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Effects of long-term in vitro culturing of transgenic bovine donor fibroblasts on cell viability and in vitro developmental potential after nuclear transfer

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Abstract Genetically modified animals have numerous applications, ranging from basic research to livestock production and agriculture. Recent progress in animal cloning by nuclear transfer has made possible the production of transgenic animals using previously genetically modified cell lineages. However, to produce such lineages, an additional time for in vitro culturing and great manipulation is needed. Herein, we aimed to characterize different aspects of genetically modified cells compared to control cells, and we also analyzed the development rate of embryos produced by nuclear transfer by using them as nuclei donors after short or long periods of in vitro culturing (early versus late passages). We hypothesized that the genetic material inserted in the genome of these cells, associated with the prolonged time in culture, ultimately alters cell growth physiology and cell viability, which leads to impaired nuclei reprogramming potential and consequent reduction in the production of cloned blastocysts. Fetal fibroblasts expressing the enhanced Green Fluorescent Protein gene (eGFP) cultured for different periods in vitro were analyzed with respect to chromosomal numeric abnormalities, nuclear DNA fragmentation, the ratio of *BAX* and *BCL2* gene transcripts, and the intensity of mitochondrial membrane potential, and they were then used as nuclei donors for somatic cell nuclear transfer (SCNT). Early passages were defined as fewer than 11 passages, and late passages were 18th passage (18^{th}p) to 21^{st}p. No differences were observed in the percentage of cells with chromosomal abnormalities or in the mitochondrial membrane potential analysis. eGFP cells in late passages and control cells in early passages were not different regarding DNA fragmentation; however, control cells in late passages presented higher fragmentation ($P<0.05$). The Bax and Bcl_2 gene expression ratio in control and transgenic cells presented different patterns regarding cell conditions during culture. For SCNT experiments, no difference was observed between groups reconstructed with early or late-passage cells when fusion (63.1% and 49%), cleavage (67.7% and 69.9%), eight-cell embryo (36.4% and 44.4%) and blastocyst (21.6% and 20.8%) rates were compared. In conclusion, culture behavior was different between control and eGFP cells. However, when different in vitro culturing periods were compared, long-term cultured transgenic fetal fibroblasts remained competent for blastocyst production when used as nuclei donors in the nuclear transfer technique, a feature needed for the genetic manipulation of cell culture experiments aiming for transgenic animal production.

Keywords Bovine · Cell culture · Cell reprogramming · Nuclear transfer · Transgenesis

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Introduction

Gene transfer technology involving animal production exhibits several potential applications such as the establishment

of models to study physiology and molecular biology, the improvement of animal traits and the use of animal bioreactors for pharmaceutical industry. Notably, the bovine model is more representative of the earliest stages of mammalian development when compared to small laboratory rodents (Berg et al. 2011), which suggests that the bovine is a suitable model of study for early human development and stem cell studies.

Pronuclear microinjection has been used for more than two decades for transgenic mice, rabbit, swine, ewe, and bovine production (Palmiter et al. 1982; Hammer et al. 1985; Bowen et al. 1994); however, the technique still has a low transgene integration rate in the host genome, uncertain offspring transmission, and variegated expression.

Somatic cell nuclear transfer (SCNT) using transgenic donor cells is an effective method for transgenic animal production such as bovine, caprine, and swine (Keefer et al. 2001; Bordignon et al. 2003; Hyun et al. 2003), and this procedure assures the presence and the expression of the transgene in the offspring using genetically modified cell lineages harboring the desired genetic feature (Bressan et al. 2011). However, additional studies are needed to optimize its efficiency and diminish the relatively high rates of embryo, fetus, and perinatal mortality or anomalies, which may still hamper the use of SCNT in the large-scale production of transgenic herds (Batchelder et al. 2007; Meirelles et al. 2010).

The production of a transgenic donor cell lineage may contribute to these outcomes. This process demands extra manipulation and additional in vitro culture periods when compared to non-modified lineages. Several passages are needed to obtain the optimal number of cells needed for genetic modification and selection procedures, leading to high passage cultures for SCNT with the probable occurrence of genetic and epigenetic alterations. Undesirable phenotypes may also be caused by random transgene insertion in the cell genome because transgene integration and expression is not usually evaluated in other techniques rather than SCNT (Bressan et al. 2011).

Nuclei from cells cultured in vitro for a short time may be more easily reprogrammed when compared to nuclei derived from long-term in vitro cultured cells (Roh et al. 2000). One possibility that may influence cell viability is an increase in apoptosis, a natural defense mechanism that promotes programmed cell death (PCD) when cell function is already damaged or inexistent. In this context, BCL-2 family proteins are important intracellular regulators of PCD by modulating the activation of pro-caspases. Some members of this family such as BCL-2 or BCL-xl inhibit apoptosis, at least partially, by blocking cytochrome c release from mitochondria. Other family members such as BAX and BAK stimulate cytochrome c release from mitochondria, which makes them pro-apoptotic factors. PCD can be easily detected using laboratorial procedures conducted in in vitro cultures, becoming a major field of exploration in cell physiology.

Herein, we aimed to produce a viable and stable eGFP-expressing fibroblast lineage through lentiviral transduction suitable for cloned embryo production. More than that, we aimed to identify possible events related to a long period of in vitro culture that may be related to low SCNT success rates, contributing to a better characterization of transgenic cell physiology in vitro and nuclear reprogramming, while diminishing the production of hampered cloned embryos due to in vitro long-term culturing or transgenesis.

Material and Methods

Production of transgenic bovine fetal fibroblasts. The isolation, genetic modification, and culturing of fetal fibroblasts were performed as detailed in Bressan et al. (2011). Briefly, a primary culture of bovine fetal fibroblasts was derived after culture of minced tissue of a 55-d Nelore (*Bos indicus*) fetus. The lentivirus (FUGW) used for transduction was produced as described by Lois et al. (2002). Cell cultures were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, and they were cultured at 38.5°C, 5% CO_2 in air, and saturated humidity. Passages were conducted when subconfluent (80–85% confluence, assessed visually) using 0.25% trypsin (Life Technologies, Carlsbad, CA).

Transduction was performed in nine wells from a 96-well dish, enabling transductions to be conducted under three different cell confluence conditions (50%, 70%, and 90%) and three different viral concentrations (1, 0.5, or 0.25 viral particles per cell) in triplicate. Each well received 8 μg/ml of polybrene. After 2 h of incubation, fresh cell culture medium was added, and after 24 h, the medium was changed. Twenty-four hours after infection, only seven wells showed healthy cells that were able to continue the experiment. Cell culture was replicated into 24-well plates and then transferred to 35- and 60-mm dishes when a sufficient number of cells were achieved. Next, the cells underwent fluorescence-activated cell sorting (FACSAria, Becton, Dickinson and Company, Franklin Lakes, NJ) to identify and recover eGFP-expressing cells. The percentage of positive cells ranged from 2.95% to 9.54%. The following experiments were developed using cells derived from only one well, guaranteeing the same background throughout experiments.

Karyotyping. The metaphase stage (n=30) of transgenic and non-transgenic fetal fibroblasts were analyzed at high passages (eGFP 20p and control 20p), and non-transgenic fetal fibroblasts were also analyzed at early passage (control, 8p) to estimate the frequency of normal metaphases.

Karyotyping was performed in 60-mm dishes when cells were approximately 70% confluent by supplementing the culture media with 16 μg/ml of colchicine for 1 h. Cells

were washed and trypsinized, and the cell pellet was resuspended and incubated in 5 ml of hypotonic solution (KCl, 0.075 M) for 20 min. Cells were fixed with 5 ml of methanol/acetic acid (3:1) for 30 min, centrifuged, and resuspended again in 500 ml of fixative solution and poured into slides at a distance of 70 cm. Slides were air dried and stained with 0.4% Giemsa in PBS for 10 min. Analyses were conducted by microscopy at ×1,000 magnification under immersion oil.

Comet assay (single cell gel electrophoresis assay). Cellular pellets of transgenic and control cells from the 20th passage and control cells from the 8th passage were resuspended in 200 μL of 1% low melt agarose at approximately 37°C. Agarose-containing cells were deposited in a thin layer of agar previously poured onto a slide. After short incubation at 4°C for polymerization, cells were lysed in proteinase K solution (10 mM Tris, pH 10, 2.5 mM NaCl, 100 mM Na_2EDTA, 1% Triton 100×, and 10 μg/mL proteinase K) at 50°C for 2 h. Slides were incubated for 20 min in electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH) and then for 20 min at 25 V. DNA staining was performed with ethidium bromide (10 ug/ml).

DNA fragmentation, which is visualized with the migration of DNA from the nucleus, resulting in a tail of damaged or broken DNA, was evaluated in 200 cells per experimental group through epifluorescence microscopy. The size of the tail is proportional to the extent of DNA damage (Takahashi et al. 2000).

Fibroblasts were categorized in five different scores, relative to the size of the DNA tail (Fig. 1): grade 0, visually inexistent fragmentation; grade 1, mildly visible fragmentation; grade 2, visible fragmentation; grade 3, advanced fragmentation; and grade 4, DNA totally fragmented.

Assessment of mitochondrial membrane potential using the MitoTracker Red assay. For mitochondrial membrane potential assay, 9th to 11th passages were used for early-passages control group, whereas 18th to 20th passages were used for late-passages GFP and control groups. Cells were cultured onto coverslips that were previously sterilized by 70% ethanol, fire, and UV exposure and placed into 35-mm culture dishes. Next, 500 nM of MitoTracker Red (Life Technologies, Carlsbad, CA) was used for cell incubation for 30 min. After incubation, coverslips were recovered from 35-mm dishes, placed on slides, and sealed. Analyses were performed by epifluorescence microscopy.

After exposure to UV, fibroblasts were categorized into four grades of DNA fragmentation. Grade 0 included cells without mitochondrial membrane potential, grade 1 cells had impaired potential, grade 2 cells had average potential, and grade 3 cells had elevated mitochondrial membrane potential. Overall, 200 cells from each passage were analyzed, and three passages comprised an experimental group. The results were analyzed using Tukey's test.

RNA extraction, cDNA production, and qPCR for BAX and BCL2 transcripts. TRIzol (Life Technologies, Carlsbad, CA) was used for RNA extraction from fibroblast cultures. For PCR analysis, fibroblasts cultures at 4th and 8th passage consisted on early-passage group, in vitro cultures with 20 passages consisted on late-passages groups, and the period between 12th and 17th passages was also analyzed for qPCR and therefore was named intermediate-passage group.

Briefly, confluent 35-mm dishes were trypsinized, centrifuged at 300×*g* for 5 min in a microtube, and the supernatant was removed. Then, 1 ml of TRIzol was used to resuspend each sample, which were vortexed and maintained at room temperature for 5 min. Next, 200 μL of chloroform was added to each sample, shaken manually for 15 s, and centrifuged at 12,000×*g* for 15 min at 8°C. The supernatant was recovered, and 500 μL of isopropanol was added, incubated at room temperature for 10 min, and centrifuged at 12,000×*g* for 10 min at 8°C. Next, 1 mL of 75% ethanol was added to the pellet, which was centrifuged at 7,500×*g* for 5 min at 8°C. Finally, the supernatant was discarded, and the pellet was air dried for 5 min. The pellet was resuspended in 10–20 μL of RNAse-free water and incubated at 60°C for 1 min. RNA was quantified in a spectrophotometer and stored at 80°C or immediately submitted to DNAseI treatment and reverse transcriptase PCR (RT-PCR).

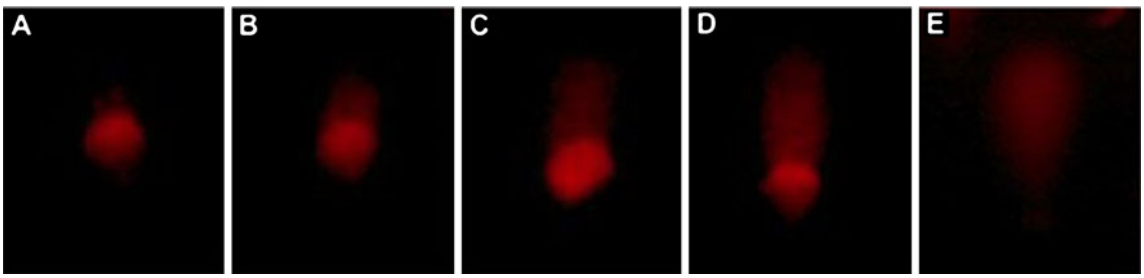

Figure 1. DNA fragmentation analyzed on Comet assay. (*A*), (*B*), (*C*), (*D*), and (*E*) correspond to zero, one, two, three, and four stages of DNA fragmentation, respectively.

DNAseI treatment consisted on the incubation of 1,000 ng of each mRNA with 1 U of DNAse1 amplification grade (Life Technologies), DNAse 1 buffer, and 10 U of RNAseout (Life Technologies) for 15 min at room temperature, followed by its inactivation with EDTA at 65°C for 10 min as suggested by the supplier. RT-PCR was performed with ImProm-II Reverse Transcriptase (Promega, Madison, WI), following the manufacturer's recommendations. cDNA amplification was performed using a 7500 Real Time PCR System (Life Technologies, Carlsbad, CA). In each reaction, Power SYBR Green® PCR Master Mix (Applied Biosystems) and 0.4 μM of each primer were used for the PCR reactions. Primers sequences are detailed in Table 1.

Quantification of target mRNA was evaluated by the 2^{DDCt} method (Livak and Schmittgen 2001), based on duplicates. Target and reference genes PCRs were equally efficient.

Somatic cell nuclear transfer. Cell-oocyte fusion, embryo cleavage rates, the number of eight-cell embryos at 3 d of in vitro culturing, and the number of blastocysts produced at 7 d of in vitro culturing were analyzed. A total of 416 embryos were reconstructed. Overall, 206 oocytes were reconstructed using early-passage cells (9th and 10th p, in triplicate), and 210 oocytes were reconstructed using late-passage cells (between 18th and 21^{st}p, in triplicate). The results were analyzed by ANOVA with 5% probability. The SCNT technique was performed as already described in Miranda et al. (2009) and is briefly reported here.

Ovary aspiration, oocyte selection, in vitro maturation, and first polar body selection. Ovaries were collected from zebu cross-bred females derived from nearby slaughterhouses. Follicles between approximately 3 and 6 mm (de Loos et al. 1989) were aspirated using syringes and needles (18 G), and only oocytes presenting homogeneous cytoplasm and layers of compact cumulus cells were used. In vitro maturation (IVM) was performed in TCM199 medium containing Earle's salts, glutamine, $NaHCO_3$ supplemented with 10% FBS, 22 μg/ml pyruvate, 50 μg/ml gentamicin, 0.5 μg/mL FSH, 50 μg/mL LH, and 1 μg/mL estradiol in 100 μl microdrops covered with mineral oil and incubated at 38.5°C, 5% CO_2 in air, and maximum humidity for 18 h.

After IVM, oocytes were recovered from microdrops, and *cumulus oophorus* cells were removed through successive pipetting in 0.1% hyaluronidase solution in PBS. Evaluation of the presence of first polar bodies was performed under stereomicroscopy in TCM199 medium with Earle's salts, glutamine, $NaHCO_3$ and HEPES buffer, supplemented with 10% FBS, 22 μg/mL pyruvate, and 50 μg/mL gentamicin.

Oocyte enucleation and embryo reconstruction. Oocytes containing the first PB were incubated for 15 min in CR2 medium containing 7.5 μg/mL cytochalasin B and 5 μg/mL Hoescht 33342 in CO_2 incubators. After incubation, they were transferred to the micromanipulation dish, already containing trypsinized fibroblasts in PBS supplemented with 22 μg/mL pyruvate, 50 μg/mL gentamicin, and 10% FBS.

Next, 21 h after the start of IVM, oocytes were enucleated with the use of micropipettes in a micromanipulator. Enucleation was performed by aspirating the first polar body and approximately 20% of the cytoplasm, assuring the removal of genetic material by exposing the aspirate to UV light. For reconstruction, one fetal fibroblast was inserted into the perivitelline space. The procedure was performed in all oocytes using different micromanipulation dishes to prevent contamination between transgenic and non-transgenic groups. The fusion of the cell and the ooplasm membrane was performed in an electroporator with mannitol (0.3 M). One electrical field of 1 V (5 s continuous field) and two pulses of 1.75 KV/cm for 65 μs were used to promote membrane fusion. Before chemical activation (5 μM ionomycin for 5 min and 2 mM of N-6186 dimethylaminopurine (6-DMAP) for 3 h) at 26 h after IVM, complexes were checked for fusion efficiency.

In vitro culture. After fusion and activation, zygotes were cultured using standard in vitro culture conditions (CR2 medium supplemented with 5% FBS and 30 mg/mL BSA in co-culture with granulosa cells) for 7 d when their potential to develop into blastocysts was evaluated.

Results

Production of transgenic bovine fetal fibroblasts. Groups transduced when presenting 50% confluence had approximately 6.05% fluorescent cells, groups with 70% had 6.83% and groups with 90% presented 5.97% positive cells. Cell cultures submitted to a cell/viral ratio of 1:1 had 9.54%

Table 1. Oligonucleotides sequences

Gene	Forward 5′-3	Reverse 5′-3
Bax	GACGGGTCCGGGGAGCAAC	ATGGTGAGCGAGGCGGTGAG
Bcl2	TTCGCCGAGATGTCCAGTCAGC	TTGACGCTCTCCACACACATGACC
Gapdh	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT

fluorescent cells, groups submitted to a 1:0.5 ratio had 7.78%, and groups submitted to 1:0.25 ratio had 3.89% positive cells. When the cell confluence results were compared independently from viral data, no differences regarding the number of fluorescent cells were observed ($P>0.05$). When the effect of the cell/viral ratio was compared independently from confluence data, differences between groups were observed ($P<0.05$), and the cell culture that received less viral particles showed a decreased number of GFP-expressing cells when compared to the other two groups. All healthy cell cultures were submitted to GFP fluorescence sorting using flow cytometry, resulting in cell lines that stably express GFP in almost 100% of cells for at least 21 passages.

Karyotyping. Chromosome preparations were performed based on the protocol described by Moorhead et al. (1960). Metaphases were analyzed from eGFP (late passages) and control cells (early and late passages; Fig. 2). The percentage of euploid cells did not differ between groups ($P>0.05$; Fig. 3).

The comet assay (single cell gel electrophoresis assay). No differences were observed between eGFP groups at late passages and the control at early passages; however, both eGFP groups differed from the control group at late passages ($P<0.05$, Fig. 4). Data from individual groups are represented in Fig. 5.

Assessment of mitochondrial membrane potential using the MitoTracker Red assay. Percentages of cells showing low or absent mitochondrial membrane potential (grades 0 and 1) were compared between groups. Cell nuclei staining with Hoechst and confirmation of eGFP expression (in the eGFP expression group) were used as references (Fig. 6). Control cell cultures in early or late passages and eGFP-expressing cultures in late passages were compared, and no difference was observed between groups ($P=1$; Fig. 7).

Estimation of relative frequency of BAX, BCL2, and BAX/BCL2 ratio transcripts. The estimation of relative frequency of *BAX* and *BCL2* gene expression were analyzed in intermediate passages as well as early and late passages. The *BAX* and *BCL2* gene expression ratio (Fig. 8) in control cells showed a tendency to decline in apoptosis during early passages, which was most likely explained by the adaptation of the cells to the in vitro culture environment. During the culture period, we observed an increased tendency in the pro-apoptotic response in intermediate and late passages. However, such tendencies were not clearly observed in GFP-expressing cells, which had an increase in the *BAX*/*BCL2* gene expression ratio in intermediate passages, followed by a minor decrease in late passages.

Developmental potential after somatic cell nuclear transfer. No difference was observed ($P>0.05$) between groups reconstructed with early- or late-passage cells when fusion (63.1% and 49%), cleavage (67.7% and 69.9%), eight-cell embryo (36.4% and 44.4%), and blastocyst (21.6% and 20.8%; Figs. 9 and 10) rates were compared.

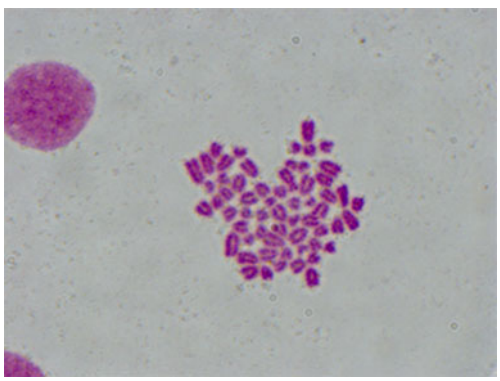

Figure 2. Typical bovine metaphase.1,000×.

Discussion

The use of gene transfer in animal production has great potential to achieve agricultural and modern biomedical improvements. In this work, lentiviruses were used for transgenesis because they offer advantages over other methods. For example, they have an intrinsic capacity to integrate into the host genome (Linney et al. 1999; Houdebine 2002), they promote efficient and stable transgene expression in a variety of cell types in vitro and in vivo, and can infect both dividing and non-dividing cells (Gropp et al. 2003; Hofmann et al. 2003). Hence, gene silencing was not observed upon lentivirus transduction (Lois et al. 2002; Gropp et al. 2003; Ikawa et al. 2003). Furthermore, they promote the evaluation of tissue-specific promoters before their use in gene therapy strategies (Lois et al. 2002).

Reporter genes are extremely important to transgenics because they allow the selection of cells that express the gene of interest. Fluorescent proteins such as Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP), and *Discosoma* sp. Red Fluorescent Protein DsRed are widely used for such purposes. For example, the GFP gene is a commonly used gene reporter that can be viewed in real time in vitro or in situ in developing embryos or adults without adverse biological effects to cell cultures (Chalfie et al. 1994) or embryonic development (Roh et al. 2000; Koo et al. 2001).

In this work, the establishment of a viable GFP transgenic cell line using the lentiviral mechanism of transgenesis was possible and efficient, and its production was independent of cell confluence; however, it was dependent on the viral concentration.

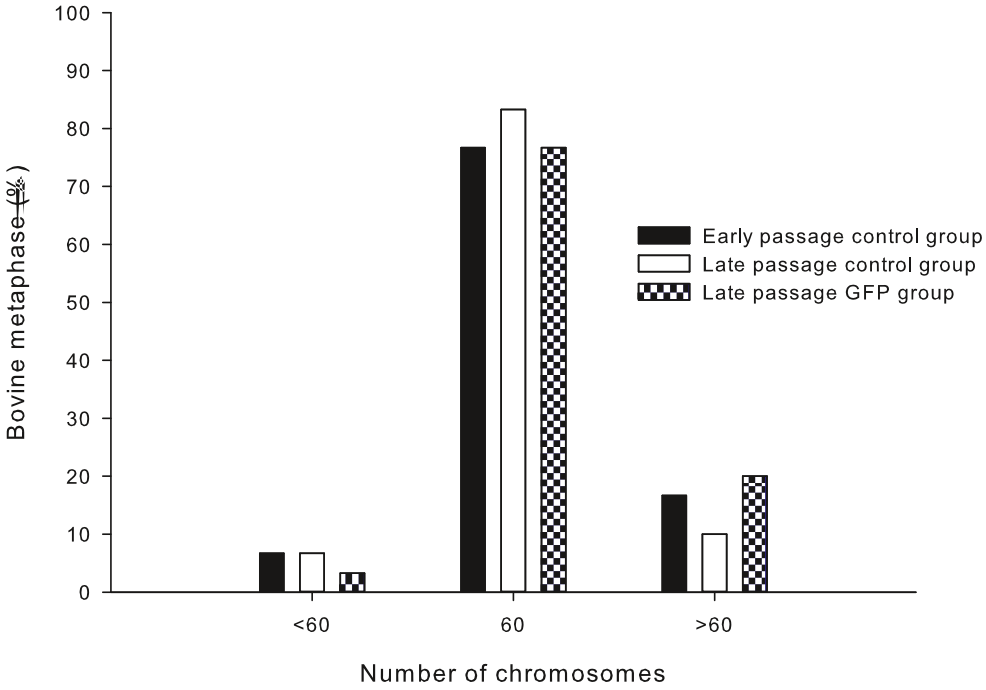

Figure 3. Percentage of metaphases showing chromosome number higher than 60, equal to 60, and lower than 60, respectively, in the three analyzed groups.

Chromosomal abnormalities were not observed in this experiment; therefore, when cell cultures were submitted to short- (10 passages) or long-term culturing (20 passages) as well as when cell cultures were submitted or not to viral transduction, no abnormalities were observed in our experimental condition. When these cultures were submitted to karyotyping, they were euploid in 90% of analyzed metaphases. Metaphases that differ from 60 chromosomes were most likely due to technical artifacts because chromosome number variation was primarily between 59 and 61. These results are in agreement with other results obtained in our SCNT routine in which fibroblasts in low passages are commonly used for SCNT.

DNA fragmentation was analyzed through the comet assay, and transgenic cells at late passages and control cells at early passages were not different; however, both groups differed from the control group at late passages. Although this result was not expected, it is in agreement with the *BAX*/*BCL2* ratio described later. Here, we discuss the possible effect of culturing in vitro for a long length of time. In this work, eGFP-expressing cells were frozen after transduction and selection, and the thawing of the cryotube and the

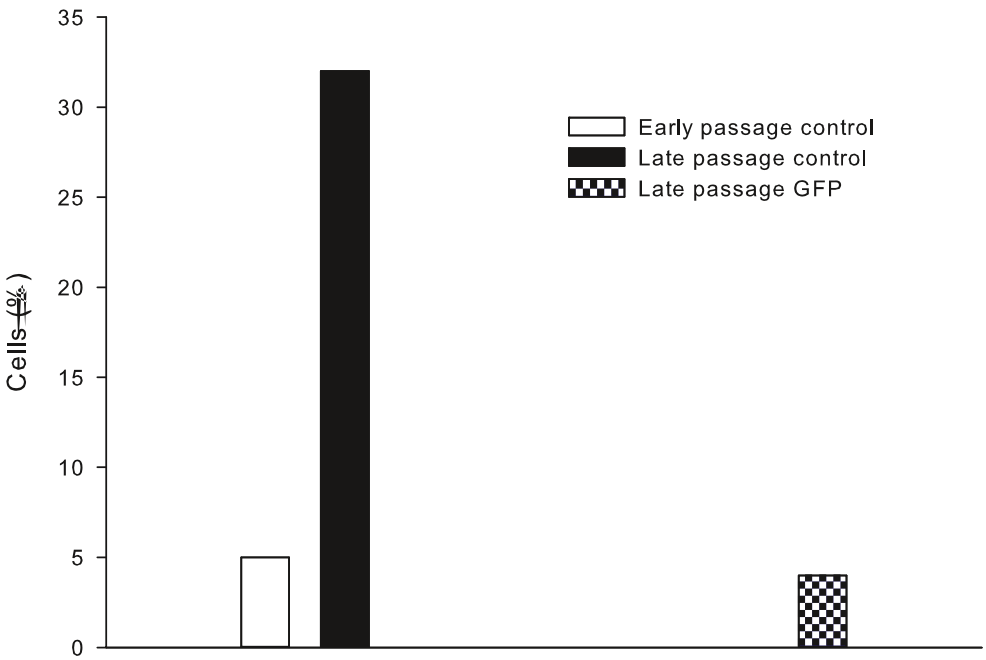

Figure 4. Percentage of cells presenting high DNA fragmentation in each of the analyzed groups when analyzed by Comet assay. Control group at 20th passage presented a higher number of cell with fragmented DNA ($P<0.05$).

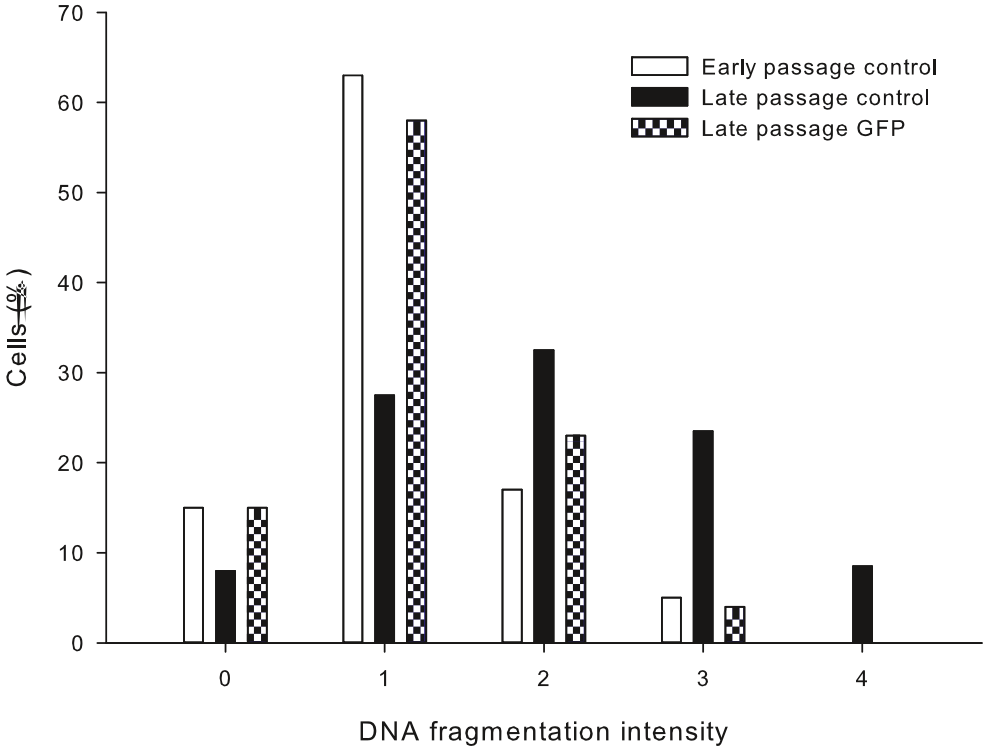

Figure 5. Percentage of cells showing different DNA fragmentation stages in each analyzed group.

restart of the in vitro culture was performed at the 11th passage. Therefore, they were manipulated for at least ten passages. However, the control cells were constantly manipulated after their first thawing, underwent a total of 18 passages, and had a longer time of non-stop culturing. It is well discussed that a long culture period may alter the conditions throughout passages (Roh et al. 2000; Li et al. 2003; Xie et al. 2013); therefore, it is presumed that after thawing, the cells need to adapt to the environment. During this adaptation period, DNA fragmentation and apoptotic findings probably diminish and then reach a plateau to finally enter into the senescence phase. In this hypothesis, control cells have passed through all stages, whereas eGFP-expressing cells would reach senescence in further passages.

Herein we suggest that a most acceptable possibility is the hypothesis that transgenic cells have already underwent great manipulation (viral incubation, culture media changes, sorting). Therefore, the most fragile cells and/or the cells prone to any disruption because of in vitro culturing will be naturally discarded.

Indeed, other hypotheses may be feasible, and further studies are needed before any final conclusions regarding the viability of this specific transgenic cell line can be made.

Although it was not observed, differences may be present between the length of culturing time or between the control and eGFP cells; however, these eventual differences regarding cell behavior and development of these in vitro cultures are most likely independent of the mitochondrial membrane potential. However, these differences may interfere with the mitochondrial membrane potential in a manner that is not revealed by the technique described here.

During the culture period, we observed an increased tendency in the pro-apoptotic response in late passages. However, such tendencies were not clearly observed in

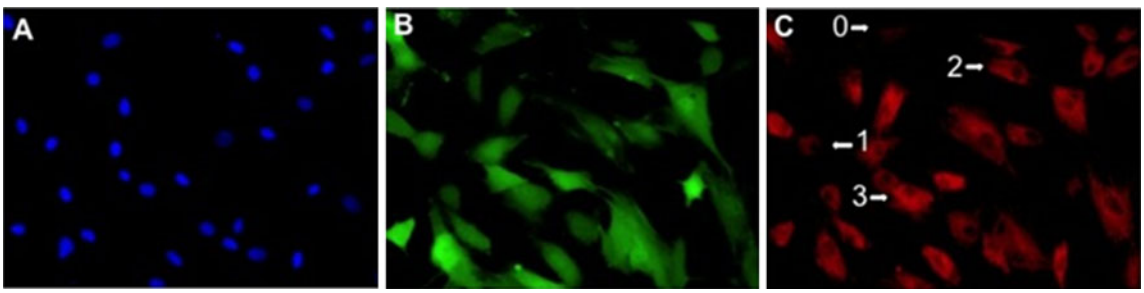

Figure 6. (*A*) Analysis of mitochondrial membrane potential. (*A*) Nuclei visualization after Hoechst33342 stainning. (*B*) eGFP fluorescence, (*C*) MitoTracker Red fluorescence. *Numbers 0–3* represent different grades of mitochondrial membrane potential: *0* none or very low, *1* low, *2* medium and *3* high. Same field for (*A*), (*B*), and (*C*), 20×.

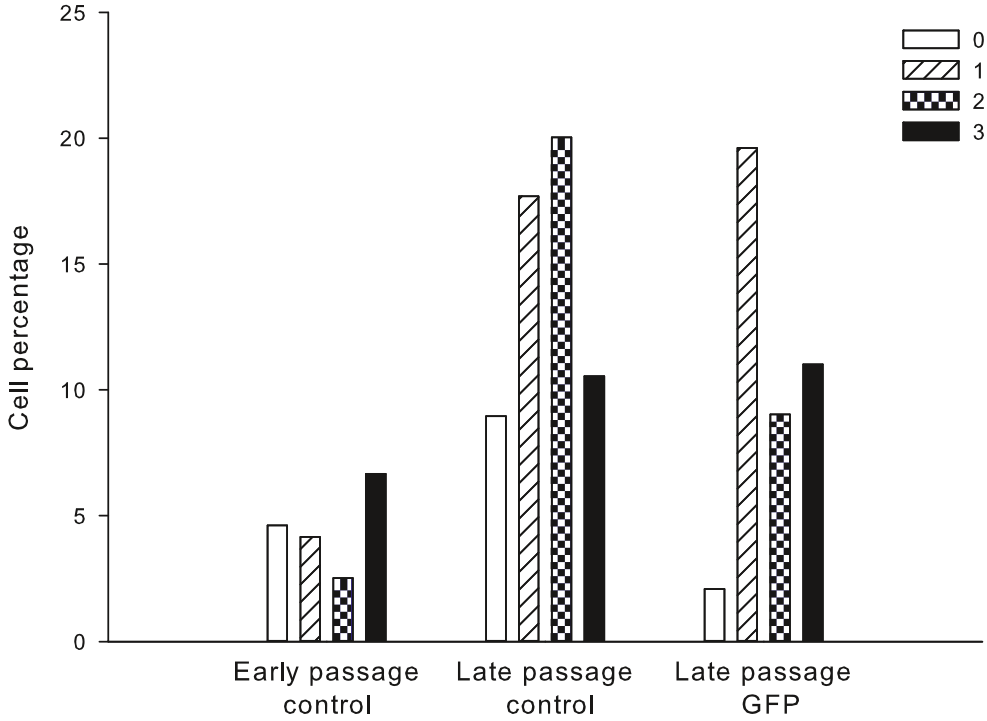

Figure 7. Percentage of control and transgenic cells with different mitochondrial membrane potential.

GFP-expressing cells, which had an increase in the Bax/Bcl_2 gene expression ratio in intermediate passages, followed by a minor decrease in late passages. These results are in agreement with the comet assay.

Complex reproductive biotechnologies, as for example, SCNT, have for long been used as promising tools to the increase of animal breeding outcomes. Their efficiency, however, is still influenced or even hampered by several factors (Alberto et al. 2012). The embryo production using somatic cells as nuclei donors is greatly relevant and interesting for basic and applied studies on animal production, reproduction, and biotechnology.

The production of apparently healthy transgenic embryos derived from greatly manipulated cell cultures may lead to

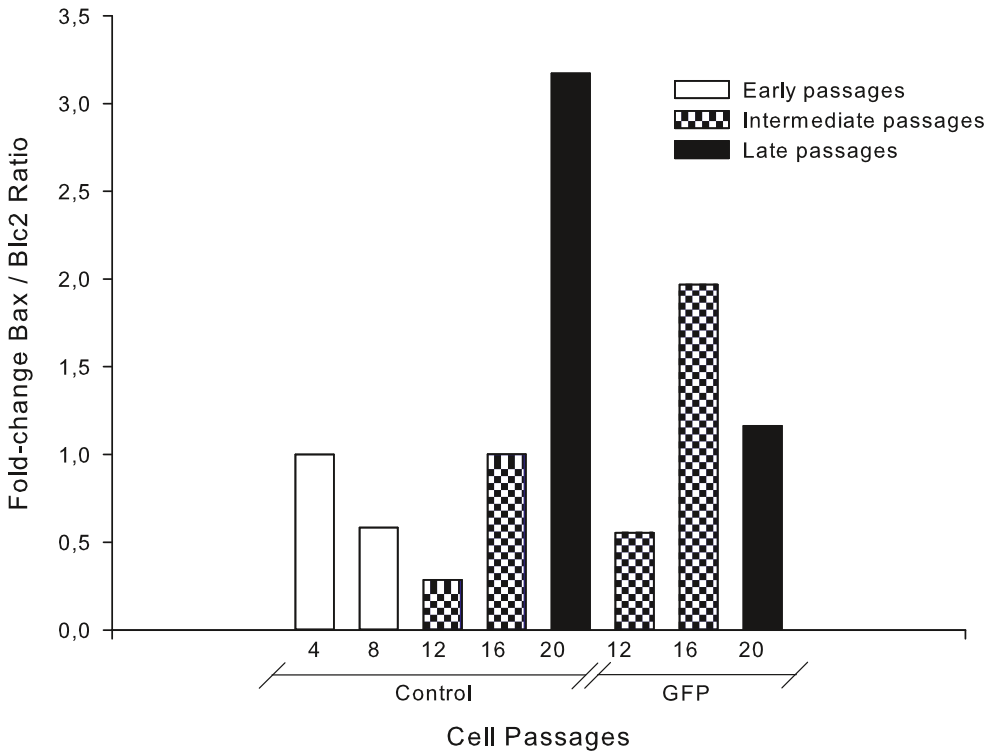

Figure 8. *BAX* and *BCL2* gene expression ratio of control and transgenic groups.

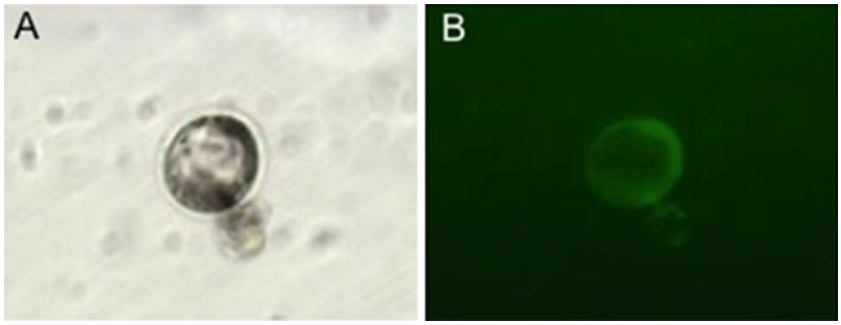

Figure 9. Hatching blastocyst produced by SCNT of eGFP expressing donor cell at late passage. (*A*) bright field, (*B*) eGFP Fluorescence under UV exposure.

the production of healthy transgenic cloned animals that are not only important for commercial purposes but are also tools for developmental physiology. Nevertheless, it is known that the condition of the donor cells used for SCNT (epigenetics, apoptosis, etc.) is crucial to determine the success of in vitro embryo production (Clark et al. 2000; Sangalli et al. 2012).

Roh et al. (2000) reported that when early passages of fetal fibroblasts were used for transgenic blastocyst production through SCNT, a better developmental rate was observed. In their study, transgenic cells underwent manipulations and cell passages, and the authors considered early passages to occur between the 8th and 16th, and the late passages were between the 17th and 32nd passages. Roh and collaborators also compared the developmental potential of blastocysts produced with fetal fibroblasts expressing GFP and non-transgenic fetal fibroblasts. In this experiment, no differences were observed, concluding that GFP insertion was not deleterious for embryo development. Conversely, in 2005, Merighe (personal communication) analyzed the developmental rate of blastocysts into embryos produced by SCNT when non-transgenic fetal fibroblasts were used as donor cells. The developmental rate of blastocysts after SCNT when early and late passages were compared differed between groups (14% and 5%, respectively) and infers that the use of cells with a minor in vitro culture period may lead to a higher success on nuclear transfer. Further studies regarding the mechanisms involving the losses that occurred during early development are necessary for the optimization of animal reproduction biotechniques.

Conclusions

The production of a viable and stable eGFP-expressing fibroblast lineage is possible and efficient after lentiviral transduction, and this lineage is suitable for cloned embryo production even after long-term in vitro culturing. Differences were observed between culture behavior when comparing cells with different passages and also when comparing control and transgenic cells. These differences are not related to the mitochondrial membrane potential; however, they relate to DNA fragmentation and the relative mRNA expression between pro- and anti-apoptotic genes. In this work, eGFP-expressing cells presented a higher DNA integrity rate and a decrease in the tendency of apoptosis occurrence when compared to control cells at the same passage.

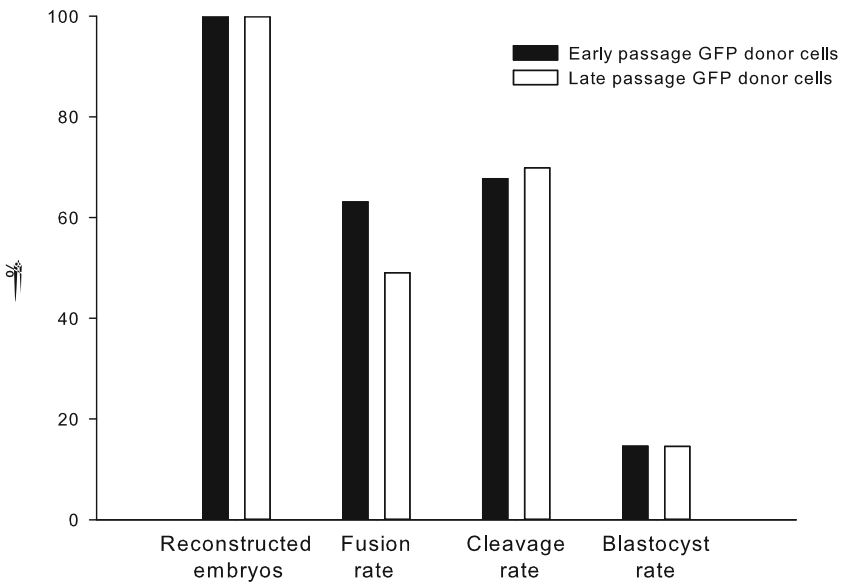

Figure 10. Developmental rates of SCNT produced bovine transgenic embryos.

This viability was confirmed by the production of viable blastocysts when these cells were used as donor cells in SCNT. The production of genetically modified embryos is not only desired on animal production fields but it is also a huge step regarding basic research of mammalian development and may become a powerful model for embryonic gene expression studies.

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