occur at 7 h postactivation. All blastomeres that were fused at the time of recipient cytoplasm activation underwent NEBD and subsequent DNA synthesis. However, when blastomeres were fused to enucleated oocytes at 7 h postactivation, no NEBD was observed; DNA was replicated in nuclei at the G1/S border, but in G2 nuclei no DNA replication was observed. The proportion of development to blastocysts of reconstructed goat embryos increased with the decline in MPF activity in fused recipient cytoplasm when reconstruction took place at 0–6 h postactivation of enucleated oocytes. Generations 1, 2, 3, 4, 5, and 6 of cloned goat embryos were produced by a combination of nuclear transplantation and in vitro techniques. After transfer to recipients, 45 kids were obtained, including three pairs of monozygotic twins, three series of monozygotic triplets, two series of monozygotic quadruplets, three series of monozygotic quintuplets, and one series of monozygotic heptaplets. The present study indicates that normal DNA replication of goat blastomere nuclei can be accomplished in enucleated oocytes when MPF activity is low, regardless of the stage of the cell cycle of donor nuclei; induction of NEBD and prematuration chromosome condensation is not essential for further development of reconstructed goat embryos.

INTRODUCTION

The reconstruction of embryos by the fusion of a blastomere from a donor embryo to an enucleated oocyte arrested at metaphase of the second meiotic division (MeII) or a pronuclear zygote has been accomplished in sheep [1], cattle [2], pigs [3], goats [4], rabbits [5], and mice [6]. The development of reconstructed embryos depends on a number of factors; an important factor among these is the stages of the donor cell cycle and recipient cytoplasm at the reconstruction. Studies in rabbits using donor blastomeres at defined cell stages have shown that development to the blastocyst is greater when blastomeres in G1 and early S phase are transplanted than when the blastomeres are in late S and G2 phases of the cell cycle [7]. Morphological examination of chromosomes of reconstructed embryos suggests that when G1- and early S-phase blastomeres are fused to MeII oocytes, there is little effect on the chromosomal constitution of the reconstructed embryos; when late S- and G2-phase blastomeres are used as the donor cell, a large proportion of blastomeres in reconstructed embryos contain both large chromosomes and other abnormalities [8]. Additionally, when MeII oocytes are used as recipient cytoplasm, a series of morphological changes are observed in the nuclei of the reconstructed embryos. These include nuclear envelope breakdown (NEBD), prematuration chromosome condensation (PCC), reformation of the nuclear envelope, and nuclear swelling [7]. In contrast, when pronuclear zygotes are used as recipient cytoplasm, neither NEBD nor PCC is observed. Induction of NEBD and PCC is mediated by maturation-promoting factor (MPF), whose activity increases during G2 phase, is maximal at metaphase, and declines rapidly after activation of fertilization [9]. A previous report has suggested that the induction of NEBD and PCC is essential for the reprogramming of gene expression and further development [7]. A more recent study showed that the phase of the cell cycle of the recipient oocyte has a significant effect on NEBD, chromosome constitution, and further development of reconstructed cattle embryos, also demonstrating the incidence of chromosomal abnormalities of reconstructed embryos in cattle is reduced when fusion takes place after the decline of MPF activity in enucleated oocytes [10]. However, a similar study in the goat has not been published. In the present study, we have examined the effect of MPF activity decline on pronuclear formation of oocytes, NEBD, and DNA replication of reconstructed embryos during the first cell cycle and cloned and recloned embryos in vivo and vitro.

MATERIALS AND METHODS

Oocyte and Embryo Collection

Guanzhong dairy goats were superovulated with eight injections of 40 IU FSH (Ningbo Hormone Factor; Ningbocyte, PR China) followed by a single administration of 100 IU LH (Ningbo Hormone Factor). Ovulated oocytes were recovered from the oviducts at 26 h after injection of LH [4]. Donor embryos were taken from Angora female goats superovulated by the same methods and mated at estrus with the same strain of males. Embryos at the 16- to 32-cell stage were recovered at 96–135 h after injection of LH by flushing of oviducts and cornua uteri. Oocytes and embryos were recovered in Dulbecco's PBS with 10% fetal calf serum (FCS).

Embryo Culture and Synchronization

Embryos were selected for synchronization at the G1/S and G2 phases of the cell cycle. For synchronization of 32-cell-stage embryos at the G1/S border, 16-cell embryos were cultured in Tissue Culture Medium (TCM) 199 con-

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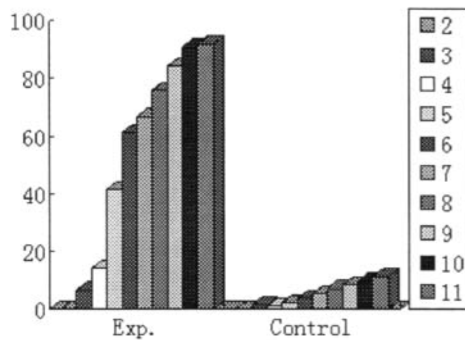

FIG. 1. Pronuclear formation of oocytes at 2–11 h postactivation using a single DC pulse in activation medium. Activated oocytes were cultured and then mounted, fixed, and stained. The number of oocytes used in each group ≥ 50. The values presented on the y-axis are percentages of oocytes that reach the pronuclear stage (0, 6.4, 14, 41.5, 61.3, 66.8, 76.1, 84.2, 90.9, and 92, respectively, for 2–11 h; controls were 1, 1.5, 1, 2, 3.6, 5, 7, 8.3, 9.6, and 11.1, respectively).

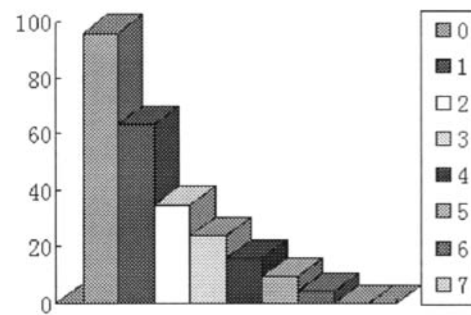

FIG. 2. Decline of MPF activity in oocyte cytoplasm was assessed by NEBD in embryos reconstructed at 0–7 h postactivation. Blastomeres from 32-cell-stage embryos were fused to enucleated oocytes at 0–7 h postactivation. Fused couplets were cultured in vitro for 5 h and then mounted, fixed, and stained. The number of reconstructed embryos used in each group ≥ 40. The values presented on the y-axis are percentages of MPF activity in the oocyte cytoplasm (96.1, 63.2, 34.8, 24.1, 15.9, 9.7, 4.2, and 0, respectively, for 0–7 h).

taining 7.5 μg/ml nocodazole (Sigma, St. Louis, MO) and 10% FCS for 12 h; then embryos were placed in fresh TCM199 with 10% FCS and 5 mM hydroxyurea (Sigma) and monitored hourly for cleavage. Cleaved embryos were placed in PBS with 10% FCS at room temperature for use. For synchronization of 32-cell embryos to G2 phase, embryos at the 32-cell stage were cultured in TCM199 with 10% FCS and 5 μg/ml cycloheximide (Sigma) for 10 h and then transferred to fresh TCM199 with 10% FCS at room temperature for use.

Enucleation, Activation, and Nuclear Transfer

Recovered oocytes were washed two times in PBS and cultured for 30 min in PBS containing 5 μg/ml cytochalasin B at 39°C. Enucleation was carried out in the same medium by aspiration of the first polar body and about one third of the cytoplasm from directly beneath the first polar body. To confirm enucleation, aspirated cytoplasm was stained with Hoescht 33342 (5 μg/ml) and checked by fluorescence microscopy. Enucleated oocytes were transferred into activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ in distilled water) in an activation chamber and given a single pulse of 1000 V/cm for 160 μsec. Activated and enucleated oocytes were cultured for 3 h in fresh TCM199 with 10% FCS and 7 μg/ml cycloheximide at 39°C in 5% CO_2 in air. Nuclear transplantation was carried out as described previously [4]. A blastomere was injected into the perivitelline space of the enucleated oocyte. The couplets were placed into PBS with 10% FCS for 0.5 h to remove the cycloheximide and then transferred to an activation chamber and aligned manually. Fusion was induced by giving a single electrical pulse of 1000 V/cm for 160 μsec in PBS.

Determination of Pronuclear Formation

Oocytes and activated oocytes were mounted on cleaned glass slides under coverslips and then fixed for 24 h in methanol:glacial acetic acid (3:1) and stained with 45% aceto-orcein. Pronuclear formation was checked by phase-contrast and differential interference contrast microscopy.

Assessment of MPF Activity

Enucleated oocytes were activated as described above and fused with blastomeres from 32-cell-stage embryos at the time of activation and at various times after activation. Fused couplets were cultured and morphological examination was carried out as described above. The number of embryos in which NEBD had occurred was determined.

Determination of DNA Synthesis

DNA synthesis was determined by incorporation and immunofluorescent detection of thymidine analogue 5-bromo-2′-deoxyuridine-5′-triphosphate (BrDU; Sigma). The primary antibody was mouse anti-BrDU (Sigma), and the secondary antibody was fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (Sigma). The experimental procedure was similar to that described previously [11]. The results were examined by fluorescence microscopy.

Serial Embryo Reconstruction

The reconstructed embryos were placed on goat oviduct epithelial cells in TCM-199 with 10% goat serum made at the second day postestrus and cultured at 39°C and in 5% CO_2 in air for 5–7 days. The embryos that developed to the 32-cell stage were serially reconstructed by the procedures of nuclear transplantation as described above. Generation 1, 2, 3, 4, 5, and 6 (G1, G2, G3, G4, G5, and G6) embryos were obtained. Cloned and recloned embryos were either cultured or transferred into recipient goats as previously described [4].

RESULTS

Pronuclear Formation

The oocytes recovered at 26 h after injection of LH were activated. Activated oocytes were cultured and then mounted, fixed, and stained at 2–11 h postactivation. The percentage of oocytes in which pronuclei were seen increased with culture time; 61% of the oocytes at 6 h and 92% at 11 h postactivation had formed a pronucleus (Fig. 1). As an activation control, oocytes were sham treated in the same activation medium (without electrical pulsing) and then cultured in TCM-199 with 10% FCS at 39°C in 5% CO_2 in air, and pronucleus formation was examined. Of these oocytes, 1%, 7%, and 11% activated spontaneously and formed a pronucleus, respectively, at 4, 8, and 11 h postactivation (Fig. 1).

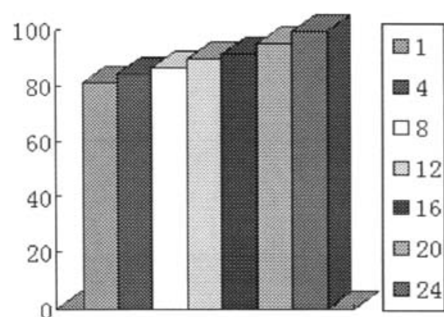

FIG. 3. Frequency of S-phase blastomeres in donor embryos at the 32-cell stage as evidenced by incorporation of BrDU (see *Materials and Methods*). The number of blastomeres used in each group ≥ 105. The values presented on the y-axis are percentages of S-phase blastomeres after culture for 1–24 h (81, 84.2, 86.1, 89.4, 91.3, 95, 99.2, respectively, for 1–24 h).

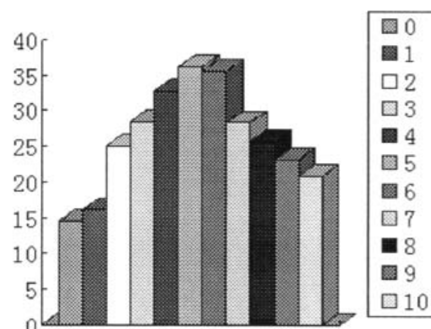

FIG. 4. Development of embryos reconstructed at 0–10 h postactivation. Blastomeres from 32-cell-stage embryos were fused to an enucleated oocyte at 0–10 h postactivation. Fused couplets were cultured in vitro for 7 days. The number of embryos used in each group ≥ 45. The values presented on the y-axis are percentages of reconstructed embryos developed to blastocysts (14.6, 16.1, 25, 28.4, 32.9, 36.2, 35.6, 28.3, 25.7, 23.2, 20.8, respectively, for 0–10 h).

Disappearance of MPF Activity

MPF activity was determined by examination of NEBD in embryos reconstructed at 0–7 h postactivation. MPF activity rapidly declined after activation, to approximately 63.2% at 1 h and 34.8% at 2 h. NEBD no longer occurred by 7 h postactivation (Fig. 2).

DNA Synthesis in Blastomeres

The proportion of S-phase blastomeres in donor embryos was determined by examining the frequency of anti-BrDU incorporation. The proportion of FITC-labeled nuclei ranged from 81% at 1 h to 99% at 24 h. The data suggest that approximately 80% or more nuclei of blastomeres were in S phase of the cell cycle in the 32-cell-stage goat embryos (Fig. 3).

TABLE 1. The development of serially reconstructed embryos in vitro.[a]

Reconstruction generation	Number of reconstructed embryos cultured in vitro	Number of embryos developing to morulae	Developmental rate[b] (%)
G1	57	18	30.9
G2	62	16	25.4
G3	55	15	27.2
G4	56	14	24.8
G5	60	17	28.3
G6	64	16	25.0

[a] Blastomeres from 32-cell-stage embryos fused to enucleated oocytes at 5–6 h postactivation; fused couplets were cultured in vitro for 6 days, and the number of embryos developed to morulae was examined.
[b] The development rate was not different among groups ($p > 0.05$).

TABLE 2. Transfer results for serially reconstructed embryos.[a]

Cloning generation	Number of recipients transferred	Number of embryos transferred	Number of recipients	Number of young produced
G1	14	28	6	9
G2	10	20	3	6
G3	11	21	4	7
G4	19	38	9	12
G5	17	34	7	11
Total	71	141	29	45

[a] Blastomeres derived from 32-cell-stage embryos were fused to enucleated oocytes at 5–6 h postactivation; fused couplets were cultured in vitro for 6 days and then reconstructed embryos that had developed to morulae were transferred.

DNA Synthesis in Reconstructed Embryos

In order to examine the DNA replication potential of nuclei at different phases of the cell cycle after reconstruction into enucleated oocytes at various times postactivation, blastomeres were fused to enucleated oocytes at 0–7 h postactivation. Fused couplets were cultured, and the proportion of FITC-labeled nuclei was examined. When blastomeres arrested at the G1/S border or at G2 phase of the cell cycle were fused to enucleated oocytes at 0 h postactivation, DNA synthesis was observed in all nuclei of blastomeres of the embryos (39 of 39, 45 of 45); when they were fused to enucleated oocytes at 7 h postactivation, DNA synthesis was observed in nuclei of blastomeres arrested at the G1/S phase (47 of 47), but not in those arrested at G2 phase of the cell cycle (0 of 52).

Development of Reconstructed Embryos

In order to compare development of embryos reconstructed at different times postactivation, 32-cell-stage blastomeres were fused to enucleated oocytes at 0–10 h postactivation. Fused couplets were cultured for 7 days. The proportion of development to blastocysts was significantly higher when reconstruction took place at 4, 5, and 6 h postactivation than at 0, 1, 9, and 10 h postactivation ($p < 0.05$). But no statistical differences were observed among the groups of embryos reconstructed at 2–8 h postactivation ($p > 0.05$, Fig. 4). Embryos were serially reconstructed by nuclear transplantation followed by culture. Every generation of cloned embryos was reconstructed by fusing 32-cell-stage blastomeres to enucleated oocytes at 5–6 h postactivation. G1, G2, G3, G4, G5, and G6 morulae were obtained in vitro, while G1, G2, G3, G4, and G5 kids were produced after transfer. A total of 45 kids were born, including three pairs of monozygotic twins, three series of monozygotic triplets, two series of monozygotic quadruplets, three series of monozygotic quintuplets, and one series of monozygotic heptaplets. The results are shown in Table 1 and Table 2.

DISCUSSION

The present study shows that when goat blastomeres are reconstructed into MeII oocytes at the time of activation, NEBD and PCC occur in all donor nuclei and that this is

followed by reformation of nuclear membrane and DNA replication regardless of the cell cycle phase of the donor blastomeres. In contrast, when blastomeres are reconstructed into enucleated oocytes in which MPF activity has declined postactivation, NEBD and PCC do not occur and DNA replication occurs only in G1 and S donor nuclei, not in G2 nuclei. The present study also shows that at 0–6 h postactivation, the proportion of development of reconstructed embryos to blastocysts increases with the decline of MPF activity in recipient cytoplasm. Additionally in the present experiments, embryos were reconstructed and serially reconstructed by fusing 32-cell-stage blastomeres to enucleated oocytes at 5–6 h postactivation, at which time MPF activity had declined to approximately 9.7–4.2%. The proportion of development to full term of reconstructed goat embryos after transfer in the present study was higher than in a previous study from our laboratory, in which embryos were reconstructed by fusing 32-cell-stage blastomeres into enucleated MeII oocytes (previous inactivation) [4]. These results suggest that NEBD and PCC induced by MPF have detrimental effects on the development of reconstructed embryos. It has been reported that the NEBD induced by MPF allows a licensing factor to enter the nucleus from the cytoplasm and to induce additional DNA synthesis [12], then resulting in chromosome abnormalities, aneuploidy and tetraploidy without polar body extrusion. In contrast, when blastomeres are reconstructed into previously activated and enucleated oocytes (after disappearance of MPF activity), coordinate and correct DNA replication occurs in G1, S, and G2 donor nuclei to maintain the correct ploidy of the reconstructed embryos because there is neither NEBD nor additional DNA synthesis. This likely resulted in the increased development of reconstructed goat embryos to blastocysts in vitro and to full term after transfer in the present study.

In the mouse, 2-cell-stage blastomere nuclei transplanted to enucleated oocytes can develop to full term, but the development potential of nuclei derived from 4- and 8-cell-stage embryos is seriously limited. It has been suggested that the ability of nuclear-transplanted embryos developed to full term in vivo is affected by the transcriptional activity of the embryo genome [13], which occurs at the 2-cell stage in the mouse. In the present study, however, not only a high proportion of development to morulae and blastocysts, but also 45 normal cloned goats, were successfully obtained from serial nuclear transplantation of embryos with nuclei from 32-cell-stage embryos. These results suggest that initiation of embryo genome transcription has little effect on the development ability of nuclear-transplanted goat embryos with nuclei, in which initiation of embryo genome transcription occurred. A study in our laboratory indicated that some genome activation occurs at the 2-cell stage but that the major transformation to embryonic expression does not begin until the 8-cell stage in the goat embryo (unpublished results). This prolonged period of transition may be the reason for the differing development potential of nuclear-transplanted embryos between the goat and the mouse.

The objective of nuclear transplantation in domestic animals is to produce genetically identical offspring. In general, 32-cell-stage embryos are used as the nuclear donor in the goat. In such embryos, only a small proportion of blastomeres are expected to be in G1 phase of the cell cycle. However, the present study included a series of experimental procedures by which more reconstructed goat embryos of normal ploidy can be produced from 32-cell-stage blastomeres that are at any point in the cell cycle through reconstruction into enucleated and previously activated oocytes in which MPF has declined. To our knowledge, this is the first report of obtaining G1, G2, G3, G4, and G5 generations of cloned embryo goats by nuclear transplantation and serial nuclear transplantation.

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