Early zygotes are suitable recipients for bovine somatic nuclear transfer and result in cloned offspring

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Abstract

Cloning by somatic cell nuclear transfer (SCNT) subverts sperm-mediated fertilization that normally leads to physiological activation of the oocyte. Therefore, artificial activation is required and it is presently unclear what developmental consequences this has. In this study, we aimed to improve cattle cloning efficiency by utilizing a more physiological method of activating SCNT reconstructs. We carried out *in vitro* fertilization (IVF) of zona-intact bovine oocytes before SCNT. We removed the zona pellucida 4 h after insemination, stained the fertilized eggs with Hoechst 33342 and mechanically removed both male and female chromatin. The enucleated pre-activated cytoplasts were fused with male adult ear skin fibroblasts ('IVF-NT' group). Chemically activated SCNT embryos, produced according to our standard operating procedure for zona-free SCNT, served as controls. After 7 days, *in vitro* development to blastocysts of morphological grade 1–3 or grade 1–2 was very similar in both groups (39 vs 40% and 20 vs 21% respectively). However, post-implantation development was improved after sperm-mediated activation. Across four replicate runs, pregnancy establishment at day 35 was significantly higher for IVF-NT than for control SCNT embryos (30/49 = 61 vs 17/41 = 42% respectively; $P < 0.05$). Development into calves at term or weaning was also higher in the IVF-NT group compared with control SCNT (9/49 = 18 vs 3/41 = 7% and 6/49 = 12 vs 3/41 = 7%; $P = 0.11$ and 0.34 respectively).

Reproduction (2006) **132** 839–848

Introduction

Hundreds of apparently normal calves have been cloned worldwide after somatic cell nuclear transfer (SCNT) with bovine donor cells. However, these surviving animals represent less than 5% of all cloned embryos transferred into recipient cows (Oback & Wells 2003). Most of the remaining 95% died at various stages of development due to developmental abnormalities, collectively referred to as the 'cloning syndrome'. The cloning syndrome manifests itself at different levels. At the organismal level, typical symptoms encompass placental problems (e.g., placentome malformation and hydroallantois), prolonged gestation, parturition difficulties (e.g., higher placental and birth weights, higher peri- and post-natal death due to dystocia, and respiratory distress), specific adult phenotypes (e.g., musculoskeletal problems and obesity), and shorter life-span (Wells *et al.* 2004). At the cellular level, cloned pre-implantation embryos suffer from morphological and physiological abnormalities, such as aberrant allocations of inner cell mass (ICM; Koo *et al.* 2002), higher incidence of apoptosis (Park *et al.* 2004), and altered metabolic requirements (Chung *et al.* 2002). At

the subcellular level, molecular defects have been described including aberrant methylation patterns of DNA (Bourc'his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2001, 2002) and histones (Santos *et al.* 2003), and dysregulation of imprinted and non-imprinted genes (Humpherys *et al.* 2002, Mann *et al.* 2003, Suemizu *et al.* 2003). At least some symptoms of the cloned phenotype are not transmitted from parent to offspring (Shimozawa *et al.* 2002, Wells 2003), indicating that the cloned phenotype does not entail changes in DNA sequence and is therefore largely epigenetic. It is believed to be mainly caused by existing epigenetic errors in the donor genome and/or faulty or incomplete reprogramming of epigenetic marks (e.g., modifications of DNA and DNA-binding proteins) imposed on the donor genome during differentiation.

A number of approaches have been devised to increase the frequency of success in epigenetic reprogramming and alleviate the somatic cloning syndrome. These include identifying a better donor cell type or cell cycle stage (Wells *et al.* 2003), treating donor cells with pharmacological agents to alter their epigenetic marks (Enright *et al.* 2003, Kishigami *et al.* 2006), fusing

transiently permeabilized cells containing artificially condensed chromatin (Sullivan *et al.* 2004), using serial NT (Ono *et al.* 2001a, 2001b), or aggregating SCNT embryos (Boiani *et al.* 2003). Here, we have focused on improving another step of the cloning procedure, namely, the method of SCNT reconstruct activation.

Cloning subverts fertilization, which normally leads to physiological activation of the oocyte. Since NT of somatic donor cells does not activate the recipient oocyte (Czolowska *et al.* 1984, Szollosi *et al.* 1986, 1988), artificial activation protocols have been employed to mimic sperm-induced activation events (Graham 1974, Tarkowski 1975, Wittingham 1980, Kaufman 1983, Yanagimachi 1994). These events include triggering of the inositol-1,4,5-trisphosphate signal transduction cascade that leads to transient intracellular calcium oscillations, initiation of the cortical granule reaction, degradation of cyclin B and Cdk 1 (formerly p34^{cdc2}) complexes (M-Cdk, formerly maturation promoting factor or MPF), and formation of the diploid zygote. Procedures were developed to activate young mammalian oocytes by completely and continuously suppressing the activity of M-Cdk (Liu & Yang 1999). In a variety of species, most SCNT activation protocols work by either administering a single artificial stimulus, e.g., electric current (Wilmut *et al.* 1997, Baguisi *et al.* 1999) or strontium (Wakayama *et al.* 1998), or sequentially applying two consecutive stimuli, e.g., electric current or calcium ionophore followed by chemicals known to suppress M-Cdk re-formation or activity, such as blockers of translation (Kato *et al.* 1998) or broad-spectrum protein kinase-inhibitors (Susko-Parish *et al.* 1994) respectively. Comparative studies have not found any significant differences in cloning efficiency between different artificial oocyte-activating agents (Kishikawa *et al.* 1999, Galli *et al.* 2002). However, the origin, frequency, and amplitude of calcium oscillations are altered in artificially activated eggs (Deguchi *et al.* 2000). An electric pulse (Sun *et al.* 1992), ethanol (Cuthbertson *et al.* 1981), or calcium ionophore (Colonna *et al.* 1989) typically induce a single, large calcium rise instead of the long-lasting repeated calcium oscillations, which are caused by sperm (Miyazaki *et al.* 1986) or sperm factors (Swann & Ozil 1994). While this is sufficient to trigger cortical granule exocytosis and resumption of meiosis, it does not, for example, down-regulate the inositol-1,4,5-trisphosphate receptor (Brind *et al.* 2000, Jellerette *et al.* 2000). Furthermore, paternal mRNAs and proteins are delivered to the oocyte during fertilization and may be beneficial for early embryonic development (Ostermeier *et al.* 2004). In summary, artificial activation and fertilization are functionally non-equivalent and one might expect different developmental consequences as a result of different activation protocols.

In this study, we aimed to improve cattle cloning efficiency by utilizing a more physiological method of

artificially activating SCNT reconstructs. In order to achieve this, we *in vitro* fertilized bovine oocytes for 4 h and enucleated them at the telophase II (TII)-stage, just before NT with adult ear skin fibroblasts (AESF-1). Artificially activated SCNT embryos produced according to our standard operating procedure (SOP) for zona-free SCNT (Oback & Wells 2003) served as controls.

Materials and Methods

Chemicals were supplied by Sigma and all embryo manipulations were carried out at 38.5 °C unless indicated otherwise. All NT experiments were direct contemporaneous comparisons, that is, within each NT experiment, all parameters other than the activation method (sperm-mediated versus chemical), were kept the same (e.g. donor cells, pool of oocytes, culture medium, and recipient population for embryo transfer). Investigations were conducted in accordance with the regulations of the New Zealand Animal Welfare Act 1999.

Nuclear donor cells

A primary male AESF-1 cell line (passage 4–6) was used for NT. Fibroblasts were isolated and cultured as described in detail previously (Oback & Wells 2003). Prior to NT, cells were seeded at $2.5 \times 10^4/\text{cm}^2$. After 17–20 h, they were washed thrice with PBS and cultured in medium containing 0.5% (v/v) fetal calf serum (FCS) for 6 days (Campbell *et al.* 1996).

In vitro maturation of oocytes (IVM)

In vitro matured non-activated metaphase II (MII)-arrested oocytes were derived as described previously (Oback & Wells 2003). Briefly, slaughterhouse ovaries were collected from mature cows, placed in saline (30 °C), and transported to the laboratory within 2–4 h. Cumulus–oocyte complexes (COCs) were collected in Hepes-buffered medium 199 (H199) (Life Technologies; Cat.-no. 31100-035) containing 15 mM Hepes, 5 mM NaHCO₃, 0.086 mM kanamycin monosulfate, with 925 IU ml⁻¹ heparin (Artex Ltd, Waipukurau, New Zealand), and 20 µl/ml 20% (w/v) albumin concentrate (Immuno-Chemical Products Tbio (ICP bio), Auckland, New Zealand) by aspirating 3–12 mm follicles into a 15 ml tube using an 18-gauge needle and negative pressure (40–50 mmHg). Only COCs with a compact, non-atretic cumulus oophorus-corona radiata, and a homogenous ooplasm were selected for IVM. COCs were washed twice in H199 with 10% (v/v) FCS (H199-10) and once in bicarbonate-buffered medium M199 with 25 mM NaHCO₃, 0.2 mM pyruvate, 0.086 mM kanamycin monosulfate, and 10% (v/v) FCS (B199-10). Ten COCs in 10 µl of B199-10 were transferred into a 40 µl drop of IVM medium comprising B199-10 with 10 µg/ml ovine

follicle-stimulating hormone (Ovagen; ICPbio), 1 µg/ml ovine luteinizing hormone (ICPbio), 1 µg/ml 17-β-estradiol, and 0.1 mM cysteamine in 6 cm dishes (Falcon 35-1007, Becton Dickinson Labware, Lincoln Park, NJ, USA) overlaid with paraffin oil (Squibb, Princeton, NJ, USA).

In vitro fertilization (IVF)

In vitro-matured oocytes were fertilized at 20–22 h post-start of maturation as described previously (Thompson *et al.* 2000). Briefly, spermatozoa were prepared from frozen-thawed semen obtained from a sire that had been characterized as suitable for IVF in the laboratory. The contents of one 0.25 ml straw (containing approximately 1×10^8 spermatozoa/ml) was layered upon a Percoll gradient (45:90%), and motile spermatozoa were collected after centrifugation at approximately 700 g for 20 min at room temperature. The motile fraction was washed once in 1 ml Hepes-buffered synthetic oviduct fluid (HSOF; 107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4 , 5 mM NaHCO_3 , 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, 1.71 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 3 mg/ml fatty-acid free bovine albumin (ABIVP, ICPbio)). Sperm concentration was adjusted to 1×10^6 sperm/ml and insemination performed in 50 µl IVF SOF medium (107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4 , 25 mM NaHCO_3 , 3.32 mM sodium lactate, 0.04 mM kanamycin monosulfate, 0.33 mM pyruvate, 1.71 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 8 mg/ml fatty-acid free bovine albumin (ABIVP, ICPbio), supplemented with 0.001 mM heparin, 0.2 mM penicillamine, and 0.1 mM hypotaurine) under oil (approximately five oocytes per drop) in a humidified modular incubation chamber (ICN Biomedicals Inc., Aurora, OH, USA) gassed with 5% CO_2 , 7% O_2 , and 88% N_2 .

In pilot experiments, the eggs were fixed in 4% paraformaldehyde in PBS, stained with 5 µg/ml Hoechst 33342, and scored for disappearance of the MII plate and second polar body extrusion at various times after insemination. In another set of pilot experiments, the proportion of cloned embryos that developed into blastocysts was quantified as a function of the time interval between insemination and NT. Both sets of initial experiments established that an interval of 4–5 h post-insemination (hpi) was optimal for achieving both high fertilization and development rates. To keep the timing between insemination and NT constant, oocytes were *in vitro* fertilized in three consecutive batches, 60 min apart, on each experimental day.

NT and artificial activation

For each IVF-batch, the cumulus-corona was dispersed 220 min post-insemination (mpi; equals 23.7–25.7 h post-IVM) by vortexing in 1 mg/ml bovine testicular

hyaluronidase (Sigma) in HSOF, followed by three washes in HSOF. The zona pellucida was removed with pronase (5 mg/ml in H199), the presumptive zygotes washed twice in HSOF and placed into drops of Hepes-buffered AgResearch (AgR)-SOF (Wells *et al.* 2003). Early zygotes were selected for second polar body extrusion at 230 mpi, then stained for 5 min in droplets of 5 µg/ml Hoechst 33342 in H199 with 1 mg/ml polyvinyl acetate (PVA, Mr: 10-30000) (H199-PVA) under oil. At 240 mpi, enucleation commenced whereby each zygote was constantly exposed to u.v.-light during removal of maternal and paternal chromatin at 100× total magnification. Bovine zona-free NT was performed using our previously described SOP (Oback & Wells 2003). Briefly, individual serum-starved cells (selected for relatively small size within the population) were attached to cytoplasts in drops of 10 µg/ml phytohemagglutinin in H199-PVA. At 270–330 mpi (this equals about 25–27 h post-IVM), couplets were electrically fused with 2×10^5 µs rectangular DC-field pulses (2.0 kV/cm) in hypoosmolar fusion buffer (165 mM mannitol, 50 µM CaCl_2 , 100 µM MgCl_2 , 500 µM Hepes, 0.05% (w/v) bovine albumin (ABIVP, ICPbio), pH 7.3) using a custom-made parallel-plate fusion chamber connected to an ECM 200 (BTX, San Diego, CA, USA) at room temperature (Gaynor *et al.* 2005). Successfully fused SCNT reconstructs were placed in *in vitro* culture drops of AgR-SOF (Wells *et al.* 2003). An overview of the IVF-NT procedure is illustrated in Fig. 1.

Standard control SCNT embryos were generated according to the same SOP. After IVM for 20.5 h, the

Figure 1 Sperm-mediated activation of NT reconstructs. The IVF-NT cloning procedure comprises five main steps. (A) MII oocytes are fertilized *in vitro*. (B) Fertilization activates the oocyte. The zona pellucida is removed. (C) Both the decondensing sperm chromatin and the maternal telophase II spindle complex are aspirated, to produce cytoplasts. (D) Lectin-mediated attachment of donor cell to cytoplast and electrical cell fusion of donor–cytoplast couplet. (E) Single *in vitro* culture of NT reconstruct to blastocyst.

cumulus-corona was dispersed as described above. After three washes in H199-PVA, the zona pellucida of oocytes with a first polar body was removed by pronase and the eggs held in H199-PVA, until start of enucleation at 21 h post-IVM. At about 22–23 h post-IVM, oocyte–donor couplets were electrically fused and reconstructed SCNT embryos were artificially activated 5–6 h post-fusion (range 310–365 min, average = 343 min post-fusion; this equals 28 h post-IVM), using a combination of 5 μ M ionomycin for 4 min and 2 mM 6-dimethylaminopurine (6-DMAP) in AgR-SOF medium with 10% (v/v) FCS (Wells *et al.* 2003). After 4 h in 6-DMAP (Susko-Parrish *et al.* 1994), reconstructs were washed thrice in HSOF and transferred into AgR-SOF culture medium droplets. Another set of control experiments utilized NT into artificially pre-activated cytoplasts or MII oocytes. Following ionomycin treatment for 4 min, 2 mM 6-DMAP was included in all media used for the production NT reconstructs except for the few minutes in fusion buffer during electrofusion. After a total of 4 h in media containing 6-DMAP, reconstructs were washed thrice in HSOF and transferred into AgR-SOF culture medium droplets.

In vitro culture (IVC)

Reconstructed embryos were cultured singularly *in vitro* for 7 days (day 0, D0 = fusion) in 5 μ l biphasic-AgR-SOF medium (Wells *et al.* 2003). On D4, embryos were changed into fresh AgR-SOF drops containing 10 μ M 2, 4-dinitrophenol (Thompson *et al.* 2000) to act as an uncoupler of oxidative phosphorylation. All cultures were overlaid with mineral oil and done in a humidified modular incubation chamber gassed with 5% CO₂, 7% O₂, and 88% N₂.

Embryo transfer, pregnancy monitoring, and controlled calving

Total embryo development into blastocysts was assessed after 7 days. Morphological grade 1 and 2 blastocysts, that is, those with symmetrical and spherical ICM cells of uniform size, color and density (Robertson & Nelson 1998) were selected for embryo transfer (ET), even though it has not yet been established that such grading criteria are at all meaningful for cloned embryos or zona-free embryos in particular. Recipient cows were synchronized as described (Oback & Wells 2003). On D7 following estrus (estrus = D0 = day of NT), a single cloned blastocyst in Emcare embryo holding solution (ICPbio) was loaded per 0.25 ml straw (Cryo-Vet, Quebriac, France) and transferred non-surgically into the uterine lumen ipsilateral to the corpus luteum. Friesian and Hereford $\times$ Friesian cows were used as recipients and embryos from each treatment were randomly allocated to each breed in roughly equal

numbers. Using ultrasonography (Aloka SSD-500 scanner with a 5 MHz linear rectal probe, Aloka Co Ltd, Tokyo, Japan) the pregnancy status of recipient cows was determined on D35 of gestation. Development throughout gestation was monitored approximately every 30 days, from D35 to D90 by ultrasonography and thereafter by palpation per rectum. Following a regime to control parturition as described (Oback & Wells 2003), cows were allowed to calve naturally if at all possible or with manual assistance varying degrees as necessary. On rare occasions, calves were delivered by Caesarean section. Calves were weaned when they weighed over 100 kg (about 3 months of age).

Statistical analysis

All values are presented as mean $\pm$ S.E.M., unless indicated otherwise. Statistical significance was accepted at $P < 0.05$ and determined using the one-tailed Fisher exact test for independence in 2×2 tables for development data or the two-tailed *t*-test with equal variance for birth weight and gestation length data.

Results

Timing of early fertilization events

Disappearance of the MII plate was used to indicate oocyte activation at hourly intervals post-insemination (Fig. 2). No activation was observed at 2 hpi, 4% at 3 hpi, 34% at 4 hpi, 69% at 5 hpi, and 75% at 6 hpi. Activation rates further increased at 8 (80%) and 10 hpi (89%). There was no further significant increase between 13 (94%) and 23 hpi (95%). In all chemically activated oocytes, the MII plate had disappeared within 1 h post-activation (Fig. 2).

Figure 2 Time course of oocyte activation. At different time points after insemination, or artificial activation, the proportion of the total number of eggs that had either lost their MII plate or extruded a second polar body was quantified after fixation and staining with Hoechst 33342. For the IVF treatment group, values are presented as mean $\pm$ S.E.M. ($n = 2$ or 3 independent experiments, $N = 39$ –82 eggs at each time point). For the artificially activated treatment group, $n = 1$ experiment and $N = 15$ eggs at each time point following artificial activation.

Effect of increased time between insemination and nuclear transfer

As the time interval between insemination and NT increased from 4.5 to 7 hpi, development to blastocysts decreased from about 50 to below 10% respectively ($N=1062$ embryos, $n=25$ separate IVF times/dates, Fig. 3). The optimal time for achieving both relatively high fertilization and development rates was determined to be between 4 and 5 hpi (Figs 2 and 3). Figure 4A shows an example of a zygote at the same stage as those used at the time of enucleation (4–5 hpi).

In vitro development of IVF-NT versus standard NT embryos

An example of an IVF-NT embryo fixed 24 h post-fusion is shown in Fig. 4B. We first compared *in vitro* development of standard NT (artificially activated after fusion) and IVF-NT embryos (Table 1) and found that cleavage was higher (98 vs 95% respectively, $P=0.05$); but development into blastocysts (B) grade 1–3 (B^{1-3}) (39 vs 40% respectively), or into B grade 1–2 (B^{1-2}) (20 vs 21% respectively) was not different. We also attempted to produce control NT embryos from artificially pre-activated cytoplasts or MII oocytes. The time between the start of artificial activation and donor cell fusion ranged from 56 to 61 min for cytoplasts and from 55 to 193 min for oocytes. Despite their cleavage being similar to that of the parthenogenote controls, none of the pre-activated NT controls developed to blastocysts (Table 2).

In vivo development of IVF-NT versus standard NT embryos

In order to correlate the results from *in vitro* development with *in vivo* developmental potential, we transferred

Figure 3 Development of IVF-NT embryos at different times between insemination and NT. Following different time intervals between insemination and electrical cell fusion, the proportion of the total number of reconstructed embryos placed into IVC that developed into blastocysts grade 1–3 (B^{1-3}) was quantified after 7 days *in vitro* ($n=25$ independent NT experiments, $N=1062$ embryos). Power curve trend line of best fit has been included ($y=58166x^{-4.7179}$, $R^2=0.7673$).

Figure 4 Morphology of IVF-NT embryos before enucleation and after NT. Embryos were fixed and stained with Hoechst 33342 immediately before enucleation (A) or 24 h after NT with serum-starved fibroblast donor cells (B). (A) The telophase II-chromosomes (arrow) and condensed sperm DNA (arrowhead) are visible. (B) A single pseudo-pronucleus (PPN) is shown. Scale bar = 50 μ m.

grades 1 and 2 NT and IVF-NT embryos into recipient cows across four replicate runs (Fig. 5). Pregnancy establishment at D35 was significantly higher for sperm-activated than for chemically-activated NT embryos ($30/49=61$ vs $17/41=42\%$ respectively, $P<0.05$). Development into live calves at term or weaning was higher in the IVF-NT group compared with standard NT (Table 3), however, this difference was no longer statistically significant ($9/49=18$ vs $3/41=7\%$ and $6/49=12$ vs $3/41=7\%$ with $P=0.11$ and $P=0.34$ respectively). The incidence of hydroallantois was not different between IVF-NT versus standard NT fetuses ($6/49=12$ vs $6/41=15\%$ respectively), but was completely absent in the artificially inseminated (AI) group. Of the nine IVF-NT calves born, three died before weaning. The first calf died at birth with a number of cloning-related pathologies (abnormalities in kidneys, thyroid and adrenals, and contracted tendons in all legs), the second died 3 days post-parturition as a result of fractured ribs (this death may have been prevented with caesarian birth), and the third died after 1 week post-parturition from rumenitis and pneumonia; this calf also had a contracted front tendon, a symptom of muscular-skeletal abnormalities common in our somatic bovine clones. Of the four standard NT calves born, one died at birth as a result of birthing difficulties from oversize; this death may have been avoided with a caesarian birth. The remaining six IVF-NT and three standard SCNT calves survived beyond weaning. All eight AI calves survived beyond weaning.

Discussion

We have evaluated the effect of sperm-mediated activation of bovine SCNT embryos on *in vitro* and *in vivo* development. We found that NT into early zygotic cytoplasts at TII resulted in similar *in vitro* development, but increased *in vivo* survival compared with NT into MII cytoplasts followed by chemical activation.

Cytoplasts prepared from non-activated MII and artificially activated TII oocytes are predominantly used for SCNT. Presently, there is no evidence that exposure of

Table 1 *In vitro* development of cloned bovine embryos from NT and IVF-NT.

Group	N	Fusion (%) ^a	nIVC	Development (%) to ≥ two-cell ^b	Development (%) to B ^{1-3b}	Development (%) to B ^{1-2b}	Development (%) to B ^{1-2c}
NT	4	322/350 (92.0)	321	313 (98)*	130 (40)	67 (21)	52
IVF-NT	4	522/571 (91.4)	522	496 (95) [†]	202 (39)	104 (20)	51

N, Total number of independent NT experiments. *†Rows with different superscripts differ significantly ($P < 0.05$).

^aProportion of total number of cytoplasm-donor couplets manipulated that fused. ^bProportion of total number of reconstructed embryos placed into IVC (nIVC) that either cleaved or developed into blastocysts (B) grades 1–3 (B¹⁻³) or into B grades 1–2 (B¹⁻²). ^cProportion of B¹⁻² of all B¹⁻³.

the donor chromatin to either MII or TII cytoplasm is inherently superior for epigenetic reprogramming. Nevertheless, it would have been more stringent to use cytoplasts of the same activation status for our study, for example, by directly comparing (i) artificially activated TII-cytoplasts versus sperm-activated early zygotes or (ii) non-activated MII cytoplasts that were artificially versus sperm-activated after NT. For various reasons, we were unable to carry out either of these comparisons. With regard to the first comparison (TII oocytes versus early zygotes), we did not obtain any blastocysts after NT of serum-starved donor cells into ionomycin/6-DMAP-activated cytoplasts (Table 2). This has been described before and is probably due to the rapid degradation of cyclin B, which destroys activity of cyclin B and Cdk 1 complexes (M-Cdk). Once M-Cdk-activity declines to basal levels, an introduced donor nucleus no longer undergoes nuclear envelope breakdown, presumably preventing efficient chromatin remodeling (Tani *et al.* 2001, 2003). We observed that within 30 min of ionomycin/6-DMAP treatment, all oocytes had lost their metaphase-plate compared with none in the fertilized group (Fig. 2 and data not shown). This is in agreement with a report on the disappearance of the MII plate and cyclin B and the decrease of M-Cdk-activity 1 h after activation with calcium ionophore A23187 and 6-DMAP (Liu & Yang 1999). A similar decrease in M-Cdk-activity has also been described in ionomycin/6-DMAP-activated cytoplasts, where the most significant drop occurred within 1 h post-activation (Tani *et al.* 2003). In contrast to our data, this drop did not immediately interfere with development to blastocyst; however, NT was done with M-phase, not with G₀/G₁-phase donor cells. When the time between activation (by electric stimulation or ionomycin followed by 4–6 h incubation in 6-DMAP or cycloheximide respectively)

and NT increased to 4–6 h, development past the eight-cell stage was almost completely abolished both with M- or G₀/G₁-phase donors (Tani *et al.* 2001, 2003). However, there are cases where artificially pre-activated TII cytoplasts have supported somatic chromatin remodeling after NT in cattle and mice (Bordignon *et al.* 1999, 2001), and even resulted in viable cloned goats (Baguisi *et al.* 1999) and cattle (Bordignon & Smith 1998, Kurosaka *et al.* 2002, Bordignon *et al.* 2003). Some of these studies differ from ours in using different species, different activation stimuli (e.g., electrical current, ionomycin, and ethanol/cycloheximide) and activation times, and oocytes of different age post-maturation at the time of telophase enucleation. Not all those studies accurately quantified M-Cdk-activity, but it is reasonable to assume that it must have been still high enough to disassemble the donor nuclear envelope and allow embryo development to proceed.

The down-regulation of M-Cdk-activity appeared to have happened even more slowly in cytoplasts pre-activated by IVF. First of all, sperm initially need to find the egg, bind to it, and penetrate the zona pellucida, then bind to and fuse with the egg plasma membrane before activation occurs. This delay of at least 2 h is evident from Fig. 2. Interestingly, even after sperm-egg fusion, the kinetics of artificial and sperm-mediated activation was different. The minimal time interval between chemical activation and NT was 56 min and development to blastocyst was nil (Table 2). In some IVF-NT groups, the interval between sperm-egg fusion and NT was longer than 56 min, however, development to blastocyst was still 20/51 = 39%, suggesting that at least some aspects of activation occur more rapidly in artificially activated eggs. This fits with previously reported morphological changes and quantitation of cyclin B abundance and M-Cdk-activity after IVF.

Table 2 *In vitro* development of cloned-bovine embryos from pre-activated cytoplasts or pre-activated oocytes.

Group	N	nIVC	Development (%) to ≥ two-cell ^a	Development (%) to B ^{1-3a}	Development (%) to B ^{1-2a}
Parthenogenote control	1	59	46 (78)	36 (61)	20 (34)
NT into activated cytoplast	1	53	36 (68)	0	0
NT into activated oocyte	2	145	99 (68)	0	0

N, Total number of independent NT experiments; nIVC, total number of embryos cultured to day 7.

^aProportion of total number of reconstructed embryos placed into IVC (nIVC) that either cleaved or developed into blastocysts (B) grades 1–3 (B¹⁻³) or into B grades 1–2 (B¹⁻²).

Figure 5 *In vivo* development of AI, NT, and IVF-NT bovine embryos. Clones were obtained after IVF-NT or control NT. Artificially inseminated cows served as additional controls (AI). Embryo survival was calculated as a proportion of total number of embryos transferred that developed into fetuses and calves ($n=4$ independent experiments, $N=49$, and 41 for IVF-NT and NT respectively). AI embryo survival was calculated as a proportion of the total number of cows inseminated, since the number of cows pregnant with grades 1 and 2 blastocysts on D7 was not determined ($N=1$, $n=12$). Values are mean $\pm$ S.E.M., * $P<0.05$.

IVF-eggs had just commenced second polar body extrusion at 4 hpi, while cyclin B disappeared completely between 3 and 6 h. A significant decrease in M-Cdk-activity started at 3 hpi was most pronounced between 4 and 5 hpi and started to plateau at basal levels after 5 h (Liu & Yang 1999). All three events correlate with our data in Fig. 2. We have not measured M-Cdk-activity in IVF-NT cytoplasts 4–5 hpi, but there must have been still enough M-Cdk-activity to allow development into blastocysts. As with artificially activated TII-cytoplasts, the timing between activation and NT was very critical for sperm-activated eggs. Cloned blastocyst development was inversely proportional to the length of time between fertilization and NT (Fig. 3). At 4.5 hpi, total blastocyst development was just under 50%, whereas at 7 hpi, it dropped to just above 5% (Fig. 3). In mouse cloning, blastocyst development was nil after SCNT into zygotes that had already reached the pronuclei-stage, about 5–6 hpi (Wakayama *et al.* 2000).

With respect to the second possible comparison (MII cytoplasts that were artificially versus sperm-activated after NT), we *in vitro* fertilized non-activated SCNT reconstructs and tried to remove the sperm chromatin

later. However, our attempts to identify and remove specifically the sperm but not the donor cell chromatin at various time points after NT were unsuccessful. We used different methods of marking and subsequently removing the sperm DNA without damaging the donor cell genome, however, they all proved to be impractical (Supplemental Fig. 1). Even in mouse, where the unstained male pronucleus can be more easily visualized in the zygote cytoplasm, its specific removal after NT was often unsuccessful (Kishikawa *et al.* 1999).

Apart from their different activation status, early zygotes differed from MII cytoplasts in several respects. First, sperm activation occurred around 25 h and artificial activation around 28 h post-IVM. In our experience with differently aged MII cytoplasts, a 3 h difference at the time of activation is unlikely to have a measurable impact on subsequent development. Second, different populations of oocytes were used for IVF-NT and standard NT. Oocytes that had extruded the first polar body were used for control NT, whereas only eggs that had extruded a second polar body 4 hpi were used for IVF-NT. By selecting eggs that had already advanced to the stage of second polar body extrusion, we might have slightly biased the *in vitro* development in favor of the IVF-NT group. Despite this, cleavage rate to two-cells was actually slightly, but significantly, lower in the IVF-NT versus control group (Table 1). Third, early zygotes are no longer developmentally arrested and may have been more compromised by the high degree of handling, e.g., the media and temperature changes that occurred during enucleation and NT. Fourth, enucleation of early zygotes was technically slightly more challenging. Aspiration of both sperm and maternal chromatin removed about twice the cytoplasmic volume as aspirating the maternal chromosomes alone, amounting to an average reduction in zygote volume of around 4% (Oback *et al.* 2003). Enucleation also took about twice as long as for MII oocytes (around 21 s), increasing the risk from u.v.-induced damage, either directly through DNA lesions (Tsunoda *et al.* 1988) in mitochondria, or effects on proteins or membrane integrity, or indirectly through activation of a DNA damage excision-repair pathway in the zygote.

Some of these confounding factors may have masked potential benefits of using sperm-mediated activation to improve *in vivo* development of clones. Even though

Table 3 *In vivo* development of cloned-bovine embryos from AI, NT, and IVF-NT.

Group	N	n	Development (%) to day 35 ^a	Development (%) to term ^b	Development (%) to weaning ^b	Age at birth (days $\pm$ S.E.M.)	Birth weight (kg $\pm$ S.E.M.)	Breed
AI	1	12	8 (67)*	8 (67)*	8 (67)*	272 $\pm$ 2 ^{b*}	39 $\pm$ 3 ^{b*}	Hereford $\times$ Friesian
NT	4	41	17 (41) [†]	4 (10) [†]	3 (7) [†]	281 $\pm$ 2 [†]	47 $\pm$ 2 [†]	Friesian
IVF-NT	4	49	30 (61)*	9 (18) [†]	6 (12) [†]	277 $\pm$ 1 [†]	49 $\pm$ 2 ^y	Friesian

N, Total number of independent NT or AI experiments; n, total number of embryo transferred (nET) or artificially inseminated (nAI). *[†]Rows with different superscripts differ significantly ($P<0.05$).

^aProportion of total number of nET or nAI that developed into fetuses and live calves at day 35 of gestation, term or weaning. ^bOnly the four male calves were included in the weight and age analysis.

pregnancy establishment was significantly higher for IVF-NT embryos, the fetal loss profile throughout development was similar in both treatment groups. For example, the incidence of hydroallantois that is typically associated with SCNT cloning did not differ between both treatments. Hydroallantois is a major animal welfare issue and we have chosen the AESF-1 cell line as it reproducibly results in higher frequency of hydrops-cases than all of our other cell lines tested (unpublished results). Any improvement on this particular phenotype should therefore have been easier to detect. Despite the occurrence of hydrops and other cloning-related phenotypes *post-partum*, there was still a clear trend for development into viable calves to be higher in the IVF-NT group due to the higher initial pregnancy rates.

There are several reasons why sperm-mediated activation may be advantageous over artificial activation and even injection of isolated sperm extracts (Knott *et al.* 2002, Hinrichs *et al.* 2006). First, sperm is the natural agent of activation. Mouse spermatozoa contain a sperm-specific member of the phospholipase C (PLC) family, PLC ζ , which induces long-lasting Ca²⁺-oscillations (Parrington *et al.* 2000, Saunders *et al.* 2002, Knott *et al.* 2005) that more closely resemble natural fertilization (Fissore *et al.* 1992, Deguchi *et al.* 2000) than the single, large Ca²⁺-rise triggered by artificial activation. Although a single Ca²⁺-rise is sufficient for the oocytes to undergo early activation events, such as cortical granule exocytosis and the resumption of meiotic division, repeated Ca²⁺-rises may be beneficial for later embryonic development (Ozil 1990). Second, the sperm delivers other factors to the egg that may be beneficial in supporting early embryonic development. These include: (i) the centrosome, an organelle which is particularly important during standard NT experiments since in bovine oocytes all microtubules are concentrated in the meiotic spindle (Schatten 1994) and are largely removed together with other centrosome-associated components during enucleation, (ii) some 3000 different kinds of mRNA (Ostermeier *et al.* 2002) at least some of which are specifically introduced into the egg after fertilization (Ostermeier *et al.* 2004), and (iii) micro-RNAs which do not code for proteins but play a role in controlling gene activity (Ostermeier *et al.* 2005). All these molecules may participate in chromatin remodeling, pronuclear formation, establishment of imprints, or embryonic genome activation (Krawetz 2005). They were likely to have entered the zygote cytoplasm to various degrees, depending on their subcellular localization in the sperm. For example, proteins in the perinuclear theca, i.e., the dense protein matrix between the sperm nucleus and plasma membrane, will take longer to release than those that with cytosolic or sub-plasma membrane localization. Third, there may be early sperm-derived transcripts that arise within the short time between sperm-egg fusion and enucleation. Even though there is no evidence for such early paternal

transcriptional activity, it has become increasingly clear that there is some minor embryonic genome activation occurring shortly after fertilization in cattle (Memili *et al.* 1998). The nature of these early transcripts is not known and it is unclear whether they come from the maternal or paternal genome or both.

During aspiration of the sperm and maternal DNA, we would have to some extent removed these factors again, which may in part explain why sperm-mediated activation did not have a more obvious effect on overcoming cloning-related phenotypes and inefficiencies. In a previous report on somatic mouse cloning, insemination of NT reconstructs did not appear to be more efficient in supporting development into live fetuses than artificial activation with either strontium, ethanol, electric current, or intracytoplasmic sperm injection, however, this may have been due to technical problems of removing the sperm genome and low numbers of fetuses analyzed (Kishikawa *et al.* 1999). Our experimental results in cattle encourage further investigation in the general role of activation during NT cloning and specifically warrant a more detailed analysis into the benefits of sperm-mediated activation. This may ultimately provide a way of improving epigenetic reprogramming and cloning success.

Acknowledgements

We would like to thank our cloning team (A Green, P Misica, F Oback, and J Oliver) and past and present farm staff (J Forsyth, M Berg, and V Prendergast) for excellent technical assistance. This work was funded by the New Zealand Foundation for Research, Science and Technology and AgResearch. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 5 June 2006

First decision 14 July 2006

Revised manuscript received 21 August 2006

Accepted 29 August 2006