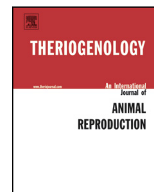
Theriogenology 80 (2013) 357–364

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com



Production of chimeric embryos by aggregation of bovine *egfp* eight-cell stage blastomeres with two-cell fused and asynchronic embryos

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ARTICLE INFO

Article history:
Received 14 February 2013
Received in revised form 18 April 2013
Accepted 19 April 2013

Keywords:
Aggregation
Chimera
Transgene
Bovine

ABSTRACT

Embryo disaggregation allows the production of two to four identical offspring from a single cow embryo. In addition, embryo complementation has become the technique of choice to demonstrate the totipotency of embryonic stem cells and induced pluripotent stem cells. Therefore, the aim of this study was to generate a new and simple method by aggregation in the well-of-the-well system to direct each single enhanced green fluorescent protein (*egfp*) eight-cell blastomere derived from bovine *in vitro* fertilization embryos to the inner cell mass (ICM) of chimeras produced with fused and asynchronic embryos. To this end, the best conditions to generate *in vitro* fertilization–fused embryos were determined. Then, the fused (F) and nonfused (NF) embryos were aggregated in two distinct conditions: synchronically (S), with both transgenic and F embryos produced on the same day, and asynchronically (AS), with transgenic embryos produced one day before F embryos. The highest fusion and blastocysts rates were obtained with two pulses of 40 V. The 2ASF and 2ASNF groups showed the best number of blastocysts expressing the EGFP protein (48% and 41%, respectively). Furthermore, the 2ASF group induced the highest localization rates of the *egfp*-expressing blastomere in the ICM (6/13, 46% of ICM transgene-expressing blastocysts). This technique will have great application for multiplication of embryos of high genetic value or transgenic embryos and also with the generation of truly bovine embryonic stem cells and induced pluripotent stem cells.

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1. Introduction

Chimeric embryos, obtained by contribution of blastomeres from two or more embryos, have several applications in basic and applied biotechnology. They serve as useful tools to study the early development and differentiation in mammals [1]. Moreover, the introduction of transgenic embryonic stem (ES) cells into blastocysts has become the preferred method to obtain transgenic mice [2–4]. In addition, with the technological development of induced pluripotent stem (iPS) cells technology [5], embryo complementation has become the technique of choice to demonstrate totipotency [6–8].

Since the early 1980s, two main techniques were employed for embryo bisection: embryo splitting [9–12] and embryo disaggregation [13–15], which allow the generation of twins from a single embryo. An alternative method, developed to reduce the damage induced by microsurgery [16,17], consisted of drilling the zona pellucida (ZP) between the inner cell mass (ICM) and the trophoblast to induce assisted hatching of the blastocyst and to ensure the extrusion of both cellular types [18]. Additionally, the well-of-the-well (WOW) system, developed for the culture of zona-free embryos [19], has facilitated complementation techniques [20] by combining disaggregated blastomeres produced by gentle pipetting. For the WOW system, small, round microdepressions are made with a needle into a plate, and these are then filled with culture medium. The culture of embryos in these small microdepressions avoids the

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0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.04.023

segregation of blastomeres, which occurs as a result of the absence of intercellular junctions in early embryonic stages.

By embryo disaggregation, it is possible to obtain two to four identical offspring from cows [14,15,20], sheep [21], and rabbits [22] from two-, four-, and eight-cell stage embryos. In humans, it has even been shown that each of the four blastomeres of a four-cell stage embryo has the potential to develop into an individual blastocyst [23]. However, both the initiation of embryonic gene expression and the size of the blastomeres could limit embryo division. Several authors have reported that embryonic gene expression in the bovine begins at the eight-cell stage [24,25]. On the other hand, despite the observation that activation of the genome occurs by the first cleavage [26,27], blastomeres of eight-cell stage mouse embryos generated stem cells lines [28,29], which indicates persistence of totipotency.

In mice, it is well known that polyploid blastomeres are selected against during the development of fetal tissues [30], but persist in extra-embryonic membranes [31]. Thus, for this reason, polyploid embryos (in particular, tetraploid embryos, 4n) have been widely used to compensate for embryo lethality of a few stem cells and to produce homogeneous offspring from chimeric embryos. In these cases, the 4n cells gave rise to the placenta and yolk sac endoderm, and the newborns were almost all completely stem cells-derived [32]. In several species, the generation of 4n embryos by electrofusion [33,34] has proved to be more efficient than the use of drugs, such as cytochalasin-B [31,35].

The aim of this study was to generate a new and simple method by aggregation in the WOW system to produce chimeras derived from one founder enhanced green fluorescent protein (*egfp*) eight-cell blastomere directed to the ICM and one or two fused embryos at different stages of development. In the first step, the best fusion conditions for the generation of putative 4n embryos were established. Then, multiplication of high-value embryos was evaluated by aggregation of *egfp*-expressing cells with aneuploid embryos or with asynchronic nonvaluable embryos in the WOW system. The use of *egfp* blastomeres allowed the easy and immediate tracking of the distribution of the valuable cells at the final chimera. This is the first study to multiply high-value embryos with the use of nonvaluable embryos for extra-embryonic tissues.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1. Determination of conditions for two-cell embryo fusion in the bovine

Two-cell embryos (Day 1) produced by *in vitro* fertilization (IVF) were subjected to two pulses of different voltage rates (20, 40, 60, 80, and 100 V) for 30 μsec at 100 msec intervals. Embryos that were visualized as a single blastomere an hour later were considered fused (F). Blastocyst development was evaluated at Day 8. Nonfused IVF embryos (NF) were used as control.

2.1.2. Experiment 2. Determination of ploidy of fused two-cell embryos

Karyotype analyses were performed to determine nuclear fusion. Spreads of fused and IVF Day 2 embryos were assessed.

2.1.3. Experiment 3. Synchronic and asynchronic aggregation of egfp-expressing blastomeres with fused or nonfused embryos and localization of transgene expression

Transgene-expressing embryos were produced by injection of oolemma vesicles coincubated with linear pCX-EGFP into IVF presumptive zygotes [36]. Blastomeres of Day 3 transgenic embryos were separated and *egfp*-expressing blastomeres were selected for aggregation with one or two IVF embryos, F or NF, at different stages of development: synchronic (S, when the age of the embryos was the same as the aggregated blastomere) or asynchronic (AS, when the embryos were one day younger than the transgene-expressing blastomere) (Fig. 1).

2.2. Chemicals

All chemicals and media were purchased from Sigma Chemical (St Louis, MO, USA), unless otherwise indicated.

2.3. Oocyte collection and maturation

Bovine ovaries were collected from slaughterhouses and transported to the laboratory at 25 °C to 30 °C. Cumulus-oocyte complexes were aspirated with 21-gauge needles from follicles with a diameter of 2 to 5 mm and collected in Hepes-buffered Tyrode's albumin (Hepes-TALP) [37]. Oocytes covered with at least three layers of granulosa cells were selected for *in vitro* maturation. The maturation medium was bicarbonate-buffered TCM-199 (31100-035; Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (013/07; Internegocios, Buenos Aires, Argentina), 10 μg/mL follicle-stimulating hormone (NIH-FSH-P1, Folltropin, Bioniche, Caufield Junction Caufield North, Victoria, Australia), 0.3 mM sodium pyruvate (P2256), 100 μM cysteamine (M9768), and 2% antibiotic-antimycotic (ATB, 15240-096; Gibco). The oocytes were incubated for 22 hours under mineral oil (M8410) in 100 μL droplets at 39 °C under 6.5% CO_2 in humidified air.

2.4. IVF procedure

Frozen semen was thawed in a 37 °C water bath for 30 seconds. Spermatozoa were then centrifuged twice (490 × *g*, 5 minutes) in Brackett-Oliphant (BO) medium [38] and resuspended in BO supplemented with 5 mM caffeine (C4144) and 20 IU/mL heparin (H3149). Spermatozoa were adjusted to 32 × 10^6/mL and diluted to half concentration (16 × 10^6/mL) with BO containing 10 mg/mL fatty acid-free bovine serum albumin (A6003). Cumulus-oocyte complexes were washed twice with Hepes-TALP and subsequently exposed to the sperm suspension for 5 hours in 100 μL droplets at 39 °C under 5% CO_2 in humidified air. Presumptive zygotes were then washed three times in Hepes-TALP. After IVF, embryos were designated into two groups: some embryos were immediately

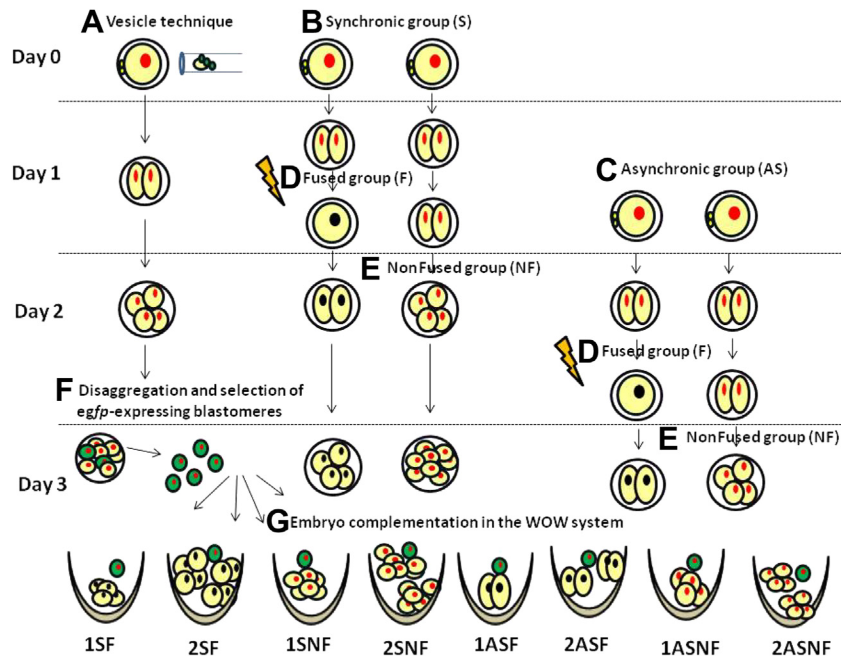

Fig. 1. Experimental design. (A) IVF presumptive zygotes were injected with oolemma vesicles coincubated with linear pCX-EGFP. (B) Two-cell stage IVF embryos were subjected to electrofusion (F) at 40 V in two ways: (B) Synchronic group (S): the age of the IVF embryos was the same as the embryo subjected to the vesicle technique or (C) asynchronic group (AS): embryos were one day younger than the transgene-expressing embryo. (E) Nonfused IVF embryos (NF) were used as control. (F) At Day 3, gentle pipetting was applied to disaggregate blastomeres from transgenic five- to eight-cell stage embryos. *Egfp*-expressing blastomeres were selected as founder blastomeres for aggregation in the WOW system. (G) After removal of the ZP of fused embryos from S or AS or from the nonfused S and AS groups, one or two embryos were aggregated with one *egfp*-expressing blastomere from five- to eight-cell stage embryos.

subjected to the vesicle technique and others were cultured for electrofusion on Day 1 as described below. Cumulus cells of the first group of presumptive zygotes were removed by vortexing for 2 minutes in hyaluronidase (H4272) (1 mg/mL in Dulbecco's PBS). Then, the presumptive zygotes were washed in Hepes-TALP, selected by visualization of at least one polar body, and immediately injected with vesicles incubated with DNA.

2.5. Vesicles production and incubation with exogenous DNA

The plasmid used was pCX-EGFP, kindly provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan) that contains the *egfp* gene under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control [39]. The plasmid pCX-EGFP employed was linear by *Hin*d III digestion.

Oocytes that were used as vesicle donors were also subjected to *in vitro* maturation, IVF, and hyaluronidase treatment. Then, the presumptive zygotes were transferred to 20 μL droplets of Hepes-TALP. Each was subjected to the technique describe by Pereyra-Bonnet et al. [36]. Briefly, a presumptive zygote was held under negative pressure with a holding pipette, while a 9-μm pipette was passed through its ZP until contact with the ooplasm. A small fraction of the ooplasm (<10 μm) was then aspirated by negative pressure, avoiding plasma membrane breakage. Approximately 15 vesicles were obtained from each donor presumptive zygote. Vesicles that formed inside the pipette were transferred into a 3 μL 10% PVP droplet containing 50 ng/μL linear pCX-EGFP and left there for 5 minutes (plasmid incubation). Finally, vesicles were aspirated into the 9-μm pipette and were directly injected into the denuded fertilized oocytes. Presumptive zygotes were subsequently cultured as described below.

2.6. Electrofusion and IVC of fused embryos

About 27 to 29 hours after the initiation of sperm-egg incubation (Day 1 embryos), two-cell stage embryos were selected. Before electrofusion, these embryos were subjected to hyaluronidase treatment, equilibrated in fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 1 mg/mL PVA) for 2 to 3 minutes and then placed in a fusion chamber (BTX Instrument Division; Harvard Apparatus, Holliston, MA, USA) containing 2 mL of the same warm medium with the cleavage plane parallel to the electrodes (0.5 mm). Fusion used different electric field levels: two electrical pulses of 20, 40, 60, 80, or 100 V for 30 μsec at 100 msec intervals. After exposure to the electric pulse, the embryos were returned to culture, and an hour later, the embryos that appeared as a single blastomere were considered fused. Embryos that were not subjected to electrofusion were cultured as a control.

2.7. Separation and selection of egfp-expressing blastomeres

Day 3 *egfp*-expressing embryos produced by the vesicle technique were treated with 1.5 mg/mL pronase (P8811) dissolved in Hepes-TALP to remove the ZP. Gentle pipetting was applied to disaggregate blastomeres from these five- to eight-cell stage embryos. *Egfp*-expressing blastomeres were selected under blue light using an excitation filter at 488 nm and an emission filter at 530 nm and then used as founder blastomeres for aggregation.

2.8. Aggregation

Two aggregation groups were included for F embryos: (1) synchronic (S), consisting of both transgenic and F embryos produced on the same day, and (2) asynchronic (AS), consisting of transgenic embryos produced one day before F embryos. Thus, embryos from the AS group were younger and had lower cell numbers. Controls consisted of S and AS aggregation with no fused IVF embryos (NF). After removal of the ZP of fused embryos from S or AS or from the nonfused S and AS groups with 1.5 mg/mL pronase, one or two embryos were aggregated in a modified WOW system [19] (see below), with one *egfp*-expressing blastomere from five- to eight-cell stage embryos (Day 3). The blastomere was put in contact with one embryo or between two embryos (Fig. 1). *In vitro* development of the aggregates and *egfp* expression of blastocysts were analyzed.

2.9. In vitro embryo culture

Presumptive zygotes derived by IVF, IVF + vesicles, and IVF + fusion were cultured in 50 μL droplets of SOF medium supplemented with 2.5% fetal bovine serum at 39 °C under 6.5% CO_2 in humidified air. Cleavage was evaluated on Day 2 only for the IVF + vesicles group (data not shown) and the number of blastocysts on Day 8 for the IVF + fusion group. Aggregates were cultured in SOF medium in a system similar to WOW [19], whereby microwells were produced using a heated glass capillary slightly pressed to the bottom of a Petri dish and then covered with 100 μL drops of SOF medium (20–30 WOW in each drop). Blastocyst formation was evaluated on Days 5–6 post-aggregation.

2.10. Statistical analysis

In vitro embryo development was compared by Fisher's exact test analysis. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Determination of conditions for two-cell embryo fusion in the bovine

In the first experiment, the best conditions to generate viable putative tetraploid embryos by two-cell embryo fusion were evaluated. To this end, different electric field intensities were assessed for two-cell stage IVF embryos (22 hours post-IVF), from which cumulus cells had been removed. Fusion at 20 V significantly reduced the numbers of fused embryos, whereas fusion at 80 V resulted in the highest fusion rates; however, embryo development to the blastocyst stage was compromised ($P < 0.05$; Table 1). Thus, 40 V was employed for the following experiments. This voltage resulted not only in high fusion rates, but also in blastocyst rates that were not significantly different from the control.

3.2. Determination of ploidy of fused two-cell embryos

Spreads of Day 1 and 2 fused embryos were analyzed. As expected, the fusion procedure induced a higher proportion of triploid (2/28, 7%) and tetraploid embryos (4/28, 14%) than observed for the control group (0/40, 0% for both cases). The numbers of aneuploid embryos were also high in fused and control treatments (8/28, 27% for fused embryos and 9/40, 22% for the IVF control). Two interesting observations of this experiment were that the IVF control showed a high rate of haploid blastomeres (16/40, 40%) and also that a higher rate of diploid embryo production was observed for the fused group compared with the IVF control (10/28, 36% vs. 8/40, 20%; respectively).

3.3. Synchronic and asynchronic aggregation of egfp-expressing blastomeres with fused or nonfused embryos and localization of transgene expression

The results in Table 2 show blastocyst development and the localization of *egfp* expression in the embryos generated by the aggregation of one blastomere of an eight-cell stage embryo with one or two IVF embryos, fused (F) or nonfused (NF) in a synchronic (S) or asynchronic (AS) way. We observed blastocyst formation in the eight experimental groups. The 2SNF group showed the highest blastocyst rate ($P < 0.05$). The 2SF, 2ASF, and 2ASNF groups showed significantly higher blastocyst rates than the 1ASNF, 1SF, and 1SNF groups (Table 2), whereas the 1ASF

Table 1
In vitro development of two-cell bovine-fused embryos with different electric field intensities. Two-cell embryos (Day 1) produced by IVF were subjected to two pulses of different voltage rates (20, 40, 60, 80, and 100 V) for 30 μsec at 100 msec intervals.

Field strength (V)	No. of treated two-cell embryos	No. of fused embryos (%)	No. of blastocysts (%)
100	43	35 (81,40)[a]	4 (11,43)[a,c]
80	61	58 (95,08)[b]	12 (20,69)[a]
60	56	45 (80,36)[a]	14 (31,11)[a,b]
40	78	60 (76,92)[a]	29 (48,33)[b,d]
20	31	5 (16,13)[c]	0 (0)[c]
Control	93	ND	44 (47,31)[d]

Embryos that were visualized as a single blastomere an hour later were considered fused. Blastocyst development was evaluated at Day 8. Nonfused IVF embryos were used as control.
[a,b,c,d] Values with different superscripts in a column are significantly different (Fisher's exact test, $P < 0.05$)

Table 2
Evaluation of blastocysts rates and localization of transgene expression in embryos produced by *egfp* blastomere aggregation with synchronic or asynchronic two-cell–fused or nonfused embryos.

Synchronicity	No. of embryos aggregated	Type of embryo aggregated	N	No. of blastocysts (%[a])	Total EGFP blastocysts (%[b])	Localized EGFP blastocysts (%[c])	Generalized EGFP blastocysts (%[c])
Asynchronic	1	Two-cells fusion	67	14 (21)^{d,e}	3 (21)^{d,f}	1 (33)^{d,e}	2 (67)^{d,e}
		No fusion	75	9 (12)^{e}	1 (11)^{d}	0 (0)^{d}	1 (100)^{d,e}
	2	Two-cells fusion	75	27 (36)^{d}	13 (48)^{e}	6 (46)^{e}	7 (54)^{e}
		No fusion	75	22 (29)^{d}	9 (41)^{e,f}	2 (22)^{d,e}	7 (78)^{e}
Synchronic	1	Two-cells fusion	52	11 (21)^{e}	1 (9)^{d}	1 (100)^{d,e}	0 (0)^{d}
		No fusion	64	13 (20)^{e}	1 (8)^{d}	0 (0)^{d}	1 (100)^{d,e}
	2	Two-cells fusion	64	22 (34)^{d}	2 (9)^{d,f}	1 (50)^{d,e}	1 (50)^{d,e}
		No fusion	75	31 (41)^{f}	3 (10)^{d,f}	0 (0)^{d,e}	3 (100)^{d,e}

[a] Percentages with respect to the number of aggregated embryos, N.
[b] Percentages with respect to the number of blastocysts obtained.
[c] Percentages with respect to the number of total EGFP blastocysts.
[d,e,f] Values with different superscripts in a column are significantly different (Fisher's exact test, $P < 0.05$).

group did not show differences. In addition, *egfp* expression and localization of *egfp* expression were evaluated in the chimeric blastocysts. According to *egfp* expression in blastocysts, the 2ASF and 2ASNF groups showed a higher number of blastocysts expressing the EGFP protein than the other groups ($P < 0.05$). As far as localization of *egfp* expression in the ICM of the blastocysts was concerned, the highest localization was observed for the 2ASF group (6/13, 46% of ICM transgene-expressing blastocysts; Fig. 2). Although the 1SF and 2SF groups had high rates of localized expression (1/1, 100% and 1/2, 50%, respectively), they did not integrate the *egfp*-expressing blastomere efficiently as a very low proportion of blastocysts exhibited total *egfp* expression (1/11 blastocysts, 9% and 2/22, 9%, respectively). The 2ASF and 2ASNF groups showed very high rates of generalized *egfp* expression in different areas of the blastocysts, including the ICM (7/13, 54% and 7/9, 78%, respectively). In some cases, the 2SNF group formed two independent blastocysts or two trophoblasts connected by only one ICM without integration of the transgenic blastomere.

4. Discussion

Conditions were evaluated to produce bovine embryos by complementation of a high-value blastomere to give rise to all embryo tissues, with fused low-value embryos, which were expected to produce only the extra-embryonic tissues. Therefore, we developed several experiments, first to produce fused embryos and then to complement these embryos to the blastomere of interest. For this, we employed *egfp*-expressing blastomeres in order to be able to visualize/locate them and to determine their final contribution to the embryo (ICM or generalized location). This strategy could have a great impact on the generation of high-value embryos and also for the production of embryos from ES and iPS cells.

In the first experiment, we tried to determine the best conditions to generate bovine tetraploid/aneuploid embryos by electrofusion. With this objective, the two-cell stage IVF embryos were fused on Day 1 post-IVF using different pulses. The fused embryos showed only one blastomere an hour after the pulse was applied. In all cases (except for the 20 V group), fused embryos developed into blastocysts. Because the highest rate of blastocyst development was generated at 40 V, we chose that voltage as the best condition for fused embryo production. This result is consistent with the result that was recently published for mice by Suo et al. [34]. They determined that the best condition to generate tetraploid embryos from two-cell stage murine embryos was an electric field of 0.8 kV/cm, equivalent to our conditions. Other researchers have also produced tetraploid bovine embryos by electrofusion [33,40]. However, the conditions and the results were very different from ours.

In the second experiment, a subset of 40 V electrofused embryos was subjected to evaluation of their chromosomal content at the time of aggregation (two- to eight-cell-stage of development). This evaluation confirmed that these fused embryos show an increased proportion of

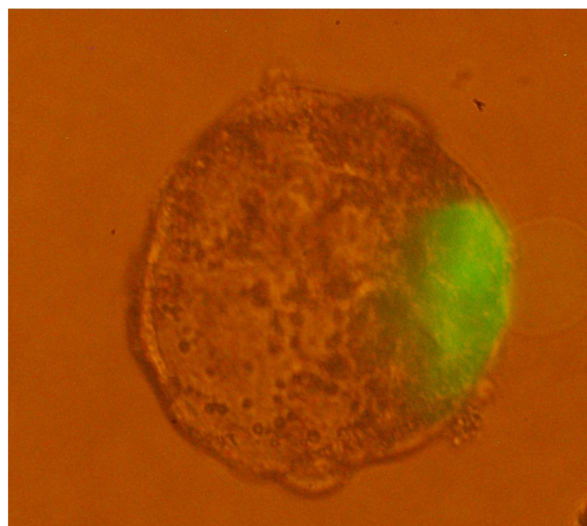

Fig. 2. Blastocyst produced in the 2ASF group with localized EGFP expression. The highest number of blastocysts expressing the EGFP protein in the ICM (green fluorescence) was obtained when two fused and asynchronic embryos (2ASF) were aggregated with the *egfp*-expressing blastomere. Embryos were visualized under blue light using an excitation filter at 488 nm and an emission filter at 530 nm.

chromosomal abnormalities that may favor those cells to be rejected from the ICM by normal diploid blastomeres [41]. In our study, the overall incidence of chromosomal abnormalities was high in both F and control NF groups. The high rates of haploid embryos (40%) in the IVF control group could be explained by the fact that chromosomal aberrations occur at high rates in the early embryos produced by IVF, but they decline in the course of their development, as previously suggested by others [41–44]. The rate of mixoploid embryos is also very high in the early stages of development, but it has minor importance for the establishment of pregnancy [45]. On the other hand, in a previous study, Curnow et al. [33] reported a high incidence (41.7%) of mosaicism between diploid and tetraploid cells in bovine blastocysts generated by electrofusion. They suggested that the asynchronic cleavage and nuclear state of the blastomeres are responsible for the mosaicism. This agrees with our observation that the F group resulted in higher triploidy (7.14%) and tetraploidy (14.29%) rates than the NF control (0% in both cases). These factors might be the reason for the cells to be rejected by the normal diploid transgene-expressing blastomeres to avoid their participation in the ICM. In the same way, Curnow et al. [33] obtained lower rates (12.5%) of truly tetraploid embryos. It remains controversial whether only tetraploid cells or any aneuploid cell can be assigned to the trophoblast. Thus, despite observing lower tetraploid rates than expected, the two-cell fused embryos were evaluated in embryo aggregation.

In the final experiment, we tested both asynchronic and synchronic complementation. A higher number of blastocysts were produced when two synchronic nonfused embryos (2SNF group) were put together with an *egfp*-expressing blastomere (31/75; 41%); however, no embryos in this group showed localized *egfp* expression in the ICM. The authors hypothesize that this observation is due to the unbalance in the number of cells between more advanced synchronized embryos (16 or >16 cells) and only one younger and bigger *egfp* blastomere of an eight-cell-stage embryo. On the other hand, two IVF asynchronic embryos (2AS) were the best condition to complement the transgene-expressing blastomere with IVF embryos and to direct the *egfp* expression to the ICM of the final chimera. The 2ASF and 2ASNF groups showed 40% to 50% of chimerism, which was detected easily through visualization of expression of the EGFP protein in the blastocysts. The AS complementation is an old technique that has not been extensively used in bovine. However, early in 1978, it was first reported in the mouse that the asynchronicity between the cells in an intact embryo indicates that the first cell to divide to the eight-cell stage tends to contribute much more to the ICM when compared with the last cell to divide to that stage [46]. Later, it was reported that in complementation, the earlier stage embryos have more chances to contribute to the ICM than the older ones and that the position of the blastomeres is a key factor to improve the generation of the chimeric embryos as well as the asynchronic embryo stage between aggregation partners [47]. So our results confirmed the hypothesis that younger aggregated embryos complement older blastomeres better than embryos in the same or in a more advanced state of development, favoring localization of blastomeres to the ICM. In particular, the 2ASF group had significantly higher complementation and orientation of the blastomere to the ICM (Fig. 2). In this case, the IVF embryos were younger than the *egfp*-expressing blastomere. Tarkowski et al. [48] have already demonstrated in mouse chimeras that the tetraploid cells were excluded from the fetus even when these cells predominated initially and that normal diploid cells contributed better to the ICM, but also to extra-embryonic tissues. The 2ASNF group also resulted in high complementation rates; however, the transgene-expressing cells were in different parts of the final chimera, including the ICM. This demonstrates that this group is also a good alternative to produce chimeric embryos, but without a specific localization of the blastomere of interest. Some researchers also discuss spatial arrangements for making chimeric embryos [46,48,49]; however, we did not test this aspect in our work.

In addition, our results indicate that the WOW system is efficient to sustain high rates of blastocyst production in all groups, especially when two IVF embryos (in any synchronicity condition evaluated) were put together into a well. The high blastocysts rates observed after embryo aggregation agree with previous SCNT studies in our laboratory [50] and in others [51,52] that have also demonstrated that the initial aggregation of embryos improves the rates of blastocyst development. An increased number of cloned bovine aggregates enhances cleavage, blastocyst rates, and number of cells per embryo [52,53] and also improves early cloned pregnancy rates [54]. In this report, we used IVF embryos, and for that reason the positive effects of embryo aggregation could be due to an increase in embryo cell numbers in the first days of development, more than to the epigenetic compensation observed for cloned embryos. Previous reports have already demonstrated that the initial number of cells influence embryo development [20,55], since techniques, such as blastomere isolation, result in reduced embryo quality and development.

A final important observation of this third experiment is that we employed *egfp*-expressing blastomeres derived from eight-cell stage bovine embryos. At this stage of embryo development in the bovine, maternal to embryonic transition occurs [24,25]; for this reason, transgene expression had not begun in all blastomeres, making it impossible to determine how many blastocysts could be produced from only one eight-cell stage embryo. In this work, the *egfp*-expressing blastomeres were produced by the vesicle technique, the main advantage of which is the possibility of producing transgene-expressing IVF embryos, with higher developmental potential than cloned embryos [39]. However, as with most available transgenesis techniques, mosaicism results due to transgene integration after the first mitosis [56]. The aim of this work was to use the transgene-expressing blastomeres to track transgene expression and to establish an efficient embryo complementation system in the bovine. Future work should test the efficiency of this technique in terms of embryo production from each embryo disaggregated. Embryo complementation and aggregation provide a method not only to multiply embryo numbers, but also to reduce or reverse mosaicism by directing transgene expression to the ICM.

4.1. Conclusion

In conclusion, this study demonstrates that asynchronic complementation with two fused embryos results in better aggregation outcome. This technique will have great application both for multiplication of embryos of high genetic value and for reversion of mosaicism of transgenic embryos produced by simple IVF technique. In addition, it will have great impact for the production of embryos from ES and iPS cells.

Acknowledgments

The authors are grateful to Ciale Alta (www.ciale.com) for their semen contribution and Dr. Elizabeth Crichton (bethiberg@aol.com) for her English assistance. The authors thank slaughterhouses EcoCarnes S.A., San Fernando, Buenos Aires, Argentina (Dr. Gilberto Llerena, Dr. Benjamín Martín Echenique, Dr. Silvia Krooft, Mr Federico Sotomayor, and especially Mr Daniel Brites and his family), and Industrias Frigoríficas Sur S.A., Loma Hermosa, Buenos Aires, Argentina (Dr. Jorge Miranda, Mrs Griselda Bulacio, and Mr Jonhatan Baez) for providing the biological material.

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