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In Vitro Development and Cell Allocation After Aggregation of Syngeneic Wild Type and Fluorescence-Expressing Bovine Cloned Embryos

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ABSTRACT

***Background*:** The *in vitro* production (IVP) of embryos by *in vitro* fertilization or cloning procedures has been known to cause epigenetic changes in the conceptus that in turn are associated with abnormalities in pre- and postnatal development. Handmade cloning (HMC) procedures and the culture of zona-free embryos in individual microwells provide excellent tools for studies in developmental biology, since embryo development and cell allocation patterns can be evaluated under a wide range of embryo reconstruction arrangements and in *in vitro* embryo culture conditions. As disturbances in embryonic cell allocation after *in vitro* embryo manipulations and unusual *in vivo* conditions during the first third of pregnancy appear to be associated with large offspring, embryo aggregation procedures may allow a compensation for epigenetic defects between aggregated embryos or even may influence more favorable cell allocation in embryonic lineages, favoring subsequent development. Thus, the aim of this study was to evaluate *in vitro* embryo developmental potential and the pattern of cell allocation in blastocysts developed after the aggregation of handmade cloned embryos produced using syngeneic wild type and/or transgenic somatic cells.

***Materials, Methods & Results*:** *In vitro*-matured bovine cumulus-oocyte complexes (COC) were manually bisected after cumulus and zona pellucida removal; then, two enucleated hemi-oocytes were paired and fused with either a wild type (WT) or a GFP-expressing (GFP) fetal skin cell at the 11th and 19th passages, respectively. Following chemical activation, reconstructed cloned embryos and zona-free parthenote embryos were *in vitro*-cultured in microwells, for 7 days, either individually (1 x 100%) or after the aggregation of two structures (2 x 100%) per microwell, as follows: (G1) one WT cloned embryo; (G2) two aggregated WT embryos; (G3) one GFP cloned embryo; (G4) two aggregated GFP embryos; (G5) aggregation of a WT embryo and a GFP embryo; (G6) one parthenote embryo; or (G7) two aggregated parthenote embryos. Fusion (clones), cleavage (Day 2), and blastocyst (Day 7) rates, and embryonic cell allocation were compared by the χ^2 or Fisher tests. Total cell number (TCN) in blastocysts was analyzed by the Student´s test ($P < 0.05$). Fusion and cleavage rates, and cell allocation were similar between groups. On a per WOW basis, development to the blastocyst stage was similar between groups, except for lower rates of development seen in G3. However, when based on number of embryos per group (one or two), blastocyst development was higher in G1 than all other groups, which were similar between one another. Cloned GFP embryos had lower *in vitro* development to the blastocyst stage than WT embryos, which had more TCN than parthenote or aggregated chimeric WT/GFP embryos. Aggregated GFP embryos had fewer cells than the other embryo groups.

Discussion: The *in vitro* development of GFP cloned embryos was lower than WT embryos, with no effects on cell allocation in resulting blastocysts. Differences in blastocyst rate between groups were likely due to lower GFP-expressing cell viability, as GFP donor cells were at high population cell doublings when used for cloning. On a per embryo basis, embryo aggregation on Day 1 resulted in blastocyst development similar to non-aggregated embryos on Day 7, with no differences in cell proportion between groups. The use of GFP-expressing cells was proven a promising strategy for the study of cell allocation during embryo development, which may assist in the elucidation of mechanisms of abnormalities after *in vitro* embryo manipulations, leading to the development of improved protocols for the *in vitro* production (IVP) of bovine embryos.

Keywords: cell allocation, animal cloning, embryo aggregation, cattle.

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INTRODUCTION

Cloning by somatic cell nuclear transfer (SCNT) is a great tool for the study of significant biological processes, such as nuclear reprogramming and epigenetics, embryo activation and development, and pre-and postnatal growth and survival. Yet, *in vitro* embryo manipulations, as in cloning by SCNT, are also related to developmental problems that often increase fetal and placental growth and affect normal development and neonatal survival [3,10,20,34].

Excessive conceptus growth after *in vitro* embryo manipulations has been associated with the occurrence of disturbed cell allocation that favors the trophectoderm (TE) rather than the inner cell mass (ICM) [33], and with changes in cell proliferation:cell death ratio [36]. Given the role of the trophectoderm in placental development, an increase in its cell population may affect placental size, shape, and/or function, and, consequently, fetal growth [3]. The culture of zona-free embryos in microwells, a common requirement after handmade cloning procedures, may be a useful approach for the study of embryonic cell allocation during early development, as embryo of distinct origins can be aggregated and tracked as development progresses [1,4,7,22,27,28,35]. The aggregation of wild type (WT) embryos with embryos expressing a reporter gene, such as the Green Fluorescent Protein (GFP), may be an effective approach to study cell allocation in early development. Thus, the aim of this study was to evaluate the developmental potential and the cell density and cell proportion (TE:ICM) of blastocysts developed after the aggregation of handmade cloned embryos produced using syngeneic WT or GFP-expressing somatic cells, with the additional evaluation of cell allocation in each embryo according to the origin of the donor cell (WT vs. GFP).

MATERIALS AND METHODS

Primary cell cultures: Wild Type (WT) and green fluorescent protein-expressing (GFP) fibroblast cells

Primary cell cultures were established from a 55-day Nellore (*Bos indicus*) fetus after *in vitro* culture of minced fetal tissue without head and organs. A batch of cells was maintained as wild type (WT), while another batch was genetically modified after lentiviral transduction for the expression of the enhanced green fluorescent protein (EGFP) driven by the ubiquitin promoter, as detailed previously [6,19]. Cells were cultured and maintained in 35-mm culture dishes[1] in Dulbecco´s modified Eagle´s Medium[2] supplemented with 0.22 mM sodium pyruvate[2], 26.2 mM sodium bicarbonate[2], 10,000 UI/mL penicillin G[2], 10 µg/mL amykacin[2], and 10% fetal bovine serum[3] (FBS), at 38.5ºC, 5% CO_2 in air and saturated humidity. Cells in high confluence (>95%) were trypsinized for 4 min, spun for 5 min at 300 g and re-suspended in culture medium to be (a) re-seeded (passages) in culture dishes (25,000 a 50,000 cells/mL); (b) stored at -196ºC after freezing; and/or (c) used for handmade cloning [12,28]. Somatic cells used for cloning were cultured up to the 11th (WT) or the 19th (GFP) passages.

In vitro embryo production

a. In vitro maturation (IVM). Selected bovine cumulus-oocyte complexes (COC) obtained from ovaries from a regional slaughterhouse[4] were *in vitro*-matured in IVM medium composed of M199[2], supplemented with 26.2 mM $NaHCO_3$[2], 25 mM HEPES[2], 0.2 mM sodium pyruvate[2], 0.01 UI/mL FSH-p[5], 0.5 µg/mL LH[5], and 10% Estrus Mare Serum, for 17 h, at 38,5ºC, 5% CO_2 in air and high humidity [12,28].

b. Somatic Cell Nuclear Transfer (SCNT) by Handmade Cloning (HMC). Bovine handmade cloned embryos were produced according to our established procedures [12,28]. After IVM, oocytes were denuded by pipetting, followed by the selection of matured oocytes (MII oocytes) based on the presence of the first polar body under a stereomicroscope. Zona pellucida was enzimatically removed by a brief exposure to 0.5% protease in M199-HEPES[2] (M199H). Zona-free oocytes were incubated in 5 µg/mL cytochalasin B[2] (CCB) in M199H + 10% FBS[3] to be manually bisected[6], in 5 µL microdrops, under mineral oil, in a stereomicroscope. Resulting hemi-oocytes were DNA screened and segregated as enucleated (hemi-cytoplasts) or nucleated (hemi-karyoplasts) hemi-oocytes in an epifluorescent inverted microscope in 10 µg/mL bisbenzimide[2] (Hoechst 33342) in M199H + 10% FBS.

Hemi-cytoplasts were exposed to 500 µg/mL phytohaemoagglutinin[2] (PHA) solution in M199H + 0.01% polyvinyl alcohol[2] (PVA). For individual embryo reconstruction, two hemi-cytoplasts and a somatic cell were adhered in a linear arrangement. Approximately half of the reconstructions used WT cells and half GFP-expressing cells. Reconstructed structures were rinsed in M199H + 10% FBS and in electrofusion medium to

be subjected to a 30-V AC pulse for 5 s, followed by two 10-μs long, 1-kV/cm DC pulses, in a 3.2-mm wide fusion chamber (BTX453)[7] coupled to an electrofusion apparatus (ECM200)[7]. Fused structures were cultured in M199 + 10% FBS for 40 to 60 min, when fusion rates for reconstructed embryos were assessed. Then, fused embryos and groups of zona-free oocytes (parthenote controls) were chemically activated in 5 μM ionomycin[2] in M199H + 10% FBS for 5 min, to be incubated in 2 mM 6-DMAP[2] in mSOFacci + 10% FBS for 4 h [28]. Zona-free parthenote embryos were used as controls for oocyte quality and *in vitro* culture conditions.

c. In vitro culture (IVC) of cloned and parthenote embryos. Cloned and parthenote embryos were *in vitro*-cultured in microwells, in the modified Well-of-the-Well system [11,32], in 4-well dishes[8] containing 400 μL modified SOFaaci medium [28], at 38.5°C, in humidified gas mixture (5% CO_2, 5% O_2 and 90% N_2), into laminated foil bags [32].

Embryos were cultured in microwells either individually (1 x 100%) or after the aggregation of two structures per microwell (2 x 100%), for each embryo type: WT cloned embryos with one embryo (G1) or two embryos (G2) per microwell; GFP cloned embryos with one embryo (G3) or two embryos (G4) per microwell; aggregation of a WT cloned embryo with a GFP cloned embryo per microwell (G5); parthenote embryos with one embryo (G6) or two embryos (G7) per microwell.

Cell density and proportion of cell lineages in blastocysts

Day-7 blastocysts from all groups were used for the estimation of the total cell number (TCN) and the proportion of cells in each embryonic cell lineage (TE:ICM) by differential staining [21], with a few modifications. In brief, embryos were exposed to a 500-μL drop containing M199H + 25 μg/mL *bis*benzimide (Hoechst 33342) + 40 μg/mL propidium iodide[2] and 5% Triton-X 100[2] for 2 to 3 min. Then, embryos were placed on a slide in a 5-μL glycerol[2] droplet, under a coverslip for immediate cell counting using an epifluorescent inverted microscope. Trophectodermal cells (TE) were estimated after visualization at the 536/617 nm (Ex/Em) (propidium iodide), TCN at the 355/465 nm (Hoechst 33342) wavelengths, and the estimation of the ICM number was obtained by the difference between TCN and TE cells. In addition, attempts to estimate GFP and/or WT blastomeres in aggregated chimeric embryos were performed at the 489/509 nm wavelengths.

Data analysis

Fusion rate 60 min after electrofusion, cleavage and blastocyst rates evaluated on Days 2 and 7 of development, and cell proportion in Day-7 developed blastocysts were compared by the χ^2 or Fisher tests, whereas quantitative data (total cell number) were analyzed by the Student´s test[9], for $P < 0.05$.

RESULTS

After three replications, 62.5% of the COCs (688/1,050) were selected by the presence of the first polar body 17-18 h after the onset of IVM. Following cumulus cells removal by pipetting, zona digestion, and manual bisection, a total of 1,047 hemi-oocytes (76.0%) were fluorescence-screened under UV light, with 578 selected as enucleated (55.0%), which were used for the reconstruction of 114 and 141 structures using WT ad GFP-expressing cells, respectively. No differences in fusion rates were seen between groups of embryos reconstructed with WT (77.2%; 88/114) or with GFP-expressing (76.2%; 108/141) somatic cells.

Results for cleavage and blastocyst rates obtained in different groups are presented in Table 1. As

Table 1. *In vitro* embryo development of zona-free parthenote or cloned embryos reconstructed with distinct karyoplast types (wild type cells, GFP-expressing cells, MII oocytes) and aggregation schemes (one or two embryos per microwell).

Embryo type	Karyoplast type	Aggregation scheme	IVC	Cleavage rate		Blastocyst rate		
			n	n	%	n	% per WOW†	% per embryo‡
Clones	WT cells	G1 (1 x 100%)	10	8	80.0[b]	5	50.0[a]	50.0[a]
		G2 (2 x 100%)	25	25	100.0[a]	10	40.0[a]	20.0[b]
	GFP cells	G3 (1 x 100%)	22	19	86.4[ab]	2	9.1[b]	9.1[b]
		G4 (2 x 100%)	24	24	100.0[a]	5	20.8[ab]	10.4[b]
	WT/GFP cells	G5 (2 x 100%)	33	30	90.9[ab]	7	21.2[ab]	10.6[b]
Parthenotes	Zona-free MII oocytes	G6 (1 x 100%)	30	25	83.3[b]	6	20.0[ab]	20.0[b]
		G7 (2 x 100%)	20	19	95.0[ab]	8	40.0[a]	20.0[b]

[a,b]Numbers in columns with distinct superscripts differ, for $P < 0.05$. †Percentage based on development per group in each microwell. ‡Percentage based on development per group on a per embryo basis.

expected, cleavage rates per microwell were higher in aggregated (2 x 100%; 96.1%, 98/102) than non-aggregated (1 x 100%; 83.9%, 52/62) cloned embryos, irrespective of the embryo or karyoplast types, being significantly lower in G1 (WT cells, 1 x 100%) and G6 (parthenote, 1 x 100%) than in G2 (WT cells, 2 x 100%) and G4 (GFP cells, 1 x 100%). On a per WOW basis, development to the blastocyst stage was similar between groups, except for lower rates of development seen in G3 (GFP cells, 1 x 100%). However, blastocyst development, when based on number of embryos per group (one or two), was surprisingly higher in G1 (WT cells, 1 x 100%) than in all other groups, which were similar between one another. Such difference was related to the cell type used for cloning, as development was higher in groups reconstructed with WT cells (G1 and G2, 25%, 15/60) than with GFP cells (G3 and G4, 10%, 7/70).

Table 2 shows results regarding TCN and TE:ICM ratio in blastocysts from embryo aggregation groups, with Figure 1 illustrating examples of fluorescence-evaluated embryos. No differences were observed between groups and between aggregation or donor cell types for any cell proportion ratio under analysis. On the other hand, WT cell-derived embryos had more TCN than parthenote or aggregated chimeric WT/GFP cell-derived embryos. In addition, aggregated GFP-derived embryos had fewer cells than the other embryo groups. Due to fluorochrome bleeding into the GFP-specific wavelength, attempts to estimate and differentiate GFP and/or WT blastomeres in aggregated embryos concurrently with the differential staining were unsuccessful under our experimental conditions.

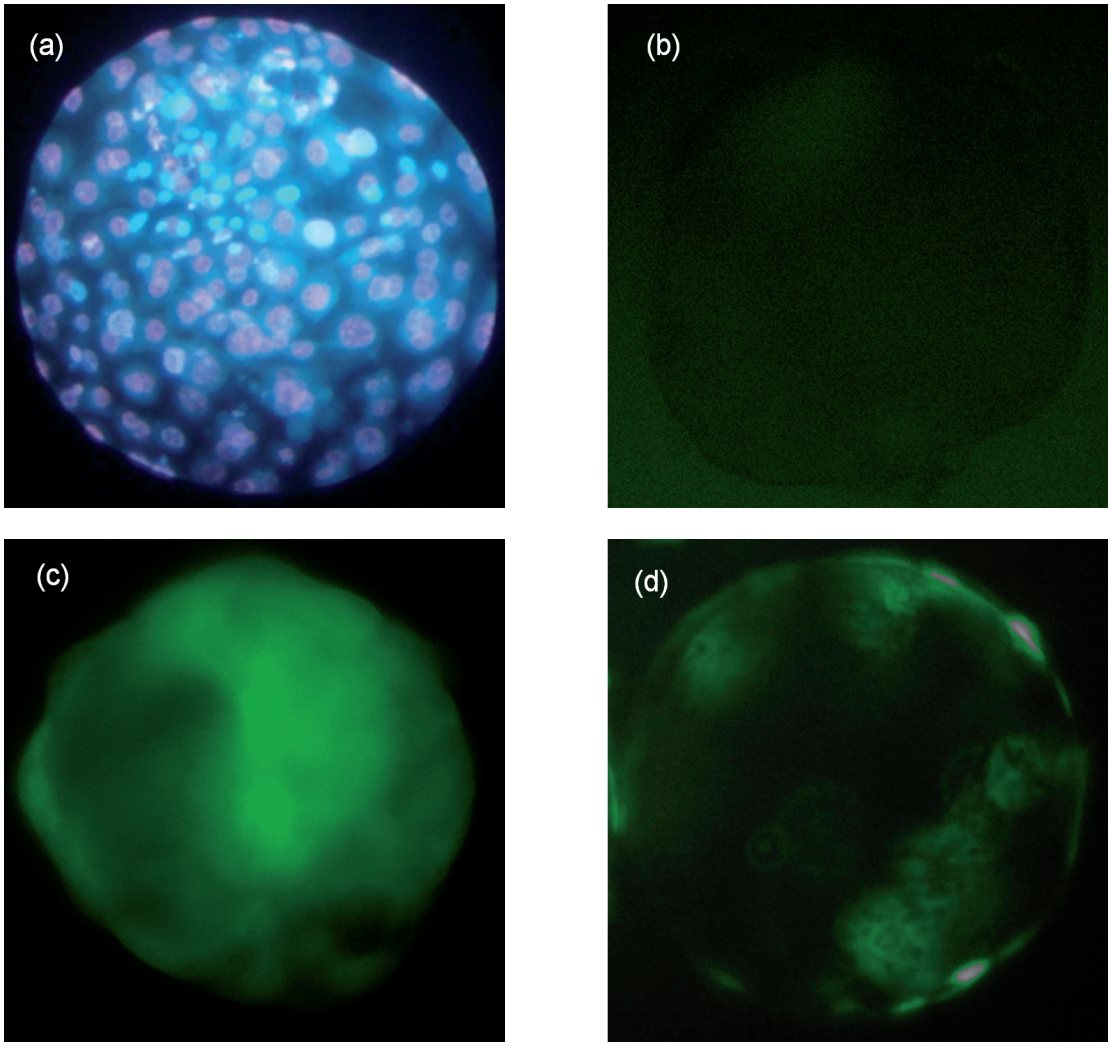

Figure 1. Bovine blastocysts cloned with WT and/or GFP-expressing fibroblast cells visualized under an epifluorescent microscope on Day-7 of development. (a) WT cell-derived non-aggregated (1 x 100%) embryo subjected to differential staining with propidium iodine (pink, trophectodermal cells) and Hoechst 33342 (blue, inner cell mass) fluorochrome dies. (b) Unstained WT cell-derived non-aggregated (1 x 100%) embryo showing a slight autofluorescence pattern. (c) Unstained GFP cell-derived non-aggregated (1 x 100%) embryo showing GFP-positive (green) blastomeres in all cell lineages. (d) Unstained GFP cell-derived aggregated (2 x 100%) chimeric embryo showing GFP-positive (green)/negative blastomeres at a random pattern.

Table 2. Total cell number (LSM ± SEM) and cell allocation ratios in blastocysts produced by the aggregation (2 x 100%) of zona-free pathenogenetic or HMC-derived embryos using distinct karyoplast types (wild type cells, GFP-expressing cells, zona-free MII oocytes).

Embryo type	Karyoplast type	Total	ICM:TE	ICM:Total	TE:Total
Clones	WT/WT cells	157.0 ± 14.7[a]	0.30[a]	0.23[a]	0.77[a]
	GFP/GFP cells	29.0 ± 20.8[c]	0.26[a]	0.21[a]	0.79[a]
	GFP/WT cells	123.0 ± 17.6[b]	0.27[a]	0.21[a]	0.79[a]
Parthenotes	MII/MII oocytes	92.3 ± 16.5[b]	0.38[a]	0.28[a]	0.72[a]

[a,b,c]Numbers in columns with distinct superscripts differ, for $P < 0.05$.

DISCUSSION

Many applications have been suggested for cloning by somatic cell nuclear transfer (SCNT) procedures, including reproductive or even therapeutic cloning, with potential implications to human health [3]. The usefulness of cloning for the production of transgenic animals using genetically engineered somatic cells in culture has been successfully demonstrated in many species [8,9,30]. However, effective transgenic production using cloning procedures still stumble in several technical problems, hindering its efficiency [3,10,34]. It has been suggested that *in vitro* embryo manipulations, such as cloning, may disturb cell allocation or the cell proliferation:cell death ratio in developing embryos [17,33,36], and such changes may affect placental size, shape or function, and, consequently, fetal growth. In this study, the use of syngeneic wild type (WT) and GFP-expressing (GFP) somatic cells to produce HMC-derived cloned embryos intended to evaluate the pattern of cell allocation after embryo aggregation at the 1-cell stage embryo. Results from a qualitatively GFP analysis revealed a trend for a random cell allocation pattern in Day-7 cloned WT/GFP blastocysts.

The green fluorescent protein (GFP) has been an important marker or tracer used in studies in cell and developmental biology, including for the production of transgenic animals by cloning [2,5,25,29], and as a tool for studies in embryonic cell allocation [7,35]. In this study, no differences in cell fusion were observed in embryos reconstructed with GFP-expressing (76.2%) or wild type control (77.2%) cells. Also, cleavage rates were high and rather similar between groups, despite the significantly higher cleavage rates seen in groups G2 and G4 (2 x 100%), which is well expected for aggregated embryos, as cleavage probability increases as more structures are aggregated within the same microwell [28]. Such findings indicate similar cell viability and membrane integrity at the time of fusion and in early development between cell types.

Embryo aggregation may be an effective alternative to increase developmental efficiency after SCNT cloning by many ways. In cattle embryos, an increase in cytoplasmic embryo volume by embryo aggregation has been shown to improve *in vitro* development [24,26,28,31]. Such observations were not observed in this study, as resulting blastocyst yield was similar between aggregated (2 x 100%) and non-aggregated (1 x 100%) embryos, irrespective of the groups, except for G1 (1 x 100%), in which a higher than expected blastocyst rate (50%) was attained on Day 7 of IVC (Table 1), when compared with the other experimental groups.

Studies on cloning continue to associate its efficiency to cell plasticity and with levels of cell differentiation and epigenetic variation within the same genotype or cell lineage [23]. Results from this study suggest the occurrence of a donor cell effect on subsequent *in vitro* embryo development possibly due to the cell´s genetic or epigenetic fingerprint. As culture conditions and prolonged time in culture are known to have detrimental effects on genetic and epigenetic stability in cells and on reconstructed mouse and bovine cloned embryos [13-16], GFP cells used in this study may have compromised embryo development to the blastocyst stage due to cell senescence, as GFP cells were at higher population doublings (19th passage) than control (WT) cells (11th passage). In fact, Giraldo *et al*. [13-15] have shown not only that an extended culture period significantly affects ploidy, chromosome stability and epigenetic features in donor cells, compromising further embryo development after cloning, but also that abnormal gene expression of an epigenetically important gene (DNA methyltransferase 1) resulted in retarded cloned embryo development likely due to inefficient nuclear reprogramming. However, cell senescence is often an issue for the production of transgenic animals, as selection and characterization of transgenic cells in culture may need many cell population doublings prior to be used for cloning.

Data from the available literature on cell allocation are usually difficult to reconcile, with authors either

detecting [22] or not [1,4,31] significant differences in the ICM:TE ratio after embryo aggregation. In this study, no differences in cell allocation in any cell lineage were observed between groups, irrespective of the aggregation scheme or donor cell type used for embryo cloning.

By using syngeneic wild type and GFP-expressing cells, one of the aims of this study was to adapt procedures to allow the concurrent evaluation of the TCN and cell lineage proportion, along with the tracking of the cell´s fate from each aggregated chimeric embryo to both cell lineages at the blastocyst stage (TE or ICM). However, the fluorescent concurrent approach was not possible to be effectively carried out in this study, in part due to the own features of the GFP marker. As our differential staining procedures use embryos treated with a detergent (triton-X 100) to induce membrane permeabilization at the TE cells, it was common to observe a GFP leakage out of the cell during the visualization and, consequently, a GFP bleaching effect. In addition, concurrent detection of GFP positive blastomeres was not possible also due to fluorochrome bleeding into GFP-specific wavelength, even when only one DNA stain (Hoechst 33342) was used in GFP-expressing embryos. Similar problems were also reported previously for pig embryos [18], and in placentas from GFP-expressing transgenic bovine concepti [6].

Usually, when syngeneic or chimeric embryos are produced by aggregation, the final cell allocation into the embryonic cell lineages is usually unknown until phenotypical dimorphism becomes apparent later in development or after birth [7]. In this study, under fluorescent microscopy and prior to embryo differential staining, most visualized WT/GFP cell-derived aggregated embryos demonstrated a random and rather homogeneous pattern of GFP-expressing blastomeres in both cell lineages (Figure 1d). However, distinct from our results, other studies with embryo aggregation at pre-compacting stages demonstrated a non-random cell allocation pattern in resulting blastocysts [7,35]. By aggregating 8-cell stage IVF- with NT-derived embryos, Wells & Powell [35] determined that NT-derived blastomeres would be preferentially diverted to the ICM. Yet, the aggregation of two NT-derived 8-cell stage embryos resulted in a random pattern of cell allocation in developing blastocysts. Conversely, by injecting a single blastomere from a 8-cell stage IVF-derived embryo into a 8-cell stage NT cloned embryo, Chen *et al*. [7] observed that 66% of the blastocysts contained IVF-derived blastomeres embryos exclusively in the ICM, whereas in the other part (34%), IVF blastomeres were present either in the ICM and/or the TE. It has been hypothesized by those authors that cells with a very distinct origin and developmental potential may have a less predictable cell allocation pattern; whereas cells originated from more competent and vigorous embryos may be directed preferentially to the ICM [7]. As signaling mechanisms that drive cell allocation in early embryo development are still uncertain, modifications in the current strategies to study cell allocation are still needed.

CONCLUSIONS

The *in vitro* development of embryos cloned with GFP-expressing cells was lower than for WT cell-derived embryos, with no apparent effects on cell allocation in resulting blastocysts. In turn, embryo aggregation on Day 1 resulted in similar blastocyst rates on Day 7 than non-aggregated embryos, on a per embryo basis, with no effect on cell density or cell proportion. The variation in blastocyst yield was likely due to cell viability in GFP-expressing cells rather than due to transgene expression, since such cells were at high population doublings than syngeneic wild type cells. Still, the use of GFP-expressing cells is a potential strategy for the study of cell allocation during development, which may assist in the elucidation of biological mechanisms and processes, as, for instance, the ones related to abnormalities after *in vitro* embryo manipulations. A better understanding of such mechanisms and processes may lead to the development of improved protocols for the IVP of bovine embryos.

SOURCES AND MANUFACTURERS
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