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Sperm genome cloning used in biparental bovine embryo reconstruction

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Abstract. The generation of androgenetic haploid embryos enables several haploid blastomeres to be obtained as identical copies of a single spermatozoon genome. In the present study, we compared the developmental ability of bovine androgenetic haploid embryos constructed by different methods, namely IVF and intracytoplasmic sperm injection (ICSI) before and after oocyte enucleation. Once obtained, the blastomeres of these androgenetic haploid embryos were used as male genome donors to reconstruct biparental embryos by fusion with matured oocytes. To verify the cytoplasmic contribution of androgenetic haploid blastomeres, we used spermatozoa incubated previously with exogenous DNA that coded for a green fluorescent protein gene (*pCX-EGFP*) and the enhanced green fluorescent protein (EGFP)-positive androgenetic haploid blastomeres generated were fused with mature oocytes. Of the reconstructed embryos reaching the cleavage and blastocyst stages, 85.1% and 9.0%, respectively, expressed EGFP ($P > 0.05$). EGFP expression was observed in 100% of reconstructed embryos, with 91.2% exhibiting homogenic expression. To confirm sperm genome incorporation, androgenetic haploid blastomeres generated by ICSI prior to enucleation and using Y chromosome sexed spermatozoa were used for biparental embryo reconstruction. Incorporation of the Y chromosome was confirmed by polymerase chain reaction and fluorescence *in situ* hybridisation analysis. In conclusion, the results of the present study prove that it is possible to use sperm genome replicates to reconstruct biparental bovine embryos and that it is a highly efficient technique to generate homogeneous transgene-expressing embryos.

Additional keywords: sexed haploid NT, sperm cloning, transgenesis.

Introduction

Uniparental androgenetic and parthenogenetic embryos are efficient models for genome imprinting research (Kaneko-Ishino *et al*. 2003; Cruz *et al*. 2008; Miki *et al*. 2009). In particular, androgenetic embryos are very useful for investigating the contribution of the paternal genome to early embryonic development. Different methods have been used for androgenetic embryo production, including: (1) removal of the female pronucleus from zygotes (Barton *et al*. 1984; McGrath and Solter 1984; Surani *et al*. 1984; Kaufman *et al*. 1989; Latham and Solter 1991; Hagemann and First 1992); (2) fertilisation of enucleated oocytes (McGrath and Solter 1984; Surani *et al*. 1984; Kaufman *et al*. 1989; Latham and Solter 1991; Hagemann and First 1992; Obata *et al*. 2000); and (3) injection of spermatozoa into oocytes, followed by removal of the maternal chromosomes (Miki *et al*. 2009). Although these methods have been used successfully in the mouse, they are less well studied in farm animal species (Lagutina *et al*. 2004; Matsukawa *et al*. 2007; Park *et al*. 2009).

The generation of androgenetic haploid embryos allows several identical haploid blastomeres to be obtained from a single spermatozoon genome (Kuznyetsov *et al*. 2007). Some of these haploid blastomeres could be used for genetic evaluation, whereas other could be used for the reconstruction of biparental embryos capable of undergoing full-term development. Haploid sperm genome replication would have high potential in livestock production, because it allows the selection of favourable characteristics using genetic markers and also makes it possible to select the sex of the offspring before biparental embryo reconstruction.

Brackett *et al*. (1971) described the ability of the spermatozoon to bind foreign DNA and its subsequent delivery into the oocyte during fertilisation. Subsequently, several sperm-mediated gene transfer (SMGT) techniques were developed, including IVF-mediated transgenesis (IVF-Tr; Lavitrano *et al*. 1989) and intracytoplasmic sperm injection-mediated transgenesis (ICSI-Tr; Perry *et al*. 1999). Contradictory results have been reported for the production of transgenic embryos or offspring

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using IVF-Tr (Brinster *et al*. 1989; Lavitrano *et al*. 1989; Yamauchi *et al*. 2007). Nevertheless, ICSI-Tr has proven to be a useful technique for the production of transgenic embryos in different mammalian species (Perry *et al*. 1999; Pereyra-Bonnet *et al*. 2008). However, a high frequency of mosaic expression has been observed in porcine (85.2%), bovine (60.4%), feline (78.7%) and equine (72.7%) embryos generated with ICSI-Tr (Pereyra-Bonnet *et al*. 2008). Currently, the challenge in transgenesis research is to find a highly efficient technique to produce transgenic animals without mosaic expression.

In the present study, we compared the developmental ability of androgenetic bovine embryos constructed using different methods, including IVF and ICSI before and after oocyte enucleation. In all treatments, the spermatozoa used had either been incubated previously incubated with exogenous DNA or not. Once obtained, the blastomeres of these haploid androgenetic embryos were used as male genome donors to reconstruct biparental embryos by fusion with matured oocytes (MII). Finally, we explored two possible applications of haploid sperm genome replication. The first was to generate homogeneous transgene-expressing embryos by fusing androgenetic haploid blastomeres that express transgene with MII oocytes. Thus, the usual mosaic expression observed in embryos produced by the traditional transgenic techniques is overcome. The second was to determine the sex of the replicated sperm nuclei before their use in biparental embryo reconstruction, thus establishing a new and advantageous way to determine the sex of the resultant embryo.

Materials and methods

Reagents

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

Oocyte collection and IVM

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory. Cumulus–oocyte complexes were aspirated from follicles 2–8 mm in diameter into 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 (ATB; 15240-096; Gibco BRL). Oocytes covered by at least three layers of granulosa cells and with an evenly granulated cytoplasm were selected for IVM. The maturation medium consisted of bicarbonate-buffered TCM-199 (31100-035; Gibco BRL), containing 2 mM glutamine (G-8540), 10% FBS, 2 μg mL^{-1} FSH (NIH-FSH-P1; Folltropin; Bioniche, Belleville, ON, Canada), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768) and 2% ATB. Oocytes were incubated in 100-μL droplets of medium covered with mineral oil (M8410) in 32-mm Petri dishes. Oocytes were allowed to mature for 22 h under 6.5% CO_2 in humidified air at 39°C. Except for those oocytes used as IVF controls, oocytes were denuded of cumulus cells by vortexing for 2 min in hyaluronidase (H-4272; 1 mg mL^{-1} in DPBS) and washed three times in HEPES-buffered Tyrode's medium (TALP-H; Bavister and Yanagimachi 1977). The MII oocytes were identified by visualisation of the first polar body and were used immediately.

Construction of DNA

The plasmid used to construct DNA was pCX-EGFP, which contains an enhanced green fluorescent protein gene (*egfp*) under the control of a chimeric cytomegalovirus IE–chicken β-actin enhancer–promoter (Ikawa *et al*. 1995).

Spermatozoa–DNA incubation

Spermatozoa-DNA (pCX-EGFP) coincubation was performed according to the method of Perry *et al*. (1999) with slight modification. Briefly, spermatozoa were washed twice (490*g*, 5 min) in Brackett-Oliphant (BO) medium (Brackett and Oliphant 1975) for IVF or in 2.8% Na citrate (F71497) with 100 mM EDTA (15576-028; Invitrogen, Carlsbad, CA, USA) for ICSI procedures. The sperm pellet was resuspended in the same medium and the concentration was adjusted to 20×10^6 spermatozoa mL^{-1}. A 10-μL of sperm suspension was incubated with 0.5 μg DNA per million spermatozoa (final concentration) for 5 min at 0°C and used immediately for ICSI.

In vitro *fertilisation*

The IVF procedures for matured oocytes and enucleated oocytes were performed as described previously (Vichera *et al.* 2011).

Intracytoplasmic sperm injection

Frozen semen was thawed in a 37°C water bath for 30 s. Spermatozoa were either incubated immediately or not (control group) with pCX-EGFP and then ICSI was performed as described by (Vichera *et al*. 2009).

Production of androgenetic haploid embryos

Androgenetic haploid embryos were produced using four different strategies: (1) IVF before oocyte enucleation; (2) IVF after enucleation; (3) ICSI before oocyte enucleation; and (4) ICSI after enucleation. The methods for IVF and ICSI were as described above. Enucleation was performed as follows. First, oocytes were held and manipulated in TALP-H supplemented with 3 mg mL^{-1} bovine serum albumin (BSA). Denuded MII oocytes were enucleated mechanically using Narishige hydraulic micromanipulators (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Diaphot microscope (Nikon, Garden City, NJ, USA) and 20-μm diameter pipettes. Metaphase chromosomes were visualised under ultraviolet light (<10 s) after staining with 5 μg mL^{-1} Hoechst 33342 for 10 min. Presumptive hemizygotes were cultured to produce embryos with blastomeres containing only replicates of the sperm genetic information (androgenetic haploid blastomeres). All treatments were performed using spermatozoa that had been incubated previously or not (control) with pCX-EGFP.

Determination of EGFP fluorescence in embryos

During *in vitro* culture, embryos were exposed to blue light using an excitation filter at 488 nm and an emission filter at 530 nm to determine *egfp* expression at different stages of

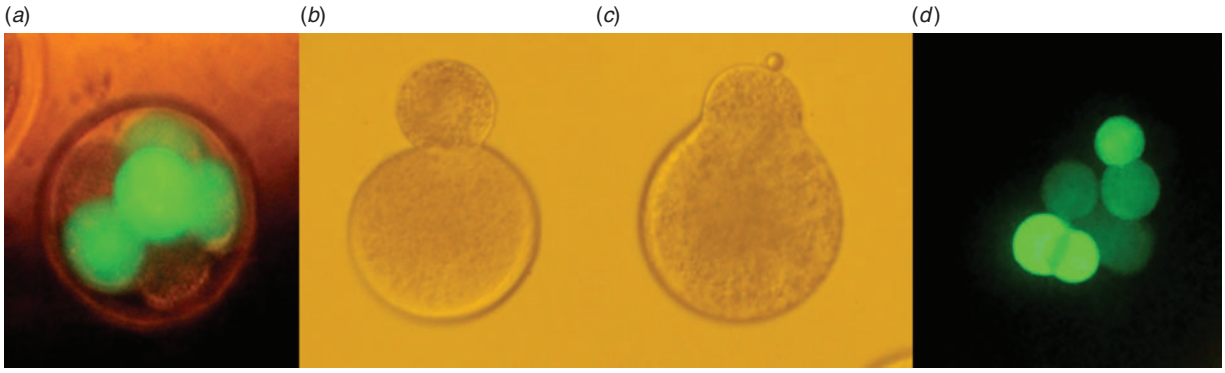

Fig. 1. (*a*) Androgenetic haploid bovine embryo (Day 3) obtained by intracytoplasmic sperm injection using spermatozoa exposed to pCX-EGFP plasmid (ICSI-Tr before enucleation) with some of its blastomeres expressing the transgene. Fluorescence was evaluated under blue light (488 nm; original magnification ×200). (*b*, *c*) Zona pellucida (ZP)-free MII oocytes attached to an androgenetic haploid blastomere (using phytohemagglutinin) before fusion (*b*) and fusing to an androgenetic haploid blastomere (*c*). (Original magnification × 200.) (*d*) Biparental reconstructed ZP-free embryo expressing the transgene in all its blastomeres. Fluorescence was evaluated under blue light (488 nm; original magnification ×200).

development. Embryos were analysed on Days 3 and 7 after IVF, ICSI or androgenetic blastomere fusion.

Karyotype analysis

Seventy-two hours after ICSI or IVF, androgenetic embryos were cultured in synthetic oviducal fluid (SOF) supplemented with 0.05 g mL^{-1} demecolcine (Colcemid; Gibco BRL) for 6 h. They were then exposed to a hypotonic 0.8% sodium citrate (F71497) solution for 10 min at 37°C. Subsequently, embryos were placed on a clean glass slide and were treated with a drop of methanol : acetic acid solution (3 : 1). After drying, slides were stained with 5% Giemsa solution (10092-013; Invitrogen) for 10 min. Chromosome spreads were evaluated at ×400 magnification.

Biparental bovine embryos reconstructed by androgenetic haploid blastomeres fusion

Androgenetic haploid embryos (Day 3) generated by ICSI and expressing EGFP or not, were treated with 1.5 mg mL^{-1} pronase (Sigma protease; P8811) dissolved in TALP-H in order to remove the zona pellucida (ZP) and disaggregate its blastomeres. Androgenetic blastomeres were fused with matured oocytes in one of two ways, namely cell fusion with or without the ZP, as described below. Androgenetic blastomeres expressing EGFP were then selected under blue light using an excitation filter at 488 nm and an emission filter at 530 nm.

Cell fusion with the ZP

Androgenetic haploid blastomeres were transferred into the perivitelline space of MII oocytes and fused electrically by manually aligning their membranes parallel to the electrodes in a fusion chamber (BTX Instrument Division; Harvard Apparatus, Holliston, MA, USA). The fusion medium used consisted of 25 μM D-sorbitol (S-1876), 100 μM CaOAc (C-1000), 5 mM MgOAc (M-0631) and 1 g L^{-1} BSA. Fusion was achieved using one electrical pulse of 120 V for 30 μs (BTX microslide 0.5 mm fusion chamber, model 450, 01-000209-01). After fusion, oocytes were activated as described below.

Zona-free cell fusion

To achieve ZP-free cell fusion, MII oocytes were incubated in 1.5 mg mL^{-1} pronase for 5–10 min on a warm plate to remove the ZP. The ZP-free oocytes were then transferred individually to a drop of 1 mg mL^{-1} phytohemagglutinin (L8754) dissolved in TCM-199 without serum for a few seconds, after which they were quickly dropped over a single androgenetic haploid blastomere resting on the bottom of a 100-μL drop of TALP-H. Following attachment, the ZP-free oocyte–androgenetic haploid blastomere pair was picked up, transferred to fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 1 mg mL^{-1} polyvinyl alcohol (PVA)), for 2–3 min and then to a fusion chamber containing 2 mL warm fusion medium. Fusion was achieved with a double direct current (DC) pulse of 65 V, each pulse of 30 ms duration, 0.1 s apart. The procedure is shown in Fig. 1.

Chemical activation protocols

The androgenetic haploid embryos produced by ICSI after enucleation, were activated immediately after ICSI with ionomycin (I24222; Invitrogen) for 4 min and then placed in 6-dimethylaminopurine (6-DMAP) (D2629) in TCM-199 for 3 h. The androgenetic haploid embryos produced by ICSI prior to enucleation were activated (immediately after ICSI) with ionomycin for 4 min and placed in TCM-199 for 1 h until the enucleation procedure. After enucleation, they were placed in 6-DMAP in TCM-199 for 3 h. The parthenogenetic diploid activation consisted of incubation in ionomycin for 4 min, followed by incubation in 6-DMAP in TCM-199 for 3 h. For parthenogenetic haploid activation and ICSI control groups, oocytes were placed in ionomycin for 4 min, then transferred to TCM-199 for 3 h to permit extrusion of their second polar body (2PB) before being placed finally in 6-DMAP in TCM-199 for 3 h. The embryos reconstructed by androgenetic blastomere fusion were activated (2 h after fusion time) with ionomycin for 4 min, subsequently moved to SOF for 3 h to permit extrusion of the 2PB and finally placed in 6-DMAP in SOF for 3 h. The extrusion of the 2PB was checked prior to incubation with 6-DMAP. In all

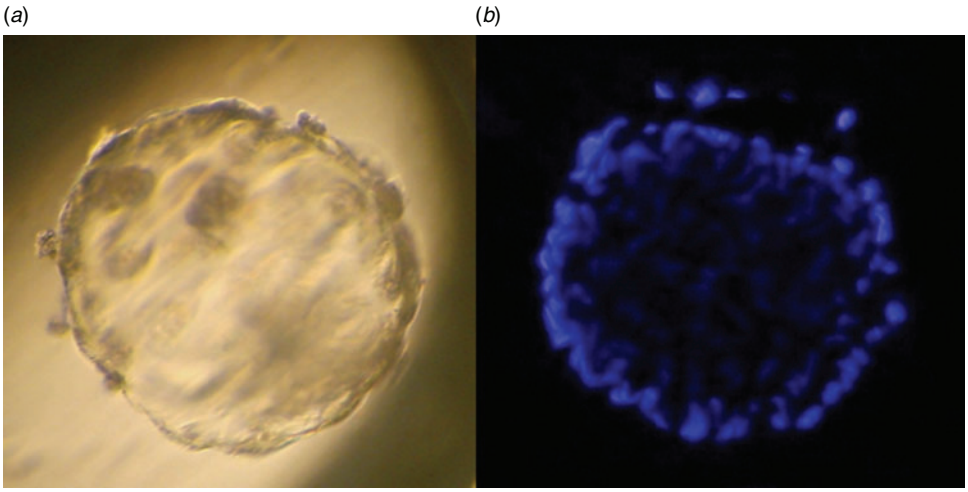

Fig. 2. (*a*) Biparental bovine blastocyst produced by fusion of an androgenetic haploid blastomere with a mature oocyte and cultured in the well-of-the-well system. (*b*) The same blastocyst stained with 4′,6′-diamidino-2-phenylindole (blue) showing the nuclei. (Original magnification ×200.)

cases, the concentrations of ionomycin in TALP-H and 6-DMAP in TCM-199 or SOF medium were 5 μM and 1.9 mM, respectively.

In vitro *culture*

In the first and second experiments, androgenetic, parthenogenetic and IVF embryos were cocultured with cumulus cells in TCM-199 medium under 6.5% CO_2 in humidified air at 39°C. Cleavage was evaluated on Day 2, whereas morula and blastocyst formation were evaluated on Days 5 and 7, after fertilisation or activation, respectively. In the third and fourth experiments, fertilised and reconstructed embryos were cultured in SOF medium (Holm *et al*. 1999) in a system similar to that of the well-of-the-well (WOW; Vajta *et al*. 2000), whereby microwells were produced using a heated glass capillary pressed gently into the bottom of a Petri dish and covered with a 100-μL microdrop of culture medium (16 WOW for each microdrop, one embryo for each WOW). For these experiments, the culture conditions were a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in air at 39°C. During embryo culture, the medium was supplemented with 10% FBS on Day 5. Cleavage was evaluated on Day 2 and blastocysts (Fig. 2) were evaluated on Day 7, after fusion.

Sex determination by fluorescence in situ *hybridisation*

Blastomeres from androgenetic haploid embryos (Day 3) generated by ICSI prior to enucleation and using sexed (Y chromosome) spermatozoa obtained by flow cytometry (CIALE, Capitán Sarmiento, Argentina) and embryos reconstructed with these blastomeres were subjected to fluorescence *in situ* hybridisation (FISH) using a specific probe for the p12 region of the bovine Y chromosome (Accession no.: AY303974). The probe was labelled with fluorescein isothiocyanate (FITC) using the Nick Translation System (18160-010; Invitrogen). The purification efficiency of the flow cytometry was >85% and was verified by FISH analysis. The embryos were cultured for 6 h in SOF medium supplemented with 0.05 g mL^{-1} demecolcine. Afterwards, they were exposed to a hypotonic 0.8% sodium citrate solution for 10 min at 37°C. Subsequently, they were placed on a clean glass slide and covered with a methanol : acetic acid solution (3 : 1). Slides containing the metaphase spreads or interphase nuclei of embryos and slides containing spermatozoa were incubated in 50 mL of 70% formamide in 2× standard saline-citrate (SSC; where 1× SSC comprises 150 mM NaCl and 15 mM sodium citrate, pH 7.0) for 2 min and dehydrated with an increasing series of ethanol. Meanwhile, 1 μL of the Y chromosome-specific probe was added to 4 μL hybridisation solution (40% of 4× SSC, 50% formamide and 3% dextran sulfate, 3.3 μg mL^{-1} salmon sperm DNA) and heated at 95°C for 5 min. The denatured probe was dropped onto the slide and sample were sealed with a coverslip using rubber cement, followed by hybridisation at 37°C for 30 min. Slides were washed with 0.3% Tween-20 in 0.4× SSC at 72°C for 2 min and subsequently with 0.1% Tween-20 in 2× SSC at room temperature for 2 min. Slides were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and examined under a fluorescence microscope (Model IX71; Olympus, Tokyo, Japan) at wavelengths of 525 and 470 nm. Images were captured by a digital camera (DP-72; Olympus) and analysed using Image Pro-Plus software (Media Cybernetics, Bethesda, MD, USA). The same procedures were used for embryos generated by IVF with sexed (Y chromosome) spermatozoa as well as for parthenogenetic embryos (used as positive and negative controls, respectively).

Sex determination analysis by polymerase chain reaction

Single androgenetic haploid blastomeres obtained from androgenetic embryos produced by ICSI prior to enucleation and using sexed (Y chromosome) spermatozoa, as well as embryos generated after fusion with an oocyte, were checked by polymerase chain reaction (PCR) using Y and X chromosome-specific sequence primers. The specific gene target of these primers was the amelogenin gene. In cattle, this gene is present in both sex chromosomes, with a 63-bp deletion in the gene on the Y chromosome (Colley *et al*. 2008). These androgenetic blastomeres and reconstructed embryos were washed in

phosphate-buffered saline (PBS), transferred in 1-μL aliquots into an Eppendorf tube, and incubated in 10 μL PCR buffer with proteinase K (2 μg mL^{-1}) at 56°C for 1 h. The proteinase K was inactivated at 95°C for 10 min. Half the reaction product was used for PCR in a final volume of 10 μL containing 1× PCR buffer, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit Taq DNA polymerase and 0.5 mM of each primer. The outer nested primers (amelF1: 5′-CATGGTGCCAGCTCAGCAG-3′; and amelR1: 5′-CCGCTTGGTCTTGTCTGTTGC-3′) produced 367- and 304-bp X and Y chromosome amplicons, respectively. One μL from the first PCR was used as a template in the second PCR. The inner nested primers (amelF2: 5′-CAGCAACCAAT GATGCCAGTTC-3′; and amelR2: 5′-GTCTTGTCTGTTG CTGGCCA-3′) produced 329- and 266-bp X and Y chromosome amplicons, respectively (Colley *et al*. 2008). The conditions of the first PCR consisted of a heating step at 95°C for 10 min, followed by 35 cycles of a second heating step at 94°C for 30 s to further denature the nucleic acid, an annealing step at 55°C for 30 s and an extension step at 72°C for 30 s. The last cycle (Cycle 35) contained an additional extension at 72°C for 7 min. The conditions of the second PCR consisted of heating step at 95°C for 10 min, followed by 25 cycles of a second heating step at 94°C for 30 s to further denature the nucleic acid, an annealing step at 60°C for 30 s and an extension step at 72°C for 30 s. The last cycle (Cycle 35) contained an additional extension at 72°C for 7 min. The PCR reaction products (5 μL) were analysed on a 2% agarose gel stained with ethidium bromide.

Statistical analysis

In vitro embryo development and transgene expression were compared by non-parametric Fisher's exact tests. All statistical analyses were performed using the SAS program (SAS Institute 1989). Differences were considered significant at $P < 0.05$.

Experimental design

In the first and second experiments we evaluated four different methods to generate androgenetic haploid bovine embryos, IVF or ICSI before and after enucleation using spermatozoa pre-incubated or not with pCX-EGFP. Embryo development and EGFP expression were compared among all treatment groups. Karyotype analysis was performed in order to determine the ploidy of the androgenetic embryos generated. In the third experiment we used haploid androgenetic blastomeres from embryos generated by ICSI prior to enucleation, to reconstruct biparental embryos. Moreover, blastomeres that expressed the transgene were used to generate homogeneous transgene-expressing embryos. To confirm sperm genome incorporation in the androgenetic blastomeres and the reconstructed biparental embryo, we used PCR and FISH analysis (Fig. 3).

Results

Experiment 1: development of androgenetic embryos generated by IVF before and after enucleation using spermatozoa exposed (or not) to pCX-EGFP plasmid

Development and EGFP expression rates of androgenetic embryos produced by IVF are summarised in Table 1. Statistical differences were observed in cleavage rates between all the androgenetic embryos groups compared with biparental IVF control, but no differences were seen in morula and blastocyst rates between these four androgenetic groups and the biparental IVF control. None of the groups contained EGFP-positive embryos. Androgenetic embryos generated by IVF after enucleation and using spermatozoa incubated with pCX-EGFP showed lower cleavage rates than those generated using spermatozoa that had not been incubated with pCX-EGFP (32.8% vs 55.2%, respectively). The blastocyst rates were also decreased in the biparental IVF control group when spermatozoa incubated with pCX-EGFP were used.

Experiment 2: development of androgenetic embryos generated by ICSI before and after oocyte enucleation using spermatozoa exposed (or not) to pCX-EGFP plasmid

Development and EGFP expression rates of androgenetic embryos produced by ICSI are summarised in Table 2. Androgenetic embryos obtained by ICSI using spermatozoa exposed to the pCX-EGFP plasmid (ICSI-Tr before and after enucleation) showed EGFP expression. Significantly lower cleavage rates were observed in the ICSI-Tr after enucleation group compared with the ICSI-Tr control, but no significant differences were observed between the ICSI-Tr prior to enucleation group and the ICSI-Tr control. In addition, development analysis between androgenetic embryo groups revealed significant differences in cleavage rates. When the ICSI-Tr was performed before enucleation, embryonic cleavage rates were higher compared with rates in the ICSI-Tr after enucleation group (61.2% vs 35.0%, respectively; $P < 0.05$). Androgenetic embryos generated by ICSI prior to enucleation and biparental ICSI controls showed lower cleavage rates when spermatozoa incubated with pCX-EGFP were used. As expected, all groups of androgenetic haploid embryos had lower percentages of morulae and blastocysts, regardless of the presence or absence of the pCX-EGFP plasmid or the timing of the enucleation.

The pattern of EGFP expression was evaluated in cleaved embryos (Day 3 after ICSI). Significant differences were observed in the number of EGFP-positive androgenetic embryos between the ICSI before and after enucleation groups (25.8% vs 11.8%, respectively). For the ICSI-Tr control group, 21.9% of embryos were EGFP positive and this did not differ from the number of EGFP-positive androgenetic embryos. In addition, a high rate of mosaic expression was seen in all ICSI-Tr EGFP-positive embryos in both the androgenetic and control groups.

Karyotype analysis of the androgenetic embryos generated by ICSI-Tr prior to enucleation, confirmed that 92% (11/12) were indeed haploid. In the group in which ICSI-Tr was performed after enucleation, 82% (9/11) of the analysed embryos had haploid chromosomal sets. In both groups, the remaining embryos were mixoploid. Moreover, the development of parthenogenetic and androgenetic haploid embryos was lower compared with parthenogenetic diploid embryos ($P < 0.05$).

Experiment 3: development and transgene expression of biparental bovine embryos reconstructed by fusion of androgenetic haploid blastomeres (EGFP-positive or negative), with matured oocytes

Development and EGFP expression rates of ZP-free biparental embryos reconstructed with androgenetic haploid blastomeres

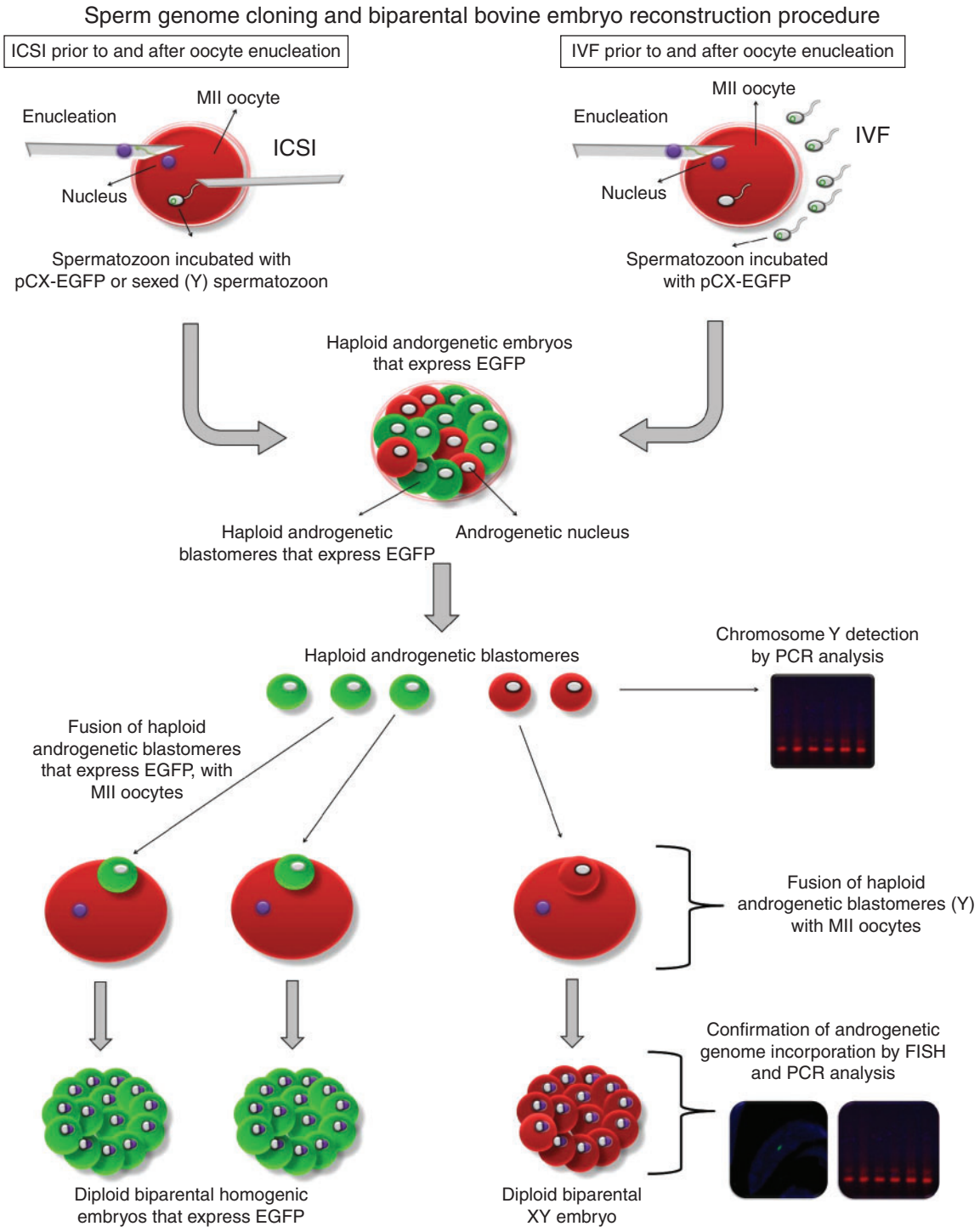

Fig. 3. Schematic diagram showing the production of haploid androgenetic embryos and biparental embryo reconstruction procedures. ICSI, intracytoplasmic sperm injection; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; FISH, fluorescence *in situ* hybridisation.

that either did or did not express the transgene are summarised in Table 3. No differences were observed in the percentage fusion or development between these groups, whereas biparental IVF controls showed a significantly higher blastocyst rate. When embryos were reconstructed with EGFP-positive androgenetic haploid blastomeres, all embryos expressed the transgene during development (57/57; 100%). Transgene expression in all embryonic cells accounted for 91.2% (52/57) of these embryos

Table 1. Androgenetic haploid embryo development and transgene expression after IVF with spermatozoa exposed (or not) to the pCX-EGFP plasmid in bovine

Data show the number in each group, with percentages given in parentheses. Values within a column with different superscripts differ significantly ($P < 0.05$, Fisher's test). NA, not applicable; +, positive; −, negative; EGFP, enhanced green fluorescent protein

Androgenetic haploid embryo production	No. replicates	Spermatozoon exposed to pCX-EGFP	*n*	Cleaved	Morulae	Blastocysts	EGFP-positive embryos (4–16 cells)
IVF before enucleation	3	+	67	22 (32.8)^{a}	8 (11.9)^{ab}	1 (1.5)^{a}	0 (0)
	4	−	105	58 (55.2)^{b}	23 (21.9)^{ab}	7 (6.6)^{ab}	NA
IVF after enucleation	3	+	61	26 (42.6)^{ab}	7 (11.5)^{a}	2 (3.3)^{ab}	0 (0)
	4	−	106	59 (55.7)^{b}	19 (17.9)^{ab}	8 (7.5)^{ab}	NA
Biparental IVF control	4	+	280	230 (82.1)^{c}	58 (20.7)^{ab}	17 (6.1)^{a}	0 (0)
	4	−	114	86 (75.4)^{c}	28 (24.6)^{b}	15 (13.1)^{b}	NA

Table 2. Androgenetic haploid embryo development and transgene expression after intracytoplasmic sperm injection with spermatozoa exposed (or not) to the pCX-EGFP plasmid

Data show the number in each group, with percentages given in parentheses. Values within a column with different superscripts differ significantly ($P < 0.05$, Fisher's exact test). ICSI, intracytoplasmic sperm injection; NA, not applicable; +, positive; −, negative; EGFP, enhanced green fluorescent protein

Production	No. replicates	Spermatozoon exposed to pCX-EGFP	*n*	Cleaved	Morulae	Blastocysts	EGFP-positive embryos (2–16 cells)	All embryo cells EGFP positive
Androgenetic haploid embryos	4	+	116	71 (61.2)^{b}	3 (2.6)^{a}	0 (0)^{a}	30 (25.8)^{b}	3/30 (10.0)
(ICSI before enucleation)	3	−	35	28 (80.0)^{c}	5 (14.3)^{bcd}	1 (2.8)^{ab}	NA	NA
Androgenetic haploid embryos	4	+	97	34 (35.0)^{a}	1 (1.0)^{a}	0 (0)^{a}	4 (11.8)^{a}	0/4 (0.0)
(ICSI after enucleation)	3	−	32	17 (53.1)^{ab}	0 (0)^{ab}	0 (0)^{a}	NA	NA
Biparental ICSI control	4	+	82	42 (51.2)^{b}	9 (10.9)^{bc}	8 (9.7)^{bc}	18 (21.9)^{ab}	1/18 (5.6)
	3	−	81	62 (76.6)^{c}	12 (14.8)^{c}	9 (11.1)^{cd}	NA	NA
Parthenogenetic haploid control	4	−	116	76 (65.5)^{cb}	18 (15.5)^{c}	4 (3.4)^{ab}	NA	NA
Parthenogenetic diploid control	4	−	113	84 (74.3)^{c}	35 (30.9)^{d}	20 (17.7)^{c}	NA	NA

Table 3. Development and transgene expression (enhanced green fluorescent protein positive) of embryos reconstructed by androgenetic blastomere fusion

Data show the number in each group, with percentages given in parentheses. Values within a column with different superscripts differ significantly ($P < 0.05$, Fisher's test). NA, not applicable; +, positive; −, negative; EGFP, enhanced green fluorescent protein

Method of production	No. replicates	Androgenetic blastomere EGFP positive	*n*	Fused	Cleaved	Blastocysts	EGFP-positive embryos (2–16 cells)	All embryo cells EGFP positive
Biparental embryo reconstructed	3	+	84	67 (79.8)^{a}	57 (85.1)^{a}	6 (9.0)^{a}	57 (100)^{a}	52/57 (91.2)
	3	−	34	27 (79.4)^{a}	24 (88.9)^{a}	2 (5.9)^{a}	NA	NA
Biparental IVF control	3	−	124	NA	98 (79.0)^{a}	37 (29.8)^{b}	NA	NA

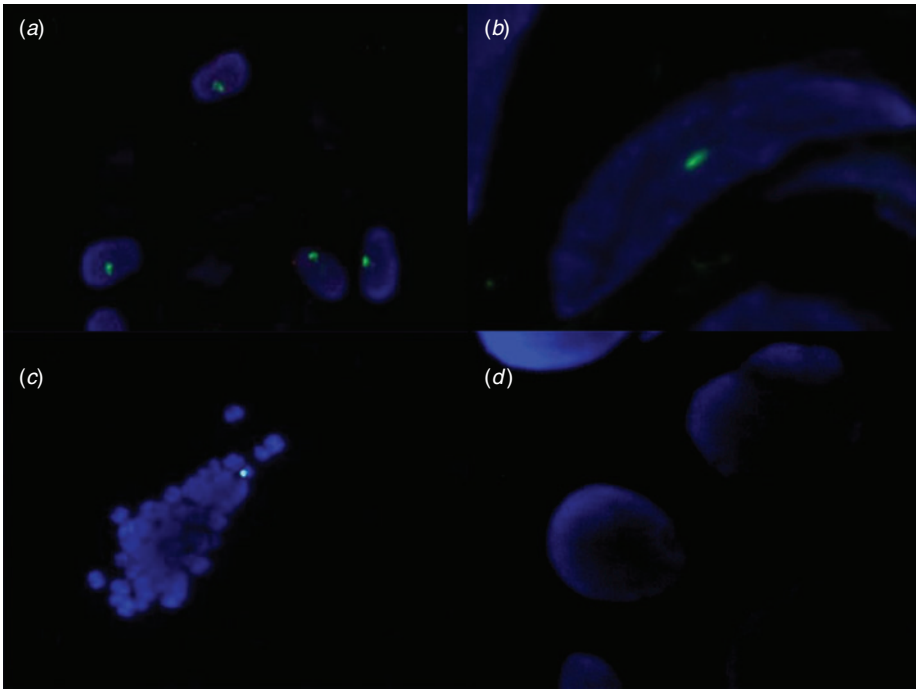

Fig. 4. Photomicrographs of fluorescence *in situ* hybridisation (FISH) labelling of the p12 region of the bovine Y chromosome (fluorescein isothiocyanate: green) and counterstaining total DNA with 4′,6′-diamidino-2-phenylindole (blue). (*a*) Sexed (Y chromosome) bovine spermatozoa with a positive green signal. (*b*) One blastocyst cell from a reconstructed embryo, confirming the presence of the sexed sperm genome. (*c*) Specific Y chromosome signal in an IVF Day 3 embryo generated with sexed spermatozoa, used as positive control. (*d*) Parthenogenetic embryos used as negative controls without any Y chromosome signal. (Original magnification ×200.)

(Table 3). In addition, we tested an alternative method (ZP included) to reconstruct biparental embryos. This involved the fusion of androgenetic haploid blastomeres previously injected into the perivitelline space of MII oocytes. However, in preliminary results this method showed low fusion and cleavage rates (53.6% (15/28) and 40.0% (6/15), respectively) and none of these embryos developed to the blastocyst stage.

Experiment 4: use of sexed spermatozoa to confirm male genome incorporation in embryos reconstructed by fusion of androgenetic haploid blastomeres with matured oocytes

To confirm male genome incorporation, 18 androgenetic haploid embryos were generated with the approaches ICSI prior to enucleation and using sexed (Y chromosome) spermatozoa. Sixty androgenetic haploid blastomeres were isolated from these embryos and 24 were used for biparental embryo reconstruction. All blastomeres were fused successfully (24/24; 100%), 91.7% cleaved (22/24) on Day 2 and 8.3% reached the blastocyst stage (2/24) on Day 7. The presence of the Y chromosome in the remaining androgenetic haploid blastomeres and embryos reconstructed with them was confirmed by PCR. In addition, FISH analysis on the blastocysts generated confirmed the presence of the sexed sperm genome in all cells (Fig. 4). Positive controls (sexed spermatozoa and the IVF embryos generated with them) similarly showed a specific Y chromosome signal (Fig. 4). Parthenogenetic embryos used as negative controls did not show a Y chromosome signal (Fig. 4).

Discussion

Initially we evaluated two methods commonly used for the generation of androgenetic embryos by IVF, namely enucleation of fertilised oocytes (IVF before enucleation) and fertilisation of enucleated oocytes (IVF after enucleation). The androgenetic embryos produced by these methods showed lower cleavage rates compared with biparental IVF controls, but there were no significant differences in morula and blastocyst rates. Previous studies have reported that most of the haploid androgenetic embryos produced by IVF are arrested after the first cell division and that only some embryos develop to compact morulae and blastocyst stages (McGrath and Solter 1984; Surani *et al*. 1986; Lagutina *et al*. 2004; Hoelker *et al*. 2007). Androgenetic embryos generated by IVF before or after enucleation with spermatozoa exposed to pCX-EGFP cleaved and developed to the blastocyst stage, but none of them expressed EGFP. When the IVF-Tr control procedure was performed using spermatozoa incubated previously with pCX-EGFP, oocytes were successfully fertilised and cleaved as well as the control group (without sperm transgene coincubation), but no embryos expressing EGFP were generated. These results agree with previous reports in mice (Brinster *et al*. 1989; Yamauchi *et al*.

2007). The absence of EGFP-positive IVF embryos could be due to plasmid loss by the spermatozoa before or during oocyte penetration as a consequence of the weak interaction between the spermatozoa and exogenous DNA, as reported previously (Szczygiel *et al*. 2003).

In the second experiment, we evaluated the development of androgenetic embryos generated by ICSI before and after oocyte enucleation. In these experiments, the higher embryonic cleavage rates obtained by ICSI prior to enucleation compared with rates in the group in which ICSI was performed after enucleation agreed with previous reports in mice (Miki *et al*. 2009). As expected, development rates of androgenetic haploid embryos to the morula and blastocyst stages were low for all groups, regardless of when the enucleation was performed. Moreover, the development of parthenogenetic and androgenetic haploid embryos was lower compared with parthenogenetic diploid embryos.

The ICSI procedure also enables the generation of embryos expressing exogenous genes (Perry *et al*. 1999). In our experiments, we demonstrated the capacity of this technique to produce haploid androgenetic embryos that express a transgene. In contrast with IVF, the ICSI procedure avoids the natural process of gamete fusion, so the weak interactions between spermatozoa and exogenous DNA are not an impediment for transgene delivery. However, the injection of spermatozoa previously incubated with pCX-EGFP had a negative effect on cleavage rates in androgenetic embryos generated by ICSI prior to enucleation and biparental ICSI control groups. Moreover, high rates of mosaic expression were seen in all EGFP-positive embryos. A possible explanation for this is that the transgene integrates into the genome after the first cell division (Lavitrano *et al*. 1989; Celebi *et al*. 2002; Kaneko *et al*. 2005; Smith and Spadafora 2005). Another explanation could be that the transgene does not integrate and is lost in some blastomeres during successive mitotic divisions.

Conversely, in biparental embryo reconstruction, the participation of both male and female gamete nuclei is essential for full-term development (McGrath and Solter 1984; Surani *et al*. 1984; Barra and Renard 1988). However, when maternal and paternal genomes are kept separately as haploid nuclei for several cycles, desynchronisation of one of them does not impair full-term development of embryos reconstructed using these nuclei (Surani *et al*. 1986). Moreover, reconstructed diploid mouse embryos from haploid parthenotes and androgenotes at the two-cell stage resulted in normal offspring (Barra and Renard 1988). This shows that male and female genomes can activate separately and still complete development when they are put together in a cytoplasm synchronised with the nuclei (Barra and Renard 1988). Our experiments show that male genomes obtained from androgenetic haploid embryos up until the 16-cell stage, generated by ICSI before enucleation, can still generate biparental embryos capable of developing to blastocysts when they are fused with MII oocytes.

When the reconstruction of biparental embryos was performed using EGFP-positive androgenetic haploid blastomeres, all embryos expressed the transgene and most showed transgene expression in all blastomeres. The transgene expression efficiency obtained with this new technique (100%) is higher compared with the other methods described previously. In the bovine, the pronuclear microinjection technique showed transgene expression in only 11.9% of embryos (Murakami *et al*. 1999), whereas an efficiency of 22.7% was obtained when ICSI-Tr was used (Pereyra-Bonnet *et al*. 2008). Another new technique to produce IVF transgene-expressing embryos achieved an efficiency of 68.4% after the injection of DNA–liposome complexes (Vichera *et al*. 2011). Development rates were not affected when the embryos were reconstructed with blastomeres that expressed EGFP, suggesting that transgene expression does not compromise *in vitro* embryonic development. Using this strategy, we could verify the cytoplasmic contribution of androgenetic haploid blastomeres in the reconstructed biparental embryos. In addition, we confirmed the sperm genome incorporation using sexed (Y chromosome) spermatozoa to generate androgenetic haploid embryos and reconstructed biparental embryos. The presence of the Y chromosome was detected by PCR and FISH.

Our results demonstrate that it is possible to multiply the haploid sperm genome from a single bovine spermatozoon and that these haploid sperm genome replicates can be used to generate biparental bovine embryos. Future research may consider the generation of stable haploid androgenetic cell lines as an alternative source of male gametes. The establishment of stable haploid cell lines from androgenetic embryos in the frog was reported by Freed and Mezger-Freed (1970). More recently, the generation of haploid embryonic stem cells was described in Mekada fish: after nuclear transfer into unfertilised oocytes, the haploid stem cells generated viable offspring capable of germline transmission (Yi *et al*. 2009). If this could be achieved in farm animals, it would have enormous potential because the analysis of recessive genes would be possible, facilitating genetic breeding programmes. Conversely, sperm genome cloning could be done by multiplying the androgenetic haploid line. This would generate an unlimited number of biparental embryos by combining these haploid cells with matured oocytes, creating a new combination of genetic traits from both parents similar to normal fertilisation (Yanagimachi 2005).

In conclusion, we have shown that it is possible to obtain a consistently high number of male haploid genome replicates from a single spermatozoon and to subsequently use these replicates to generate biparental embryos. This approach has enormous potential for use in livestock production, because it allows the sex of the sperm nucleus to be determined before its use in biparental embryo reconstruction. For this reason, it represents a new and advantageous way to determine the sex of future offspring. In addition to using genetic markers, we could also select certain favourable attributes before embryo formation. Biparental embryo reconstruction by fusion of haploid sperm genome replicates could improve transgenic animal production because, in addition to increasing the number of transgenic embryos produced from a single spermatozoon, it also generates embryos with a high rate of homogeneous transgene expression. Further studies are required to confirm the integration efficiency and the number of exogenous DNA insertions achieved using this technique. The possibility of introducing genes to modify the genome with high efficiency and homogeneous expression is projected to be a tool to increase

the generation of transgenic farm animals for livestock production, biomedicine and the pharmaceutical industry.

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