CSIRO PUBLISHING

www.publish.csiro.au/journals/rfd *Reproduction, Fertility and Development*, 2008, **20**, 741–749

A unique method to produce transgenic embryos in ovine, porcine, feline, bovine and equine species

F. Pereyra-Bonnet[A], *R. Fernández-Martín*[A], *R. Olivera*[A], *J. Jarazo*[A], *G. Vichera*[A], *A. Gibbons*[B] *and D. Salamone*[A,C]

[A]Laboratorio de Biotecnología Animal, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417 Buenos Aires, Argentina.

[B]Laboratorio de Reproducción de Rumiantes Menores, Instituto Nacional de Tecnología Agropecuaria, Box 277, EEA Bariloche, Argentina.

[C]Corresponding author. Email: salamone@agro.uba.ar

Abstract. Transgenesis is an essential tool in many biotechnological applications. Intracytoplasmic sperm injection (ICSI)-mediated gene transfer is a powerful technique to obtain transgenic pups; however, most domestic animal embryos do not develop properly after ICSI. An additional step in the protocol, namely assistance by haploid chemical activation, permits the use of ICSI-mediated gene transfer to generate transgenic preimplantation embryos in a wide range of domestic species, including ovine, porcine, feline, equine and bovine. In the present study, spermatozoa from five species were coincubated with pCX-EGFP plasmid and injected into metaphase II oocytes. The chemical activation protocol consisted of ionomycin plus 6-dimethylaminopurine. We detected high proportions of fluorescent EGFP embryos for all five species (23–60%), but with a high frequency of mosaic expression (range 60–85%). To our knowledge, this is the first study to produce exogenous DNA expression in feline and equine embryos. Chemical activation reduces the lag phase of *egfp* expression in ovine embryos. Our results show that this unique method could be used to obtain ovine, porcine, feline, bovine and equine transgenic preimplantation embryos.

Additional keywords: chemical activation, gene expression, sperm mediated, transgenic mammals.

Introduction

Transgenesis is an essential tool for the identification of gene functions and, currently, has a predominant role in many biotechnological applications. Transgenesis has been used in ovine (Schnieke *et al.* 1997), caprine (Baguisi *et al.* 1999) and bovine (Salamone *et al.* 2006) species to generate bioreactors that express recombinant proteins in milk, as well as in pigs to adapt organs for human xenotransplantation (Niemann and Kues 2003; Smolenski *et al.* 2007). One area currently in development is genetic modification to increase the agricultural production of animals that are resistant to mastitis (Wall *et al.* 2005) or bovine spongiform encephalopathy (Richt *et al.* 2007), or to produce animals that exhibit more efficient nutrient assimilation (Golovan *et al.* 2001). Microinjection of foreign DNA into the male pronuclei of zygotes (Gordon *et al.* 1980) and nuclear transfer using genetically modified somatic donor cells (Cibelli *et al.* 1998) are two methods that are commonly used in the generation of transgenic animals. However, these techniques have proved to be quite inefficient (Hammer *et al.* 1985; Eyestone 1999; Rideout *et al.* 2001).

Three decades ago, Brackett *et al*. (1971) first reported evidence of heterologous DNA introduced into a mammalian spermatozoon and subsequent delivery into an oocyte during fertilisation. Several reports have shown binding of exogenous DNA constructs on sperm cell membranes in a variety of mammalian species (Castro *et al.* 1990; Francolini *et al.* 1993; Zani *et al.* 1995; Ball *et al.* 2006; Hoelker *et al.* 2007). However, contradictory results have been reported with regard to the successful production of transgenic offspring using the sperm-mediated gene transfer method (Brinster *et al.* 1989; Lavitrano *et al.* 1989, 2002; Gandolfi *et al.* 1996; Shemesh *et al.* 2000). In 1999, Perry *et al.* (1999) complemented the transformation of spermatozoa with intracytoplasmic sperm injection (ICSI), developing a new technique called 'ICSI-mediated gene transfer', giving rise to 20% transgenic pups. However, ICSI-mediated gene transfer has had limited success in domestic animals (Robl *et al.* 2007); mosaic gene expression has been observed in mice among embryonic blastomeres (Perry *et al.* 1999) and offspring (Moreira *et al.* 2007).

The ICSI technique was developed for use in humans and mice (Palermo *et al.* 1992; Kimura and Yanagimachi 1995). In these species, injection of spermatozoa induces oocyte activation (Nakano *et al.* 1997). However, after ICSI, most domestic animals do not develop properly (McEvoy *et al.* 2003; Malcuit *et al.* 2006), and porcine and bovine embryos are reported to require electrical or chemical activation (Rho *et al.* 1998; Kolbe and Holtz 1999; Li *et al.* 1999; Chung *et al.* 2000; Nakai *et al.* 2003). The most common chemical activation protocols in ICSI

 10.1071/RD07172 1031-3613/08/070741

use ionomycin, a calcium ionophore, followed by 3 h incubation prior to treatment with 6-dimethylaminopurine (DMAP; Rho *et al.* 1998; Li *et al.* 1999; Chung *et al.* 2000).

The main aim of the present study was to use ICSI-mediated gene transfer to generate transgenic preimplantation embryos in ovine, porcine, feline, bovine and equine species. The present study reports on a unique method used for all five species. The transgenesis marker was the *egfp* gene, expressed under control of a chimeric cytomegalovirus immediate-early-chicken β-actin enhancer–promoter (Ikawa *et al.* 1995). For each species, the percentage of transgenic embryos, the incidence of mosaic expression and the timing of exogenous gene expression were determined by observation of enhanced green fluorescent protein (EGFP) fluorescence.

Materials and methods

Reagents

Unless indicated otherwise, all chemicals were obtained from Sigma Chemical (St Louis, MO, USA).

Experimental design

In the first experiment, we compared embryo development and *egfp* expression after ICSI-mediated gene transfer with or without chemical activation in prepubertal sheep (four replicates). In the second experiment, we tested the most favourable ICSI-mediated gene transfer protocol (with chemical activation) in porcine, feline, bovine and equine species (three replicates for each).

Oocyte collection and in vitro maturation

Ovaries were collected from prepubertal ewes, gilts, cows and mares at abattoirs. Gonads from female domestic cats were recovered from local veterinary clinics. All samples were transported to the laboratory in 0.9% NaCl solution at 25–30°C. Cumulus–oocyte complexes were aspirated from follicles with a diameter of 2–5 mm (ovine), 2–6 mm (porcine and bovine) or 2–8 mm (equine) in Dulbecco's phosphate-buffered saline (DPBS; 14287–072; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; 10499–044; Gibco BRL) and 2% antibiotic–antimycotic (antibiotic–antimycotic (ATB); 15240–096; Gibco BRL). In domestic cats, oocytes were released by repeatedly puncturing the ovaries with 21-gauge needles in the same medium. Oocytes covered with at least three layers of granulosa cells were selected for *in vitro* maturation (IVM) in all species. The maturation medium was bicarbonate-buffered TCM-199 (31100–035; Gibco BRL), containing 2 mM glutamine (G-8540), 10% FBS, 10 μg mL^{-1} follicle-stimulating hormone (NIH-FSH-P1; Folltropin; Bioniche, Belleville, Ontario, Canada), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% ATB. Oocytes were incubated in 500 μL medium in four-well dishes (Nunclon; Nunc, Naperville, IL, USA) covered with mineral oil (M8410). The IVM conditions were 6.5% CO_2 in humidified air at 39°C for 24 h for ovine, feline and bovine oocytes, for 36 h for equine oocytes and for 48 h for porcine oocytes. After maturation, cumulus cells were removed from all oocytes by vortexing for 2 min in hyaluronidase (H-4272; 1 mg mL^{-1} DPBS) and the oocytes were washed three times in HEPES-buffered (H4034) TCM-199. Mature oocytes were evaluated by visualising the first polar body and were used immediately for ICSI.

Semen collection, freezing and thawing

Semen was collected using an artificial vagina in ovine and bovine species. In pigs, ejaculates were obtained using the gloved-hand method, whereas in feline and equine speices, sperm was obtained from the epididymides. Samples were frozen using standard procedures: in ovine and bovine with Tris-based extender and in porcine, feline and equine with AndroMed extender (Minitub, Landshut, Germany). In all cases, frozen semen was thawed in a 37°C water bath for 30 s.

DNA construction and sperm–DNA incubation

The plasmid used was pCX-EGFP (kindly provided by Dr Masaru Okabe, Osaka University, Japan), which contains an *egfp* gene under the control of the chimeric cytomegalovirus immediate-early-chicken β-actin enhancer–promoter (Ikawa *et al.* 1995).

Sperm for DNA uptake

Coincubation sperm–DNA construction was performed according to the method of Perry *et al*. (1999) with slight modification. Briefly, spermatozoa were washed twice in 2.8% Na citrate (F71497) with 100 μM EDTA by centrifugation at 495*g* for 5 min. The sperm pellet was resuspended in 2.8% Na citrate with 100 μM EDTA, the concentration was adjusted to 20×10^6 spermatozoa mL^{-1} and a 10-μL aliquot was incubated with 0.5 μg closed circular covalent plasmid per million spermatozoa (final concentration) for 5 min at 0°C. Then, spermatozoa were used immediately for ICSI.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed in 20-μL microdroplets of Tyrode's albumin lactate pyruvate buffered with HEPES (TALP-H) under mineral oil (S66138; Fisher Scientific, Pittsburgh, PA, USA) in 100 × 20-mm tissue culture dishes (430167; Corning, NY, USA) using Narishige hydraulic micromanipulators (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA). The injection pipettes had an inner diameter of 8 μM for ovine, porcine and bovine species, and 7 μM for feline and equine species. Each injected spermatozoon was selected for its normal morphology in a 4-μL droplet of 10% polyvinylpyrrolidone (PVP; 99219; Fisher Scientific). The spermatozoon selected was immobilised by breaking its tail and then aspirated tail-first into the injection pipette. Immediately, the injection pipette was washed by passing it through a microdroplet of TALP-H to remove any spermatozoa attached and then transferred to the oocyte-containing drop. Metaphase (M) II oocytes were held under negative pressure in the holding pipette, with the polar body at the 6 or 12 o'clock position. The microinjection pipette was pushed through the zona pellucida and into the cytoplasm of the oocyte at the 3 o'clock position. Aspiration was used to break the oolemma. The spermatozoon and the aspirated ooplasm were then expelled into the oocyte with a minimal volume of PVP. In Experiment 1, we included

Sham and ICSI control groups. The Sham group was preformed as a control for mechanical activation. As for the ICSI groups, the oolema was ruptured and the ooplasm was aspirated into the injection pipette and re-injected into the oocyte with a minimum volume of spermatozoa-free medium. In the ICSI control group, frozen–thawed spermatozoa without either plasmid coincubation or chemical activation were used. The parthenogenic DNA control consisted of the injection of an equivalent volume (as used in ICSI) of 10% PVP microdroplet with 10 ng mL^{-1} pCX-EGFP into the oocyte, without a spermatozoon, followed by chemical activation.

Chemical activation

Injected oocytes were activated immediately in TALP-H with 5 μM ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) for 4 min and placed in TCM-199 for 3 h to permit second polar body extrusion. Oocytes were subsequently transferred to a drop of TCM-199 with 1.9 mM DMAP (D2629) for 3 h. The inhibitor was then removed by washing oocytes three times in TALP-H and cultures were continued as described below.

In vitro *culture*

Presumptive zygotes were returned to the original maturation medium after replacing 50% of the medium with fresh medium and were cocultured with cumulus cells. Cleavage was evaluated at Day 2 and the number of morulae and blastocysts was determined on Days 5–8 after ICSI.

Determination of EGFP fluorescence in embryos

During the period of *in vitro* culture, embryos were briefly exposed to blue light using an excitation filter at 488 nm and an emission filter at 530 nm to determine expression of the *egfp* gene at different stages of development. Embryos were analysed on Days 1, 2, 3, 4, 5 and 8 after ICSI.

Determination of embryo cell number

Embryos were stained in TCM-199 containing 1 mg mL^{-1} Hoechst 33342 (B2261) for 2 min and mounted immediately between coverslips to count total nuclei under an epifluorescence microscope.

Explants from embryos

Bovine fluorescent blastocysts were cut into four by mechanical microsurgery and each part was cultured in a feeder layer of granulose cells in 20-μL droplets of minimal essential medium (α-MEM; MTC010D; MTCELL, Buenos Aires, Argentina), containing 0.2% insulin–transferrin–selenium (41400; Gibco BRL), 5% FBS, 1000 U leukaemia inhibitory factor (LIF; L5283) and 2% ATB, under mineral oil in 5% CO_2 at 39°C. Growth and *egfp* expression in explants were evaluated daily.

Fluorescence in situ *hybridisation*

Porcine two-cell embryos were incubated for 20 h with 0.1 μg mL^{-1} Demecolcine (D1925). Then, embryos were prepared on a poly L-lysine-coated slide by treating them with a hypotonic solution (1% Na citrate in distilled water for 10 min) and fixing them *in situ* with 3 : 1 methanol : acetic acid. Signals of fluorescein isothiocyanate (FITC) anti-mouse (F6257) and anti-digoxigenin (D8156), which bind the digoxigenin-labelled pCX-EGFP (5.5 kb) probe, were labelled using the Nick Translation System (18160–010; Invitrogen). Total DNA was counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Images of each cell and its signals were captured using an Optronics (Goleta, CA, USA) camera.

Statistical analysis

In vitro embryo development, fluorescent expression and differences between replicates were compared by Fisher's exact test. In ovine, differences in mosaicism between treatments were analysed using the Chi-squared test. For all statistical analyses, the SAS program was used (SAS Institute 1989). Differences were considered significant at $P < 0.05$.

Results

Experiment 1: embryo development and EGFP expression after ICSI-mediated gene transfer with or without chemical activation in prepubertal ovine

The effects of chemical activation on ICSI-mediated gene transfer were determined in the ovine. Table 1 shows embryo development and fluorescence of injected prepubertal oocytes. Blastocysts were only obtained by ICSI plus chemical activation. Exogenous DNA–sperm incubation did not appear to affect the rate of embryo developmental. Sham control embryos did not achieve the morulae stage and their cleavage was significantly lower (14.6%) than the ICSI control. Fluorescent embryos were detected only when spermatozoa were coincubated with the plasmid. The ICSI control did not achieve the blastocyst stage and the proportion of morulae did not differ significantly from that seen for ICSI with plasmid coincubation.

The percentage of mosaic expression at Day 4 did not differ significantly between groups with and without chemical activation (69.8% and 64.6%, respectively; $P < 0.05$, Chi-squared test). Detection of *egfp* expression in ovine embryos assisted by chemical activation began at either the two-cell stage (7/39), four-cell stage (9/39) or eight-cell stage (23/39); conversely, *egfp* expression in ovine without chemical activation began only at the eight-cell stage (24/24) and never earlier.

Fluorescent and non-fluorescent 8–16-cell embryos ($n = 20$) produced by ICSI-mediated gene transfer assisted by chemical activation were selected and stained with Hoechst 33342 to count the number of nuclei. No fragmented embryos were observed.

Experiment 2: ICSI-mediated gene transfer assisted by chemical activation in porcine, feline, equine and bovine species

In the remaining four domestic species, ICSI-mediated gene transfer assisted by chemical activation was investigated. In total, 176 porcine, feline, bovine and equine oocytes were injected with spermatozoa incubated with pCX-EGFP plasmid. Table 2 shows embryo development and *egfp* expression for each species.

In total, 35 porcine and 86 bovine oocytes were injected with pCX-EGFP plasmid, followed by chemical activation

Table 1. *In vitro* development and *egfp* expression in prepubertal ovine embryos produced by intracytoplasmic sperm injection-mediated gene transfer with or without chemical activation

Values with different superscripts in a column are signficantly different ($P < 0.05$, Fisher's test). Within each group, no signficant differences were observed between replicates ($P > 0.05$, Fisher's test). Io, Ionomycin; §, transgenic; ICSI, intracytoplasmic sperm injection; DMAP, 6-dimethylaminopurine

Treatment	pCX-EGFP	No. oocytes	Cleavage (%)	Morulae (%)	Blastocysts (%)	Embryos§ (4–16 cell) (%)	Morulae§ (%)	Blastocysts§ (%)
ICSI	+	88	47 (53.4)[a]	26 (29.5)[a]	0 (0)[a]	24 (27.3)[a]	24 (27.3)[a]	0 (0)[a]
ICSI + Io + DMAP	+	86	73 (84.9)[b]	24 (27.9)[a]	3 (3.5)[a]	39 (45.3)[b]	17 (19.8)[a]	2 (2.3)[a]
ICSI control	–	63	33 (52.4)[a]	24 (38.0)[a]	0 (0)[a]	0 (0)[c]	0 (0)[b]	0 (0)[a]
Sham	–	41	6 (14.6)[c]	0 (0)[b]	0 (0)[a]	0 (0)[c]	0 (0)[b]	0 (0)[a]

Table 2. *In vitro* development and *egfp* expression in embryos produced by intracytoplasmic sperm injection-mediated gene transfer assisted by chemical activation

Values with different superscripts in a column are signficantly different ($P < 0.05$, Fisher's test). Within each group, no signficant differences were observed between replicates ($P > 0.05$, Fisher's test). §, transgenic

Species	No. oocytes	Cleavage (%)	Morulae (%)	Blastocysts (%)	Embryos§ (2–16 cell) (%)	Morulae§ (%)	Blastocysts§ (%)
Porcine	55	46 (83.6)[a]	7 (12.7)[a]	0 (0)[a]	33 (60.0)[a]	3 (5.5)[a]	0 (0)[a]
Bovine	44	25 (56.8)[b]	7 (15.9)[a]	5 (11.4)[b]	10 (22.7)[b]	5 (11.4)[a,b]	4 (9.1)[a]
Feline	35	16 (45.7)[b]	11 (31.4)[b]	1 (2.8)[a,b]	9 (25.7)[b]	7 (20.0)[b]	1 (2.8)[a]
Equine	42	28 (66.6)[a,b]	4 (9.5)[a]	0 (0)[a]	11 (22.1)[b]	3 (7.1)[a,b]	0 (0)[a]

(parthenogenic DNA control). Cleavage rates were 80.0% in the porcine and 61.2% in the bovine. Porcine embryos did not achieve the morulae stage; of bovine embryos, 8.1% (7/86) reached the morulae stage and 2.3% (2/86) reached the blastocyst stage. However, no green fluorescents embryos were observed in either species.

The percentage of embryos with mosaic expression at Day 4 was 85.2%, 60.4%, 78.7% and 72.7% for porcine, bovine, feline and equine species, respectively. The dynamics of *egfp* expression in embryos were evaluated by daily microscopic observation of fluorescence. The expression of *egfp* in porcine and feline embryos started at the two-cell stage (36.4% and 22.4%, respectively), whereas in bovine and equine embryos it started at the four-cell stage (9.6% and 54.5%, respectively).

Day 8 green expanded bovine blastocysts ($n = 3$) were stained with Hoechst 33342 and mounted immediately between coverslips to count total nuclei. The number of nuclei in each specimen was 96, 83 and 57 (mean (± s.d.) 78.7 ± 19.8 nuclei).

Different fluorescent blastocysts in ovine, feline and bovine species are shown in Fig. 1. In Fig. 2, homogenic porcine expression and ovine and equine embryos with different proportions of mosaicism are shown.

Green bovine blastocysts ($n = 2$) were cut into four and cocultured with granulose cells. After 2 days of culture, two explants from one of the two treated blastocysts showed intense adhesion and proliferation (an approximate fourfold increase) and expressed fluorescent EGFP (Fig. 3). Once the explants stopped proliferating, *egfp* expression disappeared and the adhesion of cells was lost. Conversely, preliminary fluorescence *in situ* hybridisation (FISH) analysis put forward to two integration loci in one porcine two-cell stage embryo (Fig. 4).

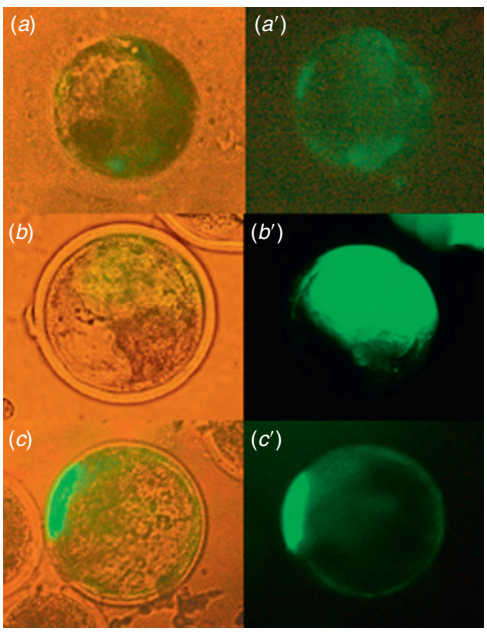

Fig. 1. Fluorescent embryos were produced by injecting spermatozoa that had been coincubated with pCX-EGFP plasmid. (*a*) Feline, (*b*) ovine and (*c*) bovine fluorescent blastocysts are shown under a combination of bright and blue (488 nm) light. (*a′*–*c′*) The same blastocysts shown under blue light only. (Original magnification ×200.)

Discussion

Herein, we describe a unique method by which transgenic preimplantation embryos in five different species can be obtained.

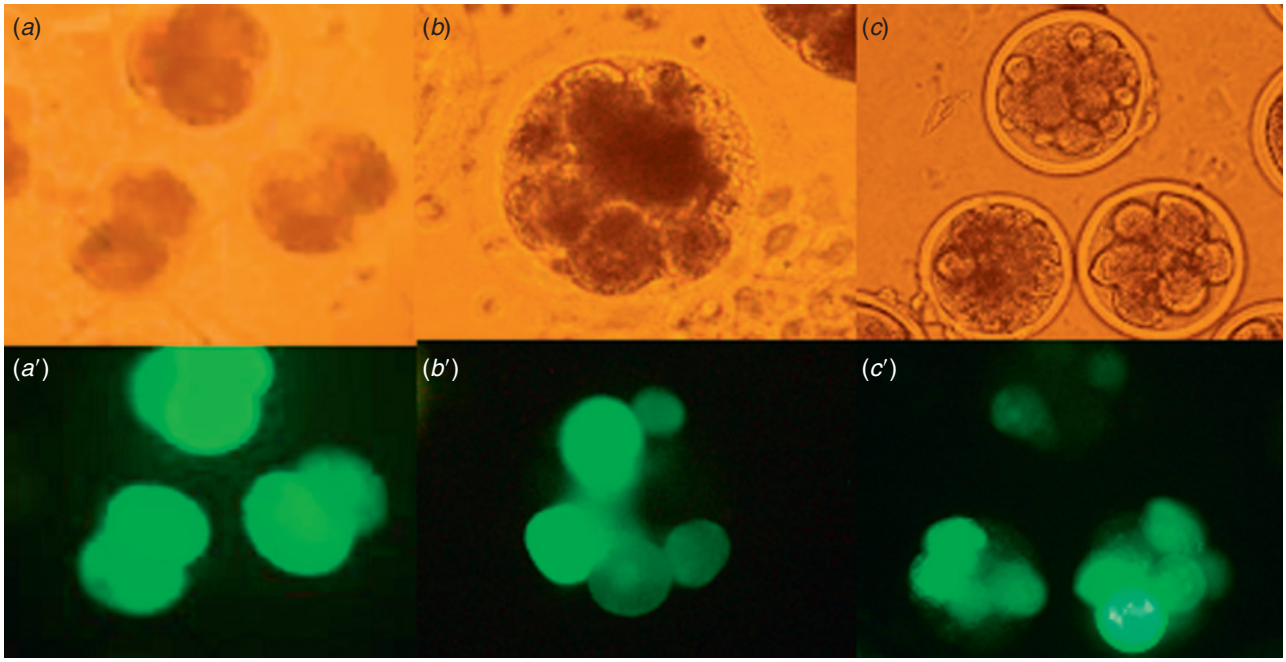

Fig. 2. Fluorescent embryos were produced by injecting spermatozoa that had been coincubated with pCX-EGFP plasmid. (*a*) Homogeneous *egfp* expression in porcine embryos and mosaic *egfp* expression in (*b*) equine and (*c*) ovine embryos. (*a*–*c*) Embryos are shown under bright light; (*a*′–*c*′) the same embryos are shown under blue light. (Original magnification ×200.)

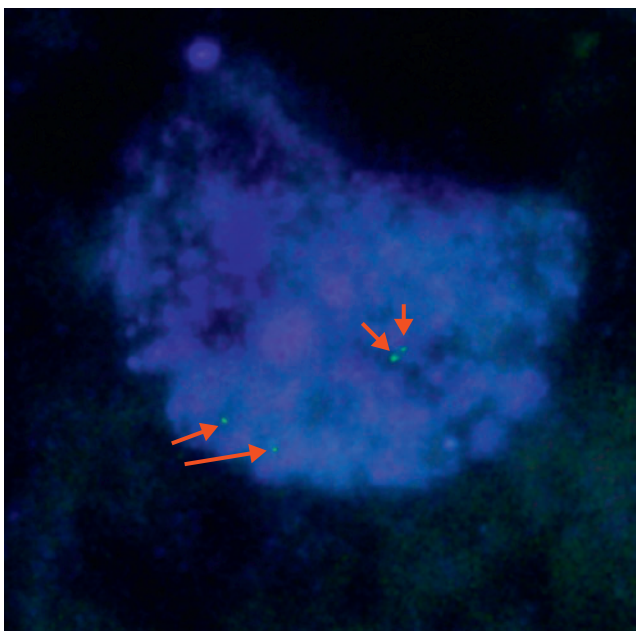

Fig. 3. Preliminary fluorescence *in situ* hybridization analysis in porcine interphase two-cell embryo with pCX-EGFP plasmid as the probe. The red arrows indicate four signals, coupled by pairs, compatible with at least two integration loci in which each signal could be associated with each sister chromatid.

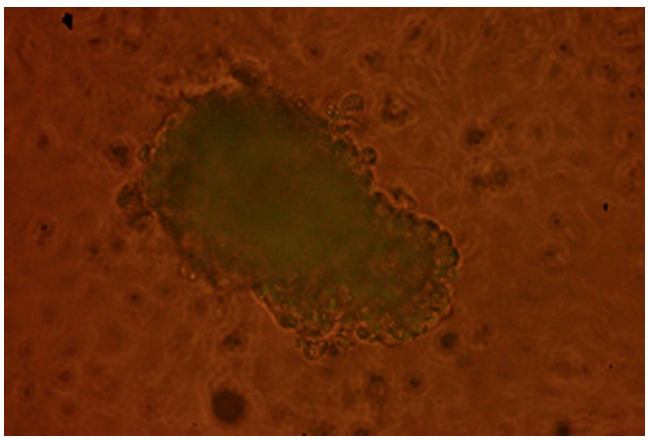

Fig. 4. Fluorescent 16-day explant generated from a 7-day intracytoplasmic sperm injection-mediated gene transfer bovine blastocyst under a combination of bright and blue (488 nm) light. (Original magnification ×100.) The explant proliferated and maintained fluorescence up to Day 20.

Even in species in which chemical activation is not necessary for embryo development after ICSI, such as the ovine, chemical activation does not negatively affect embryo development. In contrast, a slight improvement seems to be achieved in prepubertal ovine embryo development at the blastocyst stage (Table 1).

Our results for ICSI-mediated gene transfer demonstrated that the pCX-EGFP plasmid bound to spermatozoa from any source (ejaculated or from the epididymides) in a simple saline medium (citrate Na 2.8%) and without any selection of sperm cell donor. In contrast, the preparation of transgenic sperm cells for use in the sperm-mediated gene transfer technique requires specific media, carefully conditioned for the manipulation and

selection of sperm cell donors (Gandolfi 1998; Shemesh *et al.* 2000; Lavitrano *et al.* 2003; Li *et al.* 2006*a*). Results of the present study prove that the *egfp* gene from the pCX-EGFP plasmid is expressed in ovine, porcine, feline, bovine and equine preimplantation embryos, which disagrees with previous reports for the last two species (Alderson *et al.* 2006; Ball *et al.* 2006).

We detected a high proportion of fluorescent *egfp* gene expression in all five species (23–60%). To our knowledge, this is the first study to produce exogenous DNA expression in feline and equine embryos. In addition, this is the first report of successful *egfp*-expressing bovine embryo production by ICSI-mediated gene transfer, achieving 10% transgenic blastocysts. Furthermore, despite using prepubertal oocytes, we obtained a significantly higher proportion of *egfp*-expressing ovine embryos than the only other study published (Gou *et al.* 2002). Moreover, the highest proportion of *egfp*-expressing embryos was found in porcine, with the degree similar to that reported previously (Lai *et al.* 2001; Naruse *et al.* 2005). Embryo developmental results could be improved by using specific conditions for IVM/*in vitro* culture or DNA concentration for each species. Nevertheless, in conventional ICSI, without transgenesis, species-specific studies have shown that the blastocyst rate remains low (Gómez *et al.* 1998; Pope *et al.* 2006; Galli *et al.* 2007), especially when prepubertal oocytes are used (Jiménez-Macedo *et al.* 2005). The proportion of blastocysts after ICSI was only improved significantly by use of piezoelectric actuator in the bovine (Oikawa *et al*. 2005) or electric activation in porcine species (Tian *et al.* 2006).

In the present study, we observed a high frequency of mosaic expression of *egfp* in the five species (range 60–85%). A possible explanation for this is that the transgene (*tg*) is integrated into the embryo's genome after the first cell division (Perry *et al.* 1999; Szczygiel *et al.* 2003; Kaneko *et al.* 2005; Smith and Spadafora 2005). Another possibility is that the *tg* could remain extrachromosomal and be lost during successive mitotic divisions, as has been shown in two previous reports (Celebi *et al*. 2002; Li *et al.* 2006*b*). However, the presence of EGFP fluorescence at the blastocyst stage has been suggested as evidence of stable *tg* integration (Perry *et al.* 1999), especially when the plasmid is not able to replicate autonomously. Only the addition of a human scaffold/matrix attachment region (S/MAR) linked to the plasmid (Manzini *et al.* 2006), or the presence of large SV40 virus T antigen in embryos (DeLucia *et al.* 1986), would allow episomal replication of pCX-EGFP.

A simple polymerase chain reaction amplification technique or reverse transcription–polymerase chain reaction could detect the presence of the *tg* in embryos (Rieth *et al.* 2000; Alderson *et al*. 2006; Hoelker *et al.* 2007), but these methods do not distinguish between integration or episomal presence of the *tg*. It is not possible to perform Southern blot analysis in the embryos because there is not enough DNA (Szczygiel *et al.* 2003). An alternative technique, namely FISH, could be used in embryos to differentiate *tg* status, but the length of the pCX-EGFP plasmid (5.5 kb) makes the hybridisation signal detection difficult. In preliminary FISH analysis in porcine embryos, using pCX-EGFP plasmid as a probe, we found four possible signals at the two-cell stage that are compatible with at least two integration loci. This technique requires improvements in order for conclusive data to be obtained. However, we have generated explants from EGFP-expressing blastocysts and obtained a monolayer of cells and, furthermore, these cells expressed the green fluorescent protein. The explants proliferated and maintained the fluorescence up to Day 20, suggesting that the plasmid was effectively integrated in the embryos from which the explants were produced.

Our chemical activation procedure can produce haploid parthenogenic embryos in all the species studied. Therefore, non-green fluorescent morulae and blastocysts obtained in the present study could actually be parthenogenetic embryos. Bovine and porcine parthenogenic DNA controls indicate that green embryos are derived from fertilisation. None of the parthenogenic DNA control embryos exhibited transgene expression, in accordance with work in other laboratories (Perry *et al.* 1999; Yamauchi *et al.* 2007). These authors showed that injection of exogenous DNA into mice ooplasm, followed by either chemical activation or sperm injection, is not able to generate transgenic embryos. *egfp*-expressing embryos were produced only when sperm and exogenous DNA were coincubated before injection (Perry 2000). These conclusive data show that the risk of obtaining fluorescent parthenogenic embryos with our technique is very low.

Daily observation of embryo development under a fluorescent microscope permitted early detection of *egfp* expression. In the present study, *egfp* expression in the ovine was detected earlier when chemical activation was used (two- *v.* eight-cell stage). We propose that chemical activation reduces the lag phase of embryo *egfp* expression. Chemical activation enhances the speed of both pronuclear formation and cell cycle progression in sheep and bovine embryos (Susko-Parrish *et al.* 1994; Loi *et al.* 1998). Earlier pronuclear formation and enhanced cell cycle progression are two possible factors involved in the reduced lag phase of *egfp* expression in ovine preimplantation embryos. This effect was also suggested by the earlier expression of *egfp* in chemically activated porcine, bovine, feline and equine embryos in the present study. In addition, earlier expression of endogenous and exogenous genes was observed for parthenogenetic and cloned embryos assisted by chemical activation (Wrenzycki *et al.* 2006; Kasamatsu *et al*. 2007). The timing of *egfp* expression in non-activated embryos was not possible to determine because preliminary experiments indicated developmental arrest before the morula stage after ICSI without chemical activation in porcine, bovine and feline embryos (data not shown). In the future, the expression of a large battery of endogenous genes should be studied to determinate whether chemical activation reduces the lag phase for embryo gene expression in these species.

In summary, our results show that ICSI-mediated gene transfer assisted by chemical activation can be used to obtain exogenous gene expression in ovine, porcine, feline, bovine and equine preimplantation embryos, which has potential scientific and commercial interest. In addition, we found that chemical activation accelerated transgene expression in ovine prepubertal embryos. However, the incidence of mosaic transgenic expression should be minimised to optimise the production of transgenic animals.

Acknowledgements

The authors are grateful to Marcela Cueto for critical suggestions and revision of the manuscript. The authors thank Mr. Fernando and Alejandro Gonzales (El Ombu), Dr M. Lloveras (INTA Pergamino), CIALE and Dr San Martín (La Pompeya) for providing the biological material and Dr N. Judewicz (BIOSIDUS) for technical assistance. The authors extend their special thanks in memory of María Muhlmann for her generous assistance with FISH analysis. Part of this work was financed by the Instituto Nacional de Tecnología Agropecuaria (AEGR 3424).

References

Alderson, J., Wilson, B., Laible, G., Pfeffer, P., and L'Huillier, P. (2006). Protamine sulfate protects exogenous DNA against nuclease degradation but is unable to improve the efficiency of bovine sperm mediated transgenesis. *Anim. Reprod. Sci.* **91**, 23–30. doi:10.1016/J.ANIREPROSCI.2005.03.005

Baguisi, A., Behboodi, E., Melican, D., Pollock, J., Destrempes, M., *et al*. (1999). Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.* **17**, 456–461. doi:10.1038/8632

Ball, B. A., Sabeur, K., and Allen, W. R. (2006). Uptake of exogenous DNA by equine spermatozoa and applications in sperm-mediated gene transfer. *Anim. Reprod. Sci.* **94**, 115–116. doi:10.1016/J.ANIREPROSCI.2006.03.079

Brackett, B. G., Baranska, W., Sawicki, W., and Koprowski, H. (1971). Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. *Proc. Natl Acad. Sci. USA* **68**, 353–357. doi:10.1073/PNAS.68.2.353

Brinster, R. L., Sandgren, E. P., Behringer, R. R., and Palmiter, R. D. (1989). No simple solution for making transgenic mice. *Cell* **59**, 239–241. doi:10.1016/0092-8674(89)90282-1

Castro, F., Hernández, O., Uliver, C., Solano, R., Milanes, C., Aguilar, A., Perez, R., De Armas, R., Herrera, N., and Fuente, J. (1990). Introduction of foreign DNA into the spermatozoa of farm animals. *Theriogenology* **34**, 1099–1110. doi:10.1016/S0093-691X(05)80009-3

Celebi, C., Auvray, P., Benvegnu, T., Plusquellec, D., Jegou, B., and Guillaudeux, T. (2002). Transient transmission of a transgene in mouse offspring following *in vivo* transfection of male germ cells. *Mol. Reprod. Dev.* **62**, 477–482. doi:10.1002/MRD.10143

Chung, J. T., Keefer, C. L., and Downey, B. R. (2000). Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). *Theriogenology* **53**, 1273–1284. doi:10.1016/S0093-691X(00)00271-5

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, F. A., and Robl, J. M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–1258. doi:10.1126/SCIENCE.280.5367.1256

DeLucia, A. L., Deb, S., Partin, K., and Tegtmeyer, P. (1986). Functional interactions of the simian virus 40 core origin of replication with flanking regulatory sequences. *J. Virol.* **57**, 138–144.

Eyestone, W. H. (1999). Production and breeding of transgenic cattle using *in vitro* embryo production technology. *Theriogenology* **51**, 509–517. doi:10.1016/S0093-691X(98)00244-1

Francolini, M., Lavitrano, M., Lamia, C. L., French, D., Frati, L., Cotelli, F., and Spadafora, C. (1993). Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. *Mol. Reprod. Dev.* **34**, 133–139. doi:10.1002/MRD.1080340204

Galli, C., Colleoni, S., Duchi, R., Lagutina, I., and Lazzari, G. (2007). Developmental competence of equine oocytes and embryos obtained by *in vitro* procedures ranging from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim. Reprod. Sci.* **98**, 39–55. doi:10.1016/J.ANIREPROSCI.2006.10.011

Gandolfi, F. (1998). Spermatozoa, DNA binding and transgenic animals. *Transgenic Res.* **7**, 147–155. doi:10.1023/A:1008828627735

Gandolfi, F., Terqui, M., Modina, S., Brevini, T. A., Ajmone-Marsan, P., Foulon-Gauze, F., and Courot, M. (1996). Failure to produce transgenic offspring by intra-tubal insemination of gilts with DNA-treated sperm. *Reprod. Fertil. Dev.* **8**, 1055–1060. doi:10.1071/RD9961055

Golovan, S. P., Meidinger, R. G., Ajakaiye, A., Cottrill, M., Wiederkehr, M. Z., *et al*. (2001). Pigs expressing salivary phytase produce low-phosphorus manure. *Nat. Biotechnol.* **19**, 741–745. doi:10.1038/90788

Gómez, M. C., Catt, J. W., Evans, G., and Maxwell, W. M. C. (1998). Cleavage, development and competence of sheep embryos fertilized by intracytoplasmic sperm injection and *in vitro* fertilization. *Theriogenology* **49**, 1143–1154. doi:10.1016/S0093-691X(98)00062-4

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., and Ruddle, F. H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl Acad. Sci. USA* **77**, 7380–7384. doi:10.1073/PNAS.77.12.7380

Gou, K. M., An, X. R., Tian, J. H., and Chen, Y. F. (2002). Sheep transgenic embryos produced by intracytoplasmic sperm injection. *Shi Yan Sheng Wu Xue Bao* **35**, 103–108. [In Chinese with English Abstract]

Hammer, R. E., Pursel, V. G., Rexroad, C. E., Jr, Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D., and Brinster, R. L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**, 20–26.

Hoelker, M., Mekchay, S., Schneider, H., Bracket, B. G., Tesfaye, D., *et al*. (2007). Quantification of DNA binding, uptake, transmission and expression in bovine sperm mediated gene transfer by RT–PCR: effect of transfection reagent and DNA architecture. *Theriogenology* **67**, 1097–1107. doi:10.1016/J.THERIOGENOLOGY.2006.12.011

Ikawa, M., Kominamik, K., Yoshimura, Y., Tanaka, K., Nishimune, Y., and Okabe, M. (1995). A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). *FEBS Lett.* **375**, 125–128. doi:10.1016/0014-5793(95)01162-8

Jiménez-Macedo, A. R., Izquierdo, D., Anguita, B., and Paramio, M. T. (2005). Comparison between intracytoplasmic sperm injection and *in vitro* fertilisation employing oocytes derived from prepubertal goats. *Theriogenology* **64**, 1249–1262. doi:10.1016/J.THERIOGENOLOGY.2004.11.025

Kaneko, T., Moisyadi, S., Suganuma, R., Hohn, B., Yanagimachi, R., and Pelczar, P. (2005). Recombinase-mediated mouse transgenesis by intracytoplasmic sperm injection. *Theriogenology* **64**, 1704–1715. doi:10.1016/J.THERIOGENOLOGY.2005.04.003

Kasamatsu, A., Saeki, K., Tamari, T., Iwamoto, D., Tatemizo, A., Matsumoto, K., Hosoi, Y., and Iritani, A. (2007). Timing and uniformity of embryonic gene activation affect subsequent pre-implantation development of cloned bovine embryos. *J. Reprod. Dev.* **53**, 623–629.

Kimura, Y., and Yanagimachi, R. (1995). Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52**, 709–720. doi:10.1095/BIOLREPROD52.4.709

Kolbe, T., and Holtz, W. (1999). Intracytoplasmic injection (ICSI) of *in vivo* or *in vitro* matured oocytes with fresh ejaculated or frozen–thawed epididymal spermatozoa and additional calcium-ionophore activation in the pig. *Theriogenology* **52**, 671–682. doi:10.1016/S0093-691X(99)00161-2

Lai, L., Sun, Q., Wu, G., Murphy, C. N., Kuhholzer, B., Park, K. W., Bonk, A. J., Day, B. N., and Prather, R. S. (2001). Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vitro* matured oocytes. *Zygote* **9**, 339–346.

Lavitrano, M., Camaioni, A., Fazio, V. M., Dolci, S., Farace, M. G., and Spadafora, C. (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell* **57**, 717–723. doi:10.1016/0092-8674(89)90787-3

Lavitrano, M., Bacci, M. L., Forni, M., Lazzereschi, D., Di Stefano, C., *et al*. (2002). Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. *Proc. Natl Acad. Sci. USA* **99**, 14 230–14 235. doi:10.1073/PNAS.222550299

Lavitrano, M., Forni, M., Bacci, M. L., Di Stefano, C., Varzi, V., Wang, H., and Seren, E. (2003). Sperm mediated gene transfer in pig: selection of donor boars and optimization of DNA uptake. *Mol. Reprod. Dev.* **64**, 284–291. doi:10.1002/MRD.10230

Li, X., Hamano, K., Qian, X. Q., Funauchi, K., Furudate, M., and Minato, Y. (1999). Oocyte activation and parthenogenetic development of bovine oocytes following intracytoplasmic sperm injection. *Zygote* **7**, 233–237. doi:10.1017/S0967199499000611

Li, L., Shen, W., Min, L., Dong, H., Sun, Y., and Pan, Q. (2006*a*). Human lactoferrin transgenic rabbits produced efficiently using dimethylsulfoxide–sperm-mediated gene transfer. *Reprod. Fertil. Dev.* **18**, 689–695. doi:10.1071/RD06001

Li, M., Yan, A. Y., Yao, H., An, X. R., and Gou, K. M. (2006*b*). Transgenic mice produced by intracytoplasmic sperm injection. *Sheng Wu Gong Cheng Xue Bao* **22**, 784–788. [In Chinese with English Abstract]

Loi, P., Ledda, S., Fulka, J., Jr, Cappai, P., and Moor, R. M. (1998). Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. *Biol. Reprod.* **58**, 1177–1187. doi:10.1095/BIOLREPROD58.5.1177

Malcuit, C., Maserati, M., Takahashi, Y., Page, R., and Fissore, R. A. (2006). Intracytoplasmic sperm injection in the bovine induces abnormal $[Ca^{2+}]_i$ responses and oocyte activation. *Reprod. Fertil. Dev.* **18**, 39–51. doi:10.1071/RD05131

Manzini, S., Vargiolu, A., Stehle, I. M., Bacci, M. L., Cerrito, M. G., *et al*. (2006). Genetically modified pigs produced with a nonviral episomal vector. *Proc. Natl Acad. Sci. USA* **103**, 17 672–17 677. doi:10.1073/PNAS.0604938103

McEvoy, T. G., Ashworth, C. J., Rooke, J. A., and Sinclair, K. D. (2003). Consequences of manipulating gametes and embryos of ruminant species. *Reprod. Suppl.* **61**, 167–182.

Moreira, P. N., Perez-Crespo, M., Ramirez, M. A., Pozueta, J., Montoliu, L., and Gutierrez-Adan, A. (2007). Effect of transgene concentration, flanking matrix attachment regions, and RecA-coating on the efficiency of mouse transgenesis mediated by intracytoplasmic sperm injection. *Biol. Reprod.* **76**, 336–343. doi:10.1095/BIOLREPROD.106.056952

Nakai, M., Kashiwazaski, N., Takizawa, A., Hayashi, Y., Nakatsukasa, E., Fuchimoto, D., Noguchi, J., Kaneko, H., Shino, M., and Kikuchi, K. (2003). Viable piglets generated from porcine oocytes matured *in vitro* and fertilized by intracytoplasmic sperm head injection. *Biol. Reprod.* **68**, 1003–1008. doi:10.1095/BIOLREPROD.102.009506

Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y., and Miyazaki, S. (1997). Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol. Hum. Reprod.* **3**, 1087–1093. doi:10.1093/MOLEHR/3.12.1087

Naruse, K., Ishikawa, H., Kawano, H. O., Ueda, H., Kurome, M., Miyazaki, K., Endo, M., Sawasaki, T., Nagashima, H., and Makuuchi, M. (2005). Production of a transgenic pig expressing human albumin and enhanced green fluorescent protein. *J. Reprod. Dev.* **51**, 539–546. doi:10.1262/JRD.16073

Niemann, H., and Kues, W. A. (2003). Application of transgenesis in livestock for agriculture and biomedicine. *Anim. Reprod. Sci.* **79**, 291–317. doi:10.1016/S0378-4320(03)00169-6

Oikawa, T., Takada, N., Kikuchi, T., Numabe, T., Takenaka, M., and Horiuchi, T. (2005). Evaluation of activation treatments for blastocyst production and birth of viable calves following bovine intracytoplasmic sperm injection. *Anim. Reprod. Sci.* **86**, 187–194. doi:10.1016/J.ANIREPROSCI.2004.07.003

Palermo, G., Joris, H., Devroey, P., and Van Steirteghem, A. C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* **340**, 17–18. doi:10.1016/0140-6736(92)92425-F

Perry, A. C. (2000). Hijacking oocyte DNA repair machinery in transgenesis? *Mol. Reprod. Dev.* **56**, 319–324. doi:10.1002/(SICI)1098-2795(200006)56:2+<319::AID-MRD24>3.0.CO;2-P

Perry, A. C., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y., and Yanagimachi, R. (1999). Mammalian transgenesis by intracytoplasmic sperm injection. *Science* **284**, 1180–1183. doi:10.1126/SCIENCE.284.5417.1180

Pope, C. E., Gomez, M. C., and Dresser, B. L. (2006). *In vitro* production and transfer of cat embryos in the 21st century. *Theriogenology* **66**, 59–71. doi:10.1016/J.THERIOGENOLOGY.2006.03.014

Rho, G. J., Wu, B., Kawarsky, S., Leibo, S. P., and Betteridge, K. J. (1998). Activation regimens to prepare bovine oocytes for intracytoplasmic sperm injection. *Mol. Reprod. Dev.* **50**, 485–492. doi:10.1002/(SICI)1098-2795(199808)50:4<485::AID-MRD12>3.0.CO;2-1

Richt, J. A., Kasinathan, P., Hamir, A. N., Castilla, J., Sathiyaseelan, T., *et al*. (2007). Production of cattle lacking prion protein. *Nat. Biotechnol.* **25**, 132–138. doi:10.1038/NBT1271

Rideout, W. M., Eggan, K., and Jaenisch, R. (2001). Nuclear cloning and epigenetic reprogramming of the genome. *Science* **293**, 1093–1098. doi:10.1126/SCIENCE.1063206

Rieth, A., Pothier, F., and Sirard, M. A. (2000). Electroporation of bovine spermatozoa to carry DNA containing highly repetitive sequences into oocytes and detection of homologous recombination events. *Mol. Reprod. Dev.* **57**, 338–345. doi:10.1002/1098-2795(200012)57:4<338::AID-MRD5>3.0.CO;2-K

Robl, J. M., Wang, Z., Kasinathan, P., and Kuroiwa, Y. (2007). Transgenic animal production and animal biotechnology. *Theriogenology* **67**, 127–133. doi:10.1016/J.THERIOGENOLOGY.2006.09.034

Salamone, D., Baranao, L., Santos, C., Bussmann, L., Artuso, J., *et al*. (2006). High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow. *J. Biotechnol.* **124**, 469–472. doi:10.1016/J.JBIOTEC.2006.01.005

SAS Institute (1989). 'SAS/STAT: User's Guide. Version 6. Vol. 1.' 4th edn. (SAS Institute: Cary, NC.)

Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K. H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278**, 2130–2133. doi:10.1126/SCIENCE.278.5346.2130

Shemesh, M., Gurevich, M., Harel-Markowitz, E., Benvenisti, L., Shore, L. S., and Stram, Y. (2000). Gene integration into bovine sperm genome and its expression in transgenic offspring. *Mol. Reprod. Dev.* **56**(Suppl. 2), 306–308. doi:10.1002/(SICI)1098-2795(200006)56:2+<306::AID-MRD21>3.0.CO;2-3

Smith, K., and Spadafora, C. (2005). Sperm-mediated gene transfer: applications and implications. *Bioessays* **27**, 551–562. doi:10.1002/BIES.20211

Smolenski, R. T., Forni, M., Maccherini, M., Bacci, M. L., Slominska, E. M., *et al*. (2007). Reduction of hyperacute rejection and protection of metabolism and function in hearts of human decay accelerating factor (hDAF)-expressing pigs. *Cardiovasc. Res.* **73**, 143–152. doi:10.1016/J.CARDIORES.2006.10.027

Susko-Parrish, J. L., Liebfried-Rutledge, M. L., Northey, D. L., Schutzkus, V., and First, N. L. (1994). Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. *Dev. Biol.* **166**, 729–739. doi:10.1006/DBIO.1994.1351

Szczygiel, M. A., Moisyadi, S., and Ward, W. S. (2003). Expression of foreign DNA is associated with paternal chromosome degradation in intracytoplasmic sperm injection-mediated transgenesis in the mouse. *Biol. Reprod.* **68**, 1903–1910. doi:10.1095/BIOLREPROD.102.012377

Tian, J. H., Wu, Z. H., Liu, L., Cai, Y., Zeng, S. M., Zhu, S. E., Liu, G. S., Li, Y., and Wu, C. X. (2006). Effects of oocyte activation and sperm preparation on the development of porcine embryos derived from *in vitro*-matured oocytes and intracytoplasmic sperm injection. *Theriogenology* **66**, 439–448. doi:10.1016/J.THERIOGENOLOGY.2005.10.026

Wall, R. J., Powell, A. M., Paape, M. J., Kerr, D. E., Bannerman, D. D., Pursel, V. G., Wells, K. D., Talbot, N., and Hawk, H. W. (2005). Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nat. Biotechnol.* **23**, 445–451. doi:10.1038/NBT1078

Wrenzycki, C., Herrmann, D., Gebert, C., Carnwath, J. W., and Niemann, H. (2006). Gene expression and methylation patterns in cloned embryos. *Methods Mol. Biol.* **348**, 285–304.

Yamauchi, Y., Doe, B., Ajduk, A., and Ward, M. A. (2007). Genomic DNA damage in mouse transgenesis. *Biol. Reprod.* **77**, 803–812. doi:10.1095/BIOLREPROD.107.063040

Zani, M., Lavitrano, M., French, D., Lulli, V., Maione, B., Sperandio, S., and Spadafora, C. (1995). The mechanism of binding of exogenous DNA to sperm cells: factors controlling the DNA uptake. *Exp. Cell Res.* **217**, 57–64. doi:10.1006/EXCR.1995.1063

Manuscript received 24 September 2007, accepted 21 April 2008