

Efficient Production and Cellular Characterization of Sheep Androgenetic Embryos

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Abstract

The production of androgenetic embryos in large animals is a complex procedure. Androgenetic embryos have been produced so far only in cattle and sheep using pronuclear transfer (PT) between zygotes derived from in vitro fertilization (IVF) of previously enucleated oocytes. PT is required due to the poor developmental potential of androgenotes derived from IVF of enucleated oocytes. Here we compare the development to blastocyst of androgenetic embryos produced by the standard pronuclear transfer and by fertilization of oocytes enucleated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium, without pronuclear transfer. The enucleation in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium abolished almost completely the manipulation-induced activation, significantly improving the development to blastocyst of the androgenetic embryos (IVF followed by PT; 18.6%: IVF only; 17.7%, respectively). Karyotype analysis of IVF revealed a similar proportion of diploid embryos in androgenetic and control blastocysts (35% and 36%, respectively), although mixoploid blastocysts were frequently observed in both groups (64%). Androgenotes had lower total cell numbers than control and parthenogenetic embryos, but more cells in ICM cells comparing to parthenogenotes (30.42 vs. 17.15%). Higher expression of the pluripotency-associated gene *NANOG*, and trophoblastic-specific gene *CDX2*, were also observed in androgenotes compared to parthenogenotes and controls. The global methylation profile of androgenetic embryos was comparable to controls, but was lower than parthenogenetic embryos. The cell composition and methylation pattern we have detected in monoparental sheep monoparental embryos are unprecedented, and differ considerably from the standard reference mouse embryos. Altogether, these findings indicate significant differences across species in the molecular mechanisms regulating early development of monoparental embryos, and highlights the need to study postimplantation development of androgenetic embryos in sheep.

Introduction

MONOPARENTAL EMBRYOS have a diploid chromosome complement that is derived from only the mother (parthenogenetic/gynogenetic) or from the father (androgenetic embryos). Parthenogenetic embryos are produced by interfering with the meiotic reduction of the chromosomes, leading to the development of a diploid embryos with maternal chromosomes only. Gynogenetic and androgenetic embryos are produced by reconstructing fertilized oocytes (zygotes) with maternal and paternal pronuclei, respectively. Monoparental embryos were originally produced to demonstrate the complementary parental contribution to development (McGrath and Solter, 1984; Surani et al., 1984). Since

then, the mouse has become the reference model for genomic imprinting and epigenetic dynamics in normal and monoparental embryos (Dean et al., 2001). However, recent data challenge the universality of the mouse model (Haaf, 2006). Indeed, it appears that major epigenetic modifications, such as the genome-wide demethylation waves during preimplantation development, differ remarkably across mammals (Beaujean et al., 2004a; Fulka et al., 2004; Loi et al., 2008; Shi et al., 2004). Therefore, the recent interest in the production of monoparental embryos from alternative animal models to study genomic imprinting and the parental contribution to embryo development is justified.

Parthenogenetic embryos are easily obtained by artificial activation of oocytes in all species, whereas the production of

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androgenetic embryos is more complicated, particularly in large animals. We have recently produced androgenetic sheep embryos (Matsukawa et al., 2007) according to a protocol originally established in the mouse (Kono et al., 1993), and subsequently applied to the bovine (Lagutina et al., 2004). Briefly, androgenetic embryos were produced by pronuclear transfer between diploid and haploid "zygotes" that were produced by *in vitro* fertilization (IVF) of enucleated oocytes. The pronuclear transfer (PT) was required because on the contrary of the mice (Kono et al. 1993), the development of IVF enucleated oocytes was very poor (1.8% blastocyst rate) (Matsukawa et al., 2007). Although we succeeded in increasing the number of androgenetic blastocysts, the procedure is cumbersome and time consuming, thus limiting the number of androgenetic embryos that can be produced. These difficulties can explain the lack of information on androgenetic embryos in large animals.

In this work, we first set out to identify and remove the causes of the poor development of IVF-derived sheep androgenotes. We then investigated the cellular composition [i.e., the number of inner cell mass (ICM) and trophoblast (TR) cells], the global methylation profile, and chromosome composition of androgenetic, parthenogenetic, and control IVF blastocysts. Finally, we analyzed the expression levels of the totipotency genes *OCT4* and *NANOG* and trophoblast-specific gene *CDX2* in androgenetic and parthenogenetic embryos.

We demonstrate that preventing the manipulation-induced oocyte activation dramatically improves the development of androgenetic embryos to blastocyst stage. We also show that androgenetic blastocysts, like IVF controls, are diploid with a significant proportion of mixoploidy. Unexpectedly, androgenetic embryos have a higher ICM/TR cell ratio, different global methylation profiles, and expression of totipotency-associated genes than parthenogenetic embryos.

Materials and Methods

All chemicals, unless otherwise indicated, were purchased from Sigma Chemicals Co. (St. Louis, MO).

In vitro maturation

Sheep ovaries were obtained at local abattoirs and transferred at 37°C to the laboratory within 2 h. Cumulus-oocyte complexes were recovered by aspirating the follicles and cultured in four-well Nunc plastic dishes, filled with 500 μ L of TCM-199 medium without covering oil (Gibco Life Technologies, Rockville, MD) in humidified air with 5% CO₂ at 38.5°C for 24 h. TCM-199 was supplemented with 2 mM glutamine (Gibco), 100 μ M cysteamine, 0.3 mM sodium pyruvate, 10% fetal bovine serum (Gibco), 5 μ g/mL FSH (Ovagen, ICP, Auckland, New Zealand), 5 μ g/mL LH, and 1 μ g/mL estradiol. Methods for *in vitro* embryo production were as previously described (Ptak et al., 1999, 2002).

Assessment of manipulation-induced activation

Matured oocytes were stripped of granulosa cells by a combined treatment with 300 U/mL hyaluronidase and gentle pipetting. For the determination of manipulation-induced oocyte activation, 270 oocytes were divided in four

groups. Group A ($n=85$) was handled in normal manipulation medium [i.e., TCM 199 with antibiotics, 4 mg/mL bovine serum albumin (BSA), 7.5 μ g/mL Cytochalasin B] and group B ($n=60$) manipulated in Ca²⁺/Mg²⁺-free medium [i.e., Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) with antibiotics, 4 mg/mL BSA, and 7.5 μ g/mL Cytochalasin B]. For both groups manipulation consisted in the removal of a small piece of cytoplasm (opposite to the metaphase plate), as normally carried on during enucleation. In groups A1 and B1, oocytes were exposed to normal manipulation medium (group A1, $n=83$) or to Ca²⁺/Mg²⁺-free medium (group B1, $n=42$), but without manipulation. Oocytes were then cultured in standard conditions for 10–15 h, then fixed in 3:1 acetic acid:methanol overnight, and stained with 2% aceto-orcein for pronuclear identification.

Androgenetic embryo production

Androgenetic embryo production by IVF followed by pronuclear transfer (IVF-PT): *Enucleation of oocytes*. At 22 h of maturation, oocytes were denuded of granulosa cells in the presence of hyaluronidase. Oocytes with extruded first polar bodies were incubated in 10 mg/mL Hoechst 33342 in TCM 199 for 10 min, and then enucleated by aspirating the metaphase II plate in TCM 199 medium with 4 mg/mL BSA, antibiotics and 7.5 μ g/mL cytochalasin B, under UV light with a Narishige Micromanipulator fitted to an inverted Nikon microscope (Melville, NY).

IVF. The IVF procedure used for enucleated oocytes is as described below but with higher concentration of spermatozoa (25×10^6 mL).

Pronuclear Transfer IVF/PT (enucleated, *in vitro* fertilized, then pronuclear transfer). Ten hours after IVF, embryos ($n=118$) were incubated in HEPES-buffered TCM 199 with 7.5 μ g/mL cytochalasin B for 10 min, then centrifuged at $12,000 \times g$ for 5 min in order to visualize the pronuclei. Single pronuclei surrounded by a small volume of cytoplasm were aspirated with a beveled pipette from diploid androgenotes and transferred in the perivitelline space of haploid androgenotes. The reconstructed embryos were manually aligned under the micromanipulator and electro-fused using a BTX ECM 830 apparatus in 0.27 M mannitol solution, containing 50 μ M CaCl₂ and 100 μ M MgCl₂, by a single direct-current pulse (80 μ sec) of 1.2 kV/cm.

Androgenetic embryo production by IVF only (enucleated and *in vitro* fertilized). Enucleation of oocytes ($n=190$) was carried out as above, but using Ca²⁺/Mg²⁺-free manipulation medium. IVF was as described below but with higher concentration of spermatozoa (25×10^6), as above. A subset of presumptive androgenetic embryos ($n=61$) was collected at 10 h post-IVF, incubated in TCM 199 with BSA and antibiotics and 7.5 μ g/mL Cytochalasin B for 10 min and centrifuged at $12,000 \times g$ for 5 min to stratify the cytoplasm. The number of pronuclei in these embryos was scored under a microscope. The others were cultured to blastocyst stage.

Parthenogenetic embryo production

In vitro matured metaphase II oocytes ($n=190$) were activated with a combined treatment of ionomycin and

6-dimethylaminopurine, in SOF medium, as previously described (Loi et al., 1998).

IVF of control embryos

Matured oocytes ($n=443$) were partially stripped of cumulus cells by repeated pipetting. Frozen semen was rapidly thawed at 37°C and washed twice by centrifugation at $500 \times g$ for 5 min with bicarbonate-buffered SOF with 4 mg/mL BSA. IVF was carried out in 50 μ L drops, using 5×10^6 sperm/mL and a maximum of 15 oocytes per drop, at 38.5°C in 5% CO₂ for 20 h. The IVF medium was bicarbonate-buffered SOF enriched with 20% (v/v) heat-inactivated oestrous sheep serum, 2.9 mM Ca²⁺ lactate, and 16 μ M isoproterenol.

In vitro culture

All classes of embryos were transferred into 20 μ L drops of SOF enriched with 1% (v:v) Basal Medium Eagle (BME), essential amino acids, 1% (v:v) Minimum Essential Medium (MEM), nonessential amino acids (Gibco), 1 mM glutamine, and 8 mg/mL fatty acid-free BSA (SOFaa-BSA). Zygotes were cultured in a humidified atmosphere of 5% CO₂, 7% O₂, 88% N₂ at 38.5°C, and the medium changed at day 3 (supplemented with amino acids) and day 5 (supplemented with 10% FBS). Cleavage was assessed at day 2 and blastocyst formation was recorded at days 7 and 8.

Differential cell staining of parthenogenetic, androgenetic, and control blastocysts

Embryos were differentially stained as described by Thouas et al. (2001), with minor modification. Briefly, blastocysts were incubated in 500 mL of solution 1 (PBS with 1% Triton X-100 and 100 mg/mL propidium iodide) for 20 sec. Blastocyst were then directly transferred to 500 mL of solution 2 (100% ethanol with 25 mg/mL bisbensimide Hoechst 33258) and stored at 4°C overnight. Blastocysts were then mounted onto a microscope slide in a drop of glycerol and flattened with a cover slide. Cell counting was performed directly on an inverted microscope fitted with an ultraviolet lamp and excitation filter (460 nm for blue and red fluorescence).

Karyotype analysis of blastocyst stage embryos

Blastocyst stage monoparental and control embryos were incubated with 0.05 μ g/mL Colcemid (Sigma) for 3 h, then transferred to hypotonic solution, 0.8% sodium citrate (37°C) for 10 min and then into 0.56% KCl (37°C) for another 10 min. Blastocysts were fixed in 3:1 methanol:acetic acid for 2 h. Spreading was done by dropping a 1:1 methanol:acetic acid solution under an inverted phase contrast microscope. Slides were stained with 4% Giemsa for 5 min. Photographs were taken using a 100 \times objective and immersion oil.

Immunodetection of global DNA methylation

Blastocyst stage embryos were washed five times in 0.4% PBS/PVP, fixed in 4% paraformaldehyde for 15 min, and then permeabilized in 0.1% Triton X-100 for 30 min. Blastocysts were washed again and hydrolyzed in 4N HCL for 10 min, neutralized in 100 mM Tris/HCl (pH=8.5) for 15 min, washed in PBS/0.4% PVP (5 min \times 3), then blocked in

PBS plus 1% BSA, 0.05% Tween 20 at 4°C overnight. Embryos were incubated with a mouse anti-5-Methyl Cytidine antibody (Santa Cruz Biotechnology sc-56615, Santa Cruz, CA) at room temperature for 2 h, washed in blocking medium three times and incubated with goat antimouse IgG FITC conjugate antibody (Sigma F9137) at room temperature for 1 h. Mounted specimens were analyzed with an epifluorescence microscope (Nikon). The quantification of the fluorescence intensity for 5-MethylCytidine determination was carried out with the software ImageJ (Image processing and Analysis in JAVA).

Gene expression analysis

Isolation of mRNA and RT-PCR. Poly(A)⁺ RNA was isolated from single frozen (PBS and PVP 0.4%) blastocysts using the Dynabeads mRNA DIRECT Kit (Invitrogen DYNAL AS, Oslo, Norway). The procedure was carried out according to the manufacturer's instructions, with minor modifications. Briefly, single embryos (stored at -80°C) were lysed in 150 μ L lysis/binding buffer (100 mM Tris-HCl pH=8.0, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DDT). Subsequently, 10 μ L of prewashed Dynabeads oligo (dT)25 was added to each tube containing single embryos, and after a short incubation at room temperature the beads were separated using a magnetic separator (DYNAL MPC-P-12 magnet; Invitrogen DYNAL). Beads/mRNA complexes were washed once with 100 μ L washing buffer A (10 mM Tris-HCl pH=8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS) and twice with 100 μ L washing buffer B (10 mM Tris-HCl pH=8.0, 0.15 M LiCl, 1.0 mM EDTA). Poly(A)⁺ RNA was then eluted from the beads by incubation in 10 μ L of 10 mM Tris-HCl at 70°C for 3 min. RT was carried out using 80% of the eluted Poly(A)⁺ RNA in a total volume of 20 μ L using the QuantiTect Reverse Transcription Kit (Qiagen, Chatsworth, CA). Briefly, samples were first incubated in gDNA Wipeout Buffer at 42°C for 2 min to remove contaminating genomic DNA. Then, according to the manufacturer's recommended protocol, the RT reaction was carried out at 42°C for 15 min followed by a final step at 95°C for 3 min to inactivate the transcriptase.

Real-time PCR. The obtained cDNA was diluted (1:2) and then used for real-time PCR amplification to quantify the expression of *NANOG*, *OCT4*, and *CDX2* using the following primer pairs: *NANOG*: (*Ovis Aries*, FJ 970651) forward: *aaaccattgtcccacatctgc*; reverse: *tagctgaggttcaagatgttgg*; *OCT4*: (*Bos Taurus*, NM_174580) forward: *aagctcctaaagcagaagagg*; reverse: *tctctgtgtgtcagcttcc*; *CDX2*: *Bos Taurus* (XM_871005.3) forward: *aagacaaataccgggtcgtg*; reverse: *ctctcgggttctgaaccaa*.

The reaction was performed using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen) and ABI PRISM 7900 Real-Time PCR System (Applied Biosystems, Bedford, MA) according to the manufacturer's instructions. Amplification conditions were: 2 min at 50°C, 2 min at 95°C, followed by 45 cycles at 95°C for 15 sec and 56°C for 60 sec. To avoid false-positive signals, dissociation-curve analyses were performed at the end of each run; the conditions of the dissociation step were 15 sec at 95°C, 15 sec at 60°C, and 15 sec at 95°C. Relative gene expression data were calculated using the comparative threshold cycle (Ct) method with β -Actin as endogenous control.

Statistical analysis

Embryo development. Differences between the experimental groups were verified using the chi-square test. A value of $p < 0.05$ was considered significant.

Pronuclear stage and cell numbers. Differences between the experimental groups were verified with the one-way analysis of variance (ANOVA) test (GraphPad Prism). A value of $p < 0.05$ was considered significant.

Image analysis. The quantification of the fluorescence intensity for 5-MethylCytidine determination was carried out with the software ImageJ (Image processing and Analysis in JAVA). Differences between the experimental groups were verified with the one-way ANOVA test (GraphPad Prism). A value of $p < 0.05$ was considered significant.

Gene expression analysis. Data reported are the mean (\pm SEM) of at least five independent determinations, each in triplicate. Statistical analysis was performed with the non-parametric Mann-Whitney *t*-test (GraphPAD Prism). Differences were considered significant when $p < 0.05$.

Results

Assessment of manipulation-induced oocyte activation

To evaluate whether and to what extent the manipulation required to enucleate the oocytes activates them, we compared oocytes that were sham manipulated (aspiration of a portion of cytoplasm near the first polar body, group A) with oocytes that were simply maintained in the same medium with $\text{Ca}^{2+}/\text{Mg}^{2+}$, but unmanipulated (group A1). More than half of the group A oocytes (56%) showed well organized pronuclei, indicative of activation induced by the manipulation, whereas activation was only observed occasionally in the control group A1 (0.6%) (Table 1). Conversely, when $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free manipulation medium was used, activation was strongly reduced (3.3%) in sham manipulated oocytes (group B), and completely abolished in the control group (group B1, Table 1).

Number of pronuclei in zygotes obtained by IVF of oocytes enucleated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS

Based on these results, we next evaluated the fertilization rate of oocytes that were enucleated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free

TABLE 1. ACTIVATION FREQUENCIES (ASSESSED ON THE PRONUCLEAR ORGANIZATION) IN SHAM-MANIPULATED AND CONTROL OOCYTES THAT WERE FIXED IN 3:1 ACETIC ACID:METHANOL AND STAINED WITH ACETO-ORCEIN 2%

Group	Treatment	No. oocytes	Activated (pronuclei) (%)
A	TCM 199, sham manipulated	85	48 (56) ^a
A1	TCM199, control	83	4 (0.6) ^b
B	$\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS—sham	60	2 (3.3) ^b
B1	$\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS—control	42	0 (0)

Different superscripts indicate $p < 0.0001$.

medium, by counting the number of pronuclei 10 h after IVF. Most of the oocytes were fertilized (82%), with 26.2% haploid androgenotes cone pronucleus (PN), 52.42% diploid (two PN), and only a minority triploid (three PN, 3.2%; Table 2).

Development to blastocyst stage of IVF only (enucleated and in vitro fertilized) and IVF/PT (enucleated, in vitro fertilized, plus pronuclear transfer) androgenotes at days 7–8

We next compared the development potential to blastocyst stage of androgenetic embryos that were produced either by IVF of oocytes enucleated in normal manipulation medium followed by Pronuclear Transfer (IVF-PT) (Matsukawa et al., 2007), or by IVF of oocytes enucleated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium (IVF only). Biparental IVF (control) and parthenogenetic embryos were produced in every replicate ($n=6$) as controls. No differences were found between IVF only (Fig. 1) and IVF-PT androgenotes in their capacity to reach blastocyst stage (18.60 and 17.70%, respectively); the development to blastocyst stage of both groups of androgenotes was slightly lower than control and parthenogenetic embryos (20.7 and 18.9%, respectively; Table 3).

Assessment of cell composition and chromosome constitution of the IVF only androgenetic embryos

Staining with propidium iodide and Hoechst 33342 to differentiate between ICM and TR cells showed that the total cell number was comparable in control and parthenogenetic blastocysts ($n=127 \pm 28$ and $n=129 \pm 117$, respectively), but was significantly reduced in androgenotes ($n=100 \pm 51$) (Table 5). Moreover, androgenetic embryos showed a higher ICM/TR cell ratio than parthenogenetic embryos (Table 5).

We have analyzed the chromosome composition of IVF only androgenetic blastocysts ($n=56$) and control embryos ($n=23$). The analysis of the metaphase spreads revealed that 35.7% of control and 30.43% and androgenetic embryos were euploid ($2n=54$) (Fig. 2) and that in both groups mixoploidy was frequent (64.3% control and 69.57% androgenetic embryos; Table 4).

DNA methylation of monoparental and control embryos

The global methylation profile (detected using a mouse anti-5-Methyl Cytidine antibody) of androgenotes overlapped with that of control embryos, although it was significantly higher in parthenogenetic embryos (Fig. 3B). ICM and trophoblast cells were equally methylated in all classes of embryos (Fig. 3A).

TABLE 2. NUMBER OF PRONUCLEI IN LIVING ANDROGENETIC EMBRYOS PRODUCED BY IVF OF OOCYTES ENUCLEATED IN $\text{Ca}^{2+}/\text{Mg}^{2+}$ -FREE MEDIUM (OBSERVED AFTER CENTRIFUGATION AT $12,000 \times G$, 10 HOURS AFTER IVF)

No. of pronuclei	No. zygotes (%)
0 PN	10/61 (16.4%)
1 PN	16/61 (26.2%)
2 PN	32/61 (52.4%)
3 PN	2/61 (3.37%)

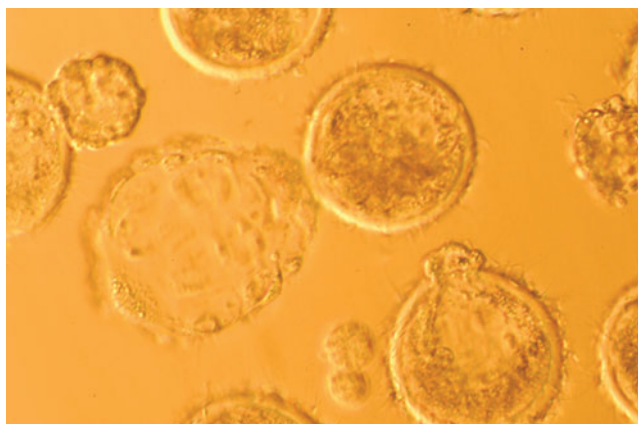


FIG. 1. Hatching androgenetic blastocysts at day 7 (40×).

Analysis of OCT4, NANOG, and CDX2 expression in individual blastocysts

The totipotency-associated genes *OCT4* and *NANOG* were expressed at higher level in androgenetic embryos, particularly *NANOG*, comparing to parthenogenetic ones (Fig. 4A–B). *CDX2*, a master gene regulating early differentiation of trophoblastic cells, was also expressed at higher level in androgenotes, comparing to parthenogenetic embryos (Fig. 4C).

Discussion

In this work we have demonstrated that the production of sheep androgenetic embryos is improved by circumventing oocyte activation during the enucleation. Originally reconstructed by PT between zygotes (Surani et al., 1984), the production of mice androgenetic embryos has been further simplified, first by fertilizing enucleated oocytes (Kono et al., 1993), and more recently by the direct injection of two round spermatids into enucleated oocytes (Miki et al., 2009; Zhao Q et al., 2010). Thus, although making androgenetic embryos is a robust procedure in mice, the situation is more complicated in other animals, particularly in sheep, where IVF of enucleated oocytes gave poor results (Matsukawa et al., 2007).

Here we suggest that the low developmental potential of sheep androgenotes obtained by IVF (Matsukawa et al., 2007) might be due to the oocyte activation caused by the enucleation procedure, which inhibits the penetration of the second spermatozoa, essential for diploidization. Moreover, we have demonstrated that the enucleation is carried out in Ca^{2+}/Mg^{2+} -free medium abolishes almost completely the activation of the oocytes (Table 1).

TABLE 3. *IN VITRO* DEVELOPMENT TO BLASTOCYST STAGE OF CONTROL AND MONOPARENTAL EMBRYOS

Group	No. oocytes	Fully expanded blastocyst
Control IVF	443	92 (20.7%) ^a
Andro IVF Only	129	24 (18.6%) ^a
Andro IVF/PT	118	21 (17.7%) ^a
Parthenogenetic	190	36 (18.9%) ^a

Different superscripts indicate $p < 0.0001$.

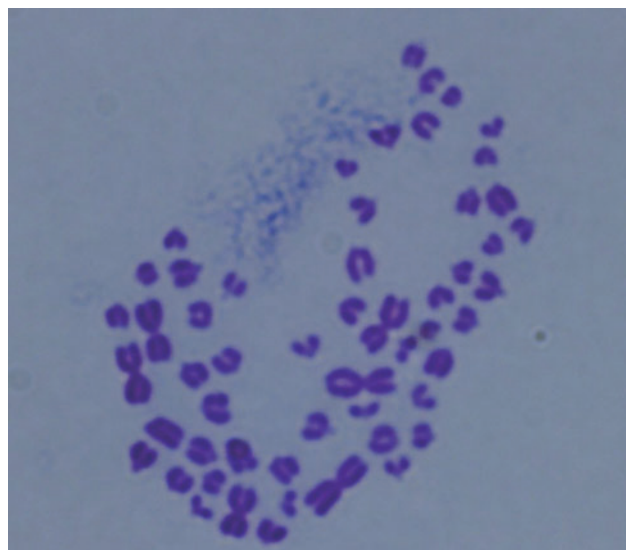


FIG. 2. Normal ($n=54$) karyotype in sheep androgenetic blastocyst (×100).

It is known that a mechanical stress triggers oocyte activation in amphibian oocytes (Yang and Sachs, 1988). Because stretch-activated ion channels are sensitive to pressure as well as suction (Yang and Sachs, 1990), it is possible that the suction applied to the oolemma during enucleation might open the stretch-activated channels, thus triggering the Ca^{2+} dependent activation. Stretch-activated ion channels are present at the oocyte membrane of *Xenopus Levis* (Yang and Sachs, 1988) and in a wide range of cells (Sachs, 2010), but no information is available concerning mammalian oocytes. If we assume that stretch-activated ion channels are present in sheep oocyte, it is plausible to find them on the mouse oocyte. How can we then explain the difference in the developmental capacity of sheep and mice IVF androgenotes (1.8 vs. 43%, respectively), given that enucleation of mice oocytes was conducted in M2, a Ca^{2+} containing medium (Kono et al., 1993)? A possible explanation might be the differences in the polyspermic block between species. (Gardner and Evans, 2006; Gardner et al., 2007). The polyspermic block occurs rapidly in sheep (Gardner and Evans, 2006), whereas mice oocytes can be penetrated by a second spermatozoa even 2h after the first one (Gardner et al., 2007). The promptness of the polyspermic block in sheep oocytes might therefore explain the low frequency of development of IVF androgenetic embryos, given that only diploid androgenotes (dispermic) develop to blastocyst stage.

The simple measure of performing the enucleation in Ca^{2+}/Mg^{2+} -free manipulation medium significantly

TABLE 4. CHROMOSOME COMPOSITION OF CONTROL AND IVF-ONLY ANDROGENETIC BLASTOCYSTS

Group	Diploid (%)	Mixoploid (%)
IVF blastocysts	20/56 (35.7%) ^a	36/56 (64.3%) ^a
Androgenetic blastocysts	7/23 (30.43%) ^a	16/23 (69.5%) ^a

Different superscripts indicate $p < 0.0001$.

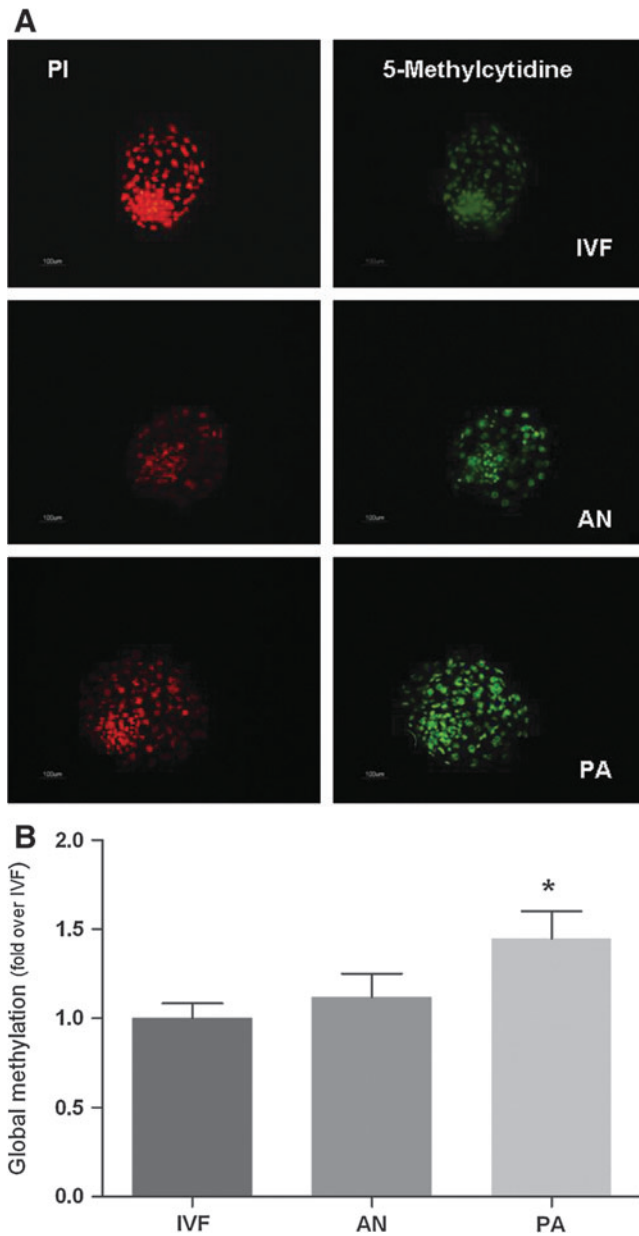


FIG. 3. Global DNA methylation of monoparental (AN, PA) and biparental (IVF-Control) sheep blastocysts. **(A)** Immunostaining anti-5-methyl. **(B)** Semiquantitative analysis of fluorescence intensity. Different superscripts indicate $p < 0.001$.

improved the development of IVF-only androgenotes, which reached blastocyst stage at rates comparable to those produced by IVF followed by Pronuclear Transfer (IVF-PT) (18.60 and 17.70%, respectively; Table 3).

The development of both classes of androgenetic embryos to blastocyst stage was lower comparing to control and parthenogenetic embryos (Table 5), consistent with the presence of the lethal YY combination in about one quarter of embryos (Latham et al., 2000). All the IVF-only androgenetic blastocysts were diploid, although a significant proportion of mixoploid embryos were also observed. The chromosome mixoploidy were found in androgenetic and control bipa-

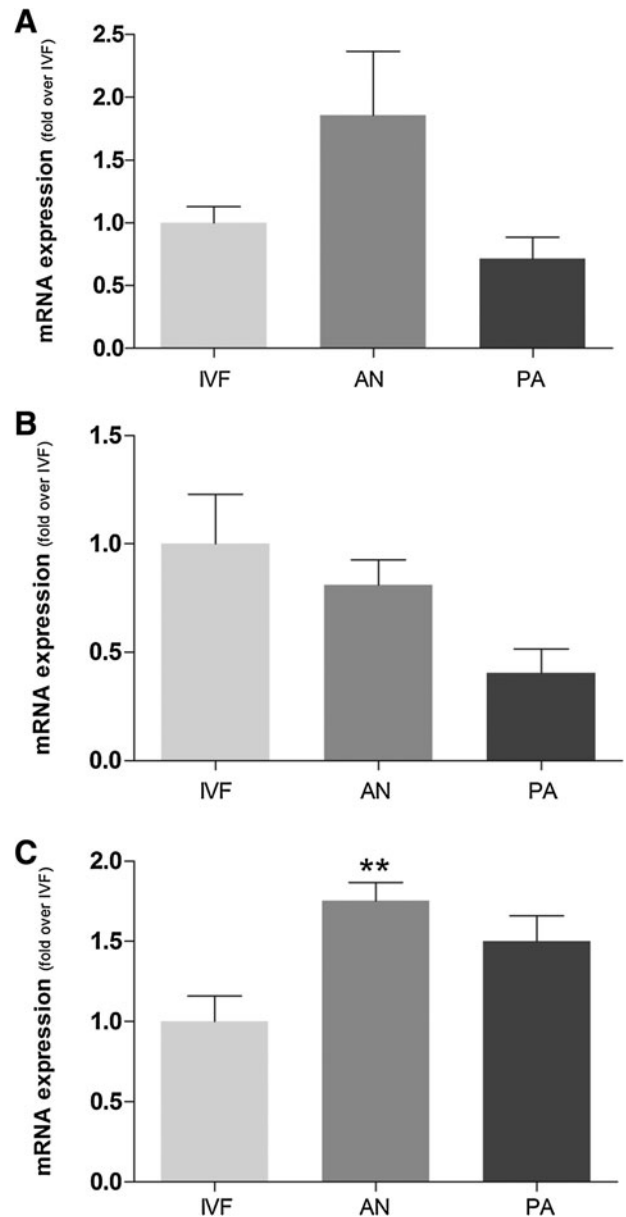


FIG. 4. Expression level of *OCT 4* **(A)**, *NANOG* **(B)**, and *CDX2* **(C)** in blastocyst stage monoparental (AN, PA) and biparental (IVF-control) embryos. Different superscripts indicate $p < 0.008$.

rental embryos (69 and 64%, respectively). These data are in agreement with previous work conducted in the bovine (Alexander et al., 2006; Van De Velde et al., 1999). The real significance of the chromosomal mixoploidy is under intense investigation at the moment, but still remains unclear (Alfarawati et al., 2010; Curlej et al., 2010; Gomez et al., 2009; Hornak et al., 2009). The fact that chromosomal abnormalities are present in control IVF (this study) and in normal human embryos (Vanneste et al., 2009) suggests that a certain degree of aneuploidy/mixoploidy should be considered as a default in early development, although further studies will be required to dissect the aneuploidy/mixoploidy between ICM and trophectoderm.

TABLE 5. DIFFERENTIAL STAINING OF MONOPARENTAL (AN, PA) AND BIPARENTAL (IVF-CONTROL) BLASTOCYSTS ON DAY 8 OF DEVELOPMENT

Blastocysts	No. embryos	Cell number			Ratio ICM/TR
		Total	ICM (%)	TR (%)	
IVF	6	127±28	38±9 (29.88±0.09)	89±24 (70.66±0.09)	0.44±0.13 ^a
AN	10	100±51	27±14 (30.42±0.18)	73±42 (69.86±0.17)	0.59±0.7 ^b
PA	10	129±117	25±15 (17.15±0.13)	105±95 (82.05±0.14)	0.44±0.2 ^a

Different superscripts indicate $p < 0.0031$.

The androgenetic embryos had a reduced number of cells and higher ICM/TR cells ratio than parthenogenetic embryos (0.59 and 0.28, respectively). A lower total cell number was also described in bovine androgenotes, but the proportion of ICM/TR cells was not evaluated (Lagutina et al., 2004). The higher ICM "cellularity" of the androgenotes might be due to their higher expression of the totipotency-associated genes *OCT4* and *NANOG* in comparison to the parthenogenetic embryos (Fig. 4A and B).

In their original article, Surani and coworkers (1984) reported that mouse androgenotes showed a preferential proliferation of trophoctoderm-derived cells and defective development of ICM-derived structures. In preimplantation sheep androgenetic embryos, we have observed an opposite trend, with a higher number of ICM cells and a very poor trophoctoderm. This finding suggests that major phenotypic differences between species in preimplantation development of monoparental embryos might exist, although further studies on postimplantation development of androgenetic embryos are required. Alternatively, the increased ICM/TR ratio in sheep androgenotes might represent a cell dosage compensation, resulting from a counting mechanism that operates in preimplantation embryos and finalized to counterbalance the absence of the maternal contribution. The reduced proliferation of trophoblastic cells in sheep androgenotes occurs despite the unregulated expression of *CDX2* (Fig. 4), the primary gene regulating trophoctoderm differentiation and proliferation (Strumpf et al., 2005). The increased expression of *CDX2* observed in androgenotes might result from either a compensatory mechanism, or from the genetic noise caused by the aneuploidy (Birchler et al., 2005) frequently observed in embryos. Whatever induced, the increased transcription of *CDX2* might be itself responsible for the defective proliferation of trophoblastic cells (Xie et al., 2010).

Finally, the global methylation profile of androgenotes overlapped with the IVF controls, whereas the parthenogenetic embryos presented higher level of methylation (Fig. 3). Both ICM and trophoblast were methylated, a feature that is conserved in other large animals (Haaf, 2006; Loi et al., 2008; Young and Beaujeau, 2004), but not in mice, where DNA methylation is confined to the ICM (Reik et al., 2003). Species-specific differences in the implantation timing and dynamics might account for the variations in methylation pattern at blastocyst stage. The higher methylation of parthenogenetic embryos was surprising. Because the paternal pronucleus is not actively demethylated in sheep zygotes (Beaujeau et al., 2004b), we would have expected higher methylation in androgenetic embryos, as they derive from two spermatozoa, normally more methylated than the oocyte (Reik et al., 2003). Again, this might reflect differences in the

epigenetic asset of preimplantation development of monoparental embryos across species.

In conclusion, we have demonstrated that it is possible to significantly improve the developmental potential of diploid androgenetic sheep embryos by simply preventing the manipulation-induced oocyte activation, a protocol that might be easily transferred to other species, including human. This procedural simplification empowered us to dispose of suitable numbers of androgenotes that allowed us to characterize the difference in cell composition and methylation profile between androgenetic, parthenogenetic, and control, fertilized embryos in sheep. The overall picture we have observed diverge significantly from the data published in monoparental mouse embryos, a finding that highlights the importance of extending the investigation on postimplantation development of androgenetic conceptuses in sheep.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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