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ZP-free enucleation for efficient haploid androgenetic embryo production and the identification of an activation-dependent barrier to hemiclone development in bovine

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Abstract

Purpose This study aimed to establish a simplified and efficient *zona pellucida* (ZP)-free protocol for bovine haploid androgenetic embryo (hAE) production and to identify molecular barriers during the embryonic genome activation (EGA) window that limit the development of reconstructed hemiclones.

Methods hAE were generated by ZP-free enucleation of zygotes following short *in vitro* fertilization (IVF). Haploidy was confirmed by chromosome analysis and commercial microsatellite profiling. Hemiclones were produced by fusing hAE blastomeres with mature oocytes followed by activation (FA) or with pre-activated oocytes (AFA). Gene expression of markers regulating chromatin status, RNA processing, metabolic activity, and pluripotency was evaluated by qRT-PCR at the 8-16 cell and blastocyst stages.

Results ZP-free enucleation simplified nuclear identification by removing adherent sperm, yielding 76.9% haploid blastomeres. Microsatellite analysis provided complete monoallelic profiles in blastocysts, validating the platform for pre-zygotic genetic screening (PZGS) using standard market tools. Hemiclone blastocyst development was markedly lower in both FA (4.6%) and AFA (1.2%) groups compared to parthenogenetic (PA) control (23.4%). Molecular analysis at the 8-16 cell stage revealed

that artificial activation (FA and PA) disrupts EGA programming and metabolic status compared to naturally activated groups (hAE and IVF).

Conclusion ZP-free enucleation provides a scalable and efficient platform for bovine hAE production and practical PZGS. While the altered gene expression resulting from artificial activation may have detrimental consequences for development, the marked developmental disparity between FA and PA embryos suggests that inherent constraints of the hemiclone condition represent the primary barrier to developmental competence.

Key words: cloning, androgenetic, parthenogenetic, hemiclones

Introduction

Androgenetic embryos (AE) contain exclusively the paternal genome and may be haploid or diploid, depending on their genetic constitution. In mammals, AE can be produced *in vitro* by mechanical removal of the maternal nucleus and have become powerful tools for research, animal production, and medicine. AE have been used to analyze the expression of imprinted genes in species lacking polymorphisms that allow discrimination of allele-specific expression [1], to derive embryonic stem cell lines [2], and to generate biparental embryos by using their blastomeres as gametes [3,4]. Moreover, uniparental embryos have been proposed as an alternative source for genome banking [5] and for the derivation of human pluripotent cell lines with therapeutic purposes [6].

To date, AE have been produced in mice [7], sheep [8], rabbits [9], cattle [10], humans [11], pigs [1] and rats [12]. However, most domestic species including bovine, present two specific challenges for the selective elimination of maternal DNA, compared with

mouse and human. First, the high lipid content of the oocyte cytoplasm requires DNA staining and subsequent exposure to UV light for accurate identification of the pronuclei (PN) [10]. Second, maternal and paternal PN are similar in size, which hinders discrimination of their parental origin. Therefore, most studies in these species have produced androgenetic embryos by enucleation of mature oocytes followed by fertilization of the ooplast by either IVF [1,10,13-15] or ICSI [10,16,17]. IVF, on the one hand, results in high fertilization and embryo development rates in cattle and other species, but the variable polyspermy rates results in uncertainty regarding the ploidy of the generated embryos. On the other hand, ICSI provides precise control over the number of sperm injected into the ooplasm and, consequently, over the ploidy of the resulting embryo, but is associated with low developmental rates in bovine. In fact, haploid androgenetic blastocysts in cattle have been obtained by IVF [10,18,19] whereas only one study has reported hAE blastocysts produced by ICSI [19] using a piezo-drill, suggesting that developmental potential is influenced by the fertilization method.

Haploid androgenetic embryos (hAE) have been proposed as means to clone individual gametes, because each of their blastomeres contains an identical genetic copy of the sperm cell used to generate the embryo. This approach has been evaluated in mice [4,20,21], rats [12], and cattle [18,22] to perform pre-zygotic genetic screening. The genetic analysis of individual blastomeres from a single hAE allows the selection of those carrying the paternal genome of interest for subsequent generation of biparental embryos. This technique holds great potential for livestock production and human reproduction. Genomes carrying valuable production traits can be selected to generate multiple calves (hemiclones, in the case of animals derived from the same hAE) or,

alternatively, human embryos can be produced using a copy of a sperm cell that does not contain a certain mutation or defective gene that is carried by the father [11].

Despite the great potential of this technique, reconstructed embryos generated using haploid androgenetic blastomeres are characterized by low developmental rates [18]. This limitation may result from inadequate cell-cycle synchronization between the haploid blastomere and the recipient cytoplast at the time of nuclear transfer. Considering that embryonic cells exhibit particularly long S phases [23], a high proportion of androgenetic blastomeres may be in this stage at the time of fusion with mature oocytes. Based on studies in somatic cell nuclear transfer, such asynchrony may lead to DNA disorganization and fragmentation [24,25], resulting from premature condensation of the replicating nucleus induced by high MPF levels in the MII-arrested ooplast [24]. Therefore, numerous nuclear transfer studies have evaluated the effect of oocyte activation prior to fusion with the donor cell, under the premise that decreasing MPF levels renders the ooplast receptive to nuclei at different cell-cycle stages ("universal receptor") [26]. Indeed, oocyte pre-activation has been shown to positively affect embryonic development of clones in sheep [27], goats [28], rabbits [29], and cattle [30], although results in mice remain controversial.

To the best of our knowledge, with the exception of a single report in mice [4], protocols for the production of hAE by micromanipulation use oocytes or zygotes with an intact zona pellucida (ZP). However, ZP–free micromanipulation in cloning protocols has been shown to be considerably simpler without compromising efficiency [31]. These studies were based on the premise that the ZP is not essential for embryonic development *in vitro* [32]. Accordingly, this approach could be applied to hAE production to simplify the procedure and improve efficiency.

The aim of this study was to establish a simplified and highly efficient ZP-free protocol for hAE production by optimizing enucleation. In addition, the effect of oocyte pre-activation as a strategy to overcome the developmental barrier in hemiclones was evaluated. Finally, gene expression analyses were performed in early embryos and blastocysts to identify factors disrupting developmental competence in reconstructed hemiclones.

Materials and methods

Oocyte collection and in vitro *maturation*

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25-30°C. Cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles of 2-8 mm diameter, into HEPES-buffered Tyrode's albumin lactate pyruvate (TALP-H), using a 21 gauge needle. Oocytes surrounded by at least three layers of granulosa cells were *in vitro* matured (IVM) in 100 µl droplets of bicarbonate-buffered TCM-199 (31100-035; Gibco), containing 10% v/v FBS, 10 µg/mL follicle stimulating hormone (Folltropin®, Bioniche, Belleville, ON, Canada), 0.3 mM sodium pyruvate (P2256), 100 mM cysteamine (M9768) and 2% v/v ATB, for 21-23 h in humidified air with 6.5% CO_2 at 39°C.

In vitro *fertilization (IVF) procedure*

The IVF procedure was performed as described previously [33]. Briefly, frozen semen was thawed in a 35°C water bath for 30 s. Sperm were washed twice by centrifugation at 400 g in Brackett-Oliphant medium (BO) supplemented with 5 mM caffeine (C4144) and 20 IU/mL heparin (H3149). Spermatozoa were adjusted to $28x10^6$/mL and diluted to half concentration ($14x10^6$/mL) with BO containing 10 mg/mL bovine serum

albumin (BSA) (A6003). Spermatozoa were then co-incubated with IVM COCs in 100 µl droplets of the sperm suspension for 3-5 h (depending on the experiment, see below) at 39 °C and 6.5% CO_2 in humidified air.

After IVF, presumptive zygotes were vortexed for 30 s in hyaluronidase (H-4272; 1 mg/mL) in Dulbecco´s phosphate buffer saline to remove cumulus cells, and washed twice in TALP-H. Presumptive zygotes were evaluated and only those with one or two polar bodies were subsequently used.

Production of hAE

Haploid androgenetic embryos were produced by mechanical enucleation of the maternal genome using Narishige hydraulic micromanipulators (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Diaphot microscope (Nikon, NJ, USA). *In vitro* produced zygotes were stained with 5 µg/mL Höechst 33342 for 15 min and briefly exposed to UV light to visualize genetic material. Two different strategies were used: (1) enucleation of zygotes with an intact *zona pellucida* (ZP), or (2) enucleation of *zona*-free (ZF) zygotes. For ZP zygotes, a 20 µm diameter pipette with bezel was used. In the case of ZF enucleation, ZP was first removed using 1.5 mg/ml pronase (P-8811) for 2-5 min on a warm plate and enucleation was performed using a 20 µm diameter blunt pipette. hAE were then *in vitro* cultured as described below.

In vitro *embryo culture*

All embryos were cultured in SOF medium supplemented with 2.5% FBS in a system similar to that of the well-of-the-well (WOW) [34], whereby microwells were produced using a heated glass capillary slightly pressed to the bottom of a Petri dish and then covered with a 100 µL droplet of culture medium (20 to 30 WOW in each droplet, one

138 embryo per WOW). Culture conditions were 39°C and 6.5% CO_2 in humidified air.
139 Cleavage, morulae and blastocysts were evaluated on days 2, 5 and 7-8, respectively.

140 *Chromosome analysis*

141 *Zona*-free four-cell-stage embryos were cultured for 6 h in SOF medium containing 0.1
142 µg/mL Nocodazole (M1404) then transferred to a trisodium citrate hypotonic solution
143 (F71497; 0.8% w/v in distilled water) for 5 min at 37°C. As the embryos lacked ZP,
144 some were disaggregated. Therefore, embryos or blastomeres were carefully placed on a
145 clean glass slide in a minimum volume of solution and a drop of cooled methanol:acetic
146 acid solution (3:1, v:v) was applied. Slides were air dried and fixed
147 embryos/blastomeres were stained with 5% v/v Giemsa solution (1.09204.1002; Merck,
148 Darmstadt, Germany) in distilled water for 10-15 min. Chromosome spreads were
149 examined under a 100x oil magnification objective and chromosome numbers were
150 determined for each blastomere to be classified as haploid (n), diploid (2n), polyploid
151 (>2n) or aneuploid. Only spreads with clear metaphases were analyzed.

152 *Biparental bovine embryo construction*

153 Blastomeres from four-cell stage hAE were fused with matured oocytes as follows. ZF
154 hAE were placed in a 100 µl droplet of TALP-H where blastomeres were disaggregated
155 by gentle pipetting. Meanwhile, ZP was removed from matured oocytes as described
156 above and these were then individually transferred to a drop of 1 mg/ml
157 phytohemagglutinin (L8754) dissolved in TCM-199 without serum for a few seconds,
158 after which they were quickly dropped over a single androgenetic haploid blastomere.
159 Following attachment, the oocyte–blastomere pair was transferred to fusion medium
160 (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 1 mg/ml polyvinyl alcohol), for

10-20 s and then to a fusion chamber (BTX Instrument Division, Harvard Apparatus, Holliston, MA, USA) containing 2 ml warm fusion medium. Fusion was achieved with a double direct current pulse of 65 V, each pulse of 30 µs duration, 0.1 s apart. The constructed embryos were then carefully transferred individually to 5 µl droplets for 20 min to confirm fusion.

Two fusion protocols were evaluated, as shown in Figure 1: (1) fusion of a haploid blastomere with a mature oocyte as described above, or (2) fusion of a haploid blastomere with a pre-activated oocyte. In the second case, ZF oocytes were treated with Ionomycin and 1.5-2 h Roscovitine (see below), fused with a haploid blastomere as described above and returned to Roscovitine treatment. These oocytes were handled in Roscovitine-free media during fusion for 4-8 min.

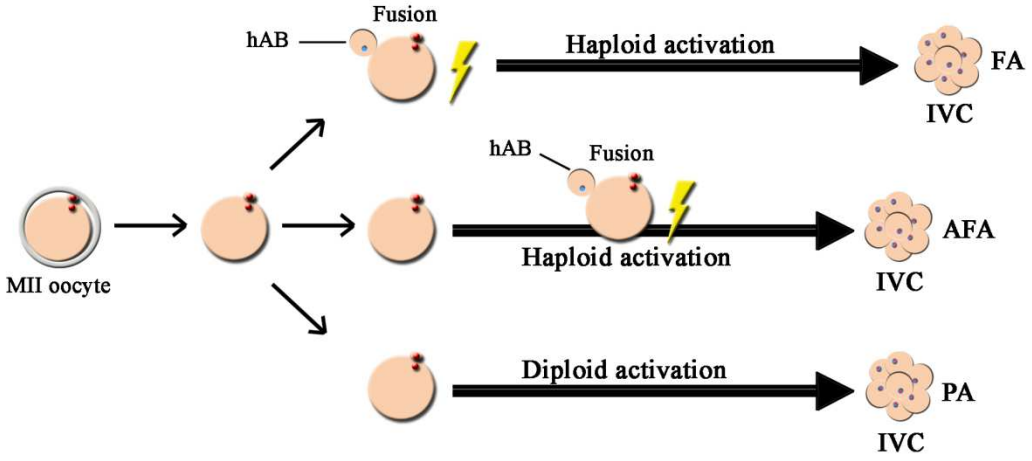


Fig 1 Production of FA, AFA and PA embryos. hAB: haploid androgenetic blastomere; FA: fusion-activation; AFA: activation-fusion-activation; PA: control diploid parthenogenetic embryos. IVC: *in vitro* culture

Chemical activation

Activation was performed with 5 µM Ionomycin (Io, I24222; Invitrogen, Carlsbad, CA, USA) in TALP-H for 4 min and subsequently transferred individually to 5 µl droplets of 50 µM Roscovitine (R7772, Sigma) in SOF medium for 5 h. For diploid activation control, matured oocytes were activated as described, but Roscovitine was supplemented with 5 µg/ml Cytochalasin B (CB, C6762) in order to inhibit second polar body extrusion. After activation treatment, zygotes were thoroughly washed in TALP-H and cultured as described above.

Microsatellite analysis

Embryonic cells were lysed by repeated freeze-thaw cycles. The resulting lysates were then tested using microsatellite markers for identification purposes. Seventeen bovine microsatellite markers, standardized according to the International Society of Animal Genetics (ISAG), were amplified in two multiplex mixes, grouped by their respective annealing temperatures: Mix 1 (60°C): AME, ETH225, ETH10, INRA23, SPS115, BRR, TGLA126, TGLA53, RME40; and Mix 2 (58°C): BM2113, BM1818, CYP21, TGLA227, TGLA122, ETH3, BL1043, BM1824, BMS510.

Each 25 µL PCR reaction contained genomic DNA, 1× PCR buffer, 2.2 mM MgCl2, 0.3 mM of dNTPs (PCR buffer, MgCl2, and dNTPs; Invitrogen), 2% dimethyl sulfoxide (DMSO), 0.15–0.5 µM of each primer (Applied Biosystems), and 1 U Platinum Taq DNA polymerase (Invitrogen). The PCR protocol was: 10 min at 95°C, followed by 31 cycles of 30 s at 95°C, 30 s at the appropriate annealing temperature, 30 s at 72°C, and a final extension step of 60 min at 72°C. PCR products were separated by electrophoresis (3130xl; Applied Biosystems) and analyzed using GeneMapper v.4.0 (Applied Biosystems).

mRNA extraction and qRT-PCR

Early embryos (8-16 cells) and blastocysts were carefully washed in PBS and stored in 20 or 10 µl of RNAlater (AM 7020, Ambion, Foster City, CA, USA), respectively, at -20°C. Samples were organized in three replicates of 11-18 early embryos and 4-6 blastocysts, so that all possible causes of variability, namely the number of cells in early embryos and the bulls used for IVF, were evenly distributed among them. Total RNA was extracted from early embryos using RNA/DNA/Protein Purification Plus Micro Kit (Norgen #51600, Canada), and Total RNA Purification Plus Kit (Norgen #48300, Canada) was used for embryos at the blastocyst stage. The cDNA was synthesized by incubation with 0.5 µl of random primers and 0,5 µl of Oligo(dT)-15 (Promega, Mannheim, Germany) for 5 min at 65°C, followed by reverse transcription with 2 µl of superscript II reverse transcriptase (50 U; Invitrogen, Karlsruhe, Germany), 4 µl of 5x first-strand buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris-HCl, pH 8.3), 2 µl of dNTPs (Roth, Karlsruhe, Germany) and 1 µl RNases inhibitor (RNasin; Promega, Mannheim, Germany). Cycling parameters were 25°C for 5 min, 37°C for 60 min and 70°C for 5 min in a PTC100 PCR (BioRad, Germany). Quantitative RT-PCR was performed using 2 µl of cDNA, 10 µl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories GmbH, Germany) and 8 µl of primers dilution, in an Applied Biosystem StepOnePlus System cycler (Thermo Fisher Scientific, Alemania) under the following amplification conditions: 95°C for 3 min, 40 amplification cycles at 95°C for 15 s and 60°C for 1 min, followed by melting curve analysis. Primers were designed using PrimerBlast from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The list of the primers used can be found in

supplementary Table 1. Results were analyzed according to $2^{-}\Delta\Delta Ct$ method, and GAPDH and β-actin from IVF embryos were used for normalization.

Statistical analysis

Embryo development was analyzed using Fisher's exact test. Quantitative data from gene expression was analyzed by one-way ANOVA, *post hoc* analysis to evaluate differences between groups was performed using Tukey's test, and graphs were created using GraphPad Prism software. Values of $p \leq 0.05$ were considered significant.

Results

Production of hAE

In a preliminary assay, the efficiency of IVF on denuded oocytes was evaluated using short co-incubation times. This experiment aimed to obtain hAE by fertilization of enucleated, thus denuded, oocytes and to avoid the exposure of the sperm DNA to binding dyes and UV light during micromanipulation. Three replicates were performed with two straws each, namely bull A (n = 61), bull B (n = 81) and a combination of bull B + bull C (n=77). After 3, 4, and 5 h of IVF, oocytes were collected and grouped as not fertilized (NF: no sperm inside de cytoplasm), fertilized (F: one sperm inside the cytoplasm) or polyspermic (P: more than one sperm inside de cytoplasm) by direct observation of nuclei with Höechst staining. Results show that the different bulls used in this study had different fertilization efficiencies after three and four hours of gamete co-incubation. Thus, replicates could not be grouped and are shown separately (Fig. 2a). After three hours, a similar proportion of F was obtained, but bull B showed a lower proportion of NF and a higher proportion of P. In contrast, after four hours, NF and P rates were similar between bulls, but bull A resulted in a lower F rate than the

246 combination of bulls B + C. No statistical differences were observed after five hours,
247 but F rate ranged between 18.8 and 44.4%. These results suggest that fertilization of
248 enucleated (denuded) oocytes would result in an uncertain proportion of androgenetic
249 embryos with the expected haploid complement, even when using short co-incubation
250 times.

251 The production of hAE was further evaluated by enucleation of fertilized oocytes, using
252 an IVF co-incubation period of four hours. Micromanipulation of zygotes with ZP or
253 ZF, and the *in vitro* development of the resulting embryos were compared. As a result of
254 the short IVF protocol, the maternal and paternal nuclei were still condensed and
255 maintained differential morphology at the time of enucleation (Fig. 2b). However,
256 attached sperm cells were observed during the ZP enucleation, interfering with the
257 correct identification of the DNA inside the oocyte. In contrast, ZP digestion necessary
258 for the ZF enucleation eliminated all the sperm that had not fused with the oocyte
259 membrane.

260 Embryo development showed that hAE-ZF resulted in higher cleavage rates (Table 1).
261 However, development to the morula and blastocyst stages was similar between hAE-
262 ZF and hAE-ZP, and lower compared to the control groups (Fig. 2c).

Group	***n***	***Cleavage (%)***	***Morulae (%)***	***Blastocysts (%)***
hAE-ZF	127	115 (90.6) [a]	27 (23.3) [a]	6 (5.2) [a]
hAE-ZP	65	50 (76.9) [b]	13 (26) [a]	7 (14) [a]
IVF-ZF	150	113 (75.3) [b]	61 (54) [b]	44 (38.9) [b]
IVF-ZP	150	115 (76.7) [b]	71 (61.7) [b]	52 (45.2) [b]

263 **Table 1.** *In vitro* development of androgenetic haploid embryos enucleated
264 and *in vitro* cultured with ZP or ZF, and IVF controls. hAE-ZF:

androgenetic haploid embryos enucleated ZF; hAE-ZP: androgenetic haploid embryos enucleated with ZP; IVF-ZP/IVF-ZF: controls with and without ZP, respectively. [a,b] Different superscripts within the same column indicate statistical differences ($p \leq 0.05$).

The chromosome count of hAE-ZF blastomeres evaluated on day 2 of *in vitro* development confirmed that 76.9% (40/52) were haploid and only 5.8% (3/52) were diploid. In contrast, 13% (3/23) of IVF-ZF control blastomeres were haploid and 65.2% (15/23) were diploid ($p < 0.05$) (Fig. 2d). Similar rates of aneuploid embryos were observed in each group (hAE-ZF: 17.3%; IVF-ZF: 21.7%).

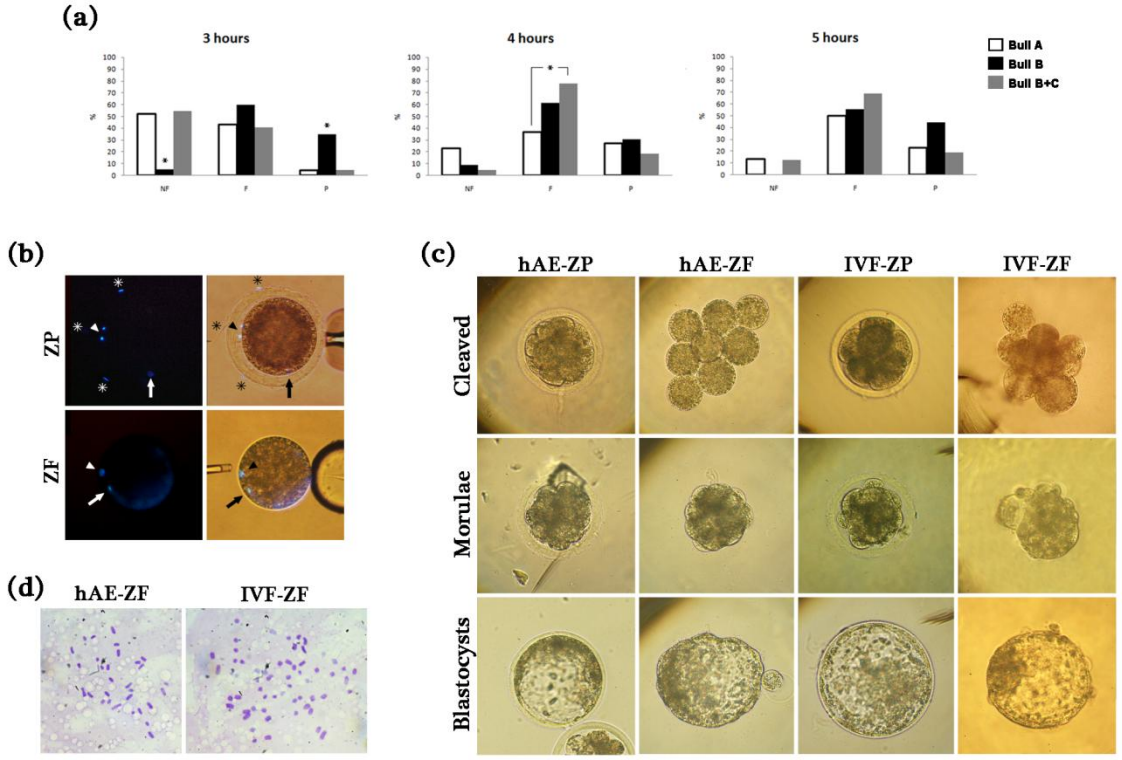


Fig 2 Haploid androgenetic embryo (hAE) production. **(a)** Fertilization efficiency of denuded oocytes. NF = not fertilized; F = fertilized, one sperm head; P = polispermic, more than one sperm head. Asterisk shows statistic differences within categories ($p \leq$

0.05). **(b)** Enucleation of zygotes with ZP or ZF. Arrows indicate paternal DNA inside the oocyte, arrowheads indicate maternal DNA, and asterisks indicate sperm cells attached to the ZP. Magnification 400 X. **(c)** hAE enucleated and *in vitro* cultured with ZP or ZF, and IVF controls. Magnification 100X. **(d)** Representative metaphase spreads. Magnification 1000X

Finally, by means of PCR analysis using a commercial platform, the number of alleles corresponding to 17 genetic markers was determined, together with their respective controls. In a preliminary assay, the amplification at hAE-ZF in 15-cell, morula, and blastocyst stages was evaluated in order to determine the minimum number of cells necessary for this study. The three embryos showed a single allele for all the markers studied, but in the case of the 15-cell stage embryo, the amplification was incomplete. Therefore, one morula and seven blastocysts from the hAE group were further analyzed, and sexing was included by analysis of the amelogenin gene (Supp. Fig. 1). Three blastocysts contained only one allele for all the markers analyzed and were female, whereas one showed amplification of three alleles for some markers. The three remaining embryos showed a diploid profile. The morula contained a single allele for all markers evaluated and was male according to the amelogenin gene analysis.

Production of diploid embryos

Blastomeres from hAE-ZF were used to produce biparental embryos by fusion with a mature oocyte. Roscovitine was used for the activation of the constructed embryos (FA group, fusion – activation). Therefore, in a preliminary assay Roscovitine was evaluated as an activating agent by assessing of the developmental potential of these embryos, and

diploid parthenogenetic embryos were used as controls (PA; n = 35). Biparental embryos showed a high cleavage rate (46/56, 82.14%), similar to PA (29/35, 82.86%), but development to the blastocyst stage decreased markedly (2/56, 3.57% vs. 7/35, 20%, Fig. 3).

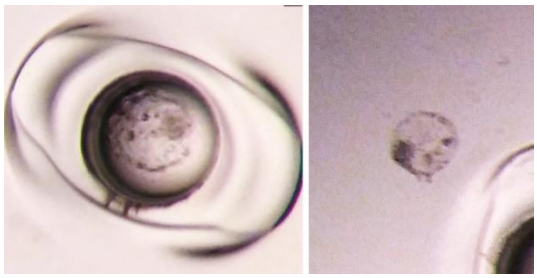


Fig 3 Blastocysts obtained by fusion of a hAE blastomere with a mature oocyte. Magnification 100X

Aiming to improve the developmental potential of biparental embryos, the effect the oocyte pre-activation was evaluated (AFA group, activation-fusion-activation) and was compared to FA group. Diploid parthenogenetic embryos were used as controls (PA group). Fusion rates were similar between FA (96%) and AFA (97.1%), so the oocyte pre-activation did not impair the correct fusion with the haploid blastomere. Cleavage rates were similar between experimental groups and the PA control, while development to morula and blastocyst stages in FA and AFA decreased (Table 2). On the fifth day of *in vitro* development, the number of cells per embryo was counted, showing that a higher proportion of cleaved embryos of the AFA group arrested their development at the two-cell stage (63.16%) compared to the FA (14.58%) and PA (7.5%) groups (Supp. Table 2).

Group	*n*	*Cleavage (%)*	*Morulae (%)*	*Blastocysts (%)*
FA	66	55 (83.33) [a]	4 (6.06) [a]	3 (4.55) [a]
AFA	66	57 (86.36) [a]	1 (1.52) [a]	1 (1.52) [a]
PA	64	57 (89.06) [a]	17 (26.56) [b]	15 (23.44) [b]

322 **Table 2.** *In vitro* development of biparental embryos obtained by fusion before or
323 during activation (FA and AFA groups, respectively) of a hAE blastomere with a
324 mature oocyte. [a,b] Different superscripts within the same column indicate statistical
325 differences ($p \leq 0.05$).

326

327 *Gene expression of haploid and diploid embryos*

328 In order to determine whether an alteration in gene expression could explain the low
329 embryonic development observed in hemiclones, the relative abundance of mRNA was
330 analyzed. The genes evaluated were previously described to have key functions during
331 genome activation, cell metabolism, and differentiation in bovine embryos. hAE and
332 biparental embryos were evaluated with their respective IVF and PA controls. Samples
333 were taken from early embryos at the 8-16-cell stage (Fig. 4a) and from blastocysts
334 (Fig. 4b). Considering that the FA group showed better development to the blastocyst
335 stage and a lower percentage of embryos arrested at the two-cell stage compared to the
336 AFA group, biparental embryos for this analysis were obtained using this protocol.

337 In early embryos, FA and PA showed similar expression of all genes analyzed. In
338 particularly, HNRNPA2B1 and Glut-1 mRNA levels were also similar to the IVF
339 control. Conversely, hAE showed a similar expression pattern to IVF and did not show
340 similarities with the FA and PA groups. hAE and FA differed significantly, as did the
341 PA and IVF controls. Although mRNA isolation and DNA synthesis were confirmed by
342 PCR, no subsequent amplification of the H2AFZ gene was obtained by qRT-PCR.

Embryos at the blastocyst stage did not show significant differences in the relative expression of genes, with the exception of Oct-4, which differed between FA and PA. On the other hand, the expression of AMPKA1 was lower in the FA and PA groups compared to the IVF control, although the difference was not significant.

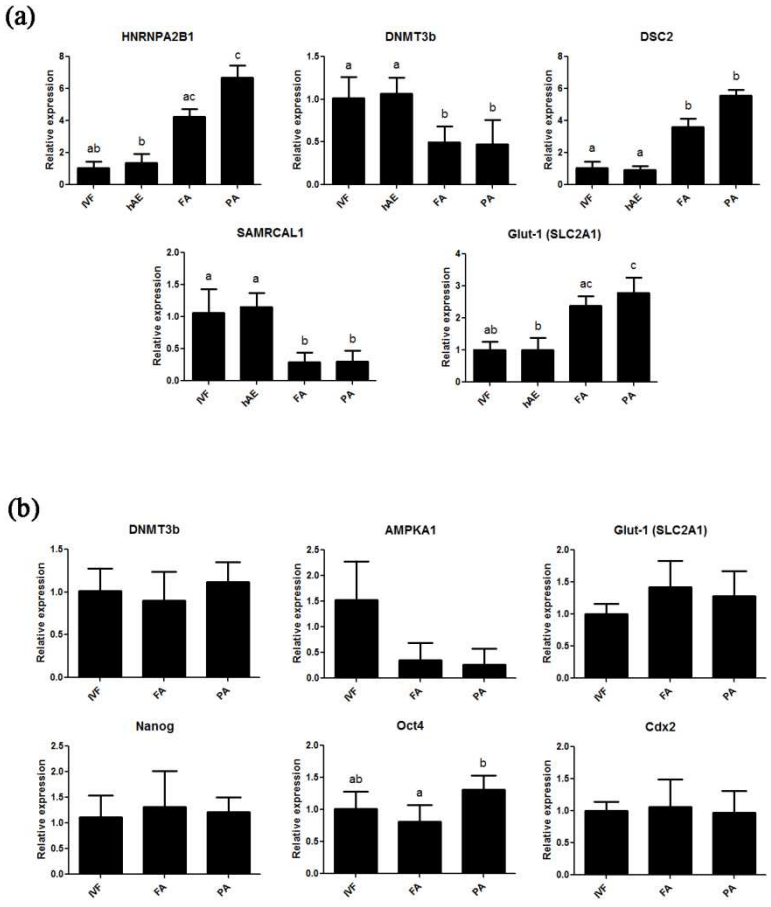


Fig 4 Relative abundance of mRNA of selected genes. (a) Relative expression in 8-16 cell stage embryos. (b) Relative expression of embryos at the blastocyst stage. IVF: *in vitro* fertilization control, hAE: haploid androgenetic embryos, FA: reconstructed embryos produced by fusion and subsequent activation of a haploid androgenetic blastomere with a mature oocyte, and PA: diploid parthenogenetic activation control

Discussion

The first objective of the present work was to improve the efficiency of hAE production by optimizing fertilization and enucleation protocols. Evaluation of IVF on denuded oocytes confirmed that, while enucleation prior to IVF prevents sperm DNA exposure to staining dyes and UV light, the resulting uncertainty regarding embryo ploidy renders this approach unreliable. Previous studies report polyspermy rates of 5.4–17% in cumulus-free IVF [10,19]. The discrepancies observed both across previous reports and within the present study are likely attributable to variations in IVF protocols (e.g., sperm concentration and co-incubation times) and the documented "bull effect" [35] on fertilization performance. Given the high and variable polyspermy rates observed, IVF of enucleated oocytes was not further evaluated.

In bovine, maternal nuclei must be removed from fertilized oocytes before DNA decondensation, as female and male PN cannot be differentiated. Given that PN form approximately 4 hours after sperm entry [36] — earlier than the 17–19 hours of a standard IVF protocol — a short, 4-hour IVF period was employed. This ensured that the paternal genome remained condensed and clearly distinguishable at the oocyte periphery during enucleation. During the subsequent enucleation step, while the hAE-ZP group presented adhered sperm that complicated the identification of the maternal nucleus, thereby increasing handling time and UV exposure, the ZP removal in hAE-ZF embryos eliminated all non-fused cells. This ZP-free method significantly simplified the enucleation process, reduced manipulation times, and ensured all observed spermatozoa were successfully incorporated within the ooplasm.

Enucleation after IVF exposes DNA to potentially mutagenic conditions, posing a significant concern for applications in breeding programs, human fertility, and *in vivo*

experiments. However, this approach has been reported to be more advantageous in terms of methodology, cost-effectiveness, and hAE development outcomes [19]. Therefore, exploring the feasibility of using dyes detectable at non-mutagenic wavelengths warrants further investigation.

In accordance with previous findings in cattle and other species, hAE exhibited reduced developmental potential to the blastocyst stage compared to controls [10,16,18,37]. This is likely due to a combination of factors, including the inherent limitations of the haploid state [16] and the hemizygous nature of the genome [10,13]. This likely explains the higher blastocyst rate observed in the hAE-ZP group, in which the presence of sperm adhered to the ZP during enucleation may have masked polyspermic fertilizations, inadvertently including more diploid embryos in culture. Moreover, the altered expression of imprinted genes resulting from uniparental origin also contributes to the reduced developmental potential of hAE [16,19]. The sex chromosome complement also plays a crucial role, as hAE carrying the Y chromosome are generally not expected to progress beyond initial divisions [7,10,19,37]. Consistently, haploid cell lines derived to date from AE of different species contain the X chromosome [38]. The results from the genetic analysis of molecular markers obtained in this work align with these reports, as the blastocysts that presented a single allele for each marker (presumed haploid) contained the X chromosome. Interestingly, the morula contained the Y chromosome, suggesting that the X chromosome may be essential for development at later stages.

Chromosome counting showed that 76.9% of the blastomeres in the hAE group were haploid on day 2 of development. Similar results were reported in human hAE using

401 ICSI as the fertilization method [11]. However, the subsequent analysis of day 7
402 blastocysts using molecular markers revealed a selective advantage: while the diploid
403 embryo population was low on day 2 (5.8%), it subsequently represented the majority of
404 blastocysts (57.1%). This is a consequence of the higher developmental potential of
405 diploid embryos, which likely resulted from polyspermic fertilization, as the elimination
406 of the maternal metaphase was confirmed through direct observation of DNA in the
407 pipette. It should be noted that, while 42.9% of blastocysts exhibited the expected
408 single-allele profile, this marker analysis cannot definitively determine whether some
409 cells underwent spontaneous diploidization, which also enhances developmental
410 capacity.

411 As previously explained, cloning the sperm genome through the production of hAE
412 would enable pre-zygotic genetic screening. This concept has been explored using
413 karyotype analysis in mice [4,20] and in humans [11]. Vichera *et al.* (2011) evaluated
414 this approach in cattle, using sex determination as a model. However, the potential of
415 this technique extends beyond chromosomal examination, as it could also be applied to
416 the analysis of specific DNA sequences for the detection paternal mutations. Therefore,
417 in this study, a commercial platform routinely used for parentage testing was employed
418 to evaluate hAE. A complete genetic profile of hAE was successfully obtained,
419 effectively representing the sperm genetic makeup, from both morula and blastocyst
420 stages. This demonstrates that hAE production is a valuable tool for obtaining precise
421 information about sperm genetic composition. Nevertheless, hAE exhibit limited
422 developmental potential at these later stages. Therefore, to utilize early embryos in
423 future experiments and obtain sufficient DNA, incorporating a genomic DNA
424 amplification step prior to marker characterization should be considered. Alternatively,

the derivation of haploid cell cultures from hAE would also yield greater availability of genomic DNA. Indeed, the production of diploid embryos using haploid cells as paternal nuclear donors has been reported in mice and rats [12,39].

The low developmental potential of biparental hemiclones remains a challenge across all studied species. Several hypotheses regarding this limitation have been evaluated. First, asynchrony between the actively dividing donor blastomere and the MII-arrested recipient oocyte—a state characterized by high MPF levels—has been proposed. However, testing this using oocyte pre-activation (AFA group) did not improve the developmental outcome, suggesting that while synchronization is important, this strategy alone does not resolve the underlying issue. Second, the possibility of failure in paternal genome incorporation is unlikely, as preliminary genetic analysis of molecular markers in two FA blastocysts obtained in this study confirmed the successful presence of the hAE DNA in the zygote, as evidenced by a heterozygous genetic constitution and the presence of a Y chromosome (data not shown). Finally, the argument that cytoplasmic dilution during fusion compromises viability is also unlikely, as demonstrated by studies in other species in which transferring donor cells with minimal cytoplasmic volume (haploid embryonic stem cells) still resulted in a similarly significant decrease in developmental potential.

To determine the quality of biparental embryos and investigate the molecular basis of their limited development, gene expression was evaluated during two key stages: the 8 – 16 cell stage (coinciding with EGA in bovine) and the blastocyst stage. At the EGA stage, six genes were analyzed, including factors related to chromatin status (DNMT3b, SMARCAL1, H2AFZ) and RNA processing (HNRNPA2B1). DSC2 (Desmocollin 2)

was included as an indicator of subsequent developmental potential, given its role in intercellular junctions [40,41]. Metabolic status was assessed via Glut-1 (glucose transporter 1) expression [42]. At the blastocyst stage, six genes were again analyzed, including DNMT3b (epigenetic marks/differentiation) and Glut-1 (trophoectoderm expression) [43,44], and three key transcription factors (Nanog, Oct4, Cdx2), along with AMPKA1 (a regulator of lipid metabolism and mitochondrial activity) as a complementary metabolic indicator [45].

Gene expression results suggest that the oocyte activation mechanism exerts a greater influence on expression pattern during early developmental stages than the genetic constitution. This is evidenced by the similar patterns observed in artificially activated FA and PA groups, which differed significantly from fertilization-activated hAE and IVF embryos. Only HNRNPA2B1 and Glut-1 in FA embryos showed no significant differences compared to IVF. This differential profile is likely a consequence of the altered Ca^{2+} oscillations triggered by artificial activation, which are known to affect EGA events, development, and implantation success [40,46]. Particularly, Ross *et al.* observed that activation via injection of PLCζ mRNA induced an expression pattern similar to that seen in IVF embryos and different from that resulting from chemical activation. Therefore, the altered expression observed in early-stage biparental embryos is attributable to the artificial activation protocol. Notably, the similar expression pattern observed in hAE and IVF embryos suggests that the absence of the maternal genome does not significantly affect EGA expression during early stages [47], confirming that hAE are molecularly similar to their diploid counterparts in this regard.

Analysis of chromatin-related genes in 8 –16 cell embryos provided molecular evidence of EGA disruption. Specifically, the altered expression of DNMT3b and SMARCAL1 in artificially activated embryos (FA and PA) compared to the IVF control suggests incorrect gene expression programming during EGA. Given the role of these genes in modulating global gene expression, their deregulation likely underlies the imbalance observed in other mRNA abundances, thereby compromising subsequent development. Contrary to observations by other researchers [42], histone H2AFZ expression was not detected in the present study. Furthermore, evaluation of metabolic status via Glut-1 expression revealed a trend towards elevated Glut-1 levels in early FA and PA embryos compared to IVF. This finding, which aligns with the "quiet embryo hypothesis" [48] linking high metabolic activity to reduced developmental potential, further supports the conclusion that the artificial activation protocol negatively impacts the quality of reconstructed embryos.

The differences observed in early embryos were not found at the blastocyst stage. This could be attributed to the fact that embryonic development itself acts as a selection factor. Analyzing embryos at early stages allows detection of alterations that compromise developmental potential, while evaluating blastocysts primarily examines the subpopulation with an initially normal expression pattern or those that successfully rectified any aberrations during development. The comparison between the results presented in this study and previous reports is limited by the variations in activation protocols employed [40], as this is the first investigation to analyze gene expression in embryos generated by activation with Roscovitine. Moreover, modifications in the culture medium used could also lead to different gene expression patterns [49].

In conclusion, this study yielded two principal advances toward improving hAE technology. First, the optimized ZP-free enucleation protocol developed in this study following short IVF effectively addressed the challenges of distinguishing parental nuclei and eliminated adherent sperm interference, establishing a more reliable, scalable, and efficient platform for bovine hAE production. The successful generation of complete monoallelic genetic profiles from these embryos using a standard commercial platform decisively underscores the potential of this technology for practical and accessible PZGS. Second, while hAE blastomere fusion successfully generated diploid hemiclones, their developmental capacity remains severely limited. The molecular findings obtained during the EGA window indicate that artificial activation disrupts key chromatin regulators and metabolic status; however, the marked developmental disparity between FA and PA embryos—despite sharing the same activation stimulus—suggests that inherent constraints of the hemiclone condition, rather than the activation protocol alone, represent the primary barrier to developmental competence. This work confirms the utility of hAE for genomic analysis and critically redirects future research toward addressing the epigenetic and regulatory limitations intrinsic to reconstructed embryos to improve hemiclone viability.

Supplementary Information The online version contains supplementary material.

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