CELLULAR REPROGRAMMING
Volume 23, Number 5, 2021

DOI: 10.1089/cell.2021.0026

Effect of Embryo Aggregation on *In Vitro* Development of Adipose-Derived Mesenchymal Stem Cell-Derived Bovine Clones

Virginia Savy,[1,2,*,†] Virgilia Alberio,[1,2,†] Geraldina Vans Landschoot,[1,2] Lucía Natalia Moro,[3] Fernanda Daniela Olea,[4] Lleretny Rodríguez-Álvarez,[5] and Daniel Felipe Salamone[1,2,i]

Abstract

Somatic cell nuclear transfer (SCNT) is a method with unique ability to reprogram the epigenome of a fully differentiated cell. However, its efficiency remains extremely low. In this work, we assessed and combined two simple strategies to improve the SCNT efficiency in the bovine. These are the use of less-differentiated donor cells to facilitate nuclear reprogramming and the embryo aggregation (EA) strategy that is thought to compensate for aberrant epigenome reprogramming. We carefully assessed the optimal time of EA by using *in vitro*-fertilized (IVF) embryos and evaluated whether the use of adipose-derived mesenchymal stem cells (ASCs) as donor for SCNT together with EA improves the blastocyst rates and quality. Based on our results, we determined that the EA improves the preimplantation embryo development per well of IVF and SCNT embryos. We also demonstrated that day 0 (D0) is the optimal aggregation time that leads to a single blastocyst with uniform distribution of the original blastomeres. This was confirmed in bovine IVF embryos and then, the optimal condition was translated to SCNT embryos. Notably, the relative expression of the trophectoderm (TE) marker KRT18 was significantly different between aggregated and nonaggregated ASC-derived embryos. In the bovine, no effect of the donor cell is observed on the developmental rate, or the embryo quality. Therefore, no synergistic effect of the use of both strategies is observed. Our results suggest that EA at D0 is a simple and accessible strategy that improves the blastocyst rate per well in bovine SCNT and IVF embryos and influence the expression of a TE-related marker. The aggregation of two ASC-derived embryos seems to positively affect the embryo quality, which may improve the postimplantation development.

Keywords: mesenchymal cells, aggregation, SCNT, bovine

Introduction

The somatic cell nuclear transfer (SCNT) allows the reprogramming of a completely differentiated somatic cell to a totipotent state (reviewed by Ogura et al., 2013) and, therefore, is a valuable tool for both basic and applied research. The SCNT has been used for the reproduction of genetically valuable farm animals, the rescue of endangered species, and the production of genetically modified large animals for pharmaceutical purposes, biomedical models (Aigner et al., 2010; Cooper et al., 2014), or agricultural applications (Liu et al., 2019; Su et al., 2018b).

Moreover, as it is possible to derive stem cells from cloned embryos, SCNT is a promising tool for regenerative medicine (Vajta, 2007). In basic science, SCNT is a suitable method to decode the mechanisms of cell reprogramming,

[1]Laboratorio Biotecnología Animal (LabBA), Dto Producción Animal, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina.
[2]Instituto de Investigaciones en Producción Animal (INPA), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina.
[3]LIAN-CONICET, Fundación FLENI, Buenos Aire, Argentina.
[4]Laboratorio de Medicina Regenerativa Cardiovascular, Instituto de Medicina Traslacional, Trasplante y Bioingeniería (IMETTYB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Favaloro, Buenos Aires, Argentina.
[5]Department of Animal Science, Faculty of Veterinary Sciences, Universidad de Concepción, Concepción, Chile.
*Present address: Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA.
[†]These authors should be considered joint first author.
[i]ORCID ID (https://orcid.org/0000-0003-0858-0313).

cell differentiation, and chromatin remodeling, among many other applications. Since the birth of Dolly, the sheep (Wilmut et al., 1997), the first mammal cloned from an adult somatic cell, SCNT has successfully been used by several laboratories worldwide in a variety of species.

To date, at least 24 species have been successfully cloned, including amphibia, fish, and mammals (Czernik et al., 2019; Matoba and Zhang, 2018); however, the efficiency of the technique remains extremely low (Czernik et al., 2019). This can be partially explained by the inappropriate reprogramming of the donor cell (Degrelle et al., 2012; Yamanaka et al., 2011; reviewed by Gouveia et al., 2020). In this regard, studies carried out on different species revealed that preimplantation cloned embryos exhibit aberrant spatiotemporal expression of master-key genes when compared with embryos produced by *in vitro* fertilization (IVF) under similar conditions (Boiani et al., 2003; Buemo et al., 2016; Bui et al., 2009; Degrelle et al., 2012; Matoba et al., 2011, 2014; Moro et al., 2015; Wrenzycki et al., 2002; Zhang et al., 2016).

Moreover, abnormal DNA methylation patterns have been reported for bovine SCNT embryos at the morula and blastocyst stages (Liu et al., 2018a), supporting the hypothesis of inefficient nuclear reprogramming (reviewed by Niemann et al., 2011; and Reik et al., 2001). Further research is essential to improve the frequency and health of live offspring and, ultimately, to facilitate the widespread use of SCNT in basic and applied research.

Significant efforts have been made to improve the efficiency of the SCNT technique. The use of chemicals or interference RNA to remodel the epigenetic landscape increases the developmental potential of cloned embryos, but only in some cases (Gonzalez-Munoz and Cibelli, 2018; Yamanaka et al., 2011). However, these techniques are usually expensive and time consuming and are not suitable for routine use. It was demonstrated that the use of less-differentiated donor cells, such as fetal cells, embryonic stem cells, or even blastomeres, results in higher rates of cloned blastocysts (Hall et al., 2013; Ono and Kono, 2006; Salamone et al., 2006; Reviewed by Liu et al., 2018b; Ogura et al., 2013). In this regard, mesenchymal cells are an attractive source of multipotent donor cells, particularly from adult animals with a valuable genotype or production traits.

In addition to the use of less differentiated donor cells, embryo aggregation (EA) is proposed as a simple and accessible alternative to improve the production of clones. This strategy consists of placing two or more zona pellucida-free (ZF) embryos in proximity during the *in vitro* culture, so that their blastomeres self-organize to form one single blastocyst of improved quality. This improvement is attributed to the higher cell number at the beginning of the development, to an ''epigenetic compensation'' between the aggregated structures or both (Boiani et al., 2003; Eckardt and McLaughlin, 2004).

Particularly in bovines, it is notable that while the embryo transfer of IVF embryos results in high pregnancy rates, the SCNT efficiency remains poor (Chavatte-Palmer et al., 2018). Furthermore, the reported delivery rate of cloned offspring per transferred embryo is highly variable between laboratories, yet it remains lower than 10% (Chavatte-Palmer et al., 2018; Long et al., 2014; reviewed by Gouveia et al., 2020). In this species, the use of mesenchymal cells as donors for SCNT, as well as the EA strategy have already been independently studied for adult animals. Interestingly, although none of the strategies alone improved the blastocyst rate, both led to an improvement on the blastocyst quality, measured in terms of total cell number, ratio of inner cell mass (ICM), and trophectoderm (TE) cells, and rescue of aberrant expression of specific transcription factors (Bang et al., 2015; da Silva et al., 2016; Picou, 2009).

However, the EA significantly improved the efficiency of handmade cloning in the bovine (Ribeiro et al., 2009). Similar results were reported for the use of mesenchymal stem cells as nuclear donors in diverse species, such as canines, pigs, and horses (Li et al., 2013; Oh et al., 2011; Olivera et al., 2016). In this sense, it remains unexplored whether the combined use of mesenchymal donor cells and EA leads to a synergistic effect that improves embryo development in bovine clones, both quantitatively and qualitatively.

The aims of this work were, first, to determine the optimal time of EA using IVF embryos and the microwell culture system and then, to evaluate whether the use of adult adipose-derived mesenchymal stem cells (ASCs) as donor for SCNT together with EA improves the blastocyst rates and embryo quality in bovine.

Materials and Methods

Chemicals

Except otherwise indicated, all chemicals were obtained from Sigma Chemicals Company (St. Louis, MO).

Animal welfare

The ovaries used in this study came from the local slaughterhouse. All aspects of animal welfare in slaughterhouses are regulated by the National Service of Health and Food Quality (SENASA), of the Ministry of Agriculture, Livestock, and Fisheries of the Argentine Republic. Regarding the collection of skin and adipose tissue samples, all procedures were performed by qualified veterinarians in accordance with the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. All efforts were made to minimize animal suffering or stress.

Donor cell isolation and in vitro *culture*

Samples, consisting of skin and subcutaneous adipose tissue, were collected aseptically, and using local anesthesia (lidocaine 2%) from the rump region of the same animal. The surgical procedure was performed with the animal standing. The area was shaved, cleaned, and sterilized with iodine–povidone. The skin biopsy was obtained using a disposable biopsy punch and immediately immersed in phosphate-buffered saline (PBS) with 4% Antibiotic–Antimycotic solution (ATB, 15240; Thermo Fisher Scientific, Grand Island, NY). Two grams of subcutaneous adipose tissue was collected from the exposed area, using sterile forceps and scissors, and immersed in PBS supplemented with 4% ATB.

In the laboratory, to obtain fibroblast from the adult bovine (FAB), the skin biopsy was excised to $1mm^2$ explants and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated bovine calf serum (FBS) and 2% ATB. FAB were obtained by

outgrowth of cells from explanted tissue pieces. For ASC isolation, the adipose tissue was enzymatically digested with 0.6% Collagenase I (4197; Worthington, NJ) and 0.25% bovine serum albumin (BSA) in Dulbecco's PBS (DPBS), for 2 hours at 37°C in a continuous shake incubator.

After digestion, cell mix was centrifuged at 264 *g* for 5 minutes, washed with DPBS, and expanded in DMEM supplemented with 5% FBS and 2% ATB. Finally, primary cell cultures with less than four passages were cryopreserved in liquid nitrogen. For their use in the SCNT procedure, cell cultures with less than six passages were used. Synchronization of the donor cells was achieved by inducing quiescence after growing cells to confluence in 0.5% FBS for 2–3 days.

Expression of surface markers

The expression of surface markers in adipose-derived cells was evaluated by direct immunofluorescence and flow cytometric analysis as was previously described (Locatelli et al., 2013). Cell cultures were trypsinized, resuspended in DPBS supplemented with 4% BSA ($5 \cdot 10^5$ cells/mL), and incubated in the dark for 30 minutes with the appropriate antibody: murine anti-human CD166 antibody conjugated with PE (BD Biosciences, CA), anti-murine antibody-CD44 ovine, conjugated with FITC (AbD Serotec, Oxford, United Kingdom) or ovine anti-CD45 murine antibody conjugated with FITC (AbD Serotec, Oxford, United Kingdom). Then, cells were centrifuged for 5 minutes at 64 *g* and the pellet was resuspended in DPBS. For each sample, at least 10,000 events were evaluated, and data were analyzed by Cyflogic 1.2.1 software (Perttu Terho and CyFlo Ltd., Turku, Finland). Unstained control as well as secondary antibody alone control were included.

In vitro *differentiation assay*

For chondrogenic, adipogenic, and osteogenic differentiation assay, the StemPro Chondrogenesis (A10071-01; Gibco, Grand Island, NY), StemPro Adipogenesis (A10070-01; Gibco), and StemPro Osteogenesis (A10072-01; Gibco) Kits were used, respectively. After 14 days of induction, cells were fixed with 4% paraformaldehyde solution for 45 minutes at room temperature. To evaluate adipogenic differentiation, fixed cells were stained with a saturated solution of Sudan Red in 70% ethanol. For osteogenic induction evaluation, calcium mineralization was assessed by 2% Alizarin Red staining. Chondrocyte induction was evaluated by staining with 1% Alcian Blue to detect glycosaminoglycans. All preparations were evaluated using an inverted Nikon Eclipse TE2000-S microscope and the images analyzed by the software Eclipse Net software.

Oocyte collection and in vitro *maturation*

Bovine cumulus–oocyte complexes (COCs) were obtained by aspiration of surface follicles from slaughterhouse ovaries and collected into Tyrode's albumin lactate pyruvate medium buffered with HEPES (TALP-H) medium. Only follicles between 2 and 8 mm diameter were aspirated and COCs surrounded by at least three layers of granulosa cells were selected.

For *in vitro* maturation (IVM), COCs were cultured for 22 hours at 38.5°C in 100 μL droplets of bicarbonate-buffered TCM-199 (31100-035; Thermo Fisher Scientific), containing 10% FBS (013/07; Internegocios, Mercedes, Argentina), 10 mg/mL follicle-stimulating hormone (NIH-FSH-P1, Folltropin; Bioniche, Victoria, Australia), 0.3 mM sodium pyruvate (P2256), 100 mM cysteamine (M9768), and 2% ATB under mineral oil, in 6.5% CO_2 in humidified air. After IVM, cumulus cells were removed by mechanical agitation of COCs with 1 mg/mL of hyaluronidase (H-4272). Those oocytes with a visible polar body under stereoscopic microscopy were selected.

Zona pellucida removal and oocyte enucleation

For zona pellucida removal, enzymatic digestion was performed with pronase 1.5 mg/mL (P8811). Before enucleation, ZF-oocytes were incubated for 30 minutes in Synthetic Oviductal Fluid (SOFaa) supplemented with 10% FBS, 1% ATB, and Demecolcine (D1925, 4 μM), followed by incubation in SOFaa containing fluorescent dye Bisbenzimide Hoechst (H33342; Sigma) 0.02 mg/mL for 15 minutes. Standard mechanical enucleation was performed in TALP-H containing 0.5 mg/mL of cytochalasin B (C6762), by aspiration of the protrusion formed after Demecolcine treatment. By means of a UV light pulse, the total removal of the DNA was confirmed.

For enucleation, a 20 μm diameter blunt pipette and a closed holding pipette were placed on hydraulic micromanipulators (Narishige, Medical Systems, Great Neck, NY) mounted on a Nikon Eclipse TE-300 microscope (Nikon, Melville, NY). The ZF-ooplasts generated were placed in 100 μL drops of SOFaa, in the incubator, until their use.

SCNT and chemical activation

Under stereoscopic microscopy, a ZF-ooplast was individually transferred to a TCM drop containing 1 mg/mL phytohemagglutinin (L8754) for 2 to 5 seconds and immediately placed over an individualized somatic cell. Fusion of ooplast–cell couplets was performed in a fusion chamber filled with 2 mL of fusion buffer (0.3 M mannitol [M9546], 0.1 mM $MgSO_4$ [M7506], 0.05 mM $CaCl_2$ [C7902], 1 mg/mL polyvinyl alcohol [P8136]) (BTX Instrument Division, Harvard Apparatus, Holliston, MA). Fusion conditions were: two pulses of 30 μs of unidirectional direct current of 75 mV, separated each other by 0.1 second (BTX microslide 0.5 mm fusion chamber, model 450, 01-000209-01).

The reconstructed embryos (REs) were individually incubated in 5-μm droplets of SOFaa supplemented with 2.5% FBS, under mineral oil at 38.5°C in 5% CO_2 in air. Couplets were evaluated for fusion under stereoscopic microscopy after 20–30 minutes' incubation. When necessary, nonfused couplets were refused. The REs were incubated for 2 hours at 38.5°C in 5% CO_2 and then chemical activation was performed by embryo treatment with 5 μM ionomycin (I24222; Thermo Fisher Scientific) in TALP-H for 4 minutes in the dark, followed by individual culture of treated embryos in 5 μL droplets of SOFaa with 1 mM 6-dimethylaminopurine (D2629) for 3 hours. As control, mature ZF-oocytes were parthenogenetically activated using the same protocol.

In vitro *embryo culture and EA strategy*

For ZF embryo culture, we relied on a modified version of the ''Well of the Well'' system (Vajta et al., 2000). Briefly, microwells were produced by pressing the base of a 35× 10 mm Petri dish with a heated glass capillary. Between 20 and 25 microwells (500 μm diameter each) were covered with

100 μL of SOFaa medium and, finally, with 3 mL of mineral oil (M8410; Sigma). Immediately after activation, embryos were placed within microwells, following two different strategies: a single embryo per microwell (group 1×, nonaggregated embryos) or two embryos were placed randomly in the same microwell (2× group, aggregated embryos). The total number of RE within a 100 μL drop was similar between 1× and 2× groups. Embryos were cultured in a humidified gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 39°C.

Half of the culture medium was renewed 2 days postactivation (p.a.) and FBS was added to the drops at a final concentration of 10% v/v on day 5 (D5) p.a. Cleavage, morula, and blastocyst rates were recorded on D2, D5, and D7 p.a., respectively. Blastocysts were stored in RNA*later* (AM 7020; Ambion Co., Austin, TX) at −20°C, until use for RNA isolation.

Embryo staining with MitoTracker

D0 ZF embryos were incubated for 45 minutes in SOFaa containing either green MitoTracker (20 mM, M7514; Invitrogen, Carlsbad, CA) or Red MitoTracker (0, 5 mM, M7512; Invitrogen), in humidified conditions at 39°C. After incubation, embryos were washed twice in TALP-H and cultured as 2× condition in the microwell system, by placing one green embryo together with one red embryo within the same well. This procedure was performed first for IVF embryos and then for clones. Contribution of both embryos to the final blastocyst was evaluated by detecting green and red fluorescence on D7 p.a.

IVF procedure

Frozen sperm straws of bulls with proven fertility were thawed in a 37°C water bath, for 30 seconds and collected in Brackett–Oliphant (BO) medium (Bracket and Oliphant, 1975). Spermatozoa were then washed twice by centrifugation at 490 *g* for 5 minutes to remove cryoprotectants and finally resuspended in BO medium, supplemented with 5 mM caffeine (C4144; Sigma) and 20 IU/mL heparin (H3149; Sigma). Spermatozoa concentration was adjusted to 1.6×10^7 spermatozoa/mL with BO medium containing fatty acid-free BSA (10 mg/mL, A6003; Sigma). A Petri dish with 100 μL droplets of sperm suspension was prepared, and 20 to 25 COCs were placed in each drop for 5 hours under 5% CO_2 in humidified air. After IVF, cumulus cells were removed by vortexing the presumptive zygotes for 2 minutes in hyaluronidase (H-4272) (1 mg/mL in Dulbecco's PBS) and cultured in 50 μL SOFaa droplets supplemented with 2.5% FBS at 38.5°C in humidified gas mixture (5% CO_2, 5% O_2, 90% N_2).

Determination of the optimal stage of development for aggregation

Fully synchronous ZF IVF embryos were aggregated at different days of development: D0 (1 cell; $n=35$), D3 (8–16 cells; $n=38$), D4 (16–32 cells; $n=39$), D5 (noncompacted morulae; $n=47$), D6 (early blastocyst; $n=48$), and D7 (blastocyst; $n=45$) in a microwell system. Embryos were cultured at the same conditions as described before. On each day, a nonaggregated group was included as reference. Aggregation and developmental rates were evaluated on D8. Nonaggregation was considered every time that the final blastocyst was derived only from one original embryo, and this includes those cases where one embryo did not develop.

RNA isolation and real-time quantitative-PCR (RTqPCR) analysis

Pools of five blastocysts were collected from the following groups: ASC1×, ASC2×, FAB1×, FAB2×, and IVF as reference. Samples were stored at −20°C in 10 μL RNAlater® (AM 7020; Ambion, Foster City, CA) until RNA extraction. For each group, three biological replicates were analyzed. An IVF control group was also included. After washing the RNAlater using DPBS, the Cells-to-cDNA TM II Kit (Ambion, Austin, TX) was used for total RNA isolation, according to the manufacturer's instruction. Reverse transcription and quantitative-PCR (qPCR) were performed as it was described in Rodríguez-Alvarez et al. (2010).

Briefly, first-strand cDNA was synthesized using 10 μL of total RNA in a 20 μL final reaction containing 5 μM random primers, 10 mM each dNTP, 2 μL first-strand buffer (10×), 10 U of RNase inhibitor, and 200 U/mL M-MuLV (Ambion, Austin, TX). Cycling parameters were: 70°C for 3 minutes, 42°C for 60 minutes, and 92°C for 10 minutes. qPCR analysis was performed by the Standard Curve Method using gene-specific primers (Table 1) in the MX3000P real-time PCR device (Agilent, Santa Clara, CA). Samples were loaded as duplicates (technical replicates) in 10 μL final volume containing 2 μL of cDNA from each sample, 1 μL of primers (10 pmol each, forward and reverse), and 5 μL of 2× Sensimix SYBR Hi-ROX (Bioline, Luckenwalde, Berlin, Germany). Melting curves (CP) and threshold values were calculated with built-in software for all the runs.

To construct each standard curve, eight points of serial 10-fold dilutions of PCR products were included, using 2 μL template and two technical replicates for each gene. PCR products used for the standard curve were purified from agarose gel, eluted using the E.Z.N.A Gel Extraction Kit (Omega, Biotek, Norcross, GA) and quantified using a

TABLE 1. PRIMER SEQUENCES USED FOR THE ANALYSIS OF EMBRYO QUALITY BY RTQPCR

Gene	*Primer sequences (5′-3′)*	*Annealing temperature (°C)*	*Product length (bp)*	*Accession number (NCBI)*
OCT4	F: 5′-GGAGAGCATGTTCCTGCAGTGC 3′ R: 5′-ACACTCGGACCACGTCCTTCTC 3′	58	95	NM_174580
SOX2	F: 5′-CGAGTGGAAACTTTTGTCCG 3′ R: 5′-GGTATTTATAATCCGGGTGTT 3′	55	101	NM_001105463
KRT18	F: 5′-GACCTGAGGGCTCAGATTTTTG 3′ R: 5′-ATGGCCAGCTCTGTCTCATAC 3′	58	119	NM_001192095.1
ACTB	F: 5′-GGCCAACCGTGAGAAGATGACC 3′ R: 5′-GAGGCATACAGGGACAGCACAG 3′	58	96	BT030480.1

spectrophotometer (Epoch; Biotek Instruments, Inc., Winooski, VT). The expression of each gene was normalized to the expression of ACTB.

Uniformity of expression withing the replicates and samples was evaluated by assessing the correlation between the expression (Ct) within replicates (meaning replicates from the same samples within a group [$r=0.99$; $p=1.40E-08$]) and also the analysis between different experimental groups ($p=0.1$). Only PCR experiments with an efficiency within the range of 90% to 110% and a correlation coefficient of at least 0.9 were used for gene expression analysis. Only samples within the quantification range of the standard curve were considered for the analysis.

Statistical analysis

The developmental rate of IVF and SCNT embryos was assessed at least three times. Differences in cleavage, morula, and blastocyst rates among groups were determined using the GraphPad Prism version 5.01 program by Fisher's exact test. Differences were considered significant at $p \leq 0.05$, with a 95% confidence interval. The analysis of gene expression data was performed using a nonparametric Kruskal–Wallis test and Chi-squared test. Differences were considered significant at $p \leq 0.1$, with a 95% confidence interval.

Results

In the present study, we aimed to elucidate whether the combined use of mesenchymal stem cells as nuclear donors together with the EA strategy improves SCNT efficiency in the bovine. We first evaluated the optimal stage of development for EA to ensure the homogeneous contribution of the aggregated embryos to the final blastocyst. Next, we successfully isolated and characterized bovine ASC and fibroblast cells from an adult cow. Finally, we determined and compared the developmental rates of aggregated embryos reconstructed either with ASC or FAB, to nonaggregated clones and we assessed the blastocyst quality by RT-qPCR.

D0 is the optimal time-window for IVF EA

IVF embryos were used to determine the most favorable stage of development for EA. Then, optimized conditions were translated to SCNT embryos. EA was considered every time the two structures cultured in close contact in a microwell system at a specific stage of development (D0, D3, D4, D5, D6, or D7) resulted in one final blastocyst, assessed at D8. Thus, EA occurred when structures were aggregated at D0 (54.2%), D3 (52.6%), D4 (48.7%), and D5 (51%) after fertilization. The effect of EA on the development of IVF bovine embryos at different stages is shown in Table 2. Surprisingly, no aggregation was observed on D6 and D7, as each independent embryo developed into a separated blastocyst. Blastocyst rates per RE did not show significant differences between groups.

However, when analyzing the blastocyst rate per well, the comparison between 2× and 1× group at each day revealed significant differences when embryos were aggregated at D0 (2×, 54.2% vs. 1×, 25.5%) and D3 (2×, 52.6% vs. 1×, 25.3%). Blastocyst rates per well were not significantly different when aggregation was performed at D4 and D5. Overall, these results suggest that there is a time window between D0 and D3 for the aggregation of two embryos that improves blastocyst rates. In view of these results, the aggregation of embryos immediately after *in vitro* production (D0) was determined as the optimal aggregation time for further experiments, to avoid manipulation of the embryos later during the *in vitro* development.

Table 2. Effects of Bovine *In Vitro* Fertilization Embryo Aggregation on *In Vitro* Development Until D8

Groups		*No. of embryos used*	*No. of aggregated embryos*	*No. of blastocysts (%)*	*% Blastocysts per well**
D0	2×	70	35	19 (27.1)	54.2[a]
	1×	43	n.a.	11 (25.5)	25.5[b]
D3	2×	76	38	20 (26.3)	52.6[a]
	1×	63	n.a.	16 (25.3)	25.3[b]
D4	2×	78	39	19 (24.3)	48.7[a]
	1×	47	n.a.	14 (29.7)	29.7[a]
D5	2×	94	47	24 (25.5)	51[a]
	1×	40	n.a.	15 (37.5)	37.5[a]

*Values with different superscripts in a column are significantly different (Fisher's exact test $p \leq 0.05$) (a, b).
n.a.; nonaggregated embryos.

Aggregation of two IVF embryos at D0 leads to homogeneous blastomere distribution at the blastocyst stage

To establish whether the aggregated embryos contribute equally and homogeneously to the whole final blastocyst, two IVF embryos were aggregated at the one-cell stage and their contribution to the blastocyst was assessed by dye staining. To do this, immediately after IVF, 50% of the zygotes were stained with Green MitoTracker, while the remaining 50% were stained with Red MitoTracker. Randomly, a green-stained zygote and a red-stained zygote were placed together within the same microwell and cultured until the blastocyst stage. The presence and distribution of green and red cells within the blastocyst were assessed under UV light exposure (Fig. 1A).

As reference, a nonaggregated (1×) green-stained blastocyst is shown in Figure 1B. The coexistence of red and green cells was confirmed in all 2× blastocysts evaluated (100%). It is noteworthy that no specific pattern was detected in the distribution of the stained cells. Instead, red and green cells were allocated homogeneously throughout the blastocyst, contributing both to the establishment of the TE and the ICM (Fig. 1). *In vitro* embryo developmental rates were within the normal parameters for this technique (data not shown) indicating that there was no compromise on the embryo development due to the use of MitoTrackers.

Bovine ASC isolation and characterization

With the goal of assessing whether the differentiation state of the adult donor cell impacts on the bovine SCNT efficiency, ASC and fibroblast with the same genetic background were obtained. ASC characterization was performed in accordance to the International Society for Cellular Therapy criteria.

The flow cytometric histograms confirmed that nearly 80% of the analyzed cells expressed CD44 and CD166 on the cell surface, and both biomarkers are associated with the mesenchymal lineage (Fig. 2). In addition, cells were

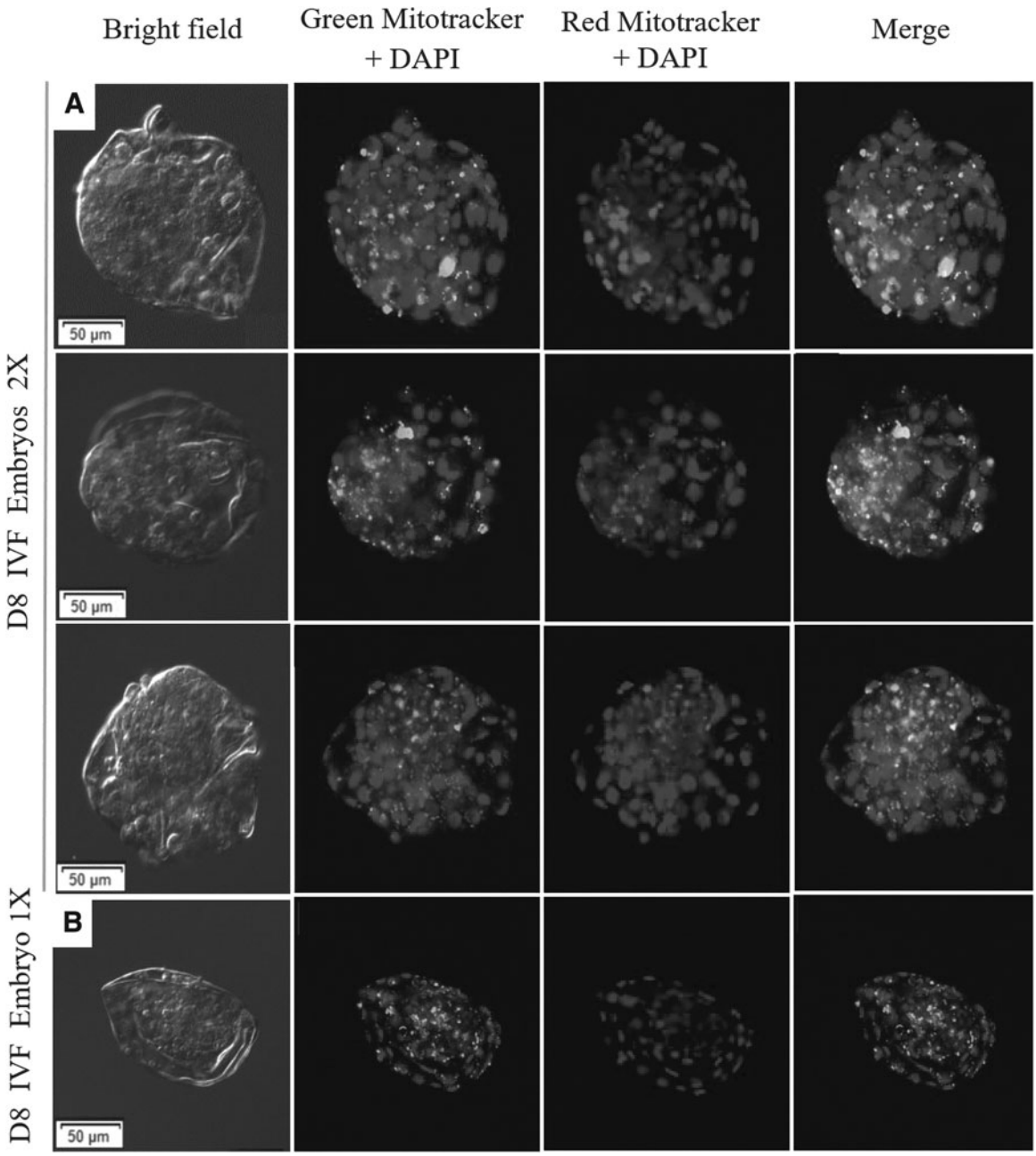

FIG. 1. Representation of the uniform blastomere distribution patterns of aggregated IVF bovine embryos, observed at D8 in a confocal microscope. **(A)** Three different IVF blastocysts aggregated (2×) at D0 stained with Mito-Tracker Green FM or Mito-Tracker Red CMXRos, and DAPI. **(B)** Nonaggregated (1×) blastocyst stained with Mito-Tracker Green FM and DAPI. Zoom 40×. IVF, *in vitro* fertilization.

negative when assessed for CD45 cell marker presence, which is specific for hematopoietic cells. This is consistent with the immunophenotype defined for ASCs.

Regarding the multipotentiality of the isolated cells, cell cultures at passage 4 were treated for 14 days to chemically induce the *in vitro* differentiation toward the adipocyte, osteocyte, and chondrocyte lineage. After the treatment, cell cultures were fixed and stained with specific dyes to evaluate their differentiation status.

After adipogenic induction, the accumulation of lipid droplets in the cytoplasm of the treated cells was detected (Fig. 3A). All microdroplets were selectively colored red-orange when using the lipophilic red, Sudan dye. Instead, cells treated with chondrogenic medium showed a blue-green coloration after fixation and staining with Alcian Blue. This dye has a high affinity for glycosaminoglycans, which are abundant in the extracellular cartilaginous matrix and surround chondrocytes cultured *in vitro* (Fig. 3B). Finally, after osteogenic induction, cells showed an intense red coloration after treatment with Alizarin Carmine, confirming the presence of calcium deposits in the treated cells (Fig. 3C).

Results from cell characterization by immunotyping and evaluation of their multipotent potential confirmed that the adipose-derived cells have a mesenchymal nature.

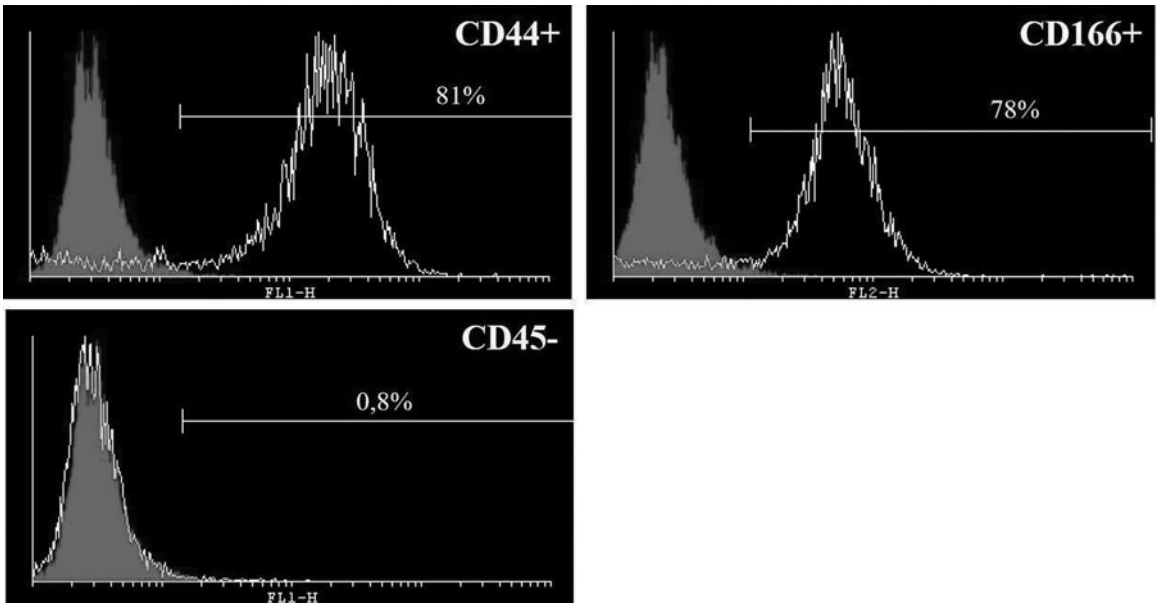

FIG. 2. Characterization of cell surface markers by flow cytometry. Histograms show cell fluorescence intensity on the x-axis and cell frequency distribution on the y-axis. Adipose-derived cells were positive for CD44 and CD166 mesenchymal markers and negative for the hematopoietic marker, CD45.

Aggregation of two SCNT embryos at D0 leads to homogeneous blastomere distribution at the blastocyst stage

To evaluate if the aggregation of SCNT embryos recapitulates the results observed after IVF EA, two cloned embryos produced from ASC donor cells were stained and aggregated at the one cell stage (D0), and their contribution to the blastocyst was assessed by fluorescence detection under UV light (Fig. 4B). The coexistence of red and green cells was confirmed in 17 out of 18 2×blastocysts evaluated (94.4%). Similar to the aggregation of IVF embryos, no specific pattern was detected in the distribution of the stained cells. Red and green cells were allocated homogeneously throughout the blastocyst, contributing both to the establishment of the TE and the ICM (Fig. 4).

Effect of the use of ASC and EA on the in vitro *development of bovine clones*

Next, we evaluated whether the combination of EA together with the use of mesenchymal stem cells as nuclear donors improves the SCNT efficiency *in vitro* in the bovine.

Aggregation of FAB RE (FAB 2×) improved the cleavage as well as the morulae rates relative to the nonaggregated group (FAB 1×; Table 3); however, although there was a trend to improve the blastocyst rate per well, blastocyst production was comparable between 1×and 2×groups. Contrarily, 1×and 2×ASC-RE showed similar rates of cleavage and morulae production; but in this case, EA significantly improved the blastocyst production per well.

When comparing the use of different donor cells with similar genetic background, the rates of cleavage, morula, and blastocyst were similar between groups, independently of the culture condition (1×or 2×). Surprisingly, the combined use of ASC and EA did not improve the blastocyst embryo production compared with the use of FAB cells in the same culture condition.

Effect of the use of ASC and EA in the quality of bovine clones

It was reported that both strategies, the use of multipotent donor cells as well as the EA technique, improve the quality of cloned embryos in several species. To evaluate the effect

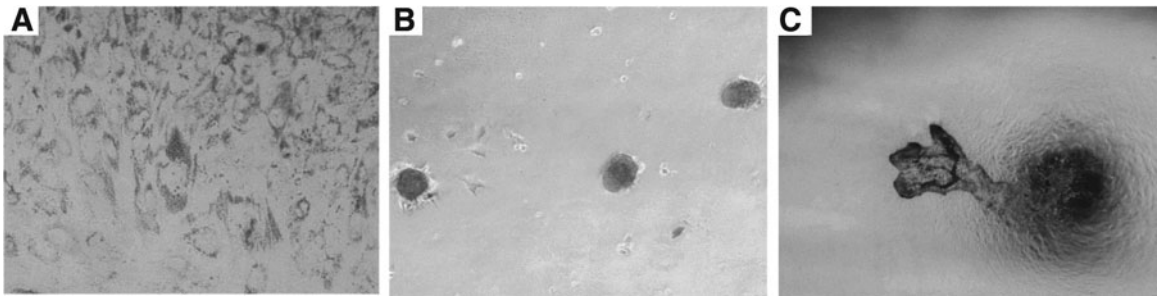

FIG. 3. *In vitro* differentiation assay of adipose-derived mesenchymal stem cells into: **(A)** chondrogenic (Alcian-Blue staining—Identification of the glycosaminoglycans in the extracellular cartilaginous matrix of the cells), **(B)** adipogenic (Sudan-Red staining—Identification of lipid droplets) and **(C)** osteogenic (Alizarin-Red staining—Presence of calcium deposits) lineages. Isolated cells were able to differentiate into these three lineages.

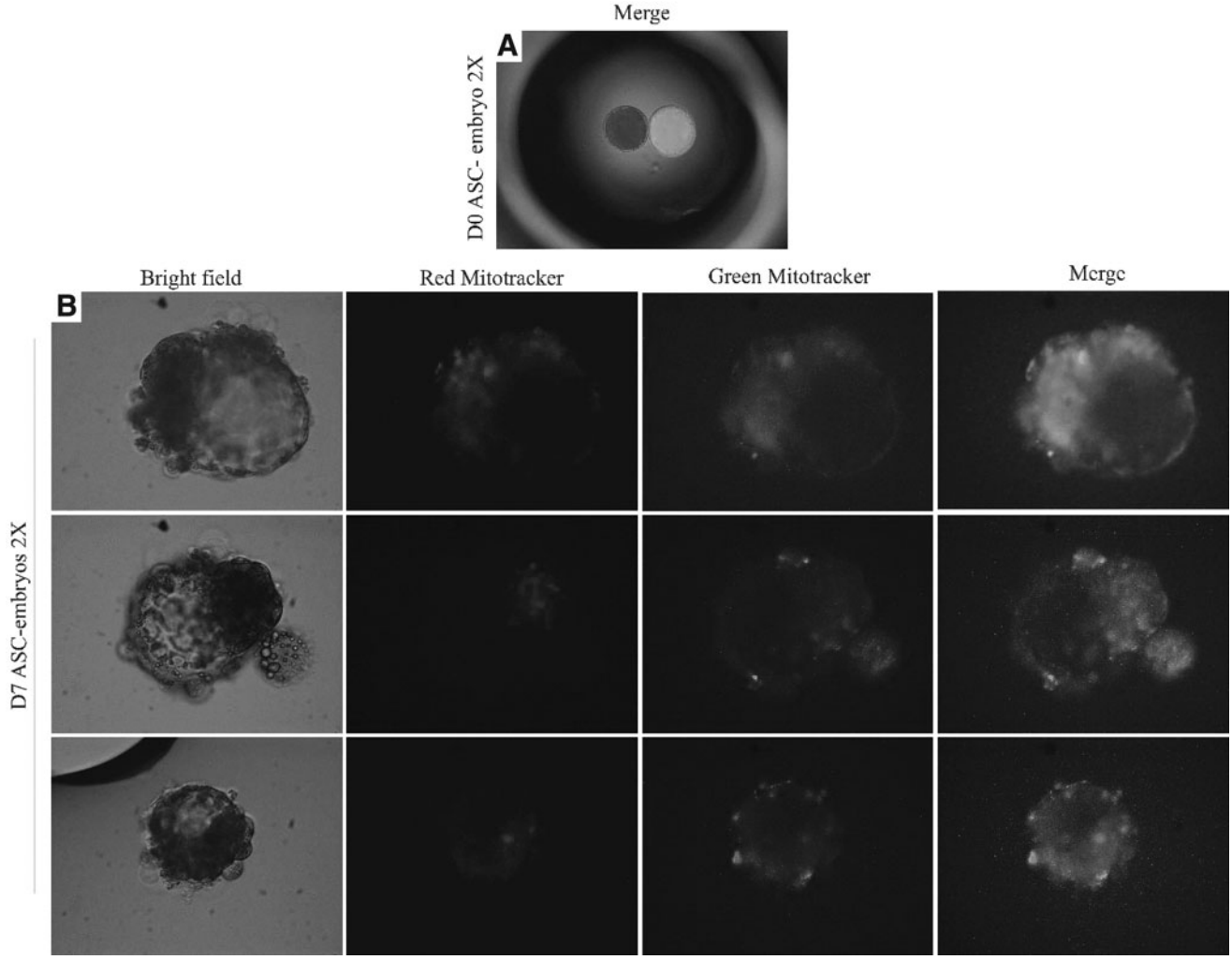

FIG. 4. Representation of the uniform blastomere distribution patterns of aggregated ASC clones. **(A)** Two D0-clones stained with Mito-Tracker Green FM or Mito-Tracker Red CMXRos placed together in a microwell. Zoom 20× **(B)** Three different SCNT blastocysts aggregated (2×) at D0, stained with Mito-Tracker Green FM or Mito-Tracker Red CMXRos, observed at D7. Zoom 40×. SCNT, somatic cell nuclear transfer.

of the combined used of multipotent donor cells and EA on the cloned blastocyst quality, we assessed the relative expression of genes that control differentiation, such as the pluripotent markers *OCT4* (Nichols et al., 1998) and *SOX2* (Avilion et al., 2003) and the TE-related marker *KRT18* (Madeja et al., 2013). IVF blastocyst was included as a reference group. As shown in Figure 5, no differences were found between groups for *SOX2* or *OCT4* expression;

Table 3. Effect of the Use of Adipose-Derived Stem Cells as Donor Cells and Embryo Aggregation on the *In Vitro* Development of Bovine Embryos Produced by Somatic Cell Nuclear Transfer Until D7

				D2	*D5*	*D7*		
Donor cell type	*IVC*	*No. of embryos reconstructed*	*No. of aggregates*	*Cleavage (%)**	*Morula (%)**	*No. of Blastocysts*	*Blastocyst formation rate of reconstructed embryos**	*Blastocysts per well**
FAB	2×	126	63	62 (98.41)^{b}	34 (53.97)^{b}	25	19.84^{a}	39.68^{a}
	1×	82	n.a.	72 (87.80)^{a}	27 (32.93)^{a}	22	26.82 ^{ab}	26.82^{ab}
ASC	2×	204	102	90 (88.24)^{a}	45 (44.12)^{ab}	34	16.66^{a}	33.33^{a}
	1×	198	n.a.	177 (89.39)^{a}	65 (32.83)^{a}	43	21.72^{a}	21.72^{b}
PA	2×	78	39	38 (97.44)^{ab}	33 (84.62)^{c}	27	34.62^{c}	69.23^{c}
	1×	160	n.a.	149 (93.13)^{ab}	83 (51.88)^{b}	56	35^{bc}	35^{a}

*Values with different superscripts in a column are significantly different (Fisher's exact test $p \leq 0.05$) (a, b, c).

ASCs, adipose-derived stem cells; FAB, fibroblasts from an adult bovine; IVC, *in vitro* culture; n.a., nonaggregated embryos; PA, parthenogenetic embryo.

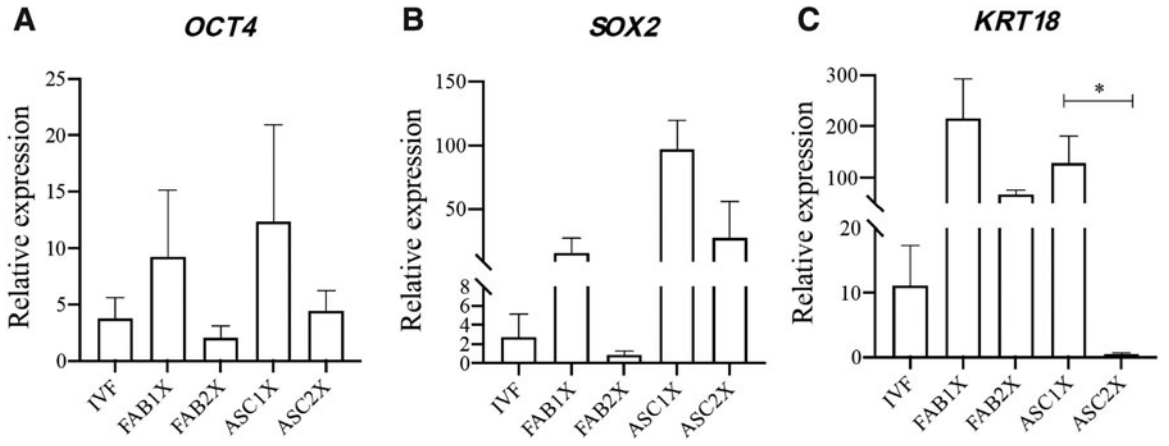

FIG. 5. Embryo quality analysis of bovine SCNT embryos. Relative abundance of transcripts **(A)** *OCT4*, **(B)** *SOX2*, and **(C)** *KRT18*. Gene expression was evaluated in aggregated (2×) and nonaggregated (1×) clones produced from ASC or FAB. Results were compared with IVF bovine embryos. Error bars in all panels display standard error of the mean (SEM). FAB, fibroblasts from an adult bovine.

however, a significant difference in *KRT18* expression was observed when ASC-derived clones were aggregated relative to the nonaggregated group (ASC1×).

Surprisingly, an interesting trend was observed for all three analyzed genes. Regardless of the donor cell, nonaggregated embryos (1×) tend to have a higher expression of the three analyzed transcription factors, relative to IVF control. Notably, for all cases, EA (2×) seems to reduce the abnormal overexpression and, therefore, gene expression closely resembles that observed for IVF controls. Taken as a whole, these results suggest that EA may improve the quality of bovine SCNT blastocyst.

Discussion

Certainly, SCNT is a widely used method for basic and applied research, unique in its ability to reprogram a fully differentiated cell. However, its efficiency remains extremely low (Czernik et al., 2019) probably due to the incomplete or inefficient epigenome reprogramming of the donor cell (reviewed by Gouveia et al., 2020). In the present work, we first established the best condition for bovine EA, a strategy known to compensate for possible epigenetic failures after donor cell reprogramming in several species (Eckardt and McLaughlin, 2004). Then, we combined the EA strategy with the use of ASC as nuclear donors for SCNT, assuming that their plasticity may facilitate the nuclear reprogramming (Ono and Kono, 2006). The ultimate goal of this work was to assess simple and accessible strategies to improve the SCNT efficiency in a simple fashion and, therefore, its widespread use.

In an initial assay, we aimed to establish the optimal time for bovine EA. IVF embryos were used for the optimization steps to then use the best experimental design for SCNT studies. Two synchronous IVF embryos were placed within the same microwell at different time points of embryo development (D0, D3, D5, D6, or D7) and the aggregation rate was evaluated at D8. Individually cultured embryos served as controls. Blastocyst rates showed no significant differences when aggregation was performed at D0, D3, D4, and D5 of development, on an RE basis.

Remarkably, significant differences were observed between 2×and 1×for D0 and D3 groups. It is worthy to note that because no differences in the blastocyst rates were observed on a per-RE basis, the aggregation did not involve the use of additional oocytes. For groups D4 and D5, 2×and 1×were not significantly different. Consequently, even though results obtained from D0 and D3 groups were comparable, D0 was considered the optimal aggregation time for further experiments to avoid extra manipulation during embryo culture, which is known to be detrimental for embryo development (Basini et al., 2008; Fatehi et al., 2005; Gaspar et al., 2015). A surprising fact that emerges from this assay and has not been previously reported in bovine embryos is that, in our culture conditions, bovine ZF synchronous embryos lose their aggregation capacity after D6 of development.

EA at D0 resulted in homogeneous blastomere allocation at the blastocyst stage. By using either green and red MitoTracker staining, we confirmed that the aggregation of two different bovine embryos performed at D0 results in a single blastocyst, in which green and red blastomeres are intermixed and homogeneously distributed. This result is true for IVF and SCNT embryos and is in agreement with that reported by our laboratory for the aggregation of feline clones (Moro et al., 2015). In bovine, it was also reported that there was no bias in the blastomere distribution when two SCNT embryos at the 6–12-cell stage were aggregated.

Contrarily, SCNT embryos aggregated with IVF or parthenogenetic embryos result in a special biased distribution of the blastomeres (Wells and Powell, 2000). Thus, the results presented in this work clearly demonstrate that the EA strategy at D0 ensures blastocyst production with homogeneous contribution of the blastomeres to both TE and ICM and, importantly, without negative consequences on embryo development.

Regarding bovine SCNT, the EA strategy or the use of mesenchymal cells as nuclear donors did not necessarily improve the blastocyst rate (Akagi et al., 2011; Colleoni et al., 2005). However, both strategies led to an improvement on embryo quality (Bang et al., 2015; da Silva et al., 2016; Picou, 2009). It is worth mentioning that in bovine, the EA strategy significantly improves the blastocyst rate per well of embryos produced by handmade cloning, even when two structures were cultured together (Ribeiro et al., 2009). Reports on bovine SCNT-EA remain inconclusive mainly

due to the different culture strategies used or the timing for EA. Moreover, to the best of our knowledge, the impact of the combined use of both strategies on the blastocyst production and embryo quality has not yet been evaluated.

In our hands, the EA strategy improves the *in vitro* embryo development per well of cloned embryos derived from both ASC and FAB cells. The cleavage as well as morula rates were significantly higher for the FAB2× embryos relative to the nonaggregated group (FAB1×). The blastocyst production followed the same trend; however, rates did not differ significantly between FAB2× and FAB1×. When ASCs were used as nuclear donor, a significantly higher blastocyst rate per well was obtained after EA (ASC2×) relative to the nonaggregated group (ASC1×). It is worthy to note that, in every replicate, the total number of embryos cultured within a 100 μL drop was comparable, regardless of the culture condition (1× and 2×).

This is important, as it has been previously shown that preimplantation embryo development is significantly improved when embryos are cultured in group, resulting in an increase in blastocyst formation, cell number, and gene expression probably due to paracrine/autocrine interactions (Gambini et al., 2014; Gopichandran and Leese, 2006). Because the embryo density was similar between 1× and 2× conditions, the improvement observed on the *in vitro* embryo production per well could be attributed to the epigenetic compensation between the aggregated structures and the higher cell number at the beginning of *in vitro* culture, as it was previously suggested for the mouse and the pig (Buemo et al., 2016; Eckardt and McLaughlin, 2004).

The positive effects of SCNT EA have been reported in several species. SCNT EA at D0 improves not only the blastocyst rate per well but also the embryo quality in domestic cat and pig (Buemo et al., 2016; Moro et al., 2015). In the horse, Gambini et al. (2012, 2014) evaluated the effect of *in vitro* aggregation of 2, 3, 4, and 5 cloned embryos and reported that the increase in the blastocyst rates per well was proportional to the number of aggregated structures, except for the 5× group. The authors propose that the EA strategy improves the developmental competence of the cloned embryos by compensating for epigenetic defects of individual cells. During SCNT reprogramming, factors present in the ooplasm erase the epigenetic identity of the donor somatic cell and reprograms it into a totipotent state, similar to zygotes.

The comparison of H3K9me3 levels or DNA methylation at 5-methylcytosine between SCNT or IVF-derived embryos suggest that some regions of the genome are resistant to this reprogramming. Moreover, the analysis of individual clones shows that each cell is reprogrammed differently (Liu et al., 2016a, 2018a). Therefore, the culture of multiple individually reprogrammed embryos may help compensate the incomplete reprogramming of the nucleus. Also, the fact that differently reprogrammed nuclei contribute to the same embryo could be beneficial, as it was recently shown that cell-to-cell heterogeneities within the early embryo are crucial for lineage differentiation in ICM or TE at the blastocyst stage (Lim et al., 2020).

Particularly in the bovine, SCNT EA remains contradictory. However, it is worth noting that the experimental methodology used in previous reports widely differs from ours. Misica-Turner et al. (2007) reported no benefits on the aggregation of three bovine cloned embryos in microdroplets. The microwell system used in our work ensures the embryo–embryo contact, improving both three-dimensional (3D) organization and communication between embryos/blastomeres to grant proper blastomere differentiation. In this sense, it was demonstrated that the spatial organization of the blastomeres at the early four-cell stage plays an important role in transcriptional and lineage differentiation in the mouse (White and Plachta, 2020). Similarly, the use of 3D culture system in microwells results critical to reconstitute early human embryos from pluripotent stem cells (Sozen et al., 2021).

Other authors opted to use different window-times for EA. Irrespective of the timing of aggregation (D0, D2, and D4), aggregated embryos developed to the blastocyst stage at a high rate, but no improvement was reported on the blastocyst rate per well (Akagi et al., 2011). Similarly, no strong conclusions were obtained in this regard by Bang and collaborators (2015). In our study, no differences were found when aggregation was performed at D0 and D3. However, blastocyst rates per well were not significantly different when aggregation was performed at D4 and D5. As it was proposed, aggregation at advanced stages of development may negatively impact the epigenetic compensation.

One caveat of our study is that the optimal window for EA was determined using IVF embryos and not SCNT embryos. It is clear that IVF and SCNT embryos are different in terms of gene expression and developmental rate, and this may impact on the optimal aggregation window. However, the differences between IVF and SCNT embryos become evident after the embryonic genome activation, which in bovines occurs at the 8C stage (Ross et al., 2010). Then, this resonates with our findings using IVF embryos that the optimized aggregation window determined is between D1 and D3, where embryos have not gone embryonic genome activation. Overall, our results provide conclusive support that the 2×EA at D0 using microwells improves the *in vitro* embryo development of bovine clones.

In our hands, the use of ASC does not increase bovine SCNT efficiency. The use of ASC as nuclear donor does not improve the *in vitro* developmental rates compared with the use of fully differentiated cells, regardless of the culture condition (1× or 2×). This observation for the cattle is contrary to what has been reported in swine and equine, where the use of mesenchymal cells, although from a different source, improved the cloning efficiency (Faast et al., 2006; Olivera et al., 2018).

However, our results are in agreement with another report on bovine (da Silva et al., 2016). A possible explanation for this could be attributed to the cell line used as nuclear donors. ASCs have a shorter cell cycle (Picou, 2009) and, therefore, its synchronization may not be as efficient as for FAB culture, which may impact directly on the overall production efficiency. Another explanation relies on the primary culture used. It is known that different primary cell cultures of the same cell type differ in their potential to be used as donor cells for SCNT and support embryo development (Salamone et al., 2006). In summary, although we extensively tested the ASC for their multipotent differentiation capability, our results suggest that there is no improvement of the SCNT blastocyst rates when clones are produced with ASCs relative to the use of FAB.

The combined use of EA and ASC as donor cells does not result in a synergistic effect on bovine embryo

production nor quality. The expression of key genes such as *SOX2*, *OCT4*, *and KRT18* was compared between groups and normalized to the expression of *ACTB*. The uniformity of expression within replicates was assessed and only those replicates with a high correlation coefficient (90%–110%) were used for gene expression analysis. No improvement was observed on the blastocyst rates nor the relative expression of key genes when EA and ASCs were used in combination, relative to the aggregation of two FAB-derived embryos.

However, a significant difference on *KRT18* expression, a TE-related marker, was detected when ASC-derived EA was performed relative to the ASC nonaggregated group. It is worth noting that an interesting trend was observed for all aggregated embryos, regardless of the donor cell used to produce the clones. EA seems to reduce the abnormal expression of *OCT4* and *SOX2* observed in 1×cloned blastocyst, so they resemble the expression levels observed in IVF blastocyst. In this regard, the chi-squared analysis of our results clearly denoted an association between the aggregation strategy and the gene expression for all analyzed genes (*SOX2*, *OCT4*, and *KRT18*, data not shown), providing additional support to the benefits of EA. Similar results were reported for EA in domestic cats, where aggregation of two embryos normalized the expression of master key genes to levels comparable to IVF embryos (Moro et al., 2015).

In pigs, the EA strategy improved the expression of *CDX2*, a TE-related gene (Buemo et al., 2016). Of note, placentation failures and placental pathologies are very common in SCNT-produced embryos (Balbach et al., 2010; Chavatte-Palmer et al., 2018, Review) and these failures may be associated with aberrant TE proliferation and differentiation during the preimplantation stage (Knott and Paul, 2014). The fact that the EA strategy directly impacts on the TE-related gene expression, as shown in this study and in previous reports, may have important implications for pregnancy establishment and gestation support after embryo transfer to surrogate cows.

Also, deciphering the molecular basis of the EA mechanism in bovines could have an enormous impact on assisted reproductive techniques in humans as the mechanisms of bovine and human TE development are closely similar (Gerri et al., 2020). Taken together, these results provide additional support to EA as a simple strategy to improve bovine SCNT efficiency.

Conclusions

In summary, after extensively and carefully controlling the aggregation conditions, our results suggest that aggregation of two embryos at D0 is a simple strategy that improves the blastocyst rate per well in both bovine SCNT and IVF embryos. In our conditions, the use of ASC as donor for SCNT has no effect on embryo development or embryo quality. Finally, the combined use of ASC and EA has no effect on the *in vitro* embryo production of bovine clones but the aggregation of two ASC-derived embryos impacts on the TE-related marker expression providing additional support to EA as a simple strategy to improve bovine SCNT efficiency.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Credit Authorship Contribution Statement

V.S. and V.A.: conceptualization, methodology, formal analysis, writing—original draft. G.V.L., L.N.M., and F.D.O.: methodology. L.R.-A.: methodology and formal analysis. D.F.S.: supervision, project administration, writing, review and editing, and funding acquisition.

Acknowledgments

The authors sincerely thank Eco Carnes S.A. (Argentina) for providing the biological material and Maimónides University for supporting this work. Also, CONICET and ANPCyT are gratefully acknowledged.

Author Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding Information

This work was supported by the ANPCyT from the Ministry of Science and Technology of Argentina PICT-2014-3288.

References

Aigner, B., Renner, S., Kessler, B., Klymiuk, N., Kurome, M., Wünsch, A., and Wolf, E. (2010). Transgenic pigs as models for translational biomedical research. J. Mol. Med. (Berl.) 88, 653–664.

Akagi, S., Yamaguchi, D., Matsukawa, K., Mizutani, E., Hosoe, M., Adachi, N., Kubo, M., and Takahashi, S. (2011). Developmental ability of somatic cell nuclear transferred embryos aggregated at the 8-cell stage or 16- to 32-cell stage in cattle. J. Reprod. Dev. 57, 500–506.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 17, 126–140.

Balbach, S.T., Esteves, T.C., Brink, T., Gentile, L., McLaughlin, K.J., Adjaye, J.A., and Boiani, M. (2010). Governing cell lineage formation in cloned mouse embryos. Dev. Biol. 343, 71–83.

Bang, J.I., Jin, J.I., Ghanem, N., Choi, B.H., Fakruzzaman, M., Ha, A.N., Lee, K.L., Uhm, S.J., Ko, D.H., Koo, B.C., Lee, J.G., and Kong, I.K. (2015). Quality improvement of transgenic cloned bovine embryos using an aggregation method: Effects on cell number, cell ratio, embryo perimeter, mitochondrial distribution, and gene expression profile. Theriogenology 84, 509–523.

Basini, G., Simona, B., Santini, S.E., and Grasselli, F. (2008). Reactive oxygen species and anti-oxidant defences in swine follicular fluids. Reprod. Fertil. Dev. 20, 269–274.

Boiani, M., Eckardt, S., Leu, N.A., Schöler, H.R., and McLaughlin, K.J. (2003). Pluripotency deficit in clones overcome by clone–clone aggregation: Epigenetic complementation? EMBO J. 22, 5304–5312.

Brackett, B.G., and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12, 260–274.

Buemo, C.P., Gambini, A., Moro, L.N., Hiriart, M.I., Fernández-Martín, R., Collas, P., and Salamone, D.F. (2016). Embryo aggregation in pig improves cloning efficiency and embryo quality. PLoS One 11, e0146390.

Bui, L.C., Evsikov, A.V., Khan, D.R., Archilla, C., Peynot, N., Henaut, A., Le Bourhis, D., Vignon, X., Renard, J.P., and Duranthon, V. (2009). Retrotransposon expression as a defining event of genome reprograming in fertilized and cloned bovine embryos. Reproduction 138, 289.

Chavatte-Palmer, P., Velazquez, M.A., Jammes, H., and Duranthon, V. (2018). Review: Epigenetics, developmental programming and nutrition in herbivores. Animal 12, s363–s371.

Colleoni, S., Donofrio, G., Lagutina, I., Duchi, R., Galli, C., and Lazzari, G. (2005). Establishment, differentiation, electroporation, viral transduction, and nuclear transfer of bovine and porcine mesenchymal stem cells. Cloning Stem Cells 7, 154–166.

Cooper, D.K., Satyananda, V., Ekser, B., van der Windt, D.J., Hara, H., Ezzelarab, M.B., and Schuurman, H.J. (2014). Progress in pig-to-non-human primate transplantation models (1998–2013): A comprehensive review of the literature. Xenotransplantation 21, 397–419.

Czernik, M., Anzalone, D.A., Palazzese, L., Oikawa, M., and Loi, P. (2019). Somatic cell nuclear transfer: Failures, successes and the challenges ahead. Int. J. Dev. Biol. 63, 123–130.

da Silva, C.G., Martins, C.F., Cardoso, T.C., Da Cunha, E.R., Bessler, H.C., Martins, G.H.L., Pivato, I., and Báo, S.N. (2016). Production of bovine embryos and calves cloned by nuclear transfer using mesenchymal stem cells from amniotic fluid and adipose tissue. Cell. Reprogram. (Formerly ''Cloning Stem Cells'') 18, 127–136.

Degrelle, S.A., Jaffrezic, F., Campion, E., Lê Cao, K.A., Le Bourhis, D., Richard, C., Rodde, N., Fleurot, R., Everts, R.E., Lecardonnel, J., Heyman, Y., Vignon, X., Yang, X., Tian, X.C., Lewin, H.A., Renard, J.-P., and Hue, I. (2012). Uncoupled embryonic and extra-embryonic tissues compromise blastocyst development after somatic cell nuclear transfer. PLoS One 7, e38309.

Eckardt, S., and McLaughlin, K.J. (2004). Interpretation of reprogramming to predict the success of somatic cell cloning. Anim. Reprod. Sci. 82, 97–108.

Faast, R., Harrison, S.J., Beebe, L.F., Mcilfatrick, S.M., Ashman, R.J., and Nottle, M.B. (2006). Use of adult mesenchymal stem cells isolated from bone marrow and blood for somatic cell nuclear transfer in pigs. Cloning Stem Cells 8, 166–173.

Fatehi, A.N., Roelen, B.A., Colenbrander, B., Schoevers, E.J., Gadella, B.M., Bevers, M.M., and Van Den Hurk, R. (2005). Presence of cumulus cells during *in vitro* fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote 13, 177.

Gambini, A., De Stefano, A., Bevacqua, R.J., Karlanian, F., and Salamone, D.F. (2014). The aggregation of four reconstructed zygotes is the limit to improve the developmental competence of cloned equine embryos. PLoS One 9, e110998.

Gambini, A., Jarazo, J., Olivera, R., and Salamone, D.F. (2012). Equine cloning: *in vitro* and *in vivo* development of aggregated embryos. Biol. Reprod. 87, 15.

Gaspar, R.C., Arnold, D.R., Corrêa, C.A., da Rocha, C.V., Jr., Penteado, J.C., Del Collado, M., Del Collado, M., Vantini, R., Garcia, J.M., and Lopes, F.L. (2015). Oxygen tension affects histone remodeling of *in vitro*–produced embryos in a bovine model. Theriogenology 83, 1408–1415.

Gerri, C., McCarthy, A., Alanis-Lobato, G., Demtschenko, A., Bruneau, A., Loubersac, S., Fogarty, N.M.E., Hampshire, D., Elder, K., Snell, P., Christie, L., David, L., Van de Velde, H., Fouladi-Nashta, A.A., and Niakan, K.K. (2020). Initiation of a conserved trophectoderm program in human, cow and mouse embryos. Nature 587, 443–447.

Gonzalez-Munoz, E., and Cibelli, J.B. (2018). Somatic cell reprogramming informed by the oocyte. Stem Cells Dev. 27, 871–887.

Gopichandran, N., and Leese, H.J. (2006). The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. Reproduction 131, 269–277.

Gouveia, C., Huyser, C., Egli, D., and Pepper, M.S. (2020). Lessons learned from somatic cell nuclear transfer. Int. J. Mol. Sci. 21, 2314.

Hall, V., Hinrichs, K., Lazzari, G., Betts, D.H., and Hyttel, P. (2013). Early embryonic development assisted reproductive technologies, and pluripotent stem cell biology in domestic mammals. Vet. J. 197, 128–142.

Knott, J.G., and Paul, S. (2014). Transcriptional regulators of the trophoblast lineage in mammals with hemochorial placentation. Reproduction 148, R121.

Li, Z., He, X., Chen, L., Shi, J., Zhou, R., Xu, W., Liu, D., and Wu, Z. (2013). Bone marrow mesenchymal stem cells are an attractive donor cell type for production of cloned pigs as well as genetically modified cloned pigs by somatic cell nuclear transfer. Cell. Reprogram. (Formerly ''Cloning Stem Cells'') 15, 459–470.

Lim, H., Alvarez, Y.D., Gasnier, M., Wang, Y., Tetlak, P., Bissiere, S., Wang, H., Biro, M., and Plachta, N. (2020). Keratins are asymmetrically inherited fate determinants in the mammalian embryo. Nature 585, 404–409.

Liu, W, Liu, X., Wang, C., Gao, Y., Gao, R., Kou, X., Zhao, Y., Li, J., Wu, Y., Xiu, W., Wang, S., Yin, J., Liu, W., Cai, T., Wang, H., Zhang, Y., and Gao, S. (2016a). Identification of key factors conquering developmental arrest of somatic cell cloned embryos by combining embryo biopsy and single-cell sequencing. Cell Discov. 2, 16010.

Liu, X., Liu, H., Wang, M., Li, R., Zeng, J., Mo, D., Cong, P., Liu, X., Chen,Y., and He, Z. (2019). Disruption of the ZBED6 binding site in intron 3 of IGF2 by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang Small Spotted pigs. Transgenic Res. 28, 141–150.

Liu, X., Wang, Y., Gao, Y., Su, J., Zhang, J., Xing, X., Zhou, C., Yao, K., An, Q., and Zhang, Y. (2018a). H3K9 demethylase KDM4E is an epigenetic regulator for bovine embryonic development and a defective factor for nuclear reprogramming. Development 145, dev158261.

Liu, Z., Cai, Y., Wang, Y., Nie, Y., Zhang, C., Xu, Y., Zhang, X., Lu, Y., Wang, Z., Poo, M., and Sun, Q. (2018b). Cloning of macaque monkeys by somatic cell nuclear transfer. Cell 172, 881–887.

Locatelli, P., Olea, F.D., Hnatiuk, A., Sepúlveda, D., Pérez Sáez, J.M., Argüello, R., and Crottogini, A. (2013). Efficient plasmid-mediated gene transfection of ovine bone marrow mesenchymal stromal cells. Cytotherapy 15, 163–170.

Long, C.R., Westhusin, M.E., and Golding, M.C. (2014). Reshaping the transcriptional frontier: Epigenetics and somatic cell nuclear transfer. Mol. Reprod. Dev. 81, 183–193.

Madeja, Z.E., Sosnowski, J., Hryniewicz, K., Warzych, E., Pawlak, P., Rozwadowska, N., Plusa, B., and Lechniak, D. (2013). Changes in sub-cellular localisation of trophoblast and inner cell mass specific transcription factors during bovine preimplantation development. BMC Dev. Biol. 13, 1–17.

Matoba, S., Inoue, K., Kohda, T., Sugimoto, M., Mizutani, E., Ogonuki, N., Nakamura, T., Abe, K., Nakano, T., Ishino, F.,

and Ogura, A. (2011). RNAi-mediated knockdown of Xist can rescue the impaired postimplantation development of cloned mouse embryos. Proc. Natl. Acad. Sci. U. S. A. 108, 20621–20626.

Matoba, S., Liu, Y., Lu, F., Iwabuchi, K.A., Shen, L., Inoue, A., and Zhang, Y. (2014). Embryonic development following somatic cell nuclear transfer impeded by persisting histone methylation. Cell 159, 884–895.

Matoba, S., and Zhang, Y. (2018). Somatic cell nuclear transfer reprogramming: Mechanisms and applications. Cell Stem Cell 23, 471–485.

Misica-Turner, P.M., Oback, F.C., Eichenlaub, M., Wells, D.N., and Oback, B. (2007). Aggregating embryonic but not somatic nuclear transfer embryos increases cloning efficiency in cattle. Biol. Reprod. 76, 268–278.

Moro, L.N., Hiriart, M.I., Buemo, C., Jarazo, J., Sestelo, A., Veraguas, D., Rodriguez-Alvarez, L., and Salamone, D.F. (2015). Cheetah interspecific SCNT followed by embryo aggregation improves *in vitro* development but not pluripotent gene expression. Reproduction 150, 1–10.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95, 379–391.

Niemann, H., Kues, W.A., Lucas-Hahn, A., and Carnwath, J.W. (2011). Somatic cloning and epigenetic reprogramming in mammals. In Principles of Regenerative Medicine. A. Atala, R. Lanza, J.A. Thomson, and R. Nerem, eds. (Elsevier) pp. 129–158. ISBN: 978-0123814227.

Ogura, A., Inoue, K., and Wakayama, T. (2013). Recent advancements in cloning by somatic cell nuclear transfer. Philos. Trans. R. Soc. Lond. B Biol. Sci. 368, 20110329.

Oh, H.J., Park, J.E., Kim, M.J., Hong, S.G., Ra, J.C., Jo, J.Y., Kang, S.K., Jang, G., and Lee, B.C. (2011). Recloned dogs derived from adipose stem cells of a transgenic cloned beagle. Theriogenology 75, 1221–1231.

Olivera, R., Moro, L.N., Jordan, R., Luzzani, C., Miriuka, S., Radrizzani, M., Donadeu, F.X., and Vichera, G. (2016). *In vitro* and *in vivo* development of horse cloned embryos generated with iPSCs, mesenchymal stromal cells and fetal or adult fibroblasts as nuclear donors. PLoS One 11, e0164049.

Olivera, R., Moro, L.N., Jordan, R., Pallarols, N., Guglielminetti, A., Luzzani, C., Miriuka, S.G., and Vichera, G. (2018). Bone marrow mesenchymal stem cells as nuclear donors improve viability and health of cloned horses. Stem Cells Cloning 11, 13.

Ono, Y., and Kono, T. (2006). Irreversible barrier to the reprogramming of donor cells in cloning with mouse embryos and embryonic stem cells. Biol. Reprod. 75, 210–216.

Picou, A. (2009). The isolation and characterization of bovine adult derived adipose stem cells for the use in nuclear transfer. LSU Master's Theses 3541. https://digitalcommons.lsu.edu/gradschool_theses/3541. Last viewed on September 17, 2021.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science 293, 1089–1093.

Ribeiro, E.S., Gerger, R.P., Ohlweiler, L.U., Ortigari, I., Jr., Mezzalira, J.C., Forell, F., Bertolini, L.R., Rodrigues, J.L., Ambrósio, C.E., Miglino, M.A., Mezzalira, A., and Bertolini, M. (2009). Developmental potential of bovine hand-made clone embryos reconstructed by aggregation or fusion with distinct cytoplasmic volumes. Cloning Stem Cells 11, 377–386.

Rodríguez-Alvarez, L., Cox, J., Tovar, H., Einspanier, R., and Castro, F.O. (2010). Changes in the expression of pluripotency-associated genes during preimplantation and peri-implantation stages in bovine cloned and in vitro produced embryos. Zygote 18, 269–279.

Ross, P.J., Wang, K., Kocabas, A., and Cibelli, J.B. (2010). Housekeeping gene transcript abundance in bovine fertilized and cloned embryos. Cell. Reprogram. 12, 709–717.

Salamone, D., Barañao, L., Santos, C., Bussmann, L., Artuso, J., Werning, C., Prync, A., Carbonetto, C., Dabsys, S., Munar, C., Salaberry, R., Berra, G.,Berra, I., Fernández, N., Papouchado, M., Foti, M., Judewicz, N., Mujica, I., Muñoz, L., Alvarez, S.F., González, E., Zimmermann, J., Criscuolo, M., and Melo, C. (2006). High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow. J. Biotechnol. 124, 469–472.

Sozen, B., Jorgensen, V., Weatherbee, B.A.T., Chen, S., Zhu, M., and Zernicka-Goetz, M. (2021). Reconstructing aspects of human embryogenesis with pluripotent stem cells. Nat. Commun. 12, 5550. DOI: 10.1038/s41467-021-25853-4. PMID: 34548496.

Su, X.,Wang, S., Su, G., Zheng, Z., Zhang, J., Ma, Y., Liu, Z., Zhou, H., Zhang, Y., and Zhang, L. (2018b). Production of microhomologous-mediated site-specific integrated LacS gene cow using TALENs. Theriogenology 119, 282–288.

Vajta, G. (2007). Somatic cell nuclear transfer in its first and second decades: Successes, setbacks, paradoxes and perspectives. Reprod. Biomed. Online 15, 582–590.

Vajta, G., Peura, T.T., Holm, P., Paldi, A., Greve, T., Trounson, A.O., and Callesen, H. (2000). New method for culture of zona-included or zona-free embryos: The Well of the Well (WOW) system. Mol. Reprod. Dev. 55, 256–264.

Wells, K.D., and Powell, A.M. (2000). Blastomeres from somatic cell nuclear transfer embryos are not allocated randomly in chimeric blastocysts. Cloning 2, 9–22.

White, M.D., and Plachta, N. (2020). Specification of the first mammalian cell lineages *in vivo* and *in vitro*. Cold Spring Harb. Perspect. Biol. 12, a035634.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810–813.

Wrenzycki, C., Lucas-Hahn, A., Herrmann, D., Lemme, E., Korsawe, K., and Niemann, H. (2002). *In vitro* production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. Biol. Reprod. 66, 127–134.

Yamanaka, K.I., Sakatani, M., Kubota, K., Balboula, A.Z., Sawai, K., and Takahashi, M. (2011). Effects of down-regulating DNA methyltransferase 1 transcript by RNA interference on DNA methylation status of the satellite I region and *in vitro* development of bovine somatic cell nuclear transfer embryos. J. Reprod. Dev. 57, 393–402.

Zhang, S., Chen, X., Wang, F., An, X., Tang, B., Zhang, X., Sun, L., and Li, Z. (2016). Aberrant DNA methylation reprogramming in bovine SCNT preimplantation embryos. Sci. Rep. 6, 1–11.

Address correspondence to:
Daniel Felipe Salamone
Laboratorio Biotecnología Animal (LabBA)
Dto Producción Animal
Facultad de Agronomía
Universidad de Buenos Aires
Buenos Aires 1417
Argentina

E-mail: salamone@agro.uba.ar