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Advances in the development of a noninvasive embryo model for the evaluation of the quality of cloned embryos subjected to different treatments

Ricardo Felmer*
Laboratorio de Biotecnología Animal
INIA-Carillanca, Casilla 58-D
Temuco, Chile
Tel: 56 45 215706. Ext. 311
Fax: 56 45 216112
E-mail: rfelmer@inia.cl

María Elena Arias
Laboratorio de Biotecnología Animal
INIA-Carillanca. Casilla 58-D
Temuco, Chile
Tel: 56 45 215706. Ext. 293
Fax: 56 45 216112
E-mail: marias@inia.cl

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Keywords: bovine embryos, HcRed, nuclear transfer.

Abbreviations: BEF: bvine embryonic fibroblasts
COCs: clus oocyte complexes
DMEM: Dulbecco's modified eagle's medium
DMSO: dmethyl sulfoxide
FCS: fal calf serum
MII: Metaphase II
PCR: polymerase chain reaction
SCNT: somatic cell nuclear transfer

Total number of cells in cloned embryos is generally lower than that of *in vivo* derived embryos and in bovines cell allocation at the blastocyst stage, has been observed to be affected in a large proportion of cloned embryos. The current embryo staining procedures are toxic for mammalian cells and thus can not be used to determine the developmental potential of a stained embryo. Therefore, in the present study we sought to assess the feasibility to develop a noninvasive embryo model that would be suitable for the evaluation of cloned embryos subjected to different nuclear transfer and embryo culture procedures. For doing this, we stably transfected a bovine embryonic fibroblast cell line and generated a number of clones that constitutively expressed a red fluorescent protein (HcRed) in the nuclear compartment of the cell. Those clones with normal chromosomal content were further used as nuclear donor in nuclear transfer procedures (SCNT) to generate transgenic cloned embryos. These embryos expressed the red fluorescent protein in each blastomere, allowing their *in vivo* evaluation during development, thus demonstrating the potential of this model as a noninvasive tool for the assessment of the quality of cloned embryos.

Cloning by SCNT has gained great interest between researchers due to the potential application of this technology in different areas including animal griculture, biotechnology, biomedicine, preservation of endangered species and basic research (Cibelli et al. 1998; Wells et al. 1999; Lanza et al. 2000; Keefer, 2004). The success of the technology originally described by Wilmut and colleges (Wilmut et al. 1997) has been replicated since then to clone the most important agricultural and domestic species (Cibelli et al. 1998; Wakayama et al. 1998; Baguisi et al. 1999; Polejaeva and Campbell, 2000; Chesne et al. 2002; Shin et al. 2002; Galli et al. 2003; Zhou et al. 2003; Li et al. 2006). However, in spite of the success in these species, the efficiency of the technology is still very low, with a success rate reported no greater than 4% (Wilmut et al. 1997; Wakayama et al. 1998; Miyoshi et al. 2001). Furthermore, a high abortion rate and mortality during pregnancy are commonly reported (Hill et al. 2000). There are a number of factors that may contribute to the low efficiency rate of

*Corresponding author

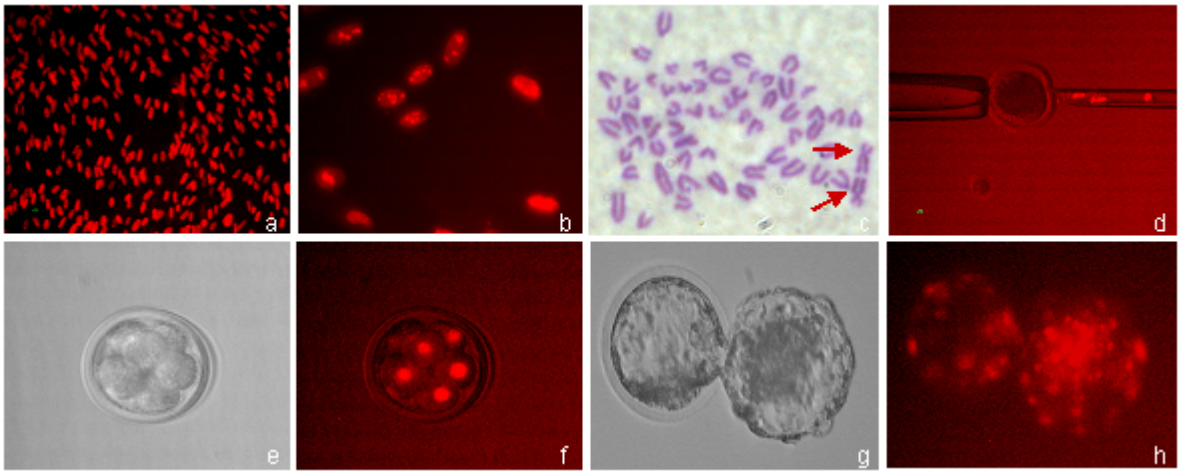

Figure 1. Developmental assessment of transgenic cloned embryos generated with bovine embryonic fibroblasts (BEF) expressing HcRed.
a) Transfected BEF expressing HcRed (20x);
b) Expression of the red fluorescent protein in the cellular nuclei (40x);
c) Karyotyping analysis of metaphase spreads of transfected BEF, with sexual chromosomes highlighted (arrows);
d) Nuclear transfer procedure with cells expressing HcRed;
e-h) Developmental assessment of transgenic cloned embryo expressing the red fluorescent protein in the blastomeres (HcRed).

this technology, including the cell cycle co-ordination between the donor nucleus and recipient oocyte, artificial activation procedure, *in vitro* culture conditions for the reconstructed embryos and an inadequate nuclear reprogramming, some of which may have a direct impact on the end quality of the generated embryo and the survival after transfer to recipients.

It has been reported that the total number of cells in cloned embryos is generally lower than that of *in vivo* derived embryos (Koo et al. 2000; Chesne et al. 2002; Chung et al. 2002; Koo et al. 2002). Furthermore, in bovines not only the total number of cells but also cell allocation at the blastocyst stage is affected in a large proportion of cloned embryos, with some observations of a reduced trophoblast to total cell number ratio compared to IVF and *in vivo* derived embryos (Koo et al. 2002). This lower number of trophoblast cells, might be responsible for the insufficient formation of the placenta observed in some nuclear transfer pregnancies. Therefore, it seems that correct allocation of cells at the blastocyst stage is necessary for a proper embryonic and fetal development. One of the limitations to assess the cell allocation and total number of cells in the embryos is that the procedure of staining is toxic for mammalian cells, thus preventing its application to determine the developmental potential of a stained embryo. Therefore, the objective of the present study was to assess the feasibility to develop a noninvasive embryo model that would be suitable for the evaluation of cloned embryos subjected to different nuclear transfer and embryo culture procedures. This will allow to determine developmental abnormalities in the embryos as result of an inadequate nuclear reprogramming or as direct consequence of sub optimal culture conditions, making possible to assess the developmental potential of these embryos post-implantation.

MATERIALS AND METHODS

Preparation of donor cell

A primary cell line of bovine embryonic fibroblasts (BEF) was isolated from a 50 days old fetus. Differentiated tissues were removed and soft tissue were cut into small pieces and the explants cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco)supplemented with 10% fetal calf serum (FCS, HyClone), 1% (v:v) penicillin/streptomycin (Gibco) at 37ºC in a humidified atmosphere of 5% CO_2. After 10 days in culture, the explants were removed, and the cells were harvested by trypsinization, counted, and seeded in 75 cm² tissue culture flasks. When the cells reached confluency, they were collected by trypsinization and frozen in DMEM supplemented with 10% FCS and 10% dimethyl sulfoxide (DMSO, Sigma).

DNA preparation, transfection and selection of clonal cell lines

The plasmid directing red fluorescence protein was a version of pHcRed1-nuc (Clontech), kindly provided by Dr. Pablo Ross. BEF were transfected with linearized plasmid-DNA using Lipofectamine according to the manufacturer's instructions (Invitrogen). Briefly, 2 x 10^5 cells were seeded in a 35-mm culture plate 1 day before transfection. Cells were transfected with 2 µg plasmid DNA using 10 µl of Lipofectamine. After transfection, the cells were selected with 800 µg/ml Geneticin (G418, HyClone) for 7-10 days. Single colonies were isolated by ring cloning and further expanded in the presence of 400 µg/ml G418. When

confluence was achieved, cells were passaged to 6-well plates and then to 100-mm plates. A portion of the cells were used for DNA isolation and confirmation of construct integration by PCR, andchromosome analysis. The remaining cells were frozen in DMEM supplemented with 10% FCS and 10% (v:v) DMSO.

Chromosome analysis

For cytogenetic analysis, each clone was grown in DMEM media containing 10% FCS for 48 hrs at 39ºC. When the cells reach confluency, they were incubated in growth media supplemented with 0.5 µg/ml KaryoMax (GIBCO) for 5-6 hrs at 39ºC. Then, cells were trypsinized and treated with hypotonic solution of 0.9% Sodium citrate for 25 min at 39ºC, fixed in methanol:acetic acid (3:1), and drops of cell suspension were spread on clean microscopic slides. The chromosomes were stained with 5% Giemsa for 10 min and examined at x 1.000 magnification under oil to determine the modal chromosome number.

Oocyte collection and maturation

Bovine ovaries were colleted from a local slaughterhouse and transported to the lab in physiological saline solution. Antral follicles of 2 to 8 mm in diameter were aspirated using an 18-gauge needle and collected into a 50 ml conical tube. Cumulus oocyte complexes (COCs), surrounded by more than four compact layers of cells and with evenly granulated cytoplasm were selected for maturation, that was

Table 1. Evaluation of transgenic cell lines expressing the red fluorescent protein (HcRed).

Cell Lines	Level of HcRed expression in cells	Karyotyping analysis	Morfology/Nº Passages	Cloned Blastocyst/ Fluorescence
clone 1	Medium	2n=60,XX	Normal/>10	Yes/Low
clone 2	Low	2n=60,XX	Normal/>10	Yes/Low
clone 3	N. Detec.	N.D.	N.D.	N.D.
clone 4	N. Detec.	N.D.	N.D.	N.D.
clone 5	N. Detec.	N.D.	N.D.	N.D.
clone 6	Low	2n=58,XX	Senescent/<5	No
clone 7	N. Detec.	N.D.	N.D.	N.D.
clone 8	Low	2n=60,XX	Normal/>10	Yes/N. Detec.
clone 9	High	2n=60,XX	Senescent/<5	No
clone 10	N. Detec.	N.D.	N.D.	N.D.
clone 11	N. Detec.	N.D.	N.D.	N.D.
clone 12	N. Detec.	N.D.	N.D.	N.D.
clone 13	N. Detec.	N.D.	N.D.	N.D.
clone 14	N. Detec.	N.D.	N.D.	N.D.
clone 15	N. Detec.	N.D.	N.D.	N.D.

Clones selected for later analysis; N. Detec.: Non Detected; N.D.: Non Determined.

carried out in Medium 199 supplemented with 10% FCS (HyClone), 1 µg/ml of FSH (Sioux Biochem), 1 µg/ml of LH (Sioux Biochem), 1 µg/ml 17b-estradiol, 2.3 mM of sodium pyruvate, and 25 µg/ml of gentamicin sulphate (Gibco) at 37ºC in a humidified atmosphere of 5% CO_2 in air.

Nuclear transfer

After maturation, oocytes were vortexed to remove expanded cumulus cells and stained using Hoechst 33342 (5 µg/ml) for 10 minutes to aid the visualization of the DNA (chromatin). Oocytes were enucleated with a 15 µm(internal diameter) glass pipette by aspirating the MII plate in a smallvolume of surrounding cytoplasm. Prior to nuclear transfer, donor cells were grown to confluency for 5 days. These cells (one per oocyte) were microsurgically placed into the perivitelline space evacuated during enucleation, ensuring intimate contact between the donor cell and the recipient oocyte. After transfer, the cell-cytoplast complexes were induced to fuse in sorbitol media with a single DC pulse of 170 volts/mm and 15 µsec by an Electrocell Manipulator 830 (BTX). Immediately after electric pulse delivery, the oocytes were activated with 5 µM Ionomicin for 5 min followed by incubation in KSOM media containing cycloheximide (10 µg/ml for 5 hrs). After activation, the NT units were cultured in 25 ml drops of KSOM media under mineral oil at 38.5ºC and at 5% CO_2, 5% O_2, 90% N_2. On day 3 (NT=day 0), the embryos were transferred to KSOM+5% FCS and cultured under the same conditions until day 7, when blastocyst rate and marker fluorescence were assayed.

RESULTS AND DISCUSSION

The present study demonstrated the feasibility of a primary cell line derived from bovine embryonic fibroblasts of being stably transfected with the marker gene pHcRed1-nuc and confirmed the suitability of these cells as nuclear donors for the generation of transgenic cloned embryos. Epifluorescence microscopy allowed to confirm expression of the red fluorescent protein in the nuclear cell compartment both in different clonal lines (Figure 1a; Figure 1b) and in the blastomeres of embryos generated with these cells used as nuclear donors (Figure 1d; Figure 1h). We generated 15 G418-resitant cell lines that were positive by PCR analysis to the marker gene (data not shown), 5 of which expressed the red fluorescent protein at different levels (Figure 1b and Table 1). The karyotyping analysis of clones expressing HcRed, showed a normal chromosome complement of 2n = 60; XX, in 4 out of 5 clones analyzed (Figure 1c and Table 1), while one of the clones (clone 6) had a decreased in one pair of somatic chromosomes, which may correlate with the premature senescence previously observed in this cell line (Table 1).

The evaluation of these cells (passage 5) as nuclear donors in SCNT, allowed the generation of transgenic cloned embryos with 3 out of 5 cell lines analyzed (Table 1). Two of the cell lines had previously shown a premature senescence in cellular growth and maintenance assessments, which may explain the inability of these cells to generate transgenic cloned blastocysts. However, with the other lines it was possible to generate transgenic cloned embryos that expressed HcRed in each blastomere, making possible their evaluation *in vivo* during the different stages of the embryonic development, including the blastocyst stage, the stage in which the embryos are suitable for transfer to synchronized recipients (Figure 1e; Figure 1h). Nevertheless, the low level of expression observed in these embryos, concomitant with the expression pattern observed in the cells prior to nuclear transfer, did not allow the accurate count of the total number of cells at the blastocyst stage, preventing in this way a quantitative evaluation of the quality of these embryos after different culture conditions. However, the generated model allowed the qualitative assessment of cloned embryos, providing a valuable tool for the selection of good quality embryos based not only in the general morphological evaluation, but also in the rough estimate of the total number of cells, information that could be useful in embryo transfer procedures, to avoid the transfer of embryos with low quality and/or low post implantation potential. Furthermore, this model may have a great potential to improve nuclear transfer efficiency, especially in bovines where the total number of cells and cell allocation at the blastocyst stage have been reported to be lower than those of *in vivo* derived embryos, attributing this as the main cause of the placentation problems observed in some nuclear transfer pregnancies (Koo et al. 2000; Chung et al. 2002; Koo et al. 2002), observations that are difficult to confirm because they invariably involved the destruction of the embryo and/or the staining with embryo toxic substances.

CONCLUDING REMARKS

The generated model demonstrated its potential as a noninvasive tool for the assessment of the end quality of cloned embryos. Although for an optimal application of this model, it is necessary to generate a cloning cell line that would ensure high level of expression of HcRed at each blastomeres of the developing embryo. In these conditions, the model could allow to identify structural abnormalities in the developing embryo as result of an inadequate nuclear reprogramming or as direct consequence of sub optimal culture conditions, making possible to assess the developmental potential of these embryos post-implantation. Further refinement of this model include the double expression of reporter genes to the trophoectoderm and inner cell mass cells, which would allow the assessment of cell allocation in the embryo as well as total number of cells, experiments that are under way.

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