

## Cloned Mice Derived from Embryonic Stem Cell Karyoplasts and Activated Cytoplasts Prepared by Induced Enucleation

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### ABSTRACT

Our objective was to induce enucleation (IE) of activated mouse oocytes to yield cytoplasts capable of supporting development following nuclear transfer. Fluorescence microscopy for microtubules, microfilaments, and DNA was used to evaluate meiotic resumption after ethanol activation and the effect of subsequent transient treatments with 0.4  $\mu\text{g/ml}$  of demecolcine. Using oocytes from B6D2F1 (C57BL/6  $\times$  DBA/2) donors, the success of IE of chromatin into polar bodies (PBs) was dependent on the duration of demecolcine treatment and the time that such treatment was initiated after activation. Similarly, variations in demecolcine treatment altered the proportions of oocytes exhibiting a reversible compartmentalization of chromatin into PBs. Treatment for 15 min begun immediately after activation yielded an optimized IE rate of 21% ( $n = 80$ ) when oocytes were evaluated after overnight recovery in culture. With this protocol, 30–50% of oocytes were routinely scored as compartmentalized when assessed 90 min postactivation. No oocytes could be scored as such following overnight recovery, with 66% of treated oocytes cleaving to the 2-cell stage ( $n = 80$ ). Activated cytoplasts were prepared by mechanical removal of PBs from oocytes whose chromatin had undergone IE or compartmentalization. These cytoplasts were compared with mechanically enucleated, metaphase (M) II cytoplasts whose activation was delayed in nuclear transfer experiments using HM-1 embryonic stem cells. Using oocytes from either B6D2F1 or B6CBAF1 (C57BL/6  $\times$  CBA) donors, the *in vitro* development of cloned embryos using activated cytoplasts was consistently inferior to that observed using MII cytoplasts. Live offspring were derived from both oocyte strains using the latter, whereas a single living mouse was cloned from activated B6CBAF1 cytoplasts.

*assisted reproductive technology, developmental biology, early development, embryo, ovum*

### INTRODUCTION

During nuclear transfer in mammals, the meiotic spindle and associated cytoplasm is removed by micromanipulation. The extent to which this procedure perturbs essential cellular processes remains unknown, although the birth of cloned offspring from several species shows that it is still

compatible with subsequent development [1–7]. As an alternative to mechanical enucleation, methods of inducing enucleation might provide superior cytoplasts for nuclear transfer by minimizing the extent to which cytoplasmic integrity is compromised. During polar body (PB) emission in meiotically maturing or activated oocytes, enucleation can be induced by treatment with reagents that interfere with chromosome segregation and spindle function. These include treatment with etoposide (ETO), which inhibits topoisomerase II-mediated DNA cutting and repair mechanisms, and cycloheximide (CHX), which inhibits protein synthesis [8, 9]. However, to date, only limited development has been observed using cytoplasts derived from mouse oocytes whose enucleation was induced by these reagents. Reconstitution of mouse 2-cell blastomeres with oocytes enucleated during first PB emission using the combination of ETO-CHX results in development only as far as the 4-cell stage [10]. In contrast, reconstitution of 2-cell blastomeres with metaphase (M) II cytoplasts enucleated by treatment with ETO alone before activation and for 4 h afterward yielded blastocysts at a low frequency [11]. Recently, the promise of producing developmentally viable cytoplasts following induced enucleation (IE) was renewed by a brief report of mice cloned from cumulus cells using oocytes enucleated by the timed administration of the microtubule depolymerizing agent, demecolcine [12].

In the present study, our aim was to evaluate the utility of demecolcine to induce the enucleation of activated mouse oocytes. In addition, the developmental competence of resulting cytoplasts was examined in nuclear transfer experiments using embryonic stem (ES) cells as nuclear donors. We report that the timed administration of demecolcine yields a proportion of oocytes whose IE is complete, although reversible compartmentalization of chromatin is also evident in a significant proportion. Cytoplasts prepared by the mechanical removal of PBs from oocytes whose chromatin has undergone IE or compartmentalization support ES cell nuclear transfer development to term, albeit at lower rates than traditional MII cytoplasts that are mechanically enucleated.

### MATERIALS AND METHODS

The present study was conducted following approval by the Roslin Institute Animal Ethics Committee and within a project license issued under the Animal (Scientific Procedures) Act 1986. Unless otherwise stated, all reagents were purchased from Sigma (Poole, U.K.).

#### *In Vivo Oocyte Collection and Embryo Culture*

Female B6D2F1 or B6CBAF1 mice (age, 8–10 wk) were superovulated by injection of 5 IU of eCG and, 48 h later, 5 IU of hCG. Oocytes

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Received: 27 June 2002.

First decision: 31 July 2002.

Accepted: 21 October 2002.

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ISSN: 0006-3363. <http://www.biolreprod.org>

were recovered as cumulus-oocyte complexes (COCs) either 13–14 or 16–18 h post-hCG, depending on whether enucleation was to be by mechanical aspiration of MII oocytes or by induction following activation, respectively. Whereas embryo culture was in CZB medium [13] at 5% CO<sub>2</sub> in air and 37°C, handling of COCs, mature and activated oocytes, and embryos was in ambient atmosphere in Hepes-buffered CZB (hCZB), in which bicarbonate buffer was replaced with 20 mM Hepes. Before enucleation or activation, oocytes were denuded of cumulus by treatment with 300 IU/ml of hyaluronidase in hCZB followed by several washes in hCZB alone.

### Induced Enucleation

Unless otherwise noted, MII oocytes with a first PB were activated for IE by exposure to 7% ethanol in hCZB for 7 min. This was followed by culture in CZB containing 0.4 µg/ml of demecolcine beginning at different times postactivation (pa) and for different durations as defined by each experiment. After demecolcine treatment, oocytes were normally incubated in CZB until 90 min pa, at which point they were either assessed for the success of IE or selected for use in nuclear transfer (see below). In experiments to optimize the efficiency of IE, the position of Hoechst 33342-stained chromatin was evaluated relative to immunostained spindle microtubules and cortical microfilaments in fixed oocytes at high magnification (1000×).

### Immunocytochemistry

Oocytes were fixed and immunostained for microtubules and microfilaments using a modification of the method described by Messinger and Albertini [14]. Briefly, oocytes were fixed and extracted for 30 min at 37°C in a microtubule-stabilizing buffer (0.1 M Pipes [pH 6.9], 5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, and 2.5 mM EGTA) containing 2% formaldehyde, 0.5% Triton X-100, 50% deuterium oxide, and 1 mM of dithiothreitol. Oocytes were then washed three times in a blocking solution of PBS containing 10% normal goat serum (NGS), 0.1% Triton X-100, and 0.02% sodium azide before being stored at 4°C until processing for immunocytochemistry. Oocytes were incubated with fluorescein isothiocyanate-conjugated anti-α-tubulin antibody (final dilution, 1:500; Sigma) and rhodamine phalloidin (1:4000; Molecular Probes, Eugene, OR) in a blocking solution of PBS containing 5% NGS in the dark at 37°C for 1 h. After three washes in the 10% NGS blocking solution, oocytes were mounted in Vectashield (Vector Laboratories Ltd., Peterborough, U.K.) containing 5 µg/ml of 4',6-diamidino-2-phenylindole and then assessed. Labeled oocytes were viewed using a Zeiss Axiovert S 100 photomicroscope (Carl Zeiss, Welwyn Garden City, U.K.) equipped with fluorescein (Zeiss 487709), Texas Red (Zeiss 487714), and Hoechst 33342 (Zeiss 487702) selective filter sets and a 50-W mercury arc lamp. Images were acquired using Kinetic Imaging System (Imaging Associates Ltd., Thame, U.K.).

### ES Cell Culture

The hypoxanthine phosphoribosyltransferase-deficient ES cell line HM-1 [15], derived from the inbred mouse strain 129/Ola, was kindly supplied by Dr. Ed Gallagher (Roslin Institute, Scotland, U.K.) at passage 19. Previously, the capacity of this cell line to yield chimeric animals and germ-line transmission was confirmed by its injection into mouse blastocysts [16]. HM-1 ES cells were cultured in Glasgow modified Eagle medium supplemented with 15% fetal calf serum (GlobePharm, Guildford, Surrey, U.K.), 1000 U/ml of leukemia inhibitory factor, 1% L-glutamine, 1% sodium pyruvate, 1% modified Eagle medium nonessential amino acids, and 0.22% β-mercaptoethanol. Cells were serum deprived for 18–20 h by reducing the concentration of serum to 5% before their use in nuclear transfer experiments. Within 1 h of being required for nuclear transfer, ES cells were lifted using trypsin-EGTA (TEG) medium consisting of 0.25% trypsin (Invitrogen Life Technologies Ltd., Paisley, U.K.), 1.1 mM EGTA, 0.01% polyvinyl alcohol (PVA; molecular weight, 30 000–70 000), 108 mM NaCl, 0.67 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1.6 mM KH<sub>2</sub>PO<sub>4</sub>, 4.5 mM KCl, 5 mM D-glucose, and 22.3 mM Tris, at pH 7.6. With the exception of EGTA and PVA, all salts in TEG medium were BDH Analar grade (BDH Laboratory Supplies, Poole, U.K.).

### Nuclear Transfer with Activated Cytoplasts

Demecolcine-treated oocytes were assessed 1.5 h pa at a magnification of 50× using a stereomicroscope. Those oocytes exhibiting either a long flat PB or two closely apposed PBs were selected for aspiration of PBs using a 12-µm pipette. Aspirations were in hCZB at room temperature on

a Nikon Eclipse TE300 microscope equipped with Nikon Narashige hydraulic micromanipulators (Nikon Ltd., Kingston Upon Thames, U.K.). Although selected oocytes were prestained with 5 µg/ml of Hoechst 33342 in hCZB for 5 min, only pipettes were normally exposed to ultraviolet light to confirm removal of spindle-associated chromatin contained in aspirated PBs. After aspiration of PBs, oocytes were collected and kept in CZB medium in the incubator before piezo-mediated injection of ES cell nuclei as described for nuclear transfer using MII cytoplasts.

### Nuclear Transfer Using MII Cytoplasts (Control)

As a positive control to evaluate the developmental competence of activated cytoplasts, nuclear transfer was also performed on mechanically enucleated MII cytoplasts according to a slightly modified version of the method of Wakayama et al. [3]. This micromanipulation and nuclear transfer was performed on a Nikon Eclipse TE300 microscope separate from that used to remove PBs from demecolcine-treated oocytes and equipped with Eppendorf TransferMan NK micromanipulators (Eppendorf UK Ltd., Cambridge, U.K.) and a Piezo Micromanipulator Controller PMM150 (Prime Tech Ltd., Ibaraki, Japan). Before enucleation, oocytes were treated for 3–5 min with 5 µg/ml of cytochalasin B (CB) in hCZB (before removal of the MII spindle and associated PB was performed using a 6- to 8-µm pipette in the same medium). Enucleated oocytes were transferred into hCZB medium before nuclear injection. Embryonic stem cells were prepared for nuclear injection by mixing an equal volume of cells suspended in hCZB with 10% polyvinyl-pyrrolidone (PVPK 90, 360 kDa; ICN, Aurora, OH). Cells with a comparatively smaller diameter (<10 µm) were selected for injection, and their membranes were ruptured using an injection pipette with an inner diameter of 5 µm to free nuclei. The same pipette was used to aspirate four to six nuclei in a row that were subsequently injected one at a time into the enucleated oocytes. Nuclear reconstructed cytoplasts derived from MII oocytes were cultured in CZB for 1–3 h before activation. Activation was by treatment for 5–6 h in calcium-free CZB medium containing 10 mM SrCl<sub>2</sub> and 5 µg/ml of CB, the latter to inhibit PB extrusion [3, 17].

### Parthenogenetic Activation

As a positive control for oocyte quality and the method of activation used with the IE protocol, a sample of oocytes in each nuclear transfer experiment were activated with 7% ethanol in hCZB for 7 min, washed in hCZB, and cultured in the presence of 5 µg/ml of CB to inhibit extrusion of the PB. After 5 h of incubation in the presence of CB, oocytes were washed in hCZB and transferred to CZB medium for culture at 37.5°C in 5% CO<sub>2</sub> in air.

### Embryo Culture and Transfer

Cloned and parthenogenetic embryos were assessed after 72 h of culture in CZB. At this point, nuclear transfer morulae/blastocysts were transferred into uteri of pseudopregnant, surrogate B6CBAF1 mothers that had been mated with vasectomized male mice 2.5 days earlier. Embryos (n = 5–10) were transferred into each uterine horn. Pups were recovered by cesarean section from recipients sacrificed at 19.5 days postcoitum.

### Statistical Analysis

The frequency of oocytes at different stages of meiosis at successive times after activation and nuclear transfer development were analyzed by one-way ANOVA. Logistic regressions were fitted to the proportions of oocytes exhibiting IE and compartmentalization of chromatin in PBs to evaluate the efficacy of treatments with demecolcine.

## RESULTS

### Meiotic Resumption Following Ethanol Activation of B6D2F1 Oocytes

The timing of meiotic progression after ethanol activation was characterized in oocytes recovered from B6D2F1 mice (Table 1 and Fig. 1). At 15 min pa, the majority of oocytes sampled (85%, n = 41) had reached anaphase (A) II. At 30 min pa, oocytes were roughly equal in terms of those still in AII and those that had progressed to telophase (T) II with the spindle tending to lie parallel to the plasma

TABLE 1. Meiotic progression after activation of B6D2F1 oocytes with ethanol.

Stage	% Frequency of stage at time postactivation (min)					
	15	30	60	90	120	180
M-II	7	2		2	2	
A-II	85	42				
T-II spindle parallel to surface		52	5	7	2	
T-II open PB	2		71	60		
T-II closed PB			12	20	91	50
Pronuclei				2		32
Degenerated	5	2	7	7	4	17
Total number of oocytes sampled	41	40	41	45	44	40

membrane (AII, 42%; TII; 52%;  $n = 40$ ). By 60 min pa, most oocytes were in various stages of TII, with the modal peak (71%,  $n = 41$ ) having undergone spindle rotation and initiation of a PB that remained open. Polar body formation was completed by virtually all oocytes (91%,  $n = 44$ ) when they were next sampled at 120 min pa. However, pronuclei were only observed in 32% of oocytes ( $n = 40$ ) at the next time of sampling (180 min pa). At this time, an increase was observed in the proportion of degenerate oocytes to 17% ( $n = 40$ ), with degeneracy manifested by vacuolized or fragmented cytoplasm. At earlier time points, the proportion of degenerate oocytes ranged from 2% to 7%.

#### *Effect of Administering Demecolcine to Ethanol-Activated B6D2F1 Oocytes*

In the next series of experiments, we hypothesized that enucleation might be induced by a transient, reversible interference with spindle function during the anaphase-telophase (A-T) II transition. Thus, the transient administration of demecolcine to ethanol-activated B6D2F1 oocytes was assessed. Because the A-TII transition was observed to occur in these oocytes between 15 and 30 min pa, a 30-min treatment initiated either immediately or 5 or 10 min after activation ( $t = 0, 5, \text{ or } 10 \text{ pa}$ ) was assessed. Activated oocytes were assessed 90 min pa, by which time first PBs emitted during meiotic maturation were invariably either degenerate or no longer detected. As a result, demecolcine-treated oocytes that lacked a PB or possessed a small, rounded PB corresponded to those that had either not yet released a second PB or had done so normally, respectively. This was confirmed by fluorescence microscopy (data not shown). Demecolcine-treatment induced three broad categories of PB phenotypes, irrespective of the treatment's time of initiation of duration. These included the presence of a single elongated PB, two closely apposed PBs, or a PB accompanied by a second, budding PB that still remained connected (Fig. 2, a, d, and g). Intermediate phenotypes could also be found, which emphasized the need to assess the effect of demecolcine by fluorescence micros-

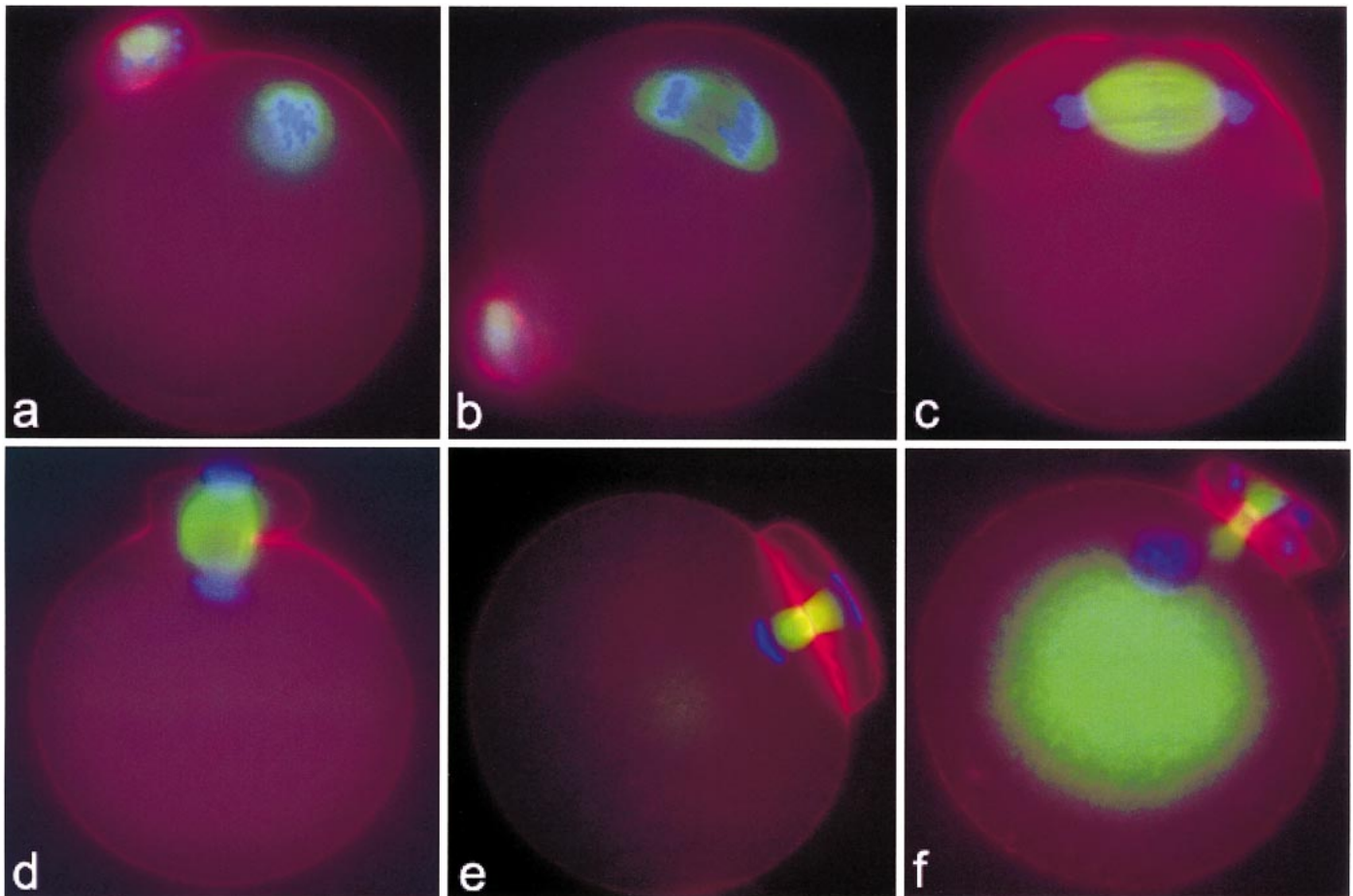
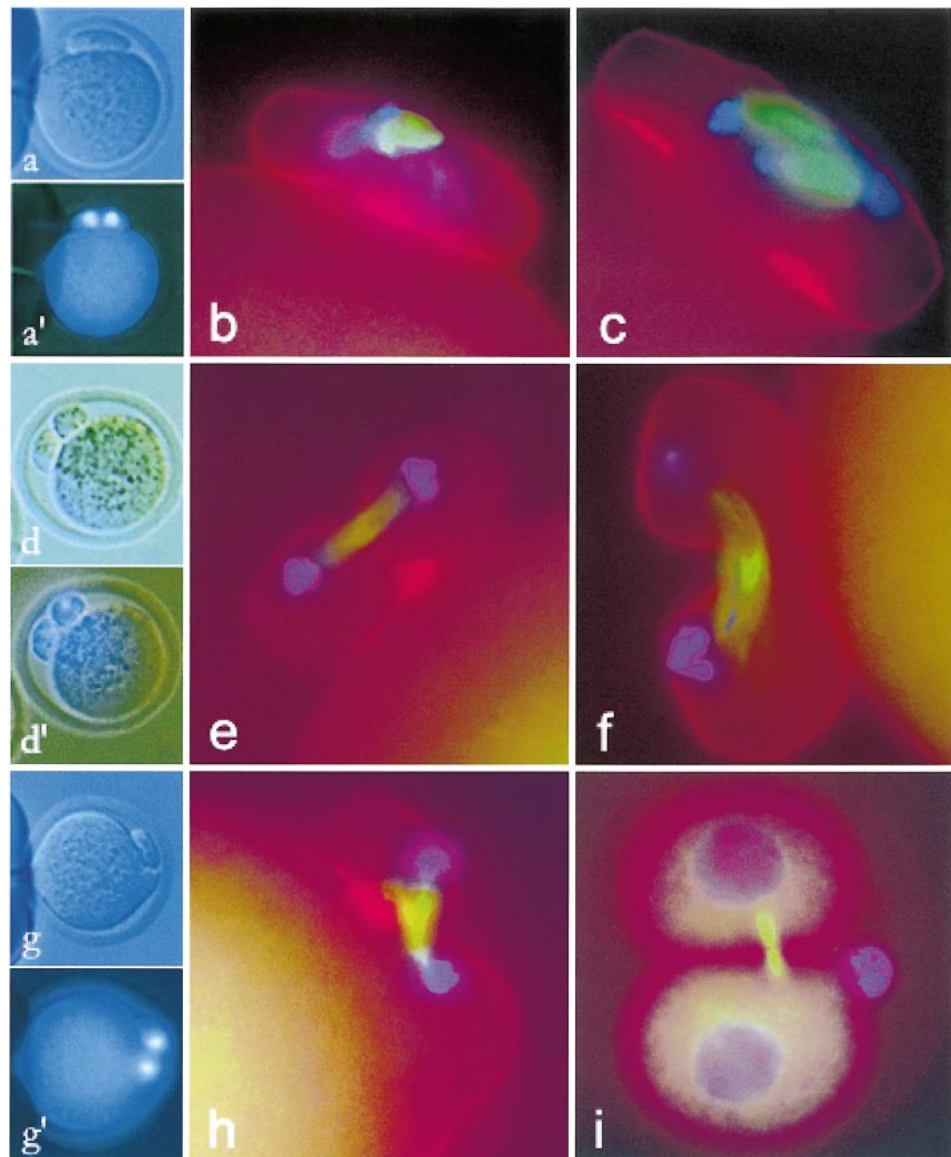


FIG. 1. Meiotic resumption of mouse oocytes following ethanol activation. Mouse B6D2F1 oocytes arrested at MII (a) and activated by culture in 7% ethanol for 7 min (b-f) were fixed and immunostained for microtubules (green) and microfilaments (red), with DNA stained blue by Hoechst 33342. Oocytes fixed at 0 (a) and 15 (b) min pa could be found at MII and AII, respectively. Oocytes fixed at 30 (c), 60 (d), and 90 (e) min pa could be found in various stages of TII, with the spindle located parallel to the surface or perpendicular to the surface before and after PB closure, respectively. At 180 min pa (f), pronuclei and a cytoplasmic network of microtubules could be observed. Magnification  $\times 1000$ .

FIG. 2. Induced enucleation and compartmentalization of oocyte chromatin. Mouse oocytes were activated with ethanol and then transiently treated with 0.4  $\mu\text{g}/\text{ml}$  of demecolcine before being fixed and immunostained for microtubules (green) and microfilaments (red), with DNA stained by Hoechst 33342 (blue). Three PB phenotypes were associated with demecolcine treatment irrespective of the time of initiation or duration of treatment or the strain of mouse oocyte. These phenotypes included the presence of a single elongated PB (a–c), two closely apposed PBs (d–f), or the former accompanied by a second, budding PB that still remained connected (g and h). Oocytes were assessed at low magnification on an inverted microscope using visible light alone (a, d, and g) or with ultraviolet light (a', d', and g'). The latter clearly revealed chromatin in the oocyte cytoplasm only in association with budding PBs (g) and not with the other PB morphologies (a and d). Visualization of cortical microfilaments and microtubule spindles in oocytes at higher magnification (b, c, e, f, and h) enabled the assessment of cytoplasmic continuity between the oocyte and their PBs. Oocytes with either a long flattened (b and c) or two closely apposed PBs (e and f) were scored as compartmentalized or IE if continuity could be observed (c and f) or not (b and e), respectively. Oocyte cytoplasm-PB continuity was invariably detected when a budding PB was observed (h). Following overnight recovery, oocytes that could not be scored as IE frequently cleaved to the 2-cell stage (i). All of the oocytes shown in all panels of the figure were from the B6CBAF1 strain, although the same results were observed in B6D2F1 strain. Magnification  $\times 360$  (a, d, and g),  $\times 1800$  (b, c, e, f, and h), and  $\times 1000$  (i).



copy. For the third category of PB phenotypes, Hoechst-stained chromatin in the oocyte at the site of the budding PB was apparent at both low and high magnification, indicating that enucleation had not been successful (Fig. 2, g' and h). In oocytes with either long flattened or two closely apposed PBs, both IE (Fig. 2, b and e) and compartmentalization (Fig. 2, c and f) of chromatin were apparent in roughly equal proportions. In two replicate preliminary experiments, the percentages of oocytes scored as IE or compartmentalized declined significantly as the onset of a 30-min treatment with demecolcine was delayed relative to the time of activation (% IE and % compartmentalized, respectively, for combined sample [n] at treatment onset time [t]: 43% and 24%,  $n = 51$  at  $t = 0$ ; 28% and 19%,  $n = 51$  at  $t = 5$  min; and 18% and 15%,  $n = 49$  at  $t = 10$  min;  $P < 0.01$ , linear logistic regression). Thus, in subsequent experiments, demecolcine treatment was initiated immediately after activation.

The effect of changing the duration of demecolcine treatment was considered next. Oocytes were treated for 15, 30, and 45 min after activation in five replicate experiments whose results were combined (Table 2). With increasing time of exposure, no significant trend was observed for the

proportion of oocytes scored as IE. However, the proportion of compartmentalized oocytes decreased as the duration of treatment increased ( $P < 0.001$ , linear logistic regression). Increasing the length of exposure to the drug also resulted in a higher incidence of DNA fragmentation (data not shown). Proportions of IE and compartmentalization in this series of experiments remained consistent between replicates, although compared with earlier trials, the average rate of IE had decreased to between 20% and 25%. This rate was observed in all subsequent experimental trials when the duration of demecolcine-treatment was standardized at 15 min.

To assess whether IE by the timed administration of de-

TABLE 2. Effect of demecolcine-treatment begun immediately after activation of B6D2F1 oocytes.

Duration of treatment (min)	Total no. activated	IE (%)	Compartmentalized (%)
15	159	24	39
30	128	17	35
45	156	19	21

TABLE 3. Effect of overnight culture after demecolcine-treatment of activated B6D2F1 oocytes.

Time of assessment	No. activated	IE (%)	Compartmentalized (%)
90 min pa	85	25	52
After overnight culture	80	21	0

mecolcine was reversible and whether oocytes scored as compartmentalized eventually became enucleated if allowed to develop further, oocytes were treated for 15 min with demecolcine beginning immediately after activation and fixed at 90 min pa or after overnight culture. In four replicate experiments, no significant difference was observed in the proportion of oocytes scored as IE at either time point (25% vs. 21%, respectively), indicating that for these oocytes, the enucleation was permanent (Table 3). No compartmentalized oocytes were observed after overnight culture, compared with 52% when assessed at 90 min pa. In the group that was left overnight, 66% of demecolcine-treated oocytes (n = 80) cleaved to the 2-cell stage, further confirming the reversibility of demecolcine-induced compartmentalization of chromatin (Fig. 2i).

*ES Cell Nuclear Transfer Using B6D2F1 Cytoplasts*

The next objective was to determine the developmental competence of ethanol-activated/demecolcine-treated oocyte cytoplasts following nuclear transfer. To make best use of the 40–50% of oocytes reversibly compartmentalizing their chromatin following our optimized protocol, cytoplasts for nuclear transfer were prepared by mechanical removal of both long flattened and closely apposed PBs. These cytoplasts were then injected with HM-1 ES cell nuclei. In each of four replicate experiments, development was compared against ethanol-activated parthenogenetic controls and embryos cloned from HM-1 ES cells by the established use of MII cytoplasts and a 2-h delay between injection and activation [3].

A total of 553 oocytes were ethanol-activated and treated with demecolcine for 15 min beginning immediately after activation. Of these, 307 (56%) possessing either a single long or two closely apposed PBs were selected by 90 min pa, and 192 (35%) survived mechanical removal of their PBs. Although not quantified, those oocytes with single long PBs tended to survive mechanical removal of PBs better. A total of 178 oocytes (32% of those activated and demecolcine-treated) were injected with HM-1 ES nuclei. Virtually 100% of MII oocytes survived mechanical enucleation. In total, 231 MII oocytes were enucleated, and 220 cytoplasts (95%) survived injection and, therefore, activated with SrCl<sub>2</sub>.

Substantial differences were also observed between cytoplasts in their rate of development after nuclear reconstruction (Table 4). Whereas high proportions of MII cytoplasts cleaved and formed morulae/blastocysts, these stages of development were only reached by 14% and 2% of activated cytoplasts, respectively. Of oocytes partheno-

TABLE 5. Meiotic progression after activation B6CBAF1 oocytes with ethanol.

Stages	% Frequency of stage at time postactivation (min)					
	15	30	60	90	120	180
M-II	97	8				
A-II	3					
T-II spindle parallel to membrane			92	15	5	
T-II open PB				59	5	
T-II closed PB				18	81	86
Pronuclei						8
Degenerated				7	8	5
Total number of oocytes sampled	35	36	27	37	37	38

genetically activated with ethanol, 85% and 75% cleaved and reached the morula/blastocyst stage, respectively (n = 40). No cloned blastocysts could be transferred into recipients when activated cytoplasts were used. However, following transfer of 99 cloned morulae/blastocysts from MII cytoplasts into four recipients, 11 pups were born, of which 6 survived.

*Meiotic Resumption Following Ethanol Activation of B6CBAF1 Oocytes*

The efficacy of IE and cytoplast developmental potential were next considered using oocytes from an alternative strain of mice (B6CBAF1). First, the timing of the A-TII transition and PB formation following ethanol activation were examined for this strain (Table 5). Compared with B6D2F1 oocytes, B6CBAF1 oocytes were slow to resume meiosis but quick to catch up. By 15 min pa, most oocytes (97%, n = 35) were still in MII, whereas by this point, we had previously observed 85% of B6D2F1 oocytes to already be at AII (Table 1). By contrast, the majority of B6CBAF1 oocytes (92%, n = 36) had reached the TII stage with the spindle lying parallel to the cell surface by 30 min pa, compared with 52% of B6D2F1 oocytes. However, by 60 min pa and onward, no difference was observed in the timing of meiotic resumption between the strains. Despite the minor initial differences in timing, the same protocol of demecolcine-treatment optimized using B6D2F1 oocytes was applied to B6CBAF1 oocytes. Immunocytochemical assessment of these oocytes at 90 min pa revealed the same PB phenotypes, IE, and compartmentalization (Fig. 2).

*Nuclear Transfer Using B6CBAF1 Cytoplasts*

In five replicate experiments using B6CBAF1 oocytes, activated and MII cytoplasts were compared in nuclear transfer experiments with HM-1 ES cell nuclei. Again, in each experiment, a sample of oocytes was also parthenogenetically activated with ethanol and cultured to the blastocyst stage. As with B6D2F1, high rates of parthenogenetic development were observed, with 92% and 84% of activated oocytes cleaving and forming morulae/blastocysts, respectively (n = 93).

A total of 1154 oocytes were activated and treated with

TABLE 4. Development following reconstruction of B6D2F1 oocyte cytoplasts with HM-1 embryonic stem cell nuclei.

Groups	No. nuclear reconstructed	Cleaved (%)	Morulae/blastocysts (%)	No. transferred	No. offspring	No. surviving
Activated cytoplasts	178	14	2	0	0	0
M-II cytoplasts	220	84	45	99	11	6

TABLE 6. Nuclear transfer in B6CBAF1 strain with HM-1 embryonic stem cells.

Groups	No. nuclear reconstructed	Cleaved (%)	Morulae/blastocysts (%)	No. transferred	No. offspring	No. surviving
Activated cytoplasts	360	36	14	47	1	1
M-II cytoplasts	223	83	44	78	4	1

demecolcine, of which 673 (58%) were selected 90 min prior for mechanical removal of PBs on the basis of having either single long flat or twin PB phenotypes. Of these oocytes, 360 (31% of the total activated and treated with demecolcine) survived PB removal and, therefore, were injected. As for B6D2F1, those oocytes possessing a long flat PB tended to survive mechanical PB removal better than those with two PBs (data not shown). Also, generally all B6CBAF1 MII oocytes survived mechanical enucleation. Of 203 enucleated MII oocytes, 193 (95%) survived injection and were activated with SrCl<sub>2</sub>. In vitro development of cloned embryos from B6CBAF1 MII cytoplasts was comparable to that previously described for B6D2F1 and also resulted in live offspring (Table 6). Activated B6CBAF1 cytoplasts yielded cleavage and morula/blastocyst rates of 36% and 14%, respectively, and unlike previous experiments with B6D2F1 oocytes, the birth and survival of a single live offspring was observed.

## DISCUSSION

We observed that transient treatment with the microtubule-destabilizing agent demecolcine during meiotic resumption resulted in the irreversible enucleation and reversible compartmentalization of chromatin in activated mouse oocytes. This treatment was effective in different mouse strains, with its efficacy being dependent on both the timing and the duration of its administration. Activated cytoplasts produced following mechanical removal of PBs associated with demecolcine-treated oocytes were competent to develop after receipt of ES cell nuclei, although at lower rates than with established methods using MII cytoplasts. However, such cytoplasts still supported development of live offspring.

We observed that resumption of meiosis following ethanol activation was both asynchronous and dependent on mouse strain. This alone was likely to account for the reduced efficacy of inducing complete enucleation to the rate of approximately 20% observed by our standardized protocol. Theoretically, the rate of IE could be improved by combining demecolcine with agents synchronizing meiotic progression. One possibility would be the protein synthesis-inhibitor CHX, which when applied to activated eggs can hasten the decline of maturation-promoting factor activity [8, 9].

Induced enucleation and compartmentalization of chromatin was likely achieved by the transient disruption of cytoplasmic and spindle microtubule dynamics following oocyte activation. One consequence of this could have been the inhibition or delay of spindle rotation during the resumption of meiosis and its subsequent uncoupling from PB emission. This was suggested by the parallel orientation of spindles with respect to the plasma membrane in demecolcine-induced PBs. In mouse oocytes, the meiotic spindle and cytoplasmic microtubules are organized by centriole-less microtubule organizing centers nucleated by  $\gamma$ -tubulin [18]. In activated mammalian oocytes, the mechanism of spindle positioning and rotation is poorly understood, but it likely involves interactions between microtu-

bule spindles and cortical microfilaments. During mouse meiotic maturation, treatment with the microfilament-inhibitor cytochalasin D disrupts microtubule spindle position within the oocyte and centrosome organization. [19]. Using the same inhibitor, cortical microfilaments have been shown to be required for spindle anchoring and rotation during maturation and activation of amphibian oocytes [20]. Although in our study the microfilament-inhibitor CB was used to suppress PB emission in embryos cloned from MII cytoplasts, CB was not used in the creation of activated/demecolcine-treated cytoplasts.

Given the relatively low rate of IE, oocytes whose chromatin would have only been incompletely compartmentalized in PBs were also used in the creation of cytoplasts for nuclear transfer experiments. Thus, the relative developmental competence of both oocyte types was not considered. In vitro development of embryos cloned by transfer of ES cell nuclei into activated/demecolcine-treated cytoplasts was generally inferior to that following transfer into unactivated MII cytoplasts, which were then activated. The latter approach has been successfully exemplified as an effective means of cloning mice, cattle, pigs, goats, and most recently, cats [3, 21–23]. However, enucleation of active spindles and emerging PBs by aspiration has also yielded viable cytoplasts capable of supporting development to term, as exemplified by the use of mouse oocytes at TI [24] and goat and bovine oocytes at TII [5, 25].

Delayed activation of MII cytoplasts has been suggested to be important for the cloning of mice and other species by allowing an increased opportunity for the removal and/or recruitment of developmentally restrictive and potentiating chromatin-associated factors, respectively. Our results suggest that this requirement may not be absolute. However, using activated/demecolcine-treated cytoplasts, the efficiency of initiating development and the production of live young were impaired. Although oocytes induced to self-enucleate could benefit by the acquisition of factors released from the spindle during meiotic resumption, the preinitiation of development limits the time over which nuclear remodeling can occur. For this reason, activated cytoplasts may have a greater requirement for previous nuclear readiness for remodeling after nuclear transfer. This may be achieved by previous synchronization of nuclear donor cells in G<sub>0</sub>/G<sub>1</sub> by serum deprivation or confluence [2]. Although ES cells used in our study were cultured under serum-reduced conditions for 1 day before nuclear transfer, with smaller cells selected as nuclear donors, their cell-cycle status was unknown.

A confounding variable in our comparison of cytoplast types was the use of ethanol versus SrCl<sub>2</sub> for activation. Ethanol was used in our study because of its apparent success in yielding cloned mice from cumulus cells following IE of B6D2F1 oocytes [12]. Activation using ethanol has also been successful in goat cloning by somatic cell nuclear transfer [5]. However, activation using SrCl<sub>2</sub> is better established in mouse cloning [3] and may well yield a superior cytoplast because of its capacity to more closely mimic calcium transients elicited by fertilization [26]. By

contrast, ethanol activation has been shown to elicit only a single calcium transient, which when it occurs in the absence of an intact microtubule spindle will fail to initiate development [27]. Although we did not evaluate microtubule spindle integrity immediately after transiently treating activated oocytes with demecolcine, a transient disruption could have impeded the cytoplasmic response to a previously applied activation stimulus. In this regard, the use of ethanol as an activating stimulus to create cytoplasts for nuclear transfer would be distinct from its capacity to make parthenogenetic embryos, the latter of which we exemplified in our study. Thus, the developmental competence of cytoplasts treated for IE possibly could be improved by activating with agents such as  $\text{SrCl}_2$ .

Although the efficacy of IE and the resulting cytoplast developmental competence in different strains of mouse oocytes were not compared directly, a slight improvement in cleavage and blastocyst development and a live offspring were obtained using B6CBAF1 oocytes. In this strain, ethanol-induced meiotic progression was initially delayed, so that by 15 min *pa*, 97% of eggs sampled were still in MII, compared with 85% of B6D2F1 observed in AII. The significance of this is unclear given the requirement for an intact MII spindle for ethanol-induced calcium oscillations [27]. However, an oocyte actively in the process of chromosome segregation would possibly be less able to recover from microtubule spindle disruption, thus yielding an inferior cytoplast for nuclear transfer development. Interestingly, impaired development of cloned mice has recently been described using, as nuclear donors, fibroblasts and ES cells arrested in M phase by microtubule destabilization with nocodazole [28, 29]. Using fibroblasts as nuclear donors in experiments where activation was delayed, serial nuclear transfer of pronuclei from cloned embryos was required to produce healthy offspring [28]. It must be noted that both demecolcine and nocodazole are likely to affect other microtubule-mediated processes, such as zygote polarity, pronucleus formation, cell cleavage, and mitosis, the extent of which may relate to their respective reversibility. The potential asynchrony in cytoplasmic events created by transient microtubule disruption during M phase may also generally diminish the developmental potential of cloned embryos.

In summary, our study exemplifies the cloning of mice from ES cells using a new method involving ethanol-activated cytoplasts induced to enucleate and compartmentalize endogenous oogenetic chromatin. Although cloned embryo development was impaired relative to the traditional method involving delayed activation, our results demonstrate that no *de facto* requirement exists in the mouse to preexpose donated nuclei to unactivated oocyte cytoplasm to attain development to term. Future improvements in the efficacy of IE and cytoplast competence may involve methods of synchronizing meiotic progression after activation, activation strategies capable of eliciting multiple transients in intracellular calcium, and enhancement of nuclear readiness for reprogramming before nuclear transfer.

## ACKNOWLEDGMENTS

The authors are grateful for the assistance of Patricia Ferrier for the provision of ES cells for nuclear transfer; Linda Harkness for assistance in embryology, delivery of cloned offspring, and preparation of figures; Norrie Russel for final preparation of figures; and Professors Dean Betts, Guelph University, and David Albertini, Tufts University, for useful discussions.

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