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Cloned Cattle Derived from a Novel Zona-Free Embryo Reconstruction System

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ABSTRACT

As the demand for cloned embryos and offspring increases, the need arises for the development of nuclear transfer procedures that are improved in both efficiency and ease of operation. Here, we describe a novel zona-free cloning method that doubles the throughput in cloned bovine embryo production over current procedures and generates viable offspring with the same efficiency. Elements of the procedure include zona-free enucleation without a holding pipette, automated fusion of 5–10 oocyte–donor cell pairs and microdrop *in vitro* culture. Using this system, zona-free embryos were reconstructed from five independent primary cell lines and cultured either singularly (single-IVC) or as aggregates of three (triple-IVC). Blastocysts of transferable quality were obtained at similar rates from zona-free single-IVC, triple-IVC, and control zona-intact embryos (33%, 25%, and 29%, respectively). In a direct comparison, there was no significant difference in development to live calves at term between single-IVC, triple-IVC, and zona-intact embryos derived from the same adult fibroblast line (10%, 13%, and 15%, respectively). This zona-free cloning method could be straightforward for users of conventional cloning procedures to adopt and may prove a simple, fast, and efficient alternative for nuclear cloning of other species as well.

INTRODUCTION

ANIMAL CLONING BY NUCLEAR TRANSFER (NT), or nuclear cloning, is now a standard methodology in dozens of laboratories around the world. It is, however, still very inefficient and fails to generate viable offspring in about 95–99% of attempts, depending on the species (Solter, 2000). It is expected that the demand for cloned embryos and offspring in commercial agriculture and basic science will increase. In the near future, cloned embryos will probably also be needed as a source of autologous embryonic stem (ES) cells for therapeutic cloning (Gurdon and Colman, 1999). Cloned embryo production methods with higher throughput are required to meet this demand. Current NT procedures are labour- and cost-intensive and require high technical skills in micromanipulation. Extensive micromanipulation may induce operator-dependant experimental variation between cloning runs, simply due to variance in technical competence. Simplified NT protocols for embryonic (Peura et al., 1998) and somatic cell cloning (Booth et al., 2001; Vajta et al., 2001) have been developed. Their major modification is the use of zona pellucida-free oocytes.

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Common elements of these zona-free methods comprise: (1) oocyte bisection to remove the maternal chromosomes, (2) UV-selection of half-oocytes without chromatin (cytoplasts), (3) fusion of one donor cell and two half cytoplasts, and (4) individual culture of single reconstructed NT embryos. These methods generate NT blastocysts of the same quality as standard zona-intact NT protocols, confirming earlier studies that the zona is dispensable for *in vitro* development (Naito et al., 1992; Thadani, 1982). Oocyte bisection methods, however, waste 50% of the oocyte starting material as they require fusion of two half cytoplasts in order to restore the original cytoplasmic volume. Consequently, embryos cloned via this procedure could contain up to three different genotypes of mitochondrial DNA within a cell, potentially increasing the incidence of mitochondrial heteroplasmy. We have developed an alternative approach of zona-free cloning that circumvents this problem and further improves throughput, ease of operation and reproducibility. Our method differs from previous zona-free approaches in three points: (1) zona-free oocytes are enucleated using a blunt aspiration pipette and no holding pipette, (2) a large number of oocyte-donor cell pairs are fused simultaneously using automated AC-alignment ("bulk fusion"), and (3) reconstructs are cultured in microdrops, either singularly or as aggregates of three. We have transferred zona-free NT embryos cloned from five different somatic cell lines, including one transgenic line. There was no difference in rates of pregnancy establishment and survival to term and weaning between (a) zona-intact and zona-free NT embryos and (b) zona-free NT embryos cultured either singularly or as aggregates. We conclude that the zona-free technology described here doubles the throughput of cloned embryo production without affecting overall cloning efficiency.

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MATERIALS AND METHODS

All chemicals were supplied by Sigma-Aldrich (Auckland, New Zealand), and all embryo manipulations were carried out at 38.5°C unless indicated otherwise.

Nuclear donor cells

Five independent primary fibroblast cell lines were used: (1) adult ear skin fibroblasts (passage 2–5) isolated from three different bulls (AESF-1, 2, and 3), (2) fetal fibroblasts (passage 3–8) derived from lung tissue of a day 60 bovine female fetus (BFF), and (3) genetically modified BFF cells (passage 11–15) carrying copies of the human myelin basic protein (TG-MBP, clonal cell strains designated TG-10.1, 10.2, and B3). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium (Life Technologies, Auckland, New Zealand) supplemented with 10% fetal calf serum (FCS, Life Technologies) and sodium pyruvate (1 mM). For NT, presumptive G_0 cells were obtained by serum deprivation (Campbell et al., 1996) in culture medium containing 0.5% FCS for 3–7 days.

Collection of mature oocytes and zona removal

In vitro maturation (IVM) of abattoir oocytes was performed as described previously (Wells et al., 1999). After IVM for 18–20 h, the cumulus-corona was removed by vortexing in 0.1% hyaluronidase in HEPES-buffered synthetic oviduct fluid (HSOF; Thompson et al., 1990). After three washes in HEPES-buffered TCM 199 (H199; Life Technologies) containing 0.1 mg/mL cold soluble PVA (M_r: 10–30000, H199/PVA), the zona pellucida was removed from oocytes with a first polar body by brief incubation in 5 mg/mL pronase dissolved in Ca- and Mg-free PBS. When the zona was dissolved, oocytes were washed in H199/10% FCS (H199/FCS) to inactivate the enzyme.

Enucleation

Zona-free oocytes were stained with 5 μg/mL Hoechst 33342 in H199/PVA before transfer into a H199/FCS droplet on the microscope stage. Using $\times$100 magnification, they were enucleated under constant UV-light exposure. As soon as the chromosomes were visible in the enucleation pipette (25–30 μm outer diameter, perpendicular break), the cytoplast was moved out of the UV light. Cytoplast and karyoplast were separated with a simple "separation needle" (100–150 μm outer diameter, perpendicular break, closed fire-polished tip). All tools were made from thin wall borosilicate capillaries (GC100T-15; Harvard Apparatus Ltd., U.K.), using a horizontal puller (P-87; Sutter Instruments, USA) and a microforge (MP-9, Narishige, Japan). Oocyte volume changes were calculated from linear measurements taken by photographing individual cytoplasts and their corresponding karyoplasts once they had re-

gained a spherical shape together with a 1-mm stage micrometer (100×0.01 mm, Graticules Ltd., U.K.).

Attachment of donor cells

Trypsinized somatic donor cells were resuspended in H199/0.5% FCS (1×10^4 cells/mL) and aliquoted into 40-μL drops covered under oil. About 5–10 individual cells were picked up with a mouth pipette and added to a drop of 10 μg/mL Phytohemagglutinin (PHA-P) in H199, already containing 5–10 cytoplasts. Cytoplasts and donor cells were pushed together with the mouth pipette, incubated for at least 5 min, then groups of 5–10 couplets were transferred into H199/PVA-washdrops without PHA-P before they could aggregate.

Electrofusion and artificial activation

Donor cells were electrically fused to cytoplasts 23–25 h post start of maturation. After equilibration in fusion buffer (0.2 M mannitol, 50 μM $CaCl_2$, 100 μM $MgCl_2$, 500 μM HEPES, 0.05% fatty acid–free BSA, pH 7.3), 5–10 couplets were placed in a fusion chamber connected to an ECM 200 (BTX, San Diego, CA). Five to 10 couplets were automatically aligned by applying an alternating current (AC)–field (60–100 V/cm) for 5–10 sec and fused using 2×10 μsec direct current (DC)–pulses (1.5–1.8 kV/cm), followed by another 5–10-sec AC-pulse (60–100 V/cm). Fusion was performed at room temperature. Couplets were removed from the fusion chamber and put back into H199/PVA to score fusion success and detect detached or lysed donor cells. Reconstructs were activated 3–6 h post-fusion, using a combination of ionomycin and 6-dimethylaminopurine (6-DMAP) as described (Wells et al., 1999).

In vitro culture

Reconstructed embryos were cultured *in vitro* for 7 days (day 0 = fusion) in biphasic AgResearch-SOF (Wrenzycki et al., 2001), either singularly or as aggregates. For single *in vitro* culture (single-IVC), one reconstruct was cultured in 5 μL of medium overlaid with mineral oil. For aggregate cultures (triple-IVC), three reconstructs were cultured per 10 μL medium droplet. Zona-intact controls were cultured in groups of 10 in 20 μL of medium.

Differential staining

Zona-free blastocysts were exposed to a 1:5 dilution in PBS of rabbit anti-bovine whole serum for 1 h, rinsed in H199/PVA and placed into a 1:5 dilution in PBS of guinea pig complement containing 40 μg/mL propidium iodide and 40 μg/mL Hoechst 33342 for 1 h. After briefly rinsing in H199/PVA, the embryos were mounted (DAKO® mounting medium, USA) on glass slides and examined using an epifluorescence microscope (AX-70, Olympus, Japan). Blue and pink colors were designated as inner cell mass (ICM) and trophectoderm (TE) cells, respectively.

Embryo transfer, pregnancy monitoring, and controlled calving

Total embryo development to compacted morula and blastocyst stages was assessed after 7 days, and grade 1 and 2 embryos (Robertson and Nelson, 1998) were non-surgically transferred singularly into synchronized recipient cows (estrus = day of fusion). Using ultrasonography, recipient cows were examined for pregnancy 35 days after embryo transfer (ET). Development throughout gestation was monitored regularly by ultrasonography and palpation per rectum. Cloned calves were delivered as described (Wells et al., 2003).

Statistical analysis

Data were analyzed using the GenStat statistical package. Statistical significance levels presented are the two-tailed p values resulting from the Fisher exact test for independence in 2×2 tables.

RESULTS

We first investigated whether removal of the zona pellucida with pronase would pre-activate the oocytes or interfere with subsequent artificial activation, as has been reported for zona-removal on mouse oocytes using acidic Tyrode's solution (Johnson et al., 1990). From the same pool of cumulus-free oocytes with a first polar body, matured for 22–24 h, the zona was either removed or left intact (controls). Zona-free oocytes and controls were washed three times in H199/FCS before half of the oocytes from each treatment were either activated using ionomycin/6-DMAP

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or left non-activated. Both activated and non-activated oocytes were cultured for 48 h in AgR-SOF, stained with Hoechst 33342 and scored for pronuclei-formation and cleavage. Spontaneous activation was not significantly different in both non-activated groups (4.8% for zona-free vs. 5.3% for zona-intact), and both artificially activated groups showed similar rates of pronuclei-formation and cleavage (>95% and >90%, respectively). This indicated that enzymatic zona-removal did not influence activation rates in cattle.

We then adapted our standard enucleation procedure to zona-free oocytes (Fig. 1). A pipette with a clean perpendicular break was positioned next to the metaphase plate (visualized by UV illumination of Hoechst 33342–labeled chromatin) or the first polar body (which can serve as a morphological landmark for the underlying spindle complex). The outer diameter of the pipette was large enough (25–30 μm) to gently aspirate both the polar body (if still present) and the metaphase plate. A simple "separation needle" was used to both prevent the oocyte from rolling away and to separate the cytoplast from the karyoplast. The accuracy of chromosome removal was practically 100%, and average reduction in oocyte volume was minimal (1.8 ± 0.3% compared to 2.4 ± 2.2% for zona-intact, $n = 25$ for each) with relatively small variation (range 1.4–3.0% compared to 0.2–8.6% for zona-intact, $n = 25$ for each). The oocytes were exposed to UV light for 10.5 ± 3.1 sec compared to 4.3 ± 6.3 sec for zona-intact, $n = 30$ for each. Average enucleation time was about 30 sec per oocyte compared to about 1 min for zona-intact oocytes.

Zona-free cytoplasts were attached to single donor cells using the lectin PHA-P. After testing 1, 5, 10, 20, 50, 100, 200, 250, and 500 μg/mL, the lowest effective concentration was found to be 10 μg/mL. At this concentration, PHA-P-attached couplets did not separate during the subsequent manipulation steps, even when considerably larger donor cells were used (e.g., mitotically arrested fibroblasts or morula blastomeres). In control experiments, this concentration did not interfere with development of *in vitro* fertilized (IVF) zygotes and parthenogenotes. Lectin agglutination allowed the production of about three couplets per minute.

During electrofusion, gain in throughput was achieved through automated AC alignment of a large number of couplets, evenly spaced between each other and the electrodes and all simultaneously exposed to the DC fusion pulse. Somatic AC alignment efficiency varied between cell lines (range 25–95%, average 78%; Table 1), and there was no significant difference in fusion efficiency (67% compared to 68% for zona-intact; Table 1).

In a direct comparison, zona-intact controls resulted in higher rates of total development into compacted morulae/blastocysts than both zona-free single- and triple-IVC conditions (56% vs. 47% and 33% for BFF; 56% vs. 52% and 21% for AESF-1, respectively; Table 1). *In vitro* development rates were much lower for triple IVC. This is, however, mainly a numerical consequence of aggregation, whereby three reconstructs placed into a drop of culture medium usually formed a single blastocyst. All embryos were graded according to the International Embryo Transfer society guidelines (Robertson and Nelson, 1998), even though it has not yet been established whether such grading criteria are at all meaningful for zona-free embryos or cloned embryos in general. Development to grade 1 and 2 embryos was either not different between zona-intact and zona-free single- and triple-IVC (28% vs. 27% and 28% for BFF; Table 1) or significantly less for triple-IVC (30% and 34% vs. 17% for AESF-1; Table 1). Of all compacted morulae and blastocysts obtained, a larger proportion was of grade 1 and 2 quality with triple-IVC than with single- and zona-intact IVC (85% vs. 58% and 50% for BFF; 81% vs. 66% and 53% for AESF-1, respectively).

Across all five cell lines, the proportion of day seven embryos of grade 1 and 2 quality was again significantly higher for triple- than for single-IVC (88% vs. 72%, $p < 0.001$; Table 1). However, the total output of grade 1 and 2 embryos was significantly higher for single- than for triple-IVC (39% vs. 25%, $p < 0.001$; Table 1), mainly because three reconstructed embryos typically formed one blastocyst. Cell counts after differential staining revealed that for two different cell lines, blastocysts derived from triple-IVC contained about twice the number of ICM, TE and total cells than those derived from single IVC, while the ICM/total cell ratio was increased (Table 2).

The gains in throughput of reconstructed NT embryos per person are summarized in Table 3. Enucleation, lectin-agglutination and bulk fusion were all easier to perform and 2× , 3× , and 2.5× faster, respectively, than the corresponding steps in our zona-intact standard system. Since NT is a sequential procedure, it is limited by its slowest

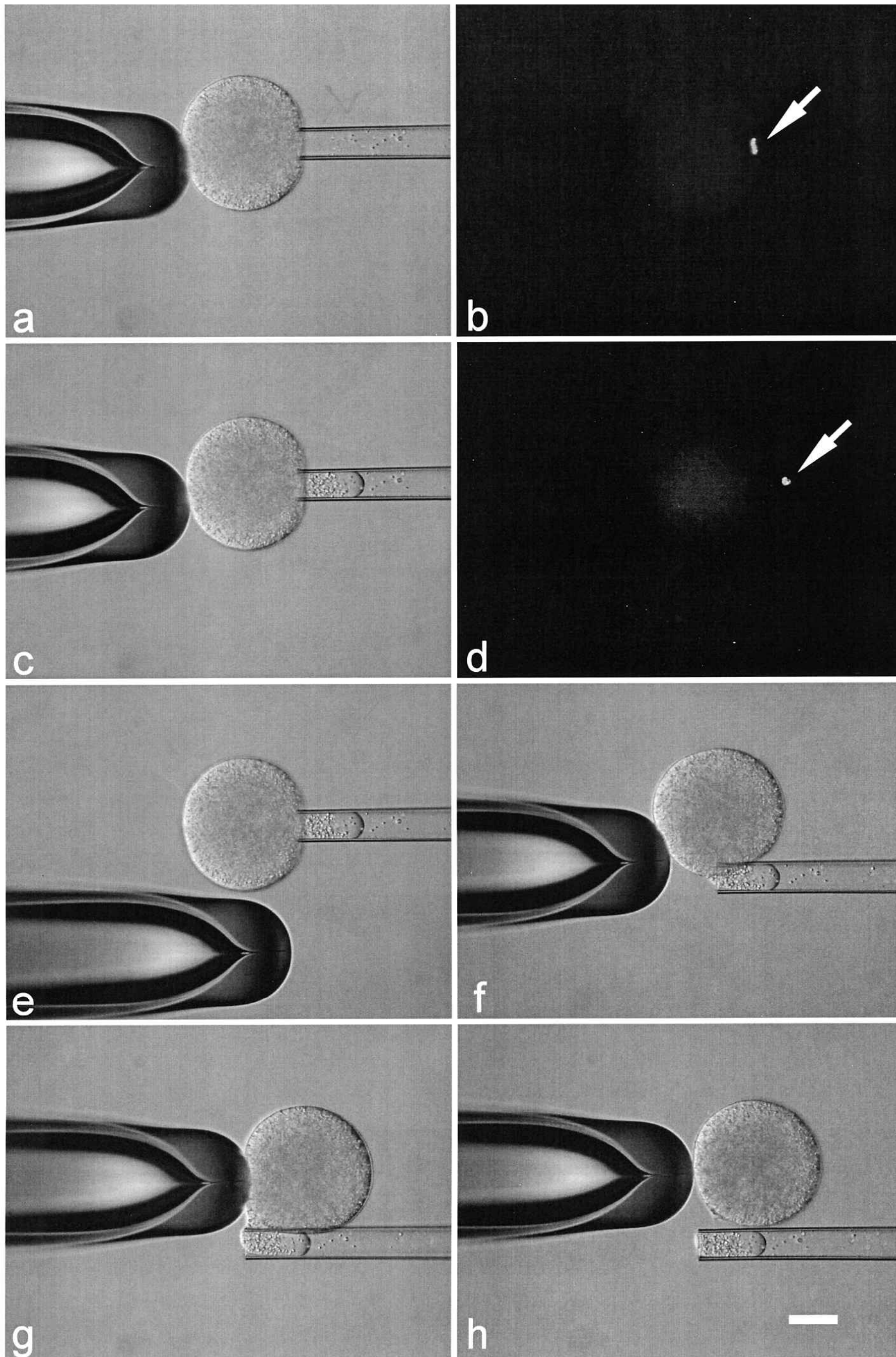

FIG. 1. Zona-free enucleation. The metaphase plate was removed using a blunt enucleation pipette and a separation needle (a,c,e–h). **(a)** The oocyte was moved until its metaphase plate was adjacent to the enucleation pipette and in the same focal plane. **(c)** The metaphase plate, visible as a clear zone of cytoplasm, was aspirated with a small amount of cytoplasm. **(e–g)** In one smooth movement, the separation needle was pushed upward (f) and rightward (g) to gently pinch off the karyoplast **(h).** Removal of the metaphase plate (arrows) was confirmed by Hoechst 33342 staining **(b,d).** Bar = 40 μm.

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step (enucleation). Thus, the overall throughput in terms of number of NT reconstructs produced was 2.4× greater and, taking into account the average development rates after single- and triple-IVC, the output of grade 1 and 2 NT embryos was 2–3× higher.

The ultimate readout of cloning efficiency is not blastocyst development but rather the *in vivo*

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Table 1. Comparison of *In Vitro* Development for Zona-Intact (zi) and Zona-Free (zf) Bovine NT Embryos

Cell type	*Cell line*	*Method*	*Automated alignment*[a]	*Fusion efficiency*[b]	*IVC*	*Total embryo development*[c]	*Development to grade 1/2*[d]
Fetal lung fibroblast	BFF	Zona-free	101/106 (95%)	249/336 (74%)	zf-single	62/132 (47%)†	36/132 (27%)
					zf-triple	39/117 (33%)*,‡	33/117 (28%)
		Zona-intact	0%	89/159 (56%)	zi-group	50/89 (56%)**	25/89 (28%)
TG fetal lung fibroblast	TG-MBP (all)	Zona-free	778/1,001 (78%)	923/1,152 (80%)	zf-single	216/336 (64%)*	174/336 (52%)*
					zf-triple	135/426 (32%)**	122/426 (29%)**
Adult skin fibroblast	AESF-1	Zona-free	Manual[e]	400/802 (50%)	zf-single	138/265 (52%)*	91/265 (34%)*
					zf-triple	26/126 (21%)**	21/126 (17%)**,†
		Zona-intact	0%	76/83 (92%)[f]	zi-group	64/115 (56%)*	34/115 (30%)‡
	AESF-2	Zona-free	163/239 (68%)	131/239 (55%)	zf-single	27/57 (47%)*	18/57 (32%)
					zf-triple	14/63 (22%)**	13/63 (21%)
	AESF-3	Zona-free	186/238 (78%)	161/238 (68%)	zf-single	20/75 (27%)	15/75 (20%)
					zf-triple	17/75 (23%)	15/75 (20%)
Total		Zona-free	1,228/1,584 (78%)[g]	1,689/2,536 (67%)	zf-single	463/865 (54%)*	334/865 (39%)*,†
					zf-triple	231/807 (29%)**	204/807 (25%)**
		Zona-intact	0%	165/242 (68%)	zi-group	114/204 (56%)*	59/204 (29%)‡

[a]Couplets that did not align automatically were manually aligned.
[b]All couplets were included, irrespective of the alignment method (automatic or manual).
[c]Proportion of total number of reconstructed embryos placed into IVC that developed into compacted morula/blastocyst.
[d]Proportion of total number of reconstructed embryos placed into IVC that developed into grade 1/2 morula/blastocysts.
[e]Since automated alignment was <25%, all couplets were aligned manually.
[f]One data set was not represented; hence, this number is smaller than the total number of reconstructs placed into culture.
[g]Excluding manually aligned AESF-1 dataset.
TG-MBP, transgenic, or carrying additional gene copies of human myelin basic protein.
*,**Rows with these different superscripts within a column differ, $p < 0.005$.
†,‡Rows with these different superscripts within a column differ, $p < 0.05$.

TABLE 2. TOTAL CELL NUMBERS AND ALLOCATIONS OF CELLS (MEAN ± SD) TO ICM AND TE IN ZONA-FREE BOVINE NT BLASTOCYSTS

			No. of cells			
Cell line	*Method*	*No. of embryos*	*ICM*	*TE*	*Total*	*ICM/total cells*
AESF-1	Single-IVC	10	31.8 ± 17.2*	61.9 ± 23.3†	93.7 ± 38.7†	0.33 ± 0.10
	Triple-IVC	4	72.5 ± 26.7**	93.3 ± 21.6‡	165.8 ± 47.6‡	0.43 ± 0.04
TG-MBP	Single-IVC	17	50.2 ± 15.3*	65.7 ± 17.5*	115.9 ± 26.8*	0.43 ± 0.09†
	Triple-IVC	12	106.7 ± 42.2**	102.8 ± 33.4**	209.4 ± 72.2**	0.50 ± 0.06‡

*,**For each cell line, rows with these different superscripts within a column differ, $p < 0.005$.
†,‡For each cell line, rows with these different superscripts within a column differ, $p < 0.05$.

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development of embryos to term and weaning (Table 4). Although numbers are low, there was no indication that development to term was significantly different between zona-intact embryos and those developed from zona-free single- and triple-IVC (15% vs. 10% and 13% for AESF-1, respectively; Table 4). Presently, every tested cell line (BFF, TG-MBP, and AESF-1,2,3) has resulted in live zona-free calves. Of the 18 live zona-free term calves so far produced, ten were derived from single-IVC and eight from triple-IVC. The birth weights of single-, triple-, and zona-intact calves were very similar (44.3 ± 6.9, 46.6 ± 9.8 and 43.9 ± 3.7, respectively).

DISCUSSION

By modifying current NT procedures, we have developed a zona-free cloning method that at least doubles the throughput in cloned embryo production while achieving the same overall efficiency in cloned offspring production. The first innovation lies in the enucleation step. Penetration of the zona is normally achieved by using either bevelled pipettes with a spike or piezo-actuated zona-drilling with a blunt pipette. For zona-free oocytes, a simple blunt enucleation pipette suffices. Instead of the conventional holding pipette, we used a fire-polished separation needle. The main advantage of this method over our standard zona-intact procedure was increased simplicity and speed: (1) both enucleation tools could each be reproducibly made in less than 2 min; (2) virtually no lysis of cytoplasts occurred during or after the process; (3) the average reduction in oocyte volume was minimal and less variable; and (4) the method was twice as fast, reducing average enucleation time to about 30 sec per oocyte. The whole procedure was done on an inverted microscope under 100× total magnification. This magnification is in the zoom-range of most modern stereomicroscopes, opening up the possibility of using cheaper equipment for enucleation. Compared to zona-intact enucleation, this technique was considerably easier to learn for staff members, especially those with no previous micromanipulation experience. A potential dis-

TABLE 3. COMPARISON IN THROUGHPUT BETWEEN ZONA-INTACT AND ZONA-FREE BOVINE NT PROCEDURE

	Throughput per person (NT reconstructs/h)			
Step	*Zona-intact*	*Zona-free*		*Increased throughput*
1. Enucleation	60	120		2×
2. Addition of donor cell	60	180		3×
3. Fusion	60	150		2.5×
Steps 1–3	20	48		2.4×[a]
4. Culture method (grade$^{1/2}$ embryos)	10/drop	1/drop	3/drop	
	29%	39%	25%	—
NT embryos (grade$^{1/2}$)	6	19	12	2–3×

[a]Overall increase in throughput was calculated using $(1/n_1 + 1/n_2 + 1/n_3) \times 1/n_{tot}$ with $n_{1,2,3\ldots}$ = increased throughput at each step and n_{tot} = total number of steps.

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TABLE 4. COMPARISON OF *IN VIVO* DEVELOPMENT FOR ZONA-INTACT (zi) AND ZONA-FREE (zf) BOVINE NT EMBRYOS

Cell type	*Cell line*	*Embryos transferred*	*D35 of gestation*	*D180 of gestation*	*Development to term*[a]	*Development to weaning*[a]
Fetal lung fibroblast	BFF	zf-single-IVC	1/4 (25%)	1/4 (25%)	1/4 (25%)	1/4 (25%)
		zf-triple-IVC	0/1	—	—	—
TG fetal lung fibroblast	TG-MBP (10.1)	zf-single-IVC	6/21 (29%)	2/21 (10%)	2/21 (10%)	not yet weaned
		zf-triple-IVC	9/20 (45%)	5/20 (25%)	3/20 (15%)	not yet weaned
	TG-MBP (10.2)	zf-single-IVC	8/15 (53%)	5/15 (33%)	4/15 (27%)	3/15 (20%)
		zf-triple-IVC	11/21 (52%)	5/21 (24%)	3/21 (14%)	2/21 (10%)
	TG-MBP (B3)	zf-single-IVC	8/22 (36%)	0/22	—	—
		zf-triple-IVC	10/25 (40%)	0/25	—	—
Adult fibroblast	AESF-1	zf-single-IVC	6/10 (60%)	2/10 (20%)	1/10 (10%)	1/10 (10%)
		zf-triple-IVC	5/8 (63%)	1/8 (13%)	1/8 (13%)	0/8
		zi-group-IVC	15/26 (58%)	6/26 (23%)	4/26 (15%)	2/26 (8%)
	AESF-2	zf-single-IVC	6/12 (50%)	1/12 (8%)	0/12	—
		zf-triple-IVC	7/13 (54%)	2/13 (15%)	1/13 (8%)	0/13
	AESF-3	zf-single-IVC	3/12 (25%)	2/12 (17%)	2/12 (17%)	1/12 (8%)
		zf-triple-IVC	5/12 (42%)	0/12	—	—

[a]Proportion of total number of embryos transferred that developed to live calves at term or weaning (about 3 months).
TG, transgenic, or carrying additional gene copies of human myelin basic protein.

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advantage was that the oocytes were exposed for a longer time to UV light. However, our data for zona-intact NT show that UV exposure using a 20× objective (numerical aperture 0.4) has no detrimental effect on the development of cloned embryos into viable calves compared to no UV exposure at all (unpublished experiments). The zona-free method utilizes a 10× objective (numerical aperture 0.3), which transmits only about 56% of the UV light intensity to the oocytes (light intensity is proportional to the square of the numerical aperture $(0.3/0.4)^2 = 0.56$), further reducing the risk of photo damage.

Lectin agglutination replaced the microinjection step of zona-intact NT methods and allowed to produce about three times more couplets per minute. It superseded the need for costly microinjection equipment and manufacturing of a different set of microinjection pipettes. Furthermore, PHA attachment prevented the separation between donor cell and cytoplast that sometimes occurs using the zona-intact microinjection method. Although lectin-attachment involved extensive mouthpipetting of zona-free oocytes, we did not encounter any handling problems compared to zona-intact oocytes at this or any other step of the procedure.

The "bulk fusion" method described here relies on automated AC alignment of a large number of oocyte–donor pairs. Reliable AC alignment has been previously achieved with embryonic blastomeres (Peura et al., 1998) but not with somatic cells as donors. Somatic AC alignment efficiency was variable between cell lines (Table 1) and depended on the size ratio between oocyte and donor cell. Large ratios (e.g., small donor cells) reduced the efficiency, whereas the best AC alignment was observed with large G_0 fibroblasts or morula stage blastomeres (data not shown). Another critical factor was the shape of the oocytes: if they were not spherical after equilibration in the fusion buffer, they tended to be distorted into an elliptical shape by the AC alignment field instead of aligning properly. This was overcome by (a) decreasing the AC-field strength while increasing the pulse time and (b) using hypoosmolar fusion buffer in order to obtain large spherical oocyte-donor pairs. The hypoosmolar fusion buffer (207 mOsm) alone increased fusion efficiency compared to our previous standard buffer (318 mOsm) about 1.4-fold (92% vs. 65.3%, respectively, $p < 0.05$) based on isofusion contours (Tatham et al., 1996) for PHA-attached oocyte–oocyte pairs. In the zona-intact system, the use of hypoosmolar buffer is problematic because swelling of the cytoplast reduces the volume of the perivitelline space, which makes it difficult to see the injected donor cell during manual alignment in the fusion chamber. Automated alignment in the presence of a zona has been unsuccessful in our hands. In the few cases where automated alignment in the absence of a zona was largely unsuccessful (Table 1), the subsequent manual alignment procedure was still considerably easier than for zona-intact couplets as the attached donor cell was more clearly visible. This also facilitated subsequent scoring of fusion success.

Reconstructed embryos were cultured either singularly or as aggregates of three. Importantly, there was no effect of embryo aggregation on implantation rates or post-implantation survival. Blastocysts from triple-IVC originated from up to three founder NT reconstructs and were probably mosaic, even though direct evidence (e.g., using cell autonomous markers for lineage-tracing) is lacking. Since only about one half of the reconstructs will develop to the morula/blastocyst stage when cultured singularly, it is unclear exactly how many reconstructs contributed to each aggregation-blastocyst. Aggregating embryos at more advanced cleavage stages could be a way of increasing the chance of mosaicism. Our rationale for the production of mosaic cloned embryos was to increase the percentage of fully reprogrammed cells per NT blastocyst in order to improve survival after embryo transfer. Increasing indirect evidence suggests that inadequate epigenetic reprogramming of the donor genome resulting in aberrant gene expression may be the major cause for the high losses during cloning (Humpherys et al., 2002; Rideout et al., 2001). Aggregation blastocysts would theoretically have a threefold higher chance of containing some fully reprogrammed cells that may be able to compensate for the presence of faulty or incompletely reprogrammed cells through direct or indirect cell-to-cell interactions. A similar epigenetic "rescue" phenomenon has been observed for chimeric embryos reconstructed through aggregation of bovine IVF-derived and parthenogenetic blastomeres, whereas as few as two IVF-derived blastomeres were capable of partially increasing overall developmental competence (Boediono et al., 1999). That we did not observe a comparable beneficial effect of aggregation may be because it was

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either too subtle to be detected in a study with relatively few animals or because it simply did not exist. If aggregation could not increase cloning efficiency, it would not be the optimal culture method due to the reduction in total throughput.

Currently, the search for biological causes underlying low cloning efficiency is confounded by technical aspects of the NT procedure. Different methods are used for different species, possibly contributing to the variety of cloning phenotypes. Somatic cell mouse cloning has proven an especially difficult technique to master (Zhou et al., 2000) and has still only been reported from a few laboratories. Mice have previously been cloned using electrofusion rather than direct microinjection (Ogura et al., 2000), which may be a particular advantage for large donor cells. We have successfully used the zona-free system presented here to efficiently reconstruct mouse NT embryos with G_0 and mitotically arrested fibroblasts and, with minor modifications, to produce live offspring from serum-starved somatic sheep donor cells (unpublished results). We expect it to be readily applicable to other species as well.

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