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ORIGINAL PAPER

Novel methods to induce exogenous gene expression in SCNT, parthenogenic and IVF preimplantation bovine embryos

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Abstract The import of exogenous DNA (eDNA) from the cytoplasm to the nucleus represents a key intracellular obstacle for efficient gene delivery in mammalian cells. In this study, cumulus cells or oolemma vesicles previously incubated with eDNA, and naked eDNA were injected into the cytoplasm of MII oocytes to evaluate their efficiency for eDNA expressing bovine embryo production. Our study evaluated the potential of short time co-incubation (5 min) of eDNA with; (1) cumulus cells, to be used as donor cells for SCNT and (2) oolemma vesicles (vesicles) to produce parthenogenic transgene expressing embryos. In addition, we included a group consisting of the injection of eDNA alone (plasmid) followed by parthenogenic activation. Two different pCX-EGFP plasmid concentrations (50 and 500 ng/μl) were employed. The results showed that embryos produced by SCNT and by vesicle injection assisted by chemical activation were able to express the eDNA in higher rates than embryos injected with plasmid alone. The lower plasmid concentration allowed the highest development rates in all groups. Using confocal microscopy, we analyzed the interaction of FITC-labeled eDNA with cumulus cells and vesicles as well as oocytes injected with labeled plasmid alone. Our images demonstrated that eDNA interacted with cumulus cells and vesicles, resulting an increase in its expression efficiency. In contrast, oocytes injected with DNA alone did not show signs of transgene accumulation, and their eDNA expression rates were lower. In a further experiment, we evaluated if transgene-expressing embryos could be produced by means of vesicle injection followed by IVF. The lower plasmid concentration (50 ng/μl) injected after IVF, produced the best results. Preliminary FISH analysis indicated detectable integration events in 1/5 of SCNT blastocysts treated. Our studies demonstrate for the first time that short term transgene co-incubation with somatic cells can produce transgene-expressing mammalian SCNT embryos and also that parthenogenic, eDNA- expressing embryos can be obtained by injection of vesicles or eDNA alone. Moreover, eDNA-expressing embryos can be also obtained by cytoplasmic injection vesicles in IVF zygotes, simplifying the traditional IVF pronuclear injection technique.

Keywords Cloned cattle parthenogenesis · GFP transgenesis

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Introduction

For successful mammalian transgenesis, the ability of exogenous DNA (eDNA) to enter into the nucleus depends on how exogenous DNA is delivered into the cells. Transgenesis by SCNT has proven to be efficient in a variety of farm animals (Cibelli et al. 1998; Salamone et al. 2006), and it has led to eDNA-expressing embryos rates of 20% (Cibelli et al. 1998). Transgenesis by SCNT requires the development of a cellular clone that contains the transgene. These donor cells are obtained by a process that involves the transformation of fetal or adult cells by electroporation, liposomes or viral agents (Lois et al. 2002; Lee et al. 2005), and their subsequent selection using drug resistance markers during successive passages. This process is time-consuming and some genetic errors can be introduced by in vitro aging of the cellular clone. The highest transgenesis efficiency in mammals was obtained using lentiviral agents, making subsequent selection or use of drug resistance markers unnecessary (Lois et al. 2002). However, limitations in the size of the introduced eDNA and the biosafety risks involved in lentiviral manipulation, render it prohibitive for most laboratories. Another method commonly used to generate eDNA-expressing embryos is microinjection of naked eDNA into the male pronucleus at the unicellular stage of embryonic development (Gordon et al. 1980). This procedure, which does not require selective culture is moderately efficient in a variety of species (Hammer et al. 1985; Eyestone 1999). However, it requires proper male pronucleus visualization which occurs only in a narrow window of time in mice. For these reasons, cheaper and easier alternatives for exogenous gene delivery into mammalian embryos are still desirable.

Lavitrano et al. (1989) reported an alternative method to produce transgenic mice called Sperm Mediated Gene Transfer (SMGT). This method uses sperm cells as vectors and consists of the production of eDNA-expressing embryos by the coincubation of live spermatozoa with plasmid constructs prior to IVF. The possibility of generating eDNA-expressing embryos by IVF is remarkably simple; however the SMGT strategy was quickly challenged (Brinster et al. 1989) and numerous studies have shown that only non-motile spermatozoa bind exogenous eDNA (in ovine: Pereyra-Bonnet et al. 2010; in bovine: Anzar and Buhr 2006; in mouse: Moreira et al. 2007), explaining the inefficiency of transgenesis by AI or IVF (Gandolfi et al. 1996; Chan et al. 2000; Yamauchi et al. 2007). The Intracytoplasmic Sperm Injection Transgenesis (ICSI-tg) method was subsequently developed. It consists of the injection of damaged spermatozoa, previously incubated with eDNA, into MII oocytes (Perry et al. 1999). The ICSI-tg method proved to be highly efficient in mice, with transgenesis rates that reach around 90% for embryos (Perry et al. 1999). Recently, our group has performed ICSI-tg assays in several domestic species and we confirmed that the interaction between eDNA and spermatozoa occurs after a brief period of time (5 min) (Pereyra-Bonnet et al. 2008; Bevacqua et al. 2010). Nevertheless, in our knowledge, no report has explored whether spontaneous and quick interaction between eDNA and sperm cells is specific to these cells or if it also applies to other cell types.

In the present study, we explored the potential of short incubation between eDNA and donor cells to act as vectors to generate transgene-expressing SCNT embryos. Cumulus cells that were exposed to pCX-EGFP plasmid for a short period of time (5 min) were injected into enucleated bovine oocytes. Additionally, fragments of ooplasm surrounded by oolemma (vesicles), without nucleous, were briefly exposed to pCX-EGFP and then injected into MII oocytes. In a control group, naked eDNA alone was injected into the cytoplasm of MII oocytes. In all groups, development was induced by chemical activation. Embryos produced by the three different treatments were observed under blue light in order to determine *egfp* expression. The interaction of cumulus cells and vesicles with labeled-eDNA and their behavior after injection into oocytes, was further examined. In addition, embryos expressing *egfp* from SCNT were subjected to FISH in order to identify their transgene status. Finally, we transferred this novel eDNA delivery technique to IVF in order to develop a robust and simple method to generate eDNA-expressing bovine preimplantation IVF embryos.

Materials and methods

Chemicals

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

DNA construction

The plasmid used was pCX-EGFP kindly provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan) that contains an enhanced green fluorescent protein gene (*egfp*) under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control (Ikawa et al. 1995). The same stock of circular plasmid was used for all these experiments.

Oocyte collection and in vitro maturation

Bovine ovaries were collected from slaughterhouses and transported to the laboratory at 25–30°C. Cumulus-oocyte-complexes (COCs) were aspirated with 21-gauge needles from follicles with a diameter of 2–5 mm into Dulbecco's phosphate buffer saline (DPBS, 14287-072; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, 013/07; Internegocios, Buenos Aires, Argentina), and 1% antibiotic–antimycotic (ATB, 15240-096; Gibco, Grand Island, NY, USA). Oocytes covered with at least 3 layers of granulosa cells were selected for in vitro maturation (IVM). The maturation medium was bicarbonate-buffered TCM-199 (31100-035; Gibco, Grand Island, NY, USA), containing 2 mM glutamine (G-8540), 10% FBS, 10 µg/ml follicle stimulating hormone (NIH-FSH-P1, Folltropin®, Bioniche, Australia), 0.3 mM sodium pyruvate (P2256), 20 µm cysteamine (M9768) and 1% ATB. The oocytes were incubated for 24 h under mineral oil (M8410) in 500 µl of medium in 4-well dishes (Nunclon® Nunc Naperville, IL, USA) (M8410), in 6.5% CO_2 in humidified air at 39°C.

Cumulus cells/exogenous DNA incubation

After IVM, cumulus cells were removed (described below) and those oocytes with 2 or 3 cumulus cells layers still attached were selected for incubation with 50 or 500 ng/µl pCX-EGFP plasmid in 10 µl of 2.8% sodium citrate (F71497) with 100 mM EDTA (15576-028, Invitrogen) for 5 min on ice. Afterwards, less than 1 µl medium containing the coincubated oocytes, was transferred to a 3 µl droplet of Hepes-buffered Tyrode's albumin lactate pyruvates (TALP-Hepes) with 10% of PVP. Cumulus cells were individually picked up from oocytes by means of a 9 µm pipette and directly injected into the cytoplasm of enucleated oocytes, as subsequently detailed in the SCNT procedure.

Vesicle production and incubation with exogenous DNA

Matured oocytes to be used as vesicle donors were denuded by hyaluronidase treatment, then transferred to 20 µl droplets of TALP-Hepes. Each oocyte was held under negative pressure with a holding pipette while a 9 µm pipette was passed through its zona pellucida until contact was made with the ooplasm. A small fraction of the ooplasm (<10 µm) was then aspirated by negative pressure, avoiding plasma membrane breakage. Vesicles that formed inside the pipette were transferred into a 3 µl of TALP-Hepes with 10% of PVP droplet containing 50 or 500 ng/ml pCX-EGFP and held there for 5 min. Finally, vesicles were aspirated into the 9 µm pipette and directly injected into the MII oocytes followed parthenogenic activation or IVF as described below.

Injection of exogenous DNA alone

Oocytes subjected to IVM and hyaluronidase treatment were transferred to 20 µl droplets of TALP-Hepes and injected using a 9 µm pipette, containing 10% PVP with 50 or 500 ng/µl pCX-EGFP, using a volume that was equivalent to that used for to the injection of cumulus cells or vesicles (<10 pl). Oocytes were chemically activated as described below.

Oocyte denudation and SCNT

After maturation, cumulus cells were removed from oocytes by vortexing for 2 min in hyaluronidase (H-4272) (1 mg/ml DPBS). Oocytes were washed three times in Hepes-buffered (H4034) TCM-199 and mature oocytes, selected by visualizing the first polar body, were immediately used for SCNT following staining (10 min) with bisbenzimidine (Hoechst 33342; 5 mg/ml). The stained chromosomes of metaphase II oocytes were visualized under ultraviolet light (<5 s) and the oocytes were mechanically enucleated using Narishige hydraulic micromanipulators and a Nikon Eclipse E 300 microscope. Metaphase chromosomes were assessed after aspiration inside the pipette. Cumulus cells previously

incubated with pCX-EGFP were injected into the enucleated oocyte followed by chemical activation described below.

Chemical oocyte activation

Embryos produced by SCNT, vesicles or eDNA alone were activated with 5 μl ionomycin (I24222; Invitrogen, California, USA) in TALP-Hepes for 4 min and subsequently transferred to a drop of TCM containing 2 mM DMAP (D2629) and held for 3 h. Embryos were then washed three times in TALP-Hepes to remove the inhibitor, and cultures were continued as described below. Prior to activation, SCNT-reconstructed embryos were held for 2 h in droplets of TCM 199 to permit the reprogramming events.

IVF procedure

Frozen semen was thawed in a 37°C water bath for 30 s. Spermatozoa were then centrifuged twice (490 g × 5 min) in Brackett-Oliphant medium (BO; Brackett and Oliphant 1975) and resuspended in BO supplemented with 5 mM caffeine (C4144) and 20 IU/ml heparin (H3149). Spermatozoa were adjusted to 40×10^6/ml and diluted to half concentration (20×10^6/ml) with BO containing 10 mg/ml fatty acid-free bovine serum albumin (A6003). COCs were washed twice with BO medium plus 5 mg/ml FAF-BSA and subsequently exposed to the sperm suspension for 5 h in a 100 μl drop at 39°C under 6.5% CO_2 in humidified air. Presumptive zygotes were then washed three times in TALP-Hepes. Vesicles were injected prior to or after IVF, and cultures were continued as described below.

In vitro embryo culture

Presumptive zygotes (15–30 per group) were cultured in 50 μl droplets of SOF medium (Tervit et al. 1972) supplemented with 2.5% FBS at 39°C in 6.5% CO_2 in humidified air. The embryos were transferred to a new droplet every 48 h. Cleavage was evaluated on day 2, and the number of blastocysts on day 7.

Confocal laser scanning microscopy

Cumulus cells and vesicles were incubated as described previously with FITC- labeled bovine DNA fragments (100–300 Kb) and analyzed by confocal microscopy (Nikon C1, Melville, NY, USA) in order to determine exogenous DNA binding sites. FITC- labeled bovine DNA fragments were also injected into oocytes to generate a group that was injected with eDNA alone. Cumulus cells and injected oocytes were fixed with paraformaldehyde and ethanol (1:3) for 10 min. The samples were mounted on slides following nuclear counterstaining for 10 min in PBS containing 10% glycerol and 0.1% propidium iodide. An excitation wavelength of 488 nm was selected from an argon-ion laser to excite the FITC- labeled DNA, and 544 nm to excite propidium iodide. Images of serial optical sections were recorded every 1.5–2 μm in a vertical step along the Z-axis of each embryo or 0.3 μm for cumulus cell being analyzed. Three-dimensional images were constructed using EZ-C1 2.20 software. At least 50 cumulus cells and 10 embryos were studied from each group.

Evaluation of EGFP fluorescence in the embryos

All the embryos produced were briefly exposed to blue light using an excitation-filter at 488 nm and an emission-filter at 530 nm to determine *egfp* gene expression on days 4 and 7 post-chemical activation or fertilization.

Fluorescence in situ hybridization (FISH)

Embryos from SCNT that expressed *egfp* were subjected to FISH using the pCX-EGFP plasmid as a probe. Embryos were incubated for 20 h with 0.1 μg/ml demelcochine (D1925). Afterward, embryos were placed on poly-L-lysine-coated slides, previously treated with a hypotonic solution (1% Na citrate in distilled water for 10 min) and fixed in situ with 3:1 methanol-acetic acid. The pCX-EGFP was labeled with Rhodamine-5-dUTP (Cat# RU-013-0135; eENZYME®) by Nick Translation System (18156-010; Invitrogen). The DNA probe was denaturated for 10 min at 85°C in a hybridization mix (H.Mix) containing 50% formamide (Merck), 40% Sulfate Dextrane (D8906), 20 × Sodium chloride-sodium citrate (SSC) 10 and 0.03% Hearing Sperm DNA. The proportion used was 5:1 (H. Mix : labeled DNA). Before application of the denatured probe, slides with fixed embryos were denatured with 70%

formamide in 2 × SSC for 2 min at 72°C and dehydrated in successive passages through 70, 95, 100% ethanol. The probe was incubated overnight in a moist dark chamber at 37°C. After incubation the coverslides were detached with a short incubation in 2 × SSC, the slides were washed at 72°C for 2 min in 0.4 × SSC with 0.3% Tween and then washed at room temperature in 2 × SSC with 0.1% Tween. The total DNA was counterstained with DAPI and the slides were mounted.

Statistical analysis

In vitro embryo development and fluorescent expression were compared by Fisher's exact test analysis. For statistical analyses, the SAS program was used (SAS Institute Inc. SAS/STAT 1989). Differences were considered to be significant at $P < 0.05$.

Results

Confocal analysis of cumulus cells, vesicles and naked eDNA

Intracellular interactions with labeled-eDNA were found in all of the cumulus cells analyzed (n = 50). Successive planes separated by 0.3 μm showed that specific eDNA signals had accumulated in the nucleus (Fig. 1a, b, c). Cumulus cells that were not previously incubated with eDNA (negative controls), were not labeled. Following injection, cumulus cells maintained eDNA labeling in most (80%) of the oocytes analyzed (8/10). Similarly, in the vesicle-injected group, 6/10 (60%) of the oocytes analyzed contained specific signals for eDNA (Fig. 1d, e, f). Vesicles were not found in the remaining oocytes (4/10). No signals were detected when naked labeled-eDNA alone was injected (n = 20).

Development and egfp expression rates in SCNT and parthenogenic embryos

Enucleated oocytes (n = 155) were submitted to SCNT, using, cumulus cells as nuclear donors, previously incubated with two plasmid concentrations (500 or 50 ng/ml). Better rates of blastocyst production and *egfp*-expressing embryos were obtained with the lower eDNA concentration (Table 1). The higher eDNA concentration negatively affected rates of blastocyst production and the percentage of embryos that expressed *egfp*, although cleavage rates were similar. Figure 1 (g, h, i), shows hatched SCNT-expressing blastocysts obtained with 50 ng/μl plasmid concentration.

Moreover, oolemma vesicles were injected into MII oocytes, after incubation with both plasmid concentrations. Developmental rates, following parthenogenetic activation, were similar between the two plasmid concentrations, but a notably high percentage of *egfp*-expressing embryos (64.2%) were obtained with the higher DNA concentration (Table 1). When eDNA alone was injected into MII oocytes using both concentrations of pCX-EGFP (Table 1), *egfp*-expressing embryos were observed, but their numbers were always significantly lower than those observed for embryos derived from SCNT or vesicle injection ($P < 0.05$). After injection of eDNA alone, the highest green embryo rates were obtained with the lower concentrations.

As a control a set of parthenogenic embryos was included. Significant differences in developmental rates were observed between controls and embryos derived from SCNT, vesicle or naked eDNA injection followed by parthenogenic activation ($P < 0.05$).

Development and egfp expression rates in IVF derived embryos

We investigated if *egfp*-expressing embryos could be obtained by IVF procedures, using the vesicle method. For this experiment vesicles were incubated with both pCX-EGFP concentrations and injected into oocytes or presumptive zygotes, either prior to IVF or immediately after IVF, respectively. The higher pCX-EGFP concentration resulted in a lower cleavage rates and fewer embryos on day 7 (Table 2). The best results were obtained when vesicles were injected immediately post-IVF (26.2%). Likewise, the percentage of expressing embryos appeared to be dependent on the time of injection.

FISH analysis of SCNT embryos

Green-expressing SCNT embryos were analyzed by FISH. One out of five blastocysts was positive using pCX-EGFP as probe. The signals were observed in pairs which could be associated with each sister

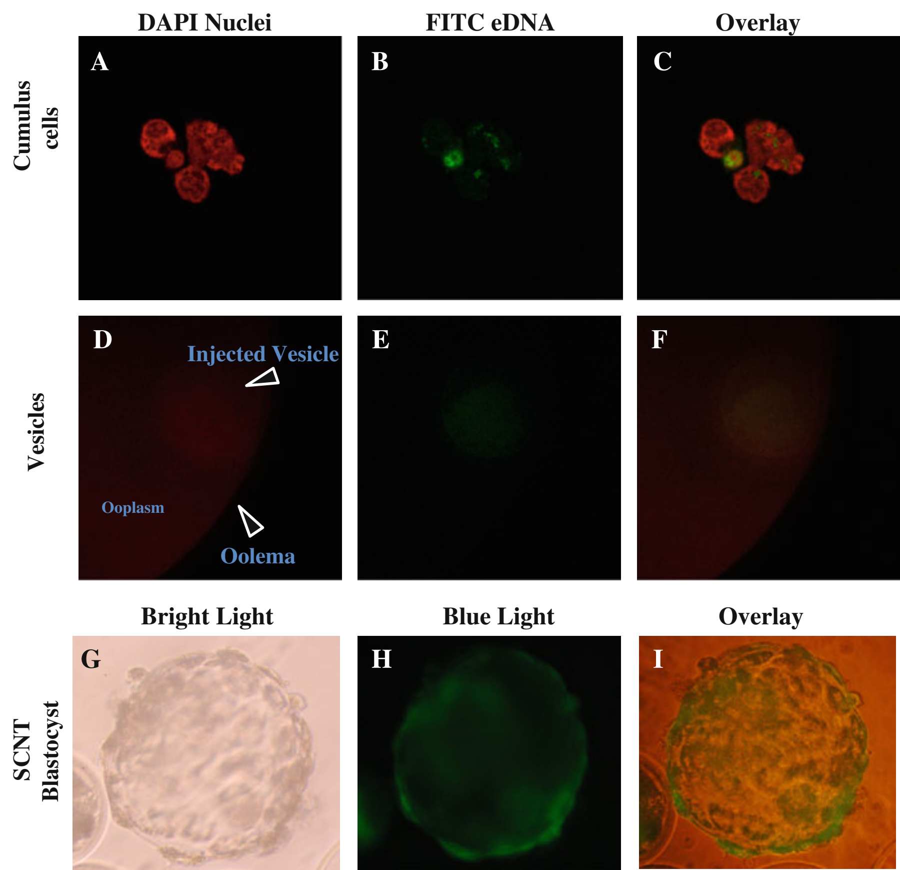

Fig. 1 Confocal microscope image of cumulus cells (**a**, **b**, **c**) and of oocytes injected with Vesicles (**d**, **e**, **f**) after a short exposure to labeled eDNA. *First panels*: DAPI staining; *Second panels*: Specific marks of the labeled eDNA; *Third panels*: Overlay of *first* and *second panels*. Note that in cumulus cells, exogenous DNA signals localize with the internal nuclear zone (0.6 μm from the top; Original magnification 400×) (**c**). In injected Vesicles the interaction with labeled eDNA is both superficial and internal (Original magnification 400×) (**f**). Expressing bovine blastocyst produced by SCNT and cumulus cell injection after a short exposure to pCX-EGFP plasmid, visualized under bright light (**g**), under blue light (**h**) and under overlay of both (**i**) (Original magnification 200×)

Table 1 In vitro development and *egfp* expression of bovine embryos derived from SCNT, and the injection of vesicles or DNA alone using two different pCX-EGFP plasmid concentrations

Technique	pCX-EGFP ng/μl	No. of oocytes injected (replicates)	Cleaved (%)	Blastocysts (%)	Green embryos at 4d (%)	Green blastocysts (%)
SCNT	50	69 (4)	57 (82.6)^{a,b}	24 (34.7)^{b,c}	34 (49.2)^{a}	23 (33.3)^{a}
	500	86 (3)	60 (69.7)^{b,c}	2 (2.3)^{d}	19 (22.0)^{b}	2 (2.3)^{c}
Vesicles	50	68 (4)	46 (67.6)^{b,c}	30 (44.1)^{b}	16 (23.5)^{b}	8 (11.7)^{b}
	500	95 (4)	72 (75.7)^{a,b,c}	25 (26.3)^{c}	61 (64.2)^{c}	23 (24.2)^{a,b}
DNA alone	50	56 (3)	33 (58.9)^{c}	16 (28.5)^{c}	14 (25.0)^{b}	4 (7.1)^{b,c}
	500	74 (3)	58 (78.3)^{a}	18 (24.3)^{c}	7 (9.4)^{d}	0 (0)^{c}
PA Control	–	100 (5)	93 (93.0)^{a}	68 (68.0)^{a}	NA	NA

Values with different superscripts in a column are significantly different (Fisher test; $P < 0.05$)

PA Parthenogenic activation

chromatid in interphase cells (Fig. 2a, b). At least in one case, one locus contained enough eDNA to be detected. The eDNA length (5.5 kb) poses a technical problem for FISH detection, and probably tandem integrations are needed for detection. Nevertheless, our positive result suggests that at least the transgene was integrated and detected in 1/5 of the SCNT embryos produced. Confirmation of this suggestion will require further analysis of cells in metaphase. Fibroblasts used as negative controls, did not show positive signals.

Table 2 In vitro development and *egfp* expression of bovine embryos after injecting vesicles coincubated with two different plasmid concentrations prior to or after IVF

Time to vesicle injection	pCX-EGFP ng/μl	No. of Oocytes injected (replicates)	Cleaved (%)	Blastocysts (%)	Green embryos at 4d (%)	Green blastocysts (%)
prior IVF	50	85 (3)	48 (56.4)[a]	20 (23.5)[a]	18 (21.1)[a]	6 (7.0)[a]
	500	83 (3)	47 (56.6)[a]	10 (12.0)[b]	12 (14.4)[a]	4 (4.8)[a]
after IVF	50	103 (3)	83 (80.5)[b]	38 (36.8)[a]	37 (35.9)[b]	27 (26.2)[b]
	500	83 (3)	54 (65.0)[a]	5 (6.0)[b]	38 (45.7)[b]	3 (3.6)[a]

Values with different superscripts in a column are significantly different ($P < 0.05$)

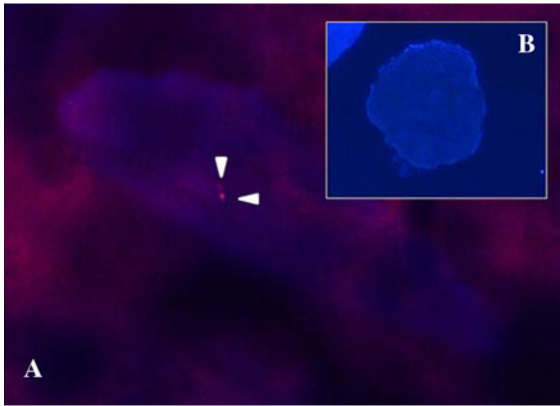

Fig. 2 FISH analysis of pCX-EGFP as a probe in one cell (*a*, 1000×) of SCNT blastocyst produced by cumulus cell injection after a short exposure to labeled pCX-EGFP plasmid (*b*, 100×). *Arrows* indicate two specific detected signals

Discussion

The import of exogenous DNA (eDNA) from the cytoplasm to the nucleus represents a key intracellular obstacle for efficient gene delivery in mammalian cells. In this study, cumulus cells, oolemma vesicles previously incubated with eDNA, and naked DNA, were injected into the ooplasm of MII oocyte (enuclated for SCNT) to evaluate their efficiency for the production of exogenous gene-expressing bovine embryos. Our results showed that the three techniques tested efficiently produced embryos positive for transgene expression demonstrating that eDNA introduced into the oocyte by means of cumulus cells, vesicles or naked DNA, could be imported to the nucleus in bovine embryos.

Our innovative SCNT protocol resulted in a twofold increase in transgene expression with respect to traditional donor cell transfection methods (Cibelli et al. 1998; Arat et al. 2002). In addition, our method simplified the production of eDNA vector cells from weeks to 5 min, and it doesn't require selection and drug resistance genes. Both plasmid concentrations tested resulted in high development and expression rates, however the lower concentration significantly increased both development and expression percentages (Table 1). These results which suggest that higher plasmid concentrations reduce embryo development, agree with reports using pronuclear microinjection (Brinster et al. 1985). During pronuclear microinjection, high amounts of plasmid molecules that are injected directly into the nucleus are deleterious. In our work confocal microscopy confirmed the presence of eDNA in cumulus cell nuclei, both before and after their injection into oocytes (Fig. 1). Although lower plasmid concentrations were better, when treatments were compared against the Control, it appeared that both plasmid concentrations (50 and 500 ng/μl) resulted in lower development rates. Additional tests would be necessary to demonstrate if the negative relationship between amount of eDNA and embryonic development, is due to a toxic effect of H_2O_2 production during GFP maturation process (Tsien 1998) or to apoptotic mechanisms triggered by the increased presence of eDNA (Moreira et al. 2007). The dissimilar *egfp* expression results observed for SCNT and naked DNA groups demonstrate that cumulus cells take an active part in the eDNA delivery process therefore rendering it easier and faster to produce eDNA-expressing SCNT bovine embryos. In addition, preliminary SCNT tests using fibroblasts and blastomere like pCX-EGFP vectors resulted in equally successful eDNA expressing bovine embryo production (data not shown), suggesting that a versatility exists in methodologies. These findings could have great implications. For example, donor nuclei cells could be used as vectors to carry induced pluripotent stem cell (iPS) genes

which could improve the early reprogramming events of SCNT embryos.

We produced parthenogenic embryos expressing *egfp* by injection of vesicles, incubated with eDNA, into MII oocytes prior to chemical activation. The lower plasmid concentration allowed the best development rates; however, the highest transgenesis rates were obtained with the higher plasmid concentration (Table 1). This result is the opposite to our SCNT observations. We demonstrated that eDNA delivery system is involved in our SCNT is more efficient that vesicle method. Confocal microscopy images of oocytes injected with vesicles revealed that vesicles encapsulated labeled-eDNA (Fig. 1). We hypothesize that vesicles retain the eDNA until it enters the nucleus which possibly occurs during pronuclear formation or successive mitoses, as proposed for ICSI-tg (Perry et al. 1999). For this reason, the efficiency of transgene import is reduced and high plasmid concentrations are not detrimental. The production of parthenogenic eDNA expressing embryos by injection of vesicles could be an ideal and inexpensive tool for the study of early maternal transcription events of development, among others.

The percentages of green embryos obtained after naked DNA injection demonstrates eDNA reach the nucleus in bovine embryos. In somatic cells, it has been demonstrated that the import of naked eDNA to the nucleus occurs via nuclear pores (Dowty et al. 1995) or Transcription Factor Complexes (Vacik et al. 1999). However, the possibility of generating transgene-expressing embryos by cytosolic DNA injection has been poorly explored in mammals. Recently, this approach has been used as a control group for the ICSI-tg technique, and reports have shown that the cytoplasmic eDNA injections are unable to produce exogenous gene expressing embryos (Perry et al. 1999; Yamauchi et al. 2007; Pereyra-Bonnet et al. 2008). Expressing embryos were produced only when sperm cells and eDNA were coincubated before injection, resulting in 94% expression rates in mice (Perry et al. 1999) and 23% rates in the bovine (Pereyra-Bonnet et al. 2008). In this respect, it has been reported that the distance between eDNA injection site and the nucleus is an important technical aspect. Dowty et al. (1995) demonstrated that β-galactosidase-positive percentages in post-mitotic myotubes increased significantly when microinjection was performed close to the nucleus (56%) compared to far from it (8%). In light of these findings, perhaps as a result of the larger oocyte volume, the efficiency of mechanisms described for somatic cells is diminished for embryos and it is only achieved when high eDNA concentrations are used or when vectors like cumulus cells or vesicles are used. For example, the concentration used by others for ICSI-tg controls (Perry et al. 1999; Yamauchi et al. 2007) is reduced several fold (50–100 times) with respect to the concentration employed in our study (5–10 vs. 50–500 ng/μl) and others (Iqbal et al. 2009). On the other hand, the construct used in this work contains the SV40 enhancer region known to bind a high number of general transcription factors (AP1, AP2, AP3, AP4, NF-kB, Oct-1, SP1 among others) that can enter the nucleus using the nuclear localization signal (NLS) machinery (Vacik et al. 1999). In the other hand, it has also been recently reported that histones (H2A, H2Av, H2B) accumulate at the surface of lipid droplets until their incorporation in dividing nuclei during early embryo development in *Drosophila* (Cermelli et al. 2006). Therefore, the NLS machinery and the cytoplasmic histone traffic (with great affinity for the DNA), could also be acting as naked DNA importers to the nucleus after cytoplasmic injection in bovine embryos. Confocal microscope images did not show signals of labeled eDNA. Therefore, the lack of labeled eDNA signals could be due to the diffusion phenomenon as described for naked DNA (Dowty et al. 1995). In contrast, cumulus cells and vesicles remained associated with eDNA after injection, enhancing eDNA expression and avoiding the diffusion phenomenon related to naked eDNA injection, as shown by confocal microscopy.

Another development of this work consisted in the production of transgene-expressing embryos by IVF using vesicles. High embryo development and *egfp*-expression rates were obtained with this innovative technique. The best protocol consisted of vesicle incubation with 50 ng/μl pCX-EGFP injected into IVF-derived zygotes. Lower cleavage rates were seen when vesicles were injected with the same plasmid concentrations, but prior to IVF (Table 2). This could be the result of cortical granule exocytosis as a consequence of mechanical effects of the injection process. The exocytosis of cortical granules can result in a lack of fertilization events. The vesicle injection technique allows high rates of eDNA expressing IVF preimplantation embryo production in the bovine. Our observations indicate that vesicles could play an

active role in the transgenesis mechanism even during the normal fertilization process.

In this work, we found a specific transgene signal in the interphase nucleus of one from five SCNT blastocysts analyzed by FISH. FISH is the only technique capable of identifying transgene status at blastocyst stage. Considering the size of our eDNA (5.5 kb), a tandem integration is required to fall into the lowest detection limit of FISH (around 100 kb). For these reasons, a higher rate of eDNA integration should be expected in our SCNT experiments.

In summary, we have shown the eDNA-spermatozoon binding is not specific for this kind of cell, and a short incubation between eDNA and donor cells can generate transgene-expressing SCNT embryos, avoiding drug resistance markers or stable cloned transgene cells culture. Additionally, eDNA incubated vesicles or naked eDNA could also generate transgene-expressing parthenogenic embryos. These new techniques are powerful tools for embryo reprogramming and gene expression research.

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