Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei

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Cloning methods are now well described and in almost routine use. However, the frequencies of production of live offspring from activated oocytes remain at < 3% and little is known about the factors that affect these frequencies. The effects of cytokinesis inhibitors, dimethylsulphoxide (DMSO) and the cell cycle of recipient cytoplasm on the cloning of mice were examined. Reconstructed oocytes, which were activated immediately after nucleus injection and cultured without cytochalasin B, developed into blastocysts at a frequency of 30–54% and into live cloned offspring at a frequency of 2–3%. Activated zygotes did not support development to full term after nuclear transfer. Reconstructed oocytes were activated 1–3 h after nuclear transfer and were exposed separately to three inhibitors of cytokinesis (cytochalasin B, cytochalasin D or nocodazole) to examine the toxicity of these

inhibitors on cloning. All of the oocytes exposed to nocodazole-containing media formed many small pseudo-pronuclei, whereas with cytochalasin-containing media most of the activated oocytes formed only two pseudo-pronuclei. Despite such differences, 42–61% of reconstructed embryos developed to the morula–blastocyst stage and 1–3% developed to full term in all groups. Addition of 1% (v/v) DMSO to the activation medium significantly improved the frequency of development to the blastocyst stage and full term; however, this improvement did not lead to a higher success rate in the generation of live cloned offspring. These results show that activated mouse oocytes/zygotes are not effective cytoplasmic recipients with the methods described and that the lack of success of cloning is not due to inhibition of cytokinesis.

Introduction

Cloning mammals by nuclear transfer began more than twenty years ago, yet the basic biology underlying this process remains unclear. A Piezo-electric nuclear transfer method has been used to generate cloned mice from cumulus cells (Wakayama *et al.*, 1998a, 2000a, 2001; Wakayama and Yanagimachi, 2001) and tail-tip cells (Wakayama and Yanagimachi, 1999) of adult animals as well as embryonic stem cells (Wakayama *et al.*, 1999; Rideout *et al.*, 2000). Ogura *et al.* (2000a) also reported cloning live mice using Sertoli cells by the same Piezo-electric injection method. Recently, other methods, such as cell fusion (Ogura *et al.*, 2000b; Ono *et al.*, 2001) or injection without a Piezo-electric device (Zhou *et al.*, 2000) were used successfully to produce live cloned mice. However, as in other mammals, the overall efficiency of mouse cloning (the percentage of live offspring obtained from all activated oocytes) in all reports is < 3%.

Why is the success rate of cloning so low? Is it because only a few per cent of any cell population are 'cloning-competent' or simply because of technical problems? It is likely that many technical factors such as cell cycle, oocyte

activation and chemical agents affect development of cloned embryos.

In our mouse cloning protocol, donor nuclei are exposed to the cytoplasm of the unfertilized oocyte for a few hours, which causes nuclear envelope breakdown and premature chromosome condensation (Czolowska *et al.*, 1984; Collas and Robl, 1991) due to the action of the maturation/metaphase promoting factor (Masui and Markert, 1971) within the cytoplasm of the oocyte. Methods that induce premature chromosome condensation in reconstructed oocytes result in increased developmental potential, indicating that these processes may promote reprogramming of gene expression in the donor nucleus (Collas and Robl, 1991; Cibelli *et al.*, 1998; Wakayama *et al.*, 1998a, 2000b; Wells *et al.*, 1999; Onishi *et al.*, 2000). In contrast, cytoplasts derived from activated oocytes or pronuclear zygotes lack active maturation/metaphase promoting factor and so do not induce nuclear envelope breakdown or premature chromosome condensation in transferred nuclei (Czolowska *et al.*, 1984; Campbell *et al.*, 1994, 1996a). In some cases, enucleated oocytes were activated before nuclear transfer, culminating in the birth of cloned pigs, cows and sheep (Prather *et al.*, 1989; Stice *et al.*, 1994; Campbell *et al.*, 1996b). Therefore, a clear understanding of the role of premature chromosome condensation and maturation/metaphase promoting factor in the development of reconstructed embryos requires further study.

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After exposure to maturation/metaphase promoting factor, reconstructed mouse oocytes must be activated for development to progress. Kishikawa *et al.* (1999) demonstrated that four different methods for oocyte activation (strontium, ethanol, electro-stimulation and sperm injection) yielded about the same rates (1–2%) of cloning success. It is interesting to note that for cloning, strontium (which we have been using routinely) is as active as sperm-born factors in oocyte activation; therefore, the choice of activation method is not critical. However, in no instance have we obtained evidence to support the possibility that enucleated zygotes can be used successfully as recipients for somatic cell nuclei. In studies in which we sought such evidence no zygotes developed to blastocysts, the transferred nuclei became extremely fragmented and development was arrested at the two-cell stage (Wakayama *et al.*, 2000b). This finding was in contrast to the very early and never independently confirmed report by Illmensee and Hoppe (1981) that zygotes could be used in this fashion (McGrath and Solter, 1984a).

In our protocol, reconstructed mouse oocytes activated by the above reagents are treated with cytochalasin to prevent the loss of somatic cell chromosomes into the pseudo-polar body (Wakayama *et al.*, 1998a). Several methods have been proposed to prevent cell division or polar body extrusion (Opas, 1977; Modlinski and McLaren, 1980; Siracusa *et al.*, 1980; Surani *et al.*, 1980; Borsuk, 1982). The most commonly used reagents are cytochalasin B or D (Snow, 1973; Siracusa *et al.*, 1980; Bos-Mikich *et al.*, 1997) and latrunculin B (Schatten *et al.*, 1986), which disrupt actin filaments. Other commonly used agents are colcemid, colchicine (Kleinfeld and Siskin, 1966; Borisy and Taylor, 1967), vinblastin (Siracusa *et al.*, 1980) and nocodazole (Johnson *et al.*, 1988), all of which interfere with microtubule assembly. These treatments prevent cytokinesis and, therefore, extrusion of the polar body. It has been reported that nocodazole is the least harmful to mouse development before and after implantation (Kato and Tsunoda, 1992; Otaegui *et al.*, 1994).

In the present study, the effects of timing of oocyte/zygote activation and duration of *in vitro* culture of cloned embryos on the success rate of cloning mice by nuclear injection methods were investigated. The effect of dimethylsulphoxide (DMSO) on embryo development was also investigated, as this reagent is included routinely in the oocyte activation medium. Finally, whether the agents used to prevent polar body extrusion affect cloning efficiency was examined.

Materials and Methods

Animals

B6D2F1 (C57BL/6 × DBA/2) female mice, aged 8–10 weeks, were used as oocyte and cumulus cell donors. Foster mothers were CD-1 females. The females were mated with vasectomized males of the same strain. All animals used in

this study were maintained in accordance with the guidelines of the Laboratory Animal Service at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEF publication no. [NIH] 80-23, revised in 1985). The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

Reagents

All inorganic and organic compounds were purchased from Sigma Chemical Co (St Louis, MO) unless otherwise stated.

Media

Oocytes, zygotes and embryos were all cultured in a bicarbonate-buffered (CZB) medium (Chatot *et al.*, 1990) at 37.5°C under 5% CO₂ in air. Oocyte manipulations were carried out in Hepes-buffered CZB (Hepes-CZB) at room temperature (25–29°C) in air. The pH of these media was approximately 7.5. Ca²⁺-free CZB containing both 10 mmol SrCl₂ l⁻¹ and 5 µg cytochalasin B ml⁻¹ was used to activate reconstructed oocytes (Bos-Mikich *et al.*, 1997). Stock solutions of Sr²⁺ (100 mmol l⁻¹ in distilled water) and cytochalasin B (500–10 000 µg ml⁻¹ in DMSO) were stored at room temperature and –20°C, respectively. Immediately before use, Sr²⁺ stock solution was diluted with Ca²⁺-free CZB to a final concentration of 10 mmol Sr²⁺ l⁻¹ (this is oocyte activation medium). Cytochalasin B, D and nocodazole were dissolved in DMSO and diluted in activation medium to final concentrations of 5, 1 and 3 µg ml⁻¹, respectively, while the DMSO concentration varied between 0.05% (v/v) and 1.00% (v/v).

Isolation of cumulus cells, oocytes and zygotes

Mature B6D2F1 female mice were induced to superovulate by consecutive injections of equine chorionic gonadotrophin (eCG; 5 iu) and hCG (5 iu) 48 h apart. Thirteen to 15 h after hCG injection, cumulus–oocyte complexes were collected from oviducts. Cumulus cells were dispersed by a 10 min treatment with 0.1% (w/v) bovine testicular hyaluronidase (300 USP units mg⁻¹; ICN Pharmaceuticals, Costa Mesa, CA) in Hepes-CZB. An aliquot (about 2 µl) of the cumulus cell suspension was transferred to 20 µl Hepes-CZB, containing 12% (w/v) polyvinylpyrrolidone (*M_r* 360 000) and kept at room temperature for up to 3 h before injection. B6D2F1 females were housed with normal CD-1 males soon after hCG injection and were killed 20 h later to collect zygotes. Zygotes collected from oviducts were freed from cumulus cells by hyaluronidase treatment, and kept in CZB at 37.5°C under 5% CO₂ in air for < 1 h before further treatment.

Enucleation of oocytes and zygotes

Oocytes. A group of oocytes (usually 20–30) was transferred to a droplet (about 10 µl) of Hepes-CZB containing

5 μg cytochalasin B ml^{-1} , which had been placed previously under mineral oil in the operation chamber on the microscope stage. After an oocyte was held by an oocyte-holding pipette, its zona pellucida was 'drilled' by applying several Piezo pulses to the tip of an enucleation pipette (Wakayama *et al.*, 1998a). The metaphase II chromosome–spindle complex, distinguished as a translucent spot in the ooplasm, was drawn into the pipette with a small amount of accompanying ooplasm and pulled gently away from the oocyte until a stretched cytoplasmic bridge was pinched off. After oocytes in one group were enucleated (which took about 10 min), they were transferred into cytochalasin B-free CZB and kept there for up to 2 h at 37.5°C (Wakayama *et al.*, 1998a). The efficiency of enucleation was 100% as assessed by fixing and staining (Yanagida *et al.*, 1991).

Zygote. Zygotes (pronuclear eggs) were placed in cytochalasin B-containing Hepes CZB. After the zona pellucida was 'drilled' by Piezo-pulses, both female and male pronuclei were drawn into the pipette, then pulled gently away from the zygote until a stretched cytoplasmic bridge was pinched off.

Parthenogenetically activated oocytes. Oocytes were activated by incubation for 1 h in Ca^{2+} -free CZB containing 10 mmol Sr^{2+} l^{-1} medium. After cumulus cell nuclei were injected into activated oocytes, the oocytes were transferred into CZB medium and kept at 37°C, 5% CO_2 in air to allow for membrane recovery. After 20–30 min, telophase II chromosomes were removed as described for metaphase II chromosomes.

Injection of cumulus cell nuclei into enucleated oocytes, zygotes and activated oocytes

A cumulus cell was drawn in and out of the injection pipette until the plasma membrane was broken. In some cases, a few Piezo pulses were applied to break the membranes. After a cumulus cell nucleus was drawn deep into the pipette, another cell nucleus was drawn into the same pipette. Within a few minutes several nuclei were lined up within a single pipette. These nuclei were injected individually into enucleated oocytes as described for the spermatid nucleus injection (Kimura and Yanagimachi, 1995a,b) except that all handling was performed at room temperature (25–29°C) instead of 17–18°C. Kimura and Yanagimachi (1995a) reported that oocytes operated on at 17–18°C survived better than at high temperature, but we later found that this is not necessarily the case. All nuclei were injected into oocytes or zygotes within 10 min after denudation.

Oocyte activation conditions and inhibition of polar body formation

Activation media condition (i). Reconstructed oocytes were activated by incubation at 37°C for 6–7 h in Ca^{2+} -free CZB containing both 10 mmol Sr^{2+} l^{-1} and 5 μg

cytochalasin B ml^{-1} . The final concentration of DMSO was 0.05%–1.00% (v/v).

When reconstructed oocytes were activated immediately after nuclear transfer, the nucleus in each oocyte enlarged without dissolution of the nuclear membrane (Czolowska *et al.*, 1984) and, therefore, cytochalasin B was not added. However, in some experiments, DMSO and cytochalasin B were added for control experiments.

Activation media condition (ii). Reconstructed oocytes were activated by incubation at 37°C for 6–7 h in Ca^{2+} -free CZB containing both 10 mmol Sr^{2+} l^{-1} and (a) 5 μg cytochalasin B ml^{-1} and 1% (v/v) DMSO, (b) 1 μg cytochalasin D ml^{-1} and 0.1% (v/v) DMSO, (c) 1 μg cytochalasin D ml^{-1} and 1% (v/v) DMSO or (d) 3 μg nocodazole l^{-1} with 1% (v/v) DMSO.

Some activated oocytes were fixed and stained to examine pseudo-pronuclei formation (Yanagida *et al.*, 1991). Activated oocytes at the pseudo-pronuclear stage were washed and cultured in Sr^{2+} -free CZB until they reached the two-cell or morula–blastocyst stage.

Oocyte activation protocols

Five different protocols were used to activate oocytes (see Fig. 1). In the first protocol (Fig. 1a: pre-activation experiment) enucleated oocytes were activated 1 h before cumulus cell nuclei were injected. In the second protocol (Fig. 1b: immediate activation experiment) enucleated oocytes were activated within 5 min of injection of cumulus cell nuclei. In the third protocol (Fig. 1c: post-activation experiment) enucleated oocytes were activated 1–3 h after injection of cumulus cell nuclei. In the fourth protocol (Fig. 1d: zygote experiment) male and female pronuclei were removed simultaneously and before injection of cumulus cell nuclei. In the fifth protocol (Fig. 1e: non-enucleated pre-activation experiment) intact oocytes were activated. Cumulus cell nuclei were injected 1 h later and within 30 min, telophase II chromosomes were removed. Oocytes in this group always contained nuclei of the oocyte or the cumulus cell or both. In other experiments, the oocytes were temporarily free of a nucleus.

Embryo transfer and examination of fetuses and placentae

Two-cell embryos (24 h after onset of activation) were transferred into oviducts of foster mothers (CD-1, albino) that had been mated with vasectomized CD-1 males 1 day previously. Morulae or blastocysts (78 h after activation) were transferred into the uteri of foster mothers (CD-1, albino) that had been mated with vasectomized CD-1 males 3 days previously. All recipient females were killed at day 19.5 post coitus and their uteri were examined for the presence of fetuses and implantation sites. Live young were raised by lactating CD-1 foster mothers. Offspring and placentae were weighed individually.

Fig. 1. Five oocyte activation protocols. (a) Protocol A: oocytes were enucleated and then activated, before injection of donor nuclei. (b) Protocol B: enucleated oocytes were activated immediately after nuclear transfer. (c) Protocol C: enucleated oocytes were activated 1–3 h after nuclear transfer. (d) Protocol D: enucleated zygotes were injected with donor nuclei. (e) Protocol E: intact oocytes were first activated and donor nuclei were injected before removal of the telophase II chromosomes of the oocyte.

Results

Timing of activation

Oocytes activated 1 h before nuclear transfer (Fig. 1a). These oocytes contained a small nucleus at the onset of activation (Fig. 2a) which changed into a small pseudo-pronucleus over the next 6 h (Fig. 2b). Most of these embryos underwent irregular cleavages and only a small percentage developed into blastocysts (Table 1). After subsequent transfer to four foster mothers (2–3 blastocysts per female), none of these blastocysts developed to term. In our control experiment (Tamashiro *et al.*, 2000), in which fertilized embryos (2–3 embryos per female) were transferred into each recipient, most females delivered one or two offspring. Therefore, the failure of cloned embryo development is not due to transfer of too few embryos.

Activation of reconstructed oocytes immediately after nuclear transfer. This resulted in a markedly different nuclear morphology and developmental potential (Fig. 1b). The reconstructed oocytes contained one small nucleus at 1 h after activation (Fig. 2c) that became a large pseudo-pronucleus during the next 6 h (Fig. 2d). Of these, 30–54% developed into blastocysts (Table 1).

Reconstructed oocytes incubated for 1–3 h before activation (Fig. 1c and Table 1). This activation protocol was used for the production of our first cloned mouse 'Cumulina' and her clones (Wakayama *et al.*, 1998a, 2000a). During this period of incubation the transferred nuclei were transformed into randomly arranged chromosomes (Fig. 2e). When the oocytes were activated in the presence of cytochalasin B, the chromosomes were transformed into two or more pseudo-pronuclei (Fig. 2f). Of these activated oocytes, 38–69% reached the blastocyst stage (Table 1).

Zygotes as recipients of donor nuclei

When enucleated zygotes were used as recipients of donor nuclei (Fig. 1d), none developed beyond the two-cell stage (Table 2). Similarly, embryos reconstructed from activated oocytes (Fig. 1e) never developed beyond the four-cell stage and upon closer examination it was observed that they contained fragmented nuclei (Table 2).

DMSO and cytokinesis inhibitors

The effects of DMSO on embryo development were examined at the same time (Table 1). The presence of 0.1–1.0% (v/v) DMSO in the oocyte activation medium further promoted the preimplantation development of embryos to the blastocyst stage. Medium containing < 0.1% (v/v) DMSO supports development to the blastocyst stage but at a reduced rate (Fig. 3; $P < 0.05$).

The results of *in vivo* experiments in which mature oocytes were enucleated and activated immediately after nuclear transfer (same method as Fig. 1b) are shown (Table

3). The oocytes were activated in the absence or presence of 1% (v/v) DMSO, or 1% (v/v) DMSO plus 5 μ g cytochalasin B ml^{-1} (activation media condition (i)). The highest frequency of preimplantation development was observed when oocytes were activated by Sr^{2+} in the presence of 1% (v/v) DMSO. The presence of cytochalasin B or absence of DMSO in the activation medium resulted in a significant decrease in the frequency of development to the blastocyst stage. Furthermore, when reconstructed oocytes were activated in cytochalasin B-containing medium, none developed to term. However, six live offspring were obtained from other experimental groups (a frequency of 2–3%, mean body weight 1.62 g and mean placental mass 0.31 g).

The results of another series of experiments in which pseudo-polar body extrusion was prevented by the separate use of three different cytokinesis inhibitors (activation media condition (ii)) are shown (Table 4). When reconstructed oocytes were treated with Sr^{2+} in the presence of cytochalasin B or cytochalasin D, reconstructed zygotes had two or three pseudo-pronuclei. In contrast, when reconstructed oocytes were activated with nocodazole, reconstructed zygotes had many small pseudo-pronuclei (Fig. 4). However, 1–3% of reconstructed oocytes developed to full term, irrespective of treatment. When reconstructed embryos were transferred to surrogate mothers at the two-cell or morula–blastocyst stage (Table 4), a mean 2.7 and 5.3% of transferred embryos developed to full term, respectively. However, when the success rates of full term development were calculated based on all reconstructed oocytes, those success rates were 2.0 and 2.5%, respectively.

Evidence that all pups were derived from somatic cell nuclei by nuclear transfer

All cloned mice were female and had black eyes. All surrogate mothers were CD-1 (albino with red eyes) and were mated with CD-1 vasectomized males, resulting in pseudopregnancy without fetuses. Even if natural mating had occurred accidentally, any resultant pups would be albino with red eyes. Furthermore, in these studies, cells from male mice were never used as nucleus donors; therefore it was expected that all 22 cloned mice would be female. This is theoretically a very unlikely outcome (occurring at a frequency of $1/2^{22}$) if the offspring had arisen via normal reproduction. Finally, all pups were born with large placentae, which is only seen in somatic cell cloned mice (Wakayama *et al.*, 1998a; Kishikawa *et al.*, 1999; Wakayama and Yanagimachi, 1999; Ogura *et al.*, 2000a,b; Tamashiro *et al.*, 2000; Wakayama *et al.*, 2000a; Ono *et al.*, 2001; Wakayama and Yanagimachi, 2001). For the above reasons, it was concluded that all pups were derived absolutely from somatic cell nuclei by nuclear transfer.

Discussion

Wakayama *et al.* (2000b) showed that embryos reconstructed using oocyte cytoplasts had normal

Fig. 2. Cumulus cell nuclei after transfer into oocytes (a) 1 and (b) 6 h after oocyte activation by protocol A, (c) 1 and (d) 6 h after oocyte activation by protocol B, and (e) 1–3 h after injection into an unactivated enucleated oocyte. Note that the nucleus is transformed into loosely arranged chromosomes by protocol C. (f) Reconstructed oocytes activated in the presence of cytochalasin B (chromosomes transform into two or more pseudo-pronuclei by protocol C). Scale bars represent 20 μ m.

Table 1. Effects of the timing of oocyte activation and DMSO on development of cumulus-injected oocytes *in vitro*

Oocyte activation	Concentration of DMSO in medium (%; v/v)	Number of enucleated oocytes	Number of surviving oocytes after nucleus injection	Number (%) of activated oocytes	Number (% ± SD) of embryos examined 96 h after oocyte activation		
					One-cell	Two- to eight-cell	Blastocyst**
1 h before nucleus injection (Fig. 1a)	0	103	99	–	85	12	4 (4.0 ± 3.2)
	1.00	104	98	–	85	10	7 (7.2 ± 7.0)
Immediately after nucleus injection (Fig. 1b)	0	103	103	94 (91.3)	27	37	38 (37.3 ± 34.3) ^a
	0.01	102	100	88 (88.0)	22	44	28 (29.8 ± 22.5) ^a
	0.10	102	99	92 (92.9)	24	31	42 (43.3 ± 31.2)
	1.00	117	114	102 (89.5)	17	34	61 (54.0 ± 26.3) ^b
	2.00	106	92	80 (87.0)	22	23	47 (51.1 ± 14.4)
	1.00*	100	72	66 (91.7)	22	22	28 (38.9 ± 19.0)
1–3 h after nucleus injection (Fig. 1c)	0.05*	164	154	138 (90.2)	20	76	58 (37.7 ± 19.4) ^c
	0.10*	126	126	124 (98.4)	8	53	65 (51.6 ± 18.5) ^{de}
	0.50*	117	113	105 (93.8)	10	31	72 (63.7 ± 16.7) ^d
	1.00*	151	144	133 (95.0)	19	26	99 (68.8 ± 15.3) ^{df}
	2.00*	142	134	134 (100)	5	56	73 (54.5 ± 9.7) ^{de}

This experiment was replicated three times.
*5 µg cytochalasin B ml⁻¹ was included in the oocyte activation medium to prevent polar body extrusion.
**Data were analysed using the chi-squared test (*P* < 0.05): a versus b, c versus d and e versus f.

Table 2. *In vitro* development of reconstructed zygote and telophase II oocytes receiving cumulus cell nuclei

Nucleus recipients	Number of prepared eggs	Number of enucleated zygotes	Number of surviving eggs after nucleus injection	Number of enucleated telophase II oocytes after nucleus injection	Number (%) of embryos examined 96 h after nucleus injection			
					One-cell	Two-cell	Four-cell	≥ Eight-cell
Zygote (Fig. 1d)	109	98	91	–	41 (45.1)	50 (54.9)	0	0
Telophase II oocytes (Fig. 1e)	105	–	101	97	45 (46.4)	46 (47.4)	6 (6.2)	0

This experiment was replicated three times.

karyotype, whether oocytes were activated before, immediately after or 1–3 h after nuclear transfer. In contrast, when enucleated zygotes supplied the recipient cytoplasm, the transferred nuclei became extremely fragmented (Wakayama *et al.*, 2000b). In the present study, embryos reconstructed using zygotes also failed to develop. These zygotes were activated 6 h before nuclear transfer and were probably in early S-phase of the first embryonic cell division cycle. This cell cycle stage may be incompatible with that of cells in an adult animal and such asynchronization could result in developmental failure of reconstructed embryos. A similar phenomenon was observed in experiments in which the second polar body was exchanged with the female pronucleus in zygotes in early developmental stages (Wakayama *et al.*, 1997); then, live offspring were obtained. However, when zygotes at late stages were used, the zygote exchanged did become fragmented, again probably due to

asynchronization of the cell cycle of the zygote and the polar body. Taken together, these results indicate that enucleated zygotes may be unsuitable for use in cloning of adult animals.
In all successful nuclear transfer experiments reported to date, nuclei of recipient oocytes were removed before fusion or injection of donor nuclei. In such experiments several hours elapsed between nuclear removal and nuclear transfer, during which time the recipient oocyte lacked nuclear contents. Unactivated oocytes are arrested in metaphase II of the cell cycle; activation relieves this arrest and numerous changes follow (for example, a decrease in maturation/metaphase promoting factor activity). If activated enucleated oocytes are used as recipient cytoplasm, this tumultuous period takes place in the absence of a nucleus. If a nucleus is essential during this change, recipient eggs might be irreparably damaged before nuclear transfer.

Fig. 3. Effect of dimethylsulphoxide (DMSO) on pre-implantation development of oocytes injected with cumulus cell nuclei by protocol B (□; see Fig. 1b) and protocol C (■; see Fig. 1c) assessed at 96 h after activation.

Cumulus cell nuclei were injected into activated oocytes and then telophase II chromosomes were removed to determine whether this lack of a nucleus prevented normal embryonic development in activated oocytes. Thus, these reconstructed oocytes never lacked a nucleus; despite this, none developed beyond the four-cell stage. This finding shows that the poor developmental capacity observed when using activated oocytes is not due to the absence of chromosomes *per se*.

When donor nuclei are exposed to oocyte cytoplasm for several hours, an inhibitor of cytokinesis must be added to the activation medium to prevent chromosome loss through extrusion of the pseudo-second polar body (Kono *et al.*, 1993; Cheong *et al.*, 1994; Wakayama *et al.*, 1998a). If the ‘immediate activation’ method is used, an inhibitor of cytokinesis is not required, as reconstructed oocytes activated immediately after nuclear transfer do not induce premature chromosome condensation or do not extrude pseudo-polar body.

With our protocol, cytochalasin B was detrimental to embryonic development when reconstructed oocytes were activated immediately after nuclear transfer. In our previous study (Wakayama *et al.*, 1998a), oocytes were exposed to cytochalasin B regardless of the time of activation. Under those conditions, no fetuses were obtained from oocytes activated immediately after nuclear transfer. Similar results were obtained in the present study. When reconstructed oocytes are activated in the presence of cytochalasin B, decreased blastocyst development is observed and none progressed to live-born young after transfer. However, live cloned mice were obtained in the absence of cytochalasin B, using the immediate activation method. It is important to note that other studies have reported that exposure to cytochalasin B resulted in an improved success rate of embryonic development in cloning of rabbits (Collas and Robl, 1990) and sheep (Smith and Wilmut, 1989). The reason for this discrepancy is unclear.

In the immediate activation experimental condition, oocytes are exposed to activation medium within 5 min after nuclear transfer. Strontium induces activation in B6D2F1 oocytes within 25 min (T. Wakayama and R. Yanagimachi, unpublished). Therefore, the introduced nucleus is exposed to high maturation/metaphase promoting factor activity for between 5 min and 30 min. If exposure of the transferred nucleus to ooplasmic maturation/metaphase promoting factor activity is indeed critical for genome reprogramming, these results indicate that the necessary changes take place rapidly. When donor nuclei are exposed to oocyte cytoplasm for > 1 h, the reprogramming of donor nuclei may be enhanced and stabilized further. Therefore, those reconstructed oocytes might develop even when activated with cytochalasin-containing medium.

Cytochalasin B may cause damage to embryo development. Therefore, if pseudo-second polar body extrusion could be prevented without cytochalasin B, the success rate of cloning might be increased. For this reason, we compared the effects of several different cytokinesis inhibitors on cloning. Cytochalasins B and D are microfilament-disrupting reagents and are often used to induce polyploidy

Table 3. Full term development of reconstructed oocytes activated immediately after nuclear injection with or without DMSO and cytochalasin B

DMSO and cytochalasin B in oocyte-activating medium	Number of enucleated oocytes	Number of surviving oocytes after nucleus injection	Number (%) of activated oocytes	Number (% ± SD) of oocytes that developed to blastocyst stage*	Number of embryos transferred (recipients)	Number (%) of live offspring
1% DMSO and cytochalasin B	172	135	102 (75.6)	59 (43.7 ± 14.8) ^a	59 (5)	0
1% DMSO	257	235	193 (82.1)	153 (65.1 ± 17.3) ^b	149 (9)	3 (1.3)
None	252	220	168 (76.4)	101 (46.0 ± 24.2) ^a	97 (7)	3 (1.4)

This experiment was performed using protocol B and was replicated five times.

DMSO: dimethylsulphoxide (v/v).

*Data were analysed using the chi-squared test ($P < 0.05$): a versus b.

Fig. 4. Pseudo-pronuclei at 6 h after activation of oocytes that had been treated with activation medium containing 3 μg nocodazole ml^{-1} . Scale bar represents 20 μm .

in preimplantation embryos (Snow, 1973; Siracusa *et al.*, 1980; Bos-Mikich *et al.*, 1997). Although these drugs do not affect development to the blastocyst stage *in vitro* (Siracusa *et al.*, 1980), their effect on full term development is unclear, as aneuploid embryos and parthenogenetic embryos can not develop to full term (Epstein, 1986; McGrath and Solter, 1984b; Surani *et al.*, 1984). Nocodazole is an inhibitor of tubulin polymerization and causes breaks in the metaphase spindle and, thus, is often used for cell cycle synchronization experiments (Johnson *et al.*, 1988). It has also been reported that this drug does not affect development to full term (Kato and Tsunoda, 1992; Otaegui *et al.*, 1994; Samake and Smith, 1996).

In the present study, all reagents succeeded in preventing pseudo-polar body extrusion. When cytochalasin B or D was used, reconstructed zygotes had two or three pseudo-pronuclei. This finding was expected because cytochalasin cannot disrupt the metaphase plate and so chromosomes were pulled to the centrosomes by actin filaments. However, when nocodazole was used, reconstructed zygotes had many small pseudo-pronuclei because the metaphase plate was broken, causing chromosomes to separate inside the oocytes. Before the present study, we had hypothesized that the low cloning success rate might be due to the use of cytochalasin, but apparently this is not the case, as the substitution of nocodazole did not increase the success rate beyond 2–3%.

Thus, neither the method of oocyte activation (Kishikawa *et al.*, 1999) nor the type of cytokinesis inhibitor (present study) can account for the low success rate of cloning.

The effect of transfer of two-cell embryos into the oviduct and transfer of morulae or blastocysts into uteri were also compared. It has been generally believed that the duration of *in vitro* culture should be minimized to promote developmental progression of manipulated embryos.

Therefore, one-cell or two-cell embryos were usually transferred into day 1 oviducts. However, recently, the *in vitro* culture system of mouse embryos was improved allowing most *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) embryos to develop to full term. In the present study, the percentage of live offspring per transfer was considerably higher when embryos were transferred into uteri of surrogate mothers at the morula–blastocyst stage (5.3%) than when embryos were transferred into oviducts at the two-cell stage (2.7%). In our laboratory, when IVF/ICSI embryos were transferred at the two-cell stage into oviducts or at the morula–blastocyst stage into uteri, the success rate of full term is almost the same (60–61% of two-cell transfers: Wakayama *et al.*, 1995; Tanemura *et al.*, 1997; Wakayama *et al.*, 1998b; 50–57% of morula–blastocyst transfers: Wakayama and Yanagimachi, 1998a,b,c). Therefore, this difference in success rate was not due to a technical problem.

When reconstructed oocytes were cultured for 1 day, 87% of cloned embryos developed to the two-cell stage, but when cultured for 4 days, 53% developed to the morula–blastocyst stage. It is possible that some of those transferred at the two-cell stage were not developmentally competent and might never develop to the morula–blastocyst stage. However, at this point, it is not possible to predict which two-cell embryos have a good chance to develop to term, and we continue to transfer all two-cell embryos into surrogate mothers. Therefore, the success rate of full term development from transfer of two-cell embryos is lower (2.7%) than from blastocyst transfer (5.3%), but the overall percentage from reconstructed oocytes was the same in both cases (2.3 versus 2.8%, respectively). These results are similar to those observed with IVF and implantation of human embryos (Gardner *et al.*, 1998).

In our experiments DMSO was used to dissolve cytochalasin B and so was present in the medium during oocyte activation. A DMSO concentration of 1% (v/v) was used in initial experiments. The effect of DMSO on embryonic development (Wood *et al.*, 1993) was assessed by decreasing the DMSO concentration to one hundredth of the original concentration. To our surprise, there was a correlation between the concentration of DMSO (in the presence of Sr^{2+}) in the activation medium and the frequency of development to the blastocyst stage and to full term development. Although Morley and Whitfield (1993) had shown that DMSO causes the release of Ca^{2+} from intracellular stores, we had been unable previously to induce oocyte activation with 1–6 h exposure to 0.05–2.00% (v/v) DMSO in the absence of Sr^{2+} (Imahie *et al.*, 1995; T. Wakayama and R. Yanagimachi, unpublished). Therefore, in our experiments, oocyte activation must have been induced by Sr^{2+} rather than by DMSO. Interestingly, DMSO is known to cause differentiation of embryonic teratocarcinoma cells into cardiac and skeletal muscle (McBurney *et al.*, 1982; Rudnicki *et al.*, 1990; Vidricaire *et al.*, 1994). In those cases, DMSO affected differentiation from embryonic teratocarcinoma cells to somatic cells, but

Table 4. Full term development of reconstructed oocytes transferred at two-cell or morula/blastocyst stage

Embryonic stage at transfer	Activation methods of cloned oocytes		Number of surviving oocytes after nucleus injection	Number (%) of activated oocytes	Number (%) of embryos that developed to		Number of transferred embryos (recipients)	Number of live offspring	% Offspring from	
	Agent*	DMSO (%; v/v)			Two-cell	Morulae/blastocysts			Transferred embryos	Nucleus-injected oocytes
Two-cell	Cytochalasin B	1.0	71	67 (94.4)	59 (84.3)	–	59 (3)	2	3.4	2.8
	Cytochalasin D	0.2	74	74 (100)	62 (83.8)	–	62 (3)	1	1.6	1.4
	Cytochalasin D	1.0	77	71 (92.2)	70 (92.1)	–	70 (4)	2	2.9	2.6
	Nocodazole	1.0	79	72 (91.1)	68 (87.2)	–	68 (4)	2	3.0	2.5
	Total		301	284 (94.4)	259 (86.9)	–	259 (14)	7	2.7	2.3
Morula/blastocyst	Cytochalasin B	1.0	77	73 (94.5)	–	47 (61.0)	47 (4)	3	6.4	3.9
	Cytochalasin D	0.2	84	83 (98.8)	–	42 (50.0)	42 (4)	1	2.4	1.2
	Cytochalasin D	1.0	78	77 (98.7)	–	46 (59.0)	46 (4)	2	4.4	2.6
	Nocodazole	1.0	83	73 (88.0)	–	35 (42.2)	35 (4)	3	8.6	3.6
	Total		322	306 (95.0)	–	170 (52.9)	170 (16)	9	5.3	2.8

This experiment was replicated three times. All reconstructed oocytes were activated 1–3 h after nuclear transfer (protocol C).
*Cytochalcasin B: 5 µg cytochalcasin B ml⁻¹; cytochalcasin D: 1 µg cytochalcasin D ml⁻¹; nocodazole: 3 µg nocodazole ml⁻¹.

for nuclear transfer, DMSO might affect reprogramming of somatic cell nuclei after or during oocyte activation.

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