Development of Nuclear Transfer and Parthenogenetic Rabbit Embryos Activated with Inositol 1,4,5-Trisphosphate

S.M. Mitalipov, K.L. White, V.R. Farrar, J. Morrey, and W.A. Reed

Animal, Dairy and Veterinary Sciences Department, Center for Developmental and Molecular Biology, Biotechnology Center, Utah State University, Logan, Utah 84322-4815

ABSTRACT

The present study was carried out to evaluate the effects of different activation protocols, enucleation methods, and culture media on the development of parthenogenetic and nuclear transfer (NT) rabbit embryos. Electroporation of 25 mM inositol 1,4,5-trisphosphate (IP3) in calcium- and magnesium-free PBS immediately induced a single intracellular calcium transient in 6 out of 14 metaphase II-stage rabbit oocytes evaluated during a 10-min recording period. The percentage of oocytes treated with IP3 followed by 6-dimethylaminopurine (IP3 + DMAP) that cleaved (83.9%) and reached the blastocyst stage (50%) was significantly higher ($p < 0.05$) than those activated with multiple pulses (61.6% and 30.1%, respectively) or treated with ionomycin + DMAP (32.9% and 5.7%, respectively). Development of IP3 + DMAP-activated rabbit oocytes and in vivo-fertilized zygotes in different culture media was studied. Development of activated oocytes to the blastocyst stage in Earle's balanced salt solution (EBSS) supplemented with MEM nonessential amino acids, basal medium Eagle amino acids, 1 mM l-glutamine, 0.4 mM sodium pyruvate, and 10% fetal bovine serum (FBS) (EBSS-complete) (40.6%) was significantly higher ($p < 0.05$) than those developed in either Dulbecco's Modified Eagle's medium (DMEM)/RPMI + 10% FBS (15.5%) or CR1aa + 10% FBS (4%) medium. In addition, 100% of in vivo-fertilized rabbit zygotes developed to the blastocyst stage in EBSS-complete. A third set of experiments was carried out to study the efficiency of blind versus stained (Hoehst 33342) enucleation of oocytes. Twenty-nine of 48 blind enucleated and IP3 + DMAP-activated oocytes cleaved (60.4%), and 15 (31.2%) subsequently reached the blastocyst stage, whereas 9 of 52 oocytes enucleated using epifluorescence (17.3%) cleaved, and none of these reached the blastocyst stage. When the above parameters that yielded the highest blastocysts were combined in an NT experiment using adult rabbit fibroblast nuclei, 72.2% (39 of 54) of the fused nuclear transplant embryos cleaved and 29.6% (16 of 54) reached the blastocyst stage.

INTRODUCTION

Cloning of embryos by nuclear transplantation has been developed in several species. Cloning involves the transfer of a nucleus from a multicellular embryo, fetal, or adult cell line into an enucleated metaphase II (MII) oocyte. This oocyte has the ability to incorporate the transferred nucleus and support development of a new embryo [1, 2]. Morphological indications of the reprogramming of the transferred nucleus are the dispersion of nucleoli [3] and swelling of the transferred nucleus [4–7]. The most conclusive evidence that oocyte cytoplasm has the ability to reprogram is the birth of offspring from nuclear transplant embryos in several species, including sheep [2, 8–10], cattle [11, 12], pigs [1], and rabbits [5, 13].

Although nuclear transplant offspring have been produced, the efficiency of the procedure remains low. In the species reported to date, the highest efficiency was approximately 4% in sheep [9], 4% in cattle [12], 1% in pigs [1], and 3.7–10% in rabbits [5, 13]. The factors affecting the efficiency of nuclear transplantation are enucleation of the recipient oocyte, fusion of the transplanted nucleus to the enucleated oocyte, activation of the oocyte, and reprogramming of the transferred nucleus. In each of the studies previously indicated, if we evaluate the efficiency of each of these steps, we find that activation may be the factor responsible for the greatest loss in efficiency [13]. In rabbits, particularly, it has been reported that activation of oocytes was the least efficient step of the nuclear transfer (NT) procedure (46%) [5]. Enucleation rates are relatively high in each of the species studied (68% in sheep [9]; 60% in cattle [11]; 74% in pigs [1]). In most reported studies, enucleation of rabbit oocytes has not been assessed by visualization. Enucleation rates in some studies varied from 60% [13] to 70% [14]. Fusion rates are also high in each of the species reported, ranging from 70% to 100%. It has been well documented that use of aged oocytes is beneficial to increase activation rates [5, 13, 15, 16], although fusion and enucleation appear to be more difficult compared to early oocytes [16, 17]. In addition, it is currently unclear what effects oocyte aging has on other aspects of nuclear transplantation and, more importantly, subsequent development.

Artificial activation of oocytes is an essential component of the current nuclear transplantation protocols. A periodic intracellular increase of calcium (transient) during fertilization is critical for activation of oocytes. Many agents that are used to induce parthenogenetic activation of mammalian oocytes cause a monotonic rise of free intracellular calcium level and do not completely mimic the sperm-induced intracellular calcium ([Ca2+]i) transients. Use of multiple electrical pulses (6 pulses, 30 min apart) in fusion medium containing calcium is the only known method of activation of rabbit oocytes [13]. As [Ca2+]i channels, at least three isoforms of both ryanodine and inositol 1,4,5-trisphosphate (IP3) receptors have been identified, and the existence of both receptors and different isoforms has been observed in excitable and nonexcitable cells [18–26]. Mechanisms that result in the repetitive release of [Ca2+]i from intracellular stores may involve IP3-induced release [25, 26].

The age of the recipient oocyte has a dramatic effect on the activation efficiency in rabbits. Aged oocytes ($\geq 24–25$ h after LH injection) can be more efficiently activated by multiple pulses than younger oocytes (18–19 h after LH injection), but the ability to develop to blastocyst decreases with age [5, 13]. Susko-Parrish et al. [27] reported that in-
duction of a [Ca$^{2+}$], transient in young bovine oocytes was sufficient to cause resumption of meiosis and extrusion of the second polar body, but not pronuclear formation; the oocytes then re-entered meiotic arrest. A combination of induction of [Ca$^{2+}$], transients (ionomycin, IP$_3$) followed by inhibition of protein phosphorylation (using 6-dimethylamino-norpurine [DMAP]) allowed young bovine oocytes to enter into embryonic cell cycles without undergoing a second reduction division [27, 28]. The dephosphorylation induced by DMAP inactivates c-mos and mitogen-activated protein kinase in mouse oocytes, resulting in the formation of a nuclear envelope after oocyte activation and subsequent normal postfertilization development [29, 30]. Treatment with DMAP after bovine oocyte activation induces pronuclear formation and entry into the interphase of the first mitotic cell cycle, presumably as a uniform diploid [27, 31]. However, no information is available on the DMAP-induced NT or parthenogenetic development of rabbit embryos.

In addition to appropriate conditions that induce activation, the culture medium supporting in vitro development of rabbit NT embryos is critical for successful cloning. Several culture systems have been used for rabbit embryos including defined media, coculture, and the use of biological fluids [32–35]. However, embryo development in most of these media is not satisfactory.

The present study was carried out to evaluate the effects of different activation protocols and culture media on the development of parthenogenetic and NT rabbit embryos.

**MATERIALS AND METHODS**

**Oocyte and Zygote Collection**

Mature New Zealand White and Dutch Belted female rabbits were superovulated with six s.c. injections of FSH (Ovagen, ovine pituitary extract; Immuno-Chemical Products Ltd., Auckland, New Zealand): two 0.3-mg and four 0.4-mg injections, given 12 h apart. LH (from bovine pituitary glands; Sioux Biochemical, Sioux, IA) at a dose of 2.5 mg was injected i.v. 12 h after the last dose of FSH. At 13–14 h after injection of LH (post-LH), the females were laparotomized, and mature MII oocytes were flushed from the oviducts with Hepes-buffered hamster embryo culture medium (HECM/Hepes, [36]) supplemented with 3 mg/ml BSA. The cumulus cells were dispersed in HECM/Hepes containing 10 mg/ml hyaluronidase, and corona cells were removed by pipetting with a small-bore Pasteur pipette. For zygote collection, superovulated does were bred concurrently with LH injection and laparotomized at 16–17 h post-LH. Zygotes were flushed from the oviducts using HECM/Hepes. Unless otherwise indicated, all experiments represent the results of a minimum of four replicates.

**Intracellular [Ca$^{2+}$], Monitoring**

Oocytes were loaded with [Ca$^{2+}$], indicator by incubation in 2 mM Fura-2 AM ester and 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR) in calcium- and magnesium-free Dulbecco’s PBS (Ca$^{2+}$-, and Mg$^{2+}$-free PBS; HyClone, Logan, UT) plus 100 mM EGTA at 37°C in darkness for 20 min. The [Ca$^{2+}$], concentration was calculated with Image-1 software (Universal Imaging, Inc., West Chester, PA) by measuring ratio changes of fluorescence intensity at emission 510-nm wavelength when excited at 340-nm and 380-nm wavelength. Images of whole oocytes were collected with a 10× fluorescence objective (Fluor; Nikon Instruments, Garden City, NY), then transmitted via a video camera to a PC 486 computer, where images were processed, saved, and displayed on the monitor in pseudocolor. We present our data in ratio with a value of 3 approximately equal to 717 nM free Ca$^{2+}$ ion concentration. For measurement of [Ca$^{2+}$], oocytes were washed in Ca$^{2+}$-, and Mg$^{2+}$-free PBS and transferred into an electroporation chamber (3.5-mm gap) mounted on a Nikon Diaphot inverted microscope. After a 20-sec baseline reading, oocytes were given two 15-μsec (1 sec apart) 1.4 kV/cm DC pulses using a BTX Electro Cell Manipulator (BTX 200; BTX, Inc., San Diego, CA). The [Ca$^{2+}$], changes were recorded for each individual oocyte for 10 min after the electroporation pulses.

**Activation of MII Oocytes**

Cumulus-free MII oocytes (18–19 h post-LH) were randomly assigned to one of four activation treatments: 1) incubation for 4 min with 5 mM ionomycin [37]; 2) electroporation (two 15-μsec DC pulses spaced 1 sec apart, 1.4 kV/cm) with IP$_3$ (25 μM d-myo-inositol 1,4,5-trisphosphate, hexapotassium salt; Molecular Probes) in Ca$^{2+}$- and Mg$^{2+}$-free PBS plus 100 mM EGTA; 3) electroporation in Ca$^{2+}$- and Mg$^{2+}$-free PBS without IP$_3$ (control); 4) electroporation in 0.25 M d-sorbitol containing 0.1 mM calcium acetate and 0.5 mM magnesium acetate (two 15-μsec DC pulses 1 sec apart, 1.4 kV/cm; six electroporations 30 min apart). Treated oocytes were then incubated with 0.2 mM DMAP for 4 h (unless indicated otherwise).

**In Vitro Culture of Activated Oocytes**

Activated oocytes were transferred into 30-μl drops of one of three culture media, which are referred to as DMEM/RPMI, CR1aa, and EBSS-complete. These media are composed as follows: 1) DMEM/RPMI is Dulbecco’s Modified Eagle medium (DMEM) + RPMI 1640 (1:1; v:v; HyClone) supplemented with MEM nonessential amino acids (NEAA; Sigma, St. Louis, MO) and 10% (v:v) fetal bovine serum (FBS; HyClone); 2) CR1aa is CR1aa medium [38] plus 10% FBS; 3) EBSS-complete is bicarbonate-buffered Earle’s balanced salt solution (EBSS) supplemented with NEAA, EAA (essential amino acids [BME]; Sigma), 1 mM L-glutamine, 0.4 mM sodium pyruvate, and 10% FBS. The drops were then covered with mineral oil. All embryos were cultured for 5–6 days in a humidified atmosphere of 5% CO$_2$ in air at 37°C. At Day 2 and Day 6 (Day 0 = day of activation), cleavage and development to the blastocyst stage, respectively, were determined and recorded.

**NT Procedures**

A donor fibroblast cell line, established from a mature Dutch Belted female rabbit, was cultured in DMEM supplemented with 10% FBS. The donor cells for NT were synchronized in the G$_0$ phase of the cell cycle by culturing in DMEM + 0.5% FBS for 5–10 days. Cumulus-free MII oocytes were incubated for 30 min with 10 μg/ml of Hoechst 33342 (unless indicated otherwise), transferred to 30 μl of HECM/Hepes manipulation medium containing 7.5 μg/ml cytochalasin B, and incubated for 10–15 min before enucleation. The first polar body and metaphase plate of an oocyte were drawn into an enucleation pipette (25–28-μm inner diameter). Enucleation was assessed by visualization of the metaphase plate under UV light (except
in the blind enucleation group) and a Chroma Technology (Brattleboro, VT) Hoechst filter set (exciter D360, emitter D460). The time of exposure to UV light was less than 10 seconds. The same enucleation pipette was used to aspirate the disaggregated donor cell and place it into the perivitelline space of the enucleated oocyte. Fusion of NT couples was induced by one 15-μsec, 2.0-kV/cm DC pulse in a 3.5-mm fusion chamber (BTX). Fusion medium was 0.25 M D-sorbitol containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.5 mM Hepes, and 1 mg/ml fatty acid-free BSA. Fused NT embryos were IP3 + DMAP-activated and cultured to the blastocyst stage in EBSS-complete medium.

Statistics

Development of activated oocytes to the blastocyst stage was evaluated, and treatments were compared using chi-square analysis. Statistical comparisons of the [Ca2+]i rise parameters were made by unpaired t-tests (Statview; Abacus Systems, Berkley, CA).

RESULTS

Intracellular calcium transients and oscillations have been reported to occur during fertilization in all species studied including rabbits [39]. To establish whether electroporation with IP3 or calcium-containing medium was able to evoke an intracellular [Ca2+]i rise, MII rabbit oocytes were pulsed either in 25 μM IP3 or in D-sorbitol buffer containing 0.1 mM Ca2+ and 0.5 mM Mg2+. Electroporation of 25 μM IP3 in Ca2+- and Mg2+-free PBS immediately induced [Ca2+]i release in 6 out of 14 rabbit oocytes as shown by Fura-2 fluorescence ratio change (Fig. 1A). As a control, oocytes were electroporated in Ca2+- and Mg2+-free PBS (vehicle buffer). Electroporation in vehicle buffer alone failed to induce any detectable [Ca2+]i elevation in rabbit oocytes (n = 5). Thus, neither contaminating Ca2+ in electroporation buffer nor the buffer itself was responsible for the observed increase in [Ca2+]i concentration. Six out of 10 oocytes responded to electroporation in D-sorbitol buffer containing 0.1 mM Ca2+ and 0.5 mM Mg2+ (Fig. 1B). The mean value of the [Ca2+]i peak in the IP3 group (3.24 ± 1.18) was higher than in D-sorbitol (1.36 ± 0.84), but the values did not differ significantly (p > 0.05). In these experiments, a ratio value of 3.0 is approximately equal to 717 nM of free Ca2+ ion concentration. Peak duration was calculated as the time interval (in seconds) between mid-peak points. The mean value of peak duration in the D-sorbitol group (30 ± 1.75) was significantly higher (p < 0.001) than in the IP3 group (20.5 ± 0.71). The oocytes in both the IP3 and D-sorbitol treatment groups failed to exhibit second [Ca2+]i rises during the recording period of 10 min. These data represent three replicates from the same population of oocytes.

Parthenogenetic development of rabbit MII oocytes induced by various activation treatments is summarized in Table 1. Oocytes (except for multiple-pulse and IP3-alone activation groups) were activated at 18–19 h post-LH, incubated for 4 h in DMAP, and transferred into EBSS-complete culture medium. Cleavage was recorded at 48 h of culture, and development to the blastocyst stage was recorded at 6 days of culture. Although the cleavage rates of embryos activated by multiple pulses or ionomycin + DMAP were comparable (61.6% and 52.9%, respectively; p > 0.2), the percentage of embryos developing to the blastocyst stage in the multiple-pulse activation group was significantly higher than that in the ionomycin + DMAP group (30.1% and 5.7%, respectively; p < 0.05). The percentages of oocytes treated with IP3 + DMAP that cleaved (83.9%) and reached the blastocyst stage (50%) were significantly higher (p < 0.05) than those activated with multiple pulses or ionomycin + DMAP. Neither electroporation with IP3 alone or vehicle medium followed by incubation in DMAP induced parthenogenetic development of MII rabbit oocytes to the blastocyst stage.

Development of IP3 + DMAP-activated rabbit oocytes and in vivo-fertilized zygotes in different culture media is summarized in Table 2. The percentage of cleaved parthenogenotes in DMEM/RPMI medium (93.7%) was significantly higher (p < 0.05) than those in CR1aa (26.5%) or EBSS-complete (71.9%) and did not significantly differ (p > 0.1)

TABLE 1. The effect of activation treatments on parthenogenetic development of rabbit oocytes.

<table>
<thead>
<tr>
<th>Activation</th>
<th>Culture medium</th>
<th>n</th>
<th>Cleavage (%)</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple pulses</td>
<td>EBSS</td>
<td>73</td>
<td>45 (61.6)a</td>
<td>22 (30.1)a</td>
</tr>
<tr>
<td>Ionomycin + DMAP</td>
<td>EBSS</td>
<td>70</td>
<td>37 (52.9)c</td>
<td>4 (5.7)c</td>
</tr>
<tr>
<td>IP3 + DMAP</td>
<td>EBSS</td>
<td>56</td>
<td>47 (83.9)b</td>
<td>28 (50)c</td>
</tr>
<tr>
<td>IP3 alone</td>
<td>EBSS</td>
<td>23</td>
<td>7 (30.4)d</td>
<td>0d</td>
</tr>
<tr>
<td>PBS + DMAP</td>
<td>EBSS</td>
<td>12</td>
<td>2 (16.7)d</td>
<td>0d</td>
</tr>
</tbody>
</table>

a-d Within a column, values with different superscripts were significantly different (p < 0.05).
TABLE 2. The effect of culture medium on the development of activated MII rabbit oocytes and in vivo-fertilized zygotes.

<table>
<thead>
<tr>
<th>Activation</th>
<th>Culture medium</th>
<th>n</th>
<th>Cleavage (%)</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP₃ + DMAP</td>
<td>DMEM/RPMI</td>
<td>32</td>
<td>30 (93.7)ᵇ</td>
<td>5 (15.6)ᵇ</td>
</tr>
<tr>
<td>IP₃ + DMAP</td>
<td>CR1aa</td>
<td>49</td>
<td>13 (26.5)ᵇ</td>
<td>2 (4.0)ᵇ</td>
</tr>
<tr>
<td>IP₃ + DMAP</td>
<td>EBSS-complete</td>
<td>32</td>
<td>23 (71.9)ᵇ</td>
<td>13 (40.6)ᵇ</td>
</tr>
<tr>
<td>Zygotes</td>
<td>EBSS-complete</td>
<td>33</td>
<td>33 (100)ᶜ</td>
<td>33 (100)ᶜ</td>
</tr>
</tbody>
</table>

Within a column, values with different superscripts were significantly different (p < 0.05).

from the cleavage rate of in vivo-produced zygotes in EBSS-complete (100%). However, development of activated oocytes to the blastocyst stage in EBSS-complete medium (40.6%) was significantly higher (p < 0.05) than those cultured in DMEM/RPMI (15.5%) or CR1aa (4%). In addition, 100% of in vivo-fertilized rabbit zygotes developed to the blastocyst stage in EBSS-complete medium.

The efficiency of blind and stained (i.e., assessed by Hoechst 33342) enucleation was studied. Recently ovulated MII rabbit oocytes were flushed from the oviducts of superovulated does at 12–13 h post-LH and enucleated at 13–14 h. The first polar body and 15–20% of adjacent cytoplasm, presumably containing the metaphase plate, were drawn into the enucleation pipette. Enucleation efficiency was assessed by IP₃ + DMAP activation and subsequent culture of enucleated oocytes in EBSS-complete medium.

If development occurred, this was evidence of incomplete enucleation. Twenty-nine of 48 blind enucleated oocytes cleaved (60.4%), and 15 (31.2%) subsequently reached the blastocyst stage. The percentages of cleavage and blastocyst development from blind enucleated oocytes did not differ (p > 0.1) from those in the control non-enucleated activated group (total of 50 oocytes) of oocytes (cleavage, n = 37, 74%; blastocyst, n = 22, 44%). To improve the enucleation efficiency, 13–14 h post-LH MII oocytes were stained with Hoechst 33342 and enucleated using an epifluorescence microscope. In more than 50% of oocytes evaluated, the metaphase plate was located far from the first polar body. This observation indicates that removal of the metaphase plate is extremely difficult without visualization (Fig. 2A).

In addition, MII oocytes from Dutch Belted does had a more opaque, dark cytoplasm compared to those from New Zealand White rabbits. It was, therefore, difficult to visualize a metaphase plate even in Hoechst-stained Dutch Belted oocytes to confirm enucleation. Only successfully enucleated New Zealand White oocytes were used (Fig. 2B) in subsequent NT experiments. Nine of 52 oocytes enucleated using epifluorescence (17.3%) subsequently cleaved, and none of these reached the blastocyst stage.

In the last experiment, we evaluated the efficiency of NT by assessing in vitro development of NT embryos using the combined parameters that yielded the highest blastocyst development in previous experiments (i.e., activation, enucleation, and culture medium). A fibroblast cell line estab-

FIG. 2. A) Hoechst-stained rabbit MII oocyte at 14 h post-LH injection. The metaphase plate (MP) is located far from the first polar body (PB). B) Visualization of polar body (PB) and metaphase plate (MP) during oocyte enucleation by Hoechst staining and epifluorescence. C) Expanded NT blastocyst. D) Hatching NT blastocyst. ×100.
lished from an adult Dutch Belted female was used as the source of nuclear donor cells. Cells were synchronized in presumable G1 phase of the cell cycle by culturing in DMEM supplemented with 0.5% FBS for 5–10 days. Fifty-seven Hoechst-stained New Zealand White oocytes were enucleated at 13–14 h post-LH injection and immediately fused to donor cells. Fused NT couples were IP3 + DMAP-activated at 18–19 h post-LH and cultured in EBSS-complete medium for 6–7 days. Fifty-four (94.7%) fused, 39 (72.2%) cleaved, and 16 (29.6%) developed to the expanded and hatched blastocyst stage (Fig. 2, C and D).

**DISCUSSION**

Fertilized rabbit oocytes exhibit repetitive [Ca\(^{2+}\)] transients over several hours after sperm-egg fusion [39]. We have shown that an electrically mediated single IP3 stimulation elicited a single [Ca\(^{2+}\)] rise, within the 10-min recording period following the pulse (Fig. 1A). The mean of amplitude and the mean value of half-time decline (duration) of IP3-induced [Ca\(^{2+}\)], rise (see *Results* section) were similar to those observed in recently fertilized eggs [39]. It has been postulated that a persistent IP3 stimulation is required to generate the multiple [Ca\(^{2+}\)] rises observed during fertilization of hamster eggs [40, 41]. Continuous intracytoplasmic injections of IP3 produced repetitive [Ca\(^{2+}\)] rises in rabbit MII oocytes, with decreasing amplitudes [39]. Electroporation in a 25 mM IP3 solution did not induce a second [Ca\(^{2+}\)] peak within a 10-min observation period. The oocytes may have either had second and subsequent [Ca\(^{2+}\)] rises, with intervals longer than 10 min or may have generated only a single [Ca\(^{2+}\)] oscillation.

Electroporation in Ca\(^{2+}\)-containing D-sorbitol medium also induced a single [Ca\(^{2+}\)] rise, with longer amplitude duration (Fig. 1B) than those observed in the IP3 treatment (*p* < 0.05). There was a relatively short response time (expressed as mean value of half-time decline) after the single electrical stimulation in either IP3 or Ca\(^{2+}\)-containing buffer (20.5 ± 0.71 and 30 + 1.75 sec, respectively). This appears to indicate that the rabbit oocyte membrane quickly recovers after electrical pulse and reduces external IP3 or Ca\(^{2+}\) influx. The rapid rabbit oocyte-cell fusion within 15–20 min after the fusion pulse as compared to cattle or sheep, which require over 1 h (unpublished results), also supports this conclusion.

A [Ca\(^{2+}\)] stimulation induces a rapid but transient decline in histone H1 kinase and, presumably, maturation promoting factor (MPF) activity in rabbit [42] and bovine oocytes [43, 44]. Premature H1 kinase and MPF reactivation in the first cell cycle may have adverse effects on embryo development. Elevated H1 kinase activity early in the cell cycle of oocytes results in premature condensation of chromat in (PCC), which in turn may induce the formation of a metaphase III oocyte [42]. Metaphase III oocytes have been observed in mice when the [Ca\(^{2+}\)] stimulus was not able to induce the formation of pronuclei [45]. Collas et al. [46] have demonstrated that PCC caused chromat damage and lethal abnormalities in embryonic chromosome structure. Multiple, subsequent [Ca\(^{2+}\)] stimulations were required to maintain basal levels of H1 kinase activity and subsequent parthenogenetic development of rabbit oocytes [13, 42]. The first [Ca\(^{2+}\)] rises are likely to mediate H1 kinase and MPF inactivation, whereas subsequent transients may be needed to maintain H1 kinase and MPF inactivity during the first cell cycle [42].

The effects of DMAP on chromatin and microtubule configurations during meiosis are mediated by the inhibition of protein kinases and, consequently, protein phosphorylation [47, 48]. Incubation in DMEM for 4 h immediately following a single IP3-mediated [Ca\(^{2+}\)], rise induced parthenogenetic development of MII rabbit oocytes to the blastocyst stage in present study (Table 1). The percentage of embryos that developed to the blastocyst stage was significantly higher (*p* < 0.005) than those in multiple [Ca\(^{2+}\)] stimulations without DMAP. Treatment with DMAP following activation with ionomycin of bovine oocytes [27] and NT embryos [37] induced blastocyst development. Treatment of rabbit oocytes in this study with ionomycin followed by DMAP resulted in a satisfactory rate of cleavage, but development to the blastocyst stage was low (Table 1). Neither elevation of intracellular calcium triggered by IP3 alone nor treatment with DMAP alone was sufficient to induce parthenogenetic development. These results support the suggestion that DMAP can induce the formation of interphase nuclei only once an initial decline in MPF has been triggered independently, in our case with IP3 [47].

Bicarbonate-buffered EBSS supplemented with 10% FBS has been successfully used to culture parthenogenetic and NT rabbit embryos [5, 13]. In the present study, we investigated the use of EBSS supplemented with NEAA, EAA, sodium pyruvate, L-glutamine, and 10% (v:v) FBS vs. DMEM/RPMI and CR1aa.

Most of Eagle’s 20 amino acids are present in rabbit uterine fluids and blastocysts [49, 50]. Inclusion of most or all of both EAA and NEAA in culture media improved embryo development in rabbits [33, 51, 52]. Supplementation with glucose and pyruvate resulted in a doubling of the cleavage rate in one-cell rabbit embryos [32]. Our results indicate that culture in EBSS-complete medium greatly enhanced parthenogenetic development of activated rabbit oocytes to the blastocyst stage compared to DMEM/RPMI or CR1aa (40.6% vs. 15.6% and 4%, respectively, Table 2). Moreover, 100% of one-cell in vivo-fertilized zygotes cultured in EBSS-complete medium developed to the blastocyst stage in this study.

The efficiency of nuclear transplantation was improved with procedures developed in this study. The results indicate that in rabbits, enucleation is more efficient with Hoechst-stained recently ovulated oocytes (see *Results* section). The cytoskeletal organization of the oocyte during postovulatory aging in vitro and in vivo has been studied in mice [53] and rabbits [54]. Postovulatory aging includes changes in the location of the second meiotic spindle, which migrates toward the center of the egg. The spindle then breaks down and the chromosomes are no longer organized in a metaphase plate [55]. Our observations support these findings. The polar body could not be used as a marker to locate the metaphase plate in recently ovulated rabbit oocytes for blind enucleation. Although others [7, 13, 15] have used blind enucleation successfully, our data indicate that unless large amounts of egg cytoplasm were removed (see Fig. 2), at least 50% of the resulting NT embryos would be polyploid. This potential for polyploidy could result in lower development to term. In cattle, pigs, and sheep, because of the darkness of the oocyte cytoplasm, the chromosomes cannot be seen and removed by blind aspiration. Enucleation efficiency has been assessed in these species by aceto-orcein [1, 11] and Hoechst staining [9]. In our experiments, Hoechst-stained, controlled enucleation was most efficient, and only successfully enucleated oocytes were used for NT experiments.

Improvements in methods of enucleation, activation, and culture of NT embryos in this study greatly improved the
efficiency of the NT procedure. In the present study, the efficiencies of each step in the nuclear transplantation procedure have been reported. The controlled enucleation using Hoechst staining and epifluorescence was highly efficient (100%) and much higher than data previously reported (60%) [13]. In addition, 94.7% of rabbit NT couples fused in this study compared to the 78–84% fusion rate previously reported in rabbits [5, 13] using 8- to 16-cell-stage blastomeres as nuclear donors. The improvement in efficiency came even though the fibroblast cells used in our experiment are more difficult to fuse because of small size. In addition, 29.6% of NT embryos developed into blastocysts.

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