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Vesicles Cytoplasmic Injection: An Efficient Technique to Produce Porcine Transgene-Expressing Embryos

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Contents

The use of vesicles co-incubated with plasmids showed to improve the efficiency of cytoplasmic injection of transgenes in cattle. Here, this technique was tested as a simplified alternative for transgenes delivery in porcine zygotes. To this aim, cytoplasmic injection of the plasmid alone was compared to the injection with plasmids co-incubated with vesicles both in diploid parthenogenic and IVF zygotes. The plasmid pcx-egfp was injected circular (CP) at 3, 30 and 300 ng/μl and linear (LP) at 30 ng/μl. The experimental groups using parthenogenetic zygotes were as follows: CP naked at 3 ng/μl (N = 105), 30 ng/μl (N = 95) and 300 ng/μl (N = 65); Sham (N = 105); control not injected (N = 223); LP naked at 30 ng/μl (N = 78); LP vesicles (N = 115) and Sham vesicles (N = 59). For IVF zygotes: LP naked (N = 44) LP vesicles (N = 94), Sham (N = 59) and control (N = 79). Cleavage, blastocyst and GFP+ rates were analysed by Fisher's test ($p < 0.05$). The parthenogenic CP naked group showed lower cleavage respect to control ($p < 0.05$). The highest concentration of plasmids to allow development to blastocyst stage was 30 ng/μl. There were no differences in DNA fragmentation between groups. The parthenogenic LP naked group resulted in high GFP rates (46%) and also allowed the production of GFP blastocysts (33%). The cytoplasmic injection with LP vesicles into parthenogenic zygotes allowed 100% GFP blastocysts. Injected IVF showed higher cleavage rates than control ($p < 0.05$). In IVF zygotes, only the use of vesicles produced GFP blastocysts. The use of vesicles co-incubated with plasmids improves the transgene expression efficiency for cytoplasmic injection in porcine zygotes and constitutes a simple technique for easy delivery of plasmids.

Introduction

The pig represents an important large animal model for biomedical and pharmaceutical research (Kues and Niemann 2011). Genetically modified pigs are mostly designed for biomedical studies. They constitute an alternative model to rodents for specific human diseases given that physiology, anatomy, pathology, genome organization, body weight and life span of pigs are more similar to those of humans (Whyte and Prather 2011; Nowak-Imialek and Niemann 2012). The development of advanced methods for genetic engineering provided the basis for the increasing numbers of biomedical pig models published in recent years (Gün and Kues 2014). Since the advent of active transgenesis, transgene delivery methods were simplified. One of the first introduced active transgenesis techniques was based on lentiviral transgene vectors. However, lentiviruses show low cargo capacity, and biosafety restrictions may apply (Hofmann et al. 2003). In recent years, alternative techniques were introduced. Among them, transposon-mediated transgenesis showed great efficiency for random integration of transgenes (Carlson et al. 2011; Jakobsen et al. 2011; Garrels et al. 2012; Marh et al. 2012). The ability of DNA transposons to move from one genomic location to another by a cut-and-paste mechanism was soon adapted as a molecular biology tool, being isolated hyperactive transposase versions and segregated the two activities of several transposons (Suganuma et al. 2005). Transposons lack the ability of viruses to traverse the plasma membrane of the cell and must be delivered directly into the zygote's cytoplasm or nucleus. Transgenic piglets and foetuses were obtained with Sleeping Beauty 100× and piggyBac transposition with high efficiency (Garrels et al. 2011; Li et al. 2014a,b (a)). More recently, CRISPR/Cas9 technique was introduced. CRISPR/Cas is a simple and effective tool for genome edition based on the defence mechanism against viruses used by bacteria and archaea (Sorek et al. 2008; Hsu et al. 2014; Westra et al. 2014). The main advantage of CRISPRs is that a single guide RNA, easily designed by base complementarity, can direct a nuclease, CRISPR-associated nuclease (Cas), to the target sequence in the genome. Genome-edited pigs and goats were efficiently produced by SCNT, using CRISPR/Cas9-edited cells as donors (Li et al. 2014a,b (b); Ni et al. 2014; Whitworth et al. 2014; Zhou et al. 2015). More recently, cytoplasmic injection of one-cell embryos resulted in genome-edited mice, rat, sheep, monkeys, pigs, goats and rabbits (Mashiko et al. 2014; Shao et al. 2014; Chen et al. 2015; Crispo et al. 2015; Honda et al. 2015; Wang et al. 2015). However, most of these animals showed mosaic integration of transgenes (Sato et al. 2015). The development of a simple and effective method that allows the introduction of exogenous DNA (eDNA) into the zygote would be a good complement to the recently introduced active transgenesis techniques. The simplest and traditional method to introduce exogenous DNA into one-cell embryos is pronuclear microinjection. Yet, pronuclear microinjection of eDNA into the male pronucleus of one-cell embryos (PNI,

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Gordon et al. 1980) shows low success in domestic species with a high ratio of lysed zygotes, due to the particular vulnerability of the pronuclei (Hirabayashi et al. 2001; Wall 2002; Niemann and Kues 2007). In addition, it requires high technical skills and proper visualization of the male pronuclei, which is difficult in pigs. To visualize pronuclei, the zygotes could be centrifuged, which may contribute to a reduced developmental capacity (Hammer et al. 1985). Also, to avoid zygotes lysis as a result of pronuclei injection, only very thin capillaries can be employed. For these new technologies, which might require the injection of high transgenes concentrations, this is a great disadvantage, as transgenes and mRNAs at high concentrations stack in the capillary and are not efficiently injected. Therefore, the ability to efficiently perform injections without puncturing the pronuclei will benefit active transgenesis and will be advantageous for the production of transgenic animals. It has been shown that cytoplasmic injection of covalently closed circular (ccc) plasmids in murine and bovine zygotes is an efficient and simple alternative for ectopic expression of foreign DNA in embryos (Iqbal et al. 2009). This technique has been also demonstrated to be useful for delivering exogenous DNA to the nucleus in bovine and ovine embryos (Pereyra-Bonnet et al. 2011; Bevacqua et al. 2012; Li et al. 2014a,b (b)). A new, economic and simple technique, the use of vesicles co-incubated with plasmids, has demonstrated to increase transgene expression using cytoplasmic injection when compared with naked plasmids in bovine and ovine embryos (Pereyra-Bonnet et al. 2011; Bevacqua et al. 2012). This method involves the production of small ooplasm surrounded by oolemma fragments (vesicles) and their short co-incubation with eDNA prior to injection into the cytoplasm of presumptive zygotes. Confocal microscopy images of oocytes injected with vesicles revealed that vesicles encapsulated labelled eDNA (Pereyra-Bonnet et al. 2011). It was hypothesized by these authors 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, high plasmid concentrations are not detrimental. A big advantage of this technique is that there is no need to introduce a foreign delivery agent; on the other hand, oolemma–ooplasm derived from other presumptive zygotes acts as the delivery agent. The use of vesicles in the cytoplasmic injection could be complementary to active methods of transgenesis. Until today, the use of vesicles has not been tested in porcine zygotes. The objective of this work was to test whether the use of vesicles in the cytoplasmic injection of the plasmid pcx-egfp in porcine embryos improves the expression rate of the green fluorescent protein (GFP).

Materials and Methods

Unless specified, all chemicals were purchased from Sigma Chemicals Company (St. Louis, MO, USA). In all culture or maturation procedures, cell culture dishes (35 × 10 mm, Nunc, Roskilde, Denmark) were used.

DNA construction

The plasmid used was pcx-egfp, kindly provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan). This plasmid contains the enhanced green fluorescent protein gene (egfp) under the chimeric cytomegalovirus–IEchicken β-actin enhancer–promoter control (Ikawa et al. 1995). The plasmid was used circular covalently closed in the first experiment and linearized with HindIII, in the subsequent experiments.

Oocyte collection and *in vitro* maturation

The oocytes used in this study were obtained from slaughtered gilt ovaries collected and transported to the laboratory at 25–35°C. The *cumulus*–oocyte complexes (COCs) were aspirated from 3- to 6-mm follicles with an 18-gauge needle attached to a 10 cm^3 syringe. Only COCs with multiple layers of intact cumulus cells and uniform cytoplasm were selected for *in vitro* maturation (IVM) and washed in Talp–HEPES containing 0.1% (w/v) polyvinyl alcohol (PVA). COCs were placed in groups of 20–30 into maturation medium 100 μl drops (M 199; Gibco BRL Grand Island, NY, USA) supplemented with 0.3 mM sodium pyruvate, 20 mM cysteamine and 1% antibiotic–antimycotic (Gibco, Grand Island, NY, USA). COCs were matured for 42–46 h at 39°C, in 5% CO_2 and 100% humidity.

Diploid parthenogenic embryos

After IVM, the *cumulus oophorus* cells were removed from COCs by vortex in Talp-HEPES medium containing 0.1% hyaluronidase and 0.01% PVA for 3 min. Only MII oocytes with uniform cytoplasm were used. MII oocytes were placed in activation medium (0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 mM HEPES, pH 7.0–7.4) at 37°C and then transferred to a fusion chamber (BTX Electro-cell Manipulator 200 (BTX, San Diego, CA, USA) covered with 2 ml activation medium. Oocytes were activated by 2 DC pulses of 60 V for 80 μs, with an interval of 0.5 s. Immediately, they were incubated in synthetic oviductal fluid medium (SOF, Tervit) 100 μl drops containing 1% 6-DMAP, covered with mineral oil for 150 min. Then, oocytes were washed four times in TL–HEPES containing 0.1% (w/v) polyvinyl alcohol (PVA). Presumptive zygotes were cultured in SOF 50 μl drops at 39°C, 5% CO_2, 5% O_2 and 100% humidity. Cleavage was evaluated on day 2 and blastocysts on days 7 and 8.

IVF embryos

After IVM, the *cumulus oophorus* cells were removed from COCs by vortex in TALP-HEPES medium

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containing 0.1% hyaluronidase and 0.01% PVA for 3 min. Only MII oocytes with uniform cytoplasm were used. Fresh fertile boar semen was centrifuged 3 times (490 × ***g***, 5 min) in modified Brackett–Oliphant medium (BO, Brackett and Oliphant 1975) supplemented with 2.5 mM glucose, 10 mM Na lactate, 5 mM caffeine and 20 IU/ml heparin. After washing three times, cumulus-free oocytes were incubated with washed and diluted sperm in 100 μl drops for 30 min at 39°C, 5% CO_2 and 100% humidity. Sperm was used for fertilization at 1×10^6 cel/ml concentration, in porcine–BO supplemented with 10 mg/ml FAF-BSA. Finally, presumptive zygotes were washed in TALP-HEPES medium and cultured in SOF 50 μl drops at 39°C, 5% CO_2, 5% O_2 and 100% humidity. Cleavage was evaluated on day 2 and blastocysts on days 7 and 8.

Vesicle production and plasmid co-incubation

Five presumptive zygotes per repetition were used as vesicle donors. These were treated equally as their counterparts. After hyaluronidase treatment, they were transferred to TALP-HEPES 20 μl droplets. Each zygote 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 10% PVP droplet containing 30 ng/μl of HindIII-linearized-pcx-egfp and held there for 5 min. Finally, vesicles were aspirated into the 9-μm pipette and directly injected into the parthenogenic or IVF presumptive zygotes. One vesicle was injected in each zygote.

Cytoplasmic injection of naked or vesicle-co-incubated plasmid

Diploid parthenogenic or IVF presumptive zygotes were placed for micromanipulation in TL-HEPES 20 μl drops covered with mineral oil. The cytoplasmic injection was performed with Narishige hydraulic micromanipulators coupled to micro-injectors (Medical Systems, Great Neck, NY, USA) mounted on a Nikon Eclipse E-300 microscope (Nikon, Melville, NY, USA). Naked plasmid or plasmid previously co-incubated with vesicles was selected from a 3-μl droplet containing 10% v/v polyvinylpyrrolidone (PVP, 99219; Fisher Scientific, Pittsburgh, PA, USA). Parthenogenetic or IVF zygotes were held under negative pressure with the holding pipette, with the polar body at the 6 or 12 o'clock position. The 9-μm microinjection pipette was pushed through the zona pellucida and into the ooplasm at the 3 o'clock position. Following breakage of the oolemma by aspiration, the naked plasmid or the plasmid co-incubated with vesicles and the aspirated ooplasm was expelled into the oocyte with a volume of less than 10 pl as measured with the microinjectors. This determination was performed injecting a volume fixed by the microinjector ruler, into a medium droplet of known volume (10 μl) and repeated several times to confirm the injection volume. Presumptive zygotes were cultured as described below. eGFP expression was evaluated under blue light using an excitation filter at 488 nm and an emission filter at 530 nm at days 4 and 7, respectively.

Experimental design

Experiments were first performed using parthenogenic porcine embryos to determine the best conditions for cytoplasmic injection of the plasmid (Fig. 1). In experiment 1, three different concentrations of the plasmid were assayed and the concentration of 30 ng/μl was selected for further experiments. This experiment was repeated four times. Experimental groups were as follows: CP (naked circular plasmid), injected with 10% PVP + plasmid at 3 ng/μl (N = 105), 30 ng/μl (N = 95) and 300 ng/μl (N = 65); Sham, injected with 10% PVP alone (N = 105) and control, not injected (N = 223). The results of this experiment are shown in Table 1. In experiments 2 and 3, linear conformation of the plasmid was tested and then this conformation was chosen for the next experiments. Then, naked plasmid was compared with plasmid co-incubated with vesicles. The use of vesicles gave better results than naked plasmid. These experiments were repeated three times. Experimental groups were as follows: LP vesicles (linear plasmid co-incubated with vesicles), injected with 10% PVP + linear plasmid at 30 ng/μl previously co-incubated with vesicles (N = 115); LP naked (linear plasmid naked), injected with 10% PVP + plasmid at 30 ng/μl (N = 78); Sham vesicles, injected with PVP + vesicles (N = 59) and control, not injected (N = 198). The results of these experiments are presented in Table 2. Then, experimental conditions selected in parthenogenic embryos were assayed in IVF porcine embryos: linear plasmid at 30 ng/μl co-incubated with vesicles. This experiment was repeated three times. Experimental groups were as follows: LP vesicles injected with 10% PVP + linear plasmid at 30 ng/μl previously co-incubated with vesicles (N = 94); LP naked, injected with 10% PVP + plasmid at 30 ng/μl (N = 44); Sham, injected with 10% PVP alone (N = 59) and control, not injected (N = 79). The results of this experiment are shown in Table 3.

Evaluation of DNA fragmentation: TUNEL assay (Tdt-mediated dUTP nick-end labelling)

Blastocysts at day 8 were fixed in 4% paraformaldehyde and treated with 0.5% Triton X-100/0.1% sodium citrate. Controls were incubated with 0.1 U/μl DNase. TUNEL reaction was made according to the kit

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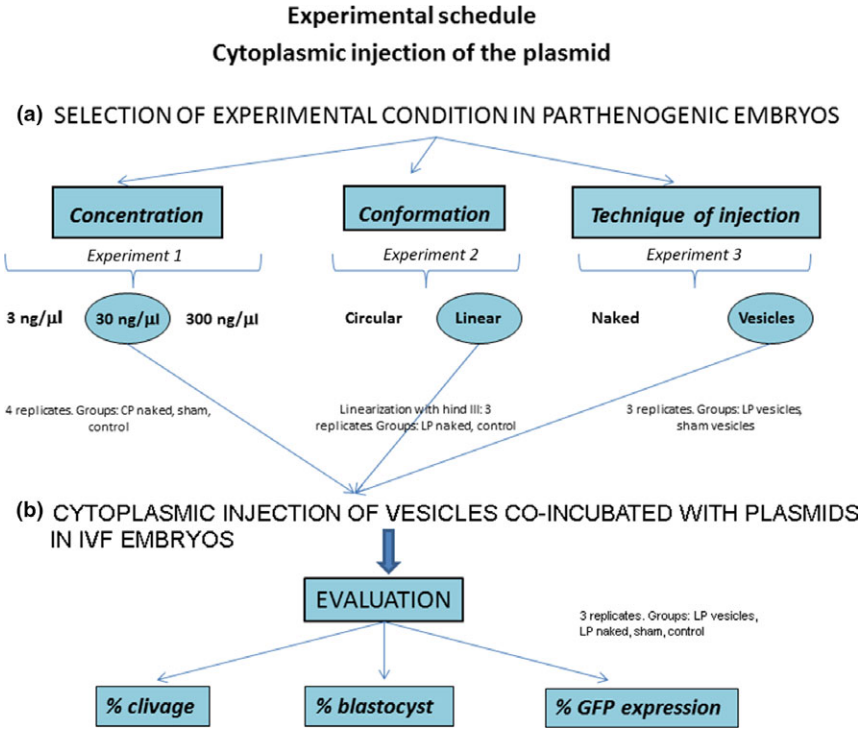

Fig. 1. Experimental design. (a) Experiments performed using parthenogenic porcine embryos to determine the best conditions for cytoplasmic injection of the plasmid. Experiment 1: the concentration of the plasmid 30 ng/µl was chosen for further experiments. Experiment 2: linear conformation of the plasmid was selected for next experiments. Experiment 3: the plasmid co-incubated with vesicles was chosen for the future experiment. (b) The experimental conditions selected in section (a) were assayed in IVF porcine embryos. CP, circular plasmid; LP, linear plasmid

Table 1. Embryo development and egfp expression of porcine diploid parthenogenic zygotes after cytoplasmic injection with 3, 30 and 300 ng/µl of circular pcx-egfp plasmid

Experimental group	N	Cleaved/ Total (%)	Blastocysts/ Cleaved (%)	eGFP+/ Total (%)	Blastocysts eGFP+/Total Blastocyst (%)
300 ng/µl	65	37 (57)*	0	17 (26)*	0
30 ng/µl	95	38 (40)*	3 (8)	17 (18)	0
3 ng/µl	105	64 (61)*	9 (14)	11 (11)	0
Sham	105	68 (65)*	5 (7)*	–	–
Control	223	170 (76)	37 (22)	–	–

Asterisk indicates significant differences vs control (Fisher's exact test: *: $p < 0.05$).

Table 2. Embryo development and egfp expression of porcine diploid parthenogenic zygotes after cytoplasmic injection with naked 30 ng/µl linear pcx-egfp plasmid and vesicles co-incubated with plasmid

Experimental group	N	Cleaved/ Total (%)	Blastocysts/ Cleaved (%)	eGFP+/ Total (%)	Blastocysts eGFP+/Total Blastocyst (%)
30 ng/µl vesicles	115	94 (82)	7 (7)*	87 (76)*	7 (100)
30 ng/µl naked	78	49 (84)	3 (6)*	36 (46)	1 (33)
Sham vesicles	59	43 (73)	2 (5)*	–	–
Control	198	147 (74)	32 (22)	–	–

Asterisk indicates significant differences vs control (Fisher's exact test: *: $p < 0.05$).

manufacturers protocol (*In Situ* Cell Death Detection Kit 1684795 Roche®, Mannheim, Germany). About 10 µG/ml propidium iodide (PI) was used as a contrast stain, and samples were mounted on slides for their observation with an epifluorescence microscope (DM4000B and capture software LASZ; Leica Microsystems, Wetzlar, Germany.) at 488 (excitation) and 518 (emission). TUNEL positive cells were counted.

Statistical analysis

Experiments were repeated at least three times, and data expressed as proportions (percentages). Row data were analysed with chi-square test. Fisher′s exact test was applied to specifical differences. GraphPad Prism software was used, version 5.0 (GraphPAD software, San Diego, CA, USA).

Results

Cytoplasmic injection of naked circular plasmid into porcine parthenogenetic zygotes

Diploid parthenogenic zygotes were injected with circular covalently closed pcx-egfp plasmid into the cytoplasm with an ICSI needle. Three different plasmid concentrations were assayed as follows: 3, 30 and 300 ng/µl. The three concentrations induced eGFP expression, although 300 ng/µl (26%) showed higher rates than 30 ng/µl (18%) and 3 ng/µl (11%, Table 1).

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Table 3. Embryo development and egfp expression of porcine IVF zygotes after cytoplasmic injection with naked 30 ng/μl linear pcx-egfp plasmid and vesicles co-incubated with plasmid

Experimental group	N	Cleaved/Total (%)	Blastocysts/Cleaved (%)	eGFP+/Total (%)	Blastocysts eGFP+/Total Blastocyst (%)
30 ng/μl vesicles	94	79/94 (84)*	15/79 (19)	48/94 (51)	8/15 (53)
30 ng/μl naked	44	40/44 (91)*	5/40 (13)	16/44 (36)	0 (0)
Sham	59	48/59 (81)*	10/48 (21)	–	–
Control	79	41/79 (52)	6/41 (15)	–	–

Asterisk indicates significant differences vs control (Fisher's exact test: *: $p < 0.05$).

The injection had a deleterious effect on embryo development, reflected on lower cleavage rates in the experimental groups than in the control (Table 1). The injection with vehicle alone (Sham) or with 300 ng/μl plasmid affected the rate of blastocyst/cleaved embryos when compared to control. The highest concentration of plasmid that allowed development to blastocyst stage was 30 ng/μl. However, blastocysts expressing the transgene were not obtained.

Detection of DNA fragmentation in porcine parthenogenetic zygotes cytoplasmically injected with naked circular plasmids by TUNEL assay

To determine whether cytoplasmic injection induces DNA fragmentation, TUNEL assay was performed in blastocysts of each experimental group. It was not possible to test TUNEL in the group injected with 300 ng/μl because in this group, there were no blastocyst production. The proportion of TUNEL positive cells was not different between treatments [control: 2 ± 1 positive/38 ± 8 total cells (N = 3); 3 ng/μl: 2 ± 1 positive/40 ± 9 total cells (N = 3); 30 ng/μl: 3 ± 1 positive/40 ± 8 total cells (N = 2)]. Figure 2 shows a representative picture of TUNEL in a control blastocyst.

Cytoplasmic injection of linear naked and vesicles co-incubated plasmids into porcine parthenogenetic zygotes

Diploid parthenogenic zygotes were injected into the cytoplasm with naked pcx-egfp plasmid linearized with HindIII or with vesicles previously co-incubated with linearized plasmid. The concentration used was 30 ng/μl because it was the higher concentration tested in this study that allowed embryo development to blastocyst stage. The injection did not affect cleavage rate (Table 2). On the other hand, the blastocyst/cleavage rate decreased in all groups compared to the control (Table 2). Both groups injected with linear plasmid expressed eGFP. The use of vesicles increased eGFP expression when compared to naked plasmid (vesicles 76% vs naked 46%). Using naked linear plasmid, only one blastocyst expressed transgene. The use of vesicles co-incubated with plasmid increased the transgene-expressing blastocysts rate to 100% (Table 2). Figure 3 shows a representative picture of an eGFP-expressing blastocyst produced by cytoplasmic injection of vesicles.

Cytoplasmic injection of linear naked plasmid and vesicles co-incubated with plasmid to IVF embryos

IVF zygotes were injected with naked linear pcx-egfp plasmid or with vesicles previously co-incubated with linear plasmid into the cytoplasm. The concentration used was 30 ng/μl. The cytoplasmic injection increased cleavage rates compared to the control (Table 3). There were no significant differences in blastocysts/cleaved embryos between groups. Both groups injected with plasmid, naked or vesicles co-incubated showed transgene expression. The group injected with vesicles showed 53% of GFP+ blastocysts, whereas the group injected with naked plasmid showed no GFP+ blastocysts.

Discussion

In this work, vesicles previously co-incubated with plasmids were used for the first time to improve the

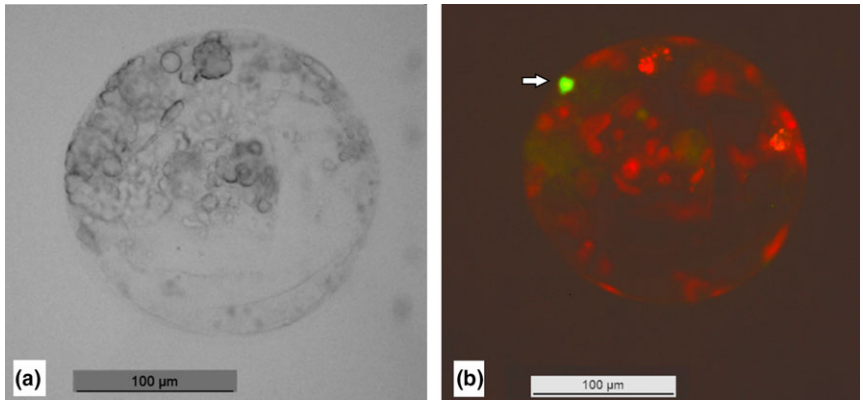

Fig. 2. Representative picture of TUNEL assay in an 8 days parthenogenic porcine control blastocyst. (a) Under bright light. (b) Under fluorescent light. Arrow indicates a TUNEL positive cell

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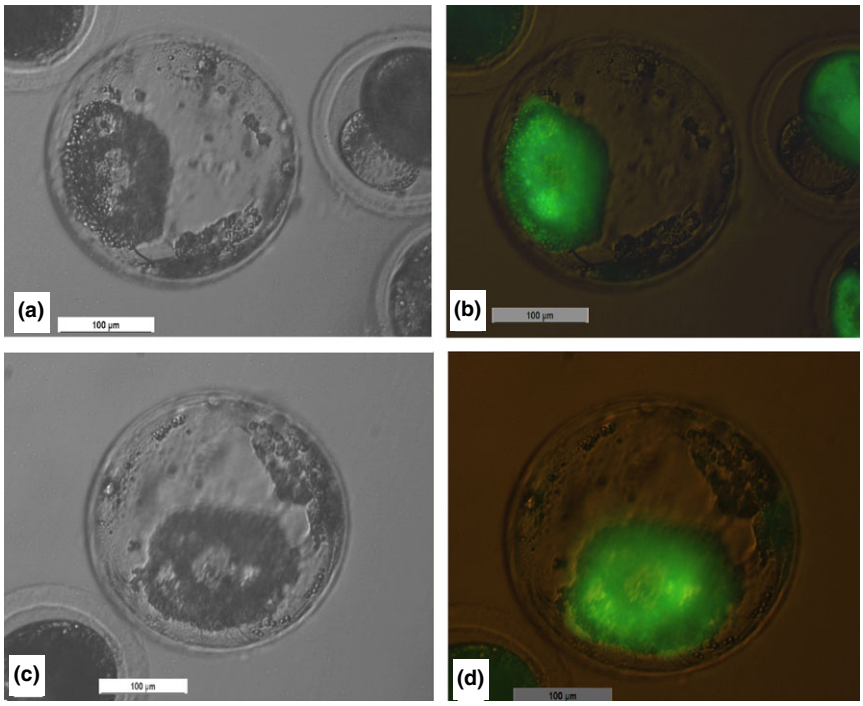

Fig. 3. Representative pictures of egfp expressing parthenogenic porcine blastocysts at day 8 produced by cytoplasmic injection of vesicles co incubated with pcx-egfp (30 ng/µl). (a and c) Under bright light. (b and d) Under blue light (488 nm)

efficiency of cytoplasmic injection of transgenes into porcine parthenogenic and IVF zygotes. In a series of experiments conducted in diploid parthenogenetic embryos, it was shown that the cytoplasmic injection of naked DNA at three different concentrations results in the expression of the green fluorescent protein gene (gfp). This confirmed that the exogenous DNA (eDNA) is delivered from the cytoplasm to the nucleus, but does not give information about the integration of the transgene in the embryonic genome. Most likely, expression might be transient, from episomal plasmids. It remains unclear how the plasmid DNA translocates into the nucleus after the cytoplasmic injection. However, after nuclear membrane disassembly in the first cell cycle, some plasmid molecules might be taken up in the newly forming nucleus. Although the foreign DNA plasmids are mainly maintained as episomal entities during pre-implantation development, they accurately behave like nuclear DNA (Iqbal et al. 2009). The injection of foreign DNA into the cytoplasm of zygotes has demonstrated to be a simple and reliable technique to induce its transcription and expression of genes of interest. Cytoplasmic injection of plasmids allowed the production of mice, cattle, sheep and buffalo expressing embryos (Iqbal et al. 2009; Pereyra-Bonnet et al. 2011; Bevacqua et al. 2012; Meng et al. 2015). Using cytoplasmic injection in combination with active transgenesis technologies, transgenic embryos and animals were produced in different species, including mice (Page et al. 1995; Dunlap-Brown et al. 2012), rats (Shao et al. 2014), cynomolgus monkey (Niu et al. 2014), cattle (Bevacqua et al. 2013) and pigs (Garrels et al. 2011; Ivics et al. 2014; Li et al. 2014a,b). Our results also showed that high plasmid concentrations (300 ng/µl) significantly increased transgene expression, but compromised blastocyst production. This phenomenon coincides with the observations made by Pereyra-Bonnet et al. (2011) in bovine and ovine embryos and Meng et al. (2015) in buffaloes and agrees with the findings of Brinster et al. (1985) where high concentrations of plasmid after the pronuclear or cytoplasmic injection reduce embryonic development. It could be due to a toxic effect of the H_2O_2 produced by GFP (Tsien 1998) or to apoptotic mechanisms triggered by the increased presence of eDNA (Moreira et al. 2007). In this sense, the present study also showed a decrease in the cleavage of zygotes injected with the plasmid or with vehicle alone (Sham). This could indicate damage exerted by the cytoplasmic injection technique, independently of the plasmid introduced. As it was not possible to observe morphological parameters that clearly indicate injury, DNA damage was studied. This was performed in blastocysts because this is the stage in which cells with DNA fragmentation can be better identified using the TUNEL technique. All experimental groups were examined, except 300 ng/ml group, which did not result in blastocysts. No differences in the proportion of cells with DNA fragmentation were observed between groups. The highest concentration to result in development to blastocysts was 30 ng/µl. A previous report (Bevacqua et al. 2012) demonstrated that pcx-egfp plasmid linearization results in improved transgene expression in bovine. Here, as in that work, linearization of the plasmid resulted in high expression rates and allowed transgene expression at the blastocyst stage. In contrast to observations for the circular

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plasmids, cleavage was not affected when the linear plasmid was used. However, the rate of blastocysts was hampered by linear plasmid injection. The use of vesicles previously co-incubated with plasmids was effectively reported for bovine and ovine zygotes (Pereyra-Bonnet et al. 2011; Bevacqua et al. 2012). The efficiency of gfp plasmid binding to the vesicles was previously determined by our group (Pereyra-Bonnet et al. 2011). In that report, confocal microscopy images of oocytes injected with vesicles revealed that vesicles encapsulated labelled eDNA and that 60% of the oocytes analysed contained specific signals for eDNA (vesicles were not found in the remaining oocytes). In this study, the use of vesicles was tested in porcine zygotes, and in agreement with observations for bovine and ovine, it improved the efficiency of cytoplasmic injection. Besides, it increased the transgene expression rate when compared to naked plasmid and also increased the percentage of blastocysts expressing the transgene. The greatest value of the present work is that the vesicles could exert a protective effect on the plasmids introduced, preventing their degradation by cytoplasmic enzymes until they reach the nucleus. This was clearly reflected in the fact that embryos injected with the naked plasmid showed significantly less expression than embryos injected with vesicles. Assays performed in parthenogenic embryos are useful for preliminary trials. Yet, only IVF embryos are potentially useful for embryo transfer and live piglets production. For that reason, in the final experiment, we tested the injection with vesicles co-incubated with plasmids in IVF embryos employing the experimental conditions determined for parthenogenetic embryos. Both naked and co-incubated plasmids were useful to generate embryos expressing the transgene, but only the use of vesicles allowed transgene-expressing blastocysts production. From the 51% of embryos expressing the transgene with the use of vesicles, 53% reached the blastocyst stage. Interestingly, cytoplasmic injection improved the cleavage rate in IVF porcine embryos. This can be seen in the Sham, LP naked and LP vesicles groups. An additional mechanical activating effect of the cytoplasmic injection could exist in these groups. In conclusion, the use of vesicles results effective and simple and represents a good choice for introducing transgenes into porcine embryos. New tools for transgenesis such as transposases and CRISPR could be complemented to the use of vesicles for generating transgenic pigs with a high efficiency.

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Author contributions

Dr Luchetti has designed the study, made the experimental procedures, analysed data and drafted the study. Dr Bevacqua has contributed to design the study, made experimental procedures and drafted the study. Dr Lorenzo has contributed to make experimental procedures and drafted the study. Dr Tello and Willis have contributed to make experimental procedures. Dr Buemo has contributed to analyse data. Dr Lombardo and Dr Salamone have supervised all the work, designed the study and drafted the study.

Conflict of interest

None of the authors have any conflict of interest to declare.

References

Bevacqua RJ, Pereyra-Bonnet OR, Hiriart MI, Sipowicz P, Fernandez-Martín R, Radrizzani M, Salamone DF, 2012: Production of IVF transgene-expressing bovine embryos using a novel strategy based on cell cycle inhibitors. Theriogenology **78**, 57–68.

Bevacqua RJ, Canel NG, Hiriart MI, Sipowicz P, Rozenblum GT, Vitullo A, Radrizzani M, Fernandez Martin R, Salamone DF, 2013: Simple gene transfer technique based on I-SceI meganuclease and cytoplasmic injection in IVF bovine embryos. Theriogenology **80**, 104–113.

Brackett BG, Oliphant G, 1975: Capacitation of rabbit spermatozoa in vitro. Biol Reprod **12**, 260–274.

Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD, 1985: Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc Natl Acad Sci USA **8**, 4438–4442.

Carlson DF, Garbe JR, Tan W, Martin MJ, Dobrinsky JR, Hackett PB, Clark KJ, Fahrenkrug SC, 2011: Strategies for selection marker-free swine transgenesis using the Sleeping Beauty transposon system. Transgenic Res **20**, 1125–1137.

Chen Y, Zheng Y, Kang Y, Yang W, Guo X, Tu Z, Si C, Wang H, Xing R, Pu X, Yang SH, Li S, Ji W, Li XJ, 2015: Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. Hum Mol Genet **24**, 3764–3774.

Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, Dos Santos-Neto PC, Nguyen TH, Crénéguy A, Brusselle L, Anegón I, Menchaca A, 2015: Efficient generation of myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. PLoS ONE **10**, e0136690.

Dunlap-Brown M, Butler S, Velander W, Gwazdauskas F, 2012: Murine embryo development following cytoplasmic injection of linear and condensed DNA. Open J Animal Sci **2**, 244–252.

Garrels W, Mates L, Holler S, Dalda A, Taylor U, Petersen B, Niemann H, Izsvak Z, Ivics Z, Kues WA, 2011: Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLoS ONE **6**, 23573.

Garrels W, Kues WB, Herrmann D, Holler S, Baulain U, Niemann H, 2012: Ectopic expression of human telomerase RNA component results in increased telomerase activity and elongated telomeres in bovine blastocysts. Biol Reprod **87**, 95.

Gordon JW, Scangos GA, Plotkin DJ, Barbarosa JA, Ruddle FH, 1980: Genetic transformation of mouse embryos by microinjection of purified DNA. PNAS **77**, 7380–7384.

Gün G, Kues WA, 2014: Current progress of genetically engineered pig models for biomedical research. Biores Open Access **3**, 255–264.

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Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL, 1985: Production of transgenic rabbits, sheep and pigs by microinjection. Nature **315**, 680–683.

Hirabayashi M, Takahashi R, Ito K, Kashiwazaki N, Hirao M, Hirasawa K, Hochi S, Ueda M, 2001: A comparative study on the integration of exogenous DNA into mouse, rat, rabbit, and pig genomes. Exp Anim **50**, 125–131.

Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A, 2003: Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep **4**, 1054–1060.

Honda A, Hirose M, Sankai T, Yasmin L, Yuzawa K, Honsho K, Izu H, Iguchi A, Ikawa M, Ogura A, 2015: Single-step generation of rabbits carrying a targeted allele of the tyrosinase gene using CRISPR/Cas9. Exp Anim **64**, 31–37.

Hsu PD, Lander ES, Zhang F, 2014: Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell **157**, 1262–1278.

Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M, 1995: A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). FEBS Lett **375**, 125–128.

Iqbal K, Barg-Kues B, Broll S, Bode J, Niemann H, Kues W, 2009: Cytoplasmic injection of circular plasmids allows targeted expression in mammalian embryos. Biotechniques **47**, 959–968.

Ivics Z, Garrels W, Mátés L, Yau TY, Bashir S, Zidek V, Landa V, Geurts A, Pravenec M, Rülicke T, Kues WA, Izsvák Z, 2014: Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. Nat Protoc **9**, 810–827.

Jakobsen JE, Li J, Kragh PM, Moldt B, Lin L, Liu Y, Schmidt M, Winther KD, Schyth BD, Holm IE, Vajta G, Bolund L, Callesen H, Jørgensen AL, Nielsen AL, Mikkelsen JG, 2011: Pig transgenesis by Sleeping Beauty DNA transposition. Transgenic Res **20**, 533–545.

Kues WA, Niemann H, 2011: Advances in farm animal transgenesis. Prev Vet Med **102**, 146–156.

Li Z, Zeng F, Meng F, Xu Z, Zhang X, Huang X, Tang F, Gao W, Shi J, He X, Liu D, Wang C, Urschitz J, Moisyadi S, Wu Z, 2014a: Generation of transgenic pigs by cytoplasmic injection of piggyBac transposase-based pmGENIE-3 plasmids. Biol Reprod **90**, 93.

Li P, Estrada JL, Burlak C, Montgomery J, Butler JR, Santos RM, Wang ZY, Paris LL, Blankenship RL, Downey SM, Tector M, Tector AJ, 2014b: Efficient generation of genetically distinct pigs in a single pregnancy using multiplexed single-guide RNA and carbohydrate selection. Xenotransplantation **21**, 376–384.

Marh J, Stoytcheva Z, Urschitz J, Sugawara A, Yamashiro H, Owens JB, Stoytchev I, Pelczar P, Yanagimachi R, Moisyadi S, 2012: Hyperactive self-inactivating piggyBac for transposase-enhanced pronuclear microinjection transgenesis. Proc Natl Acad Sci U S A **109**, 19184–19189.

Mashiko D, Young SA, Muto M, Kato H, Nozawa K, Ogawa M, Noda T, Kim YJ, Satouh Y, Fujihara Y, Ikawa M, 2014: Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. Dev Growth Differ **56**, 122–129.

Meng F, Li H, Wang X, Qin G, Oback B, Shi D, 2015: Optimized production of transgenic buffalo embryos and offspring by cytoplasmic zygote injection. J Animal Sci Biotechnol **6**, 44.

Moreira PN, Perez-Crespo M, Ramirez MA, Pozueta J, Montoliu L, Gutierrez-Adan A, 2007: Effect of transgene concentration, flanking matrix attachment regions, and RecA-coating on the efficiency of mouse transgenesis mediated by intracytoplasmic sperm injection. Biol Reprod **76**, 336–343.

Ni W, Qiao J, Hu S, Zhao X, Regouski M, Yang M, Polejaeva IA, Chen C, 2014: Efficient gene knockout in goats using CRISPR/Cas9 system. PLoS ONE **9**, e106718.

Niemann H, Kues WA, 2007: Transgenic farm animals: an update. Reprod Fertil Dev **19**, 762–770 Review.

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J, 2014: Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell **156**, 836–843.

Nowak-Imialek M, Niemann H, 2012: Pluripotent cells in farm animals: state of the art and future perspectives. Reprod Fertil Dev **25**, 103–128.

Page RL, Butler SP, Subramanian A, Gwazdauskas FC, Johnson JL, Velander WH, 1995: Transgenesis in mice by cytoplasmic injection of polylysine/DNA mixtures. Transgenic Res **4**, 353–360.

Pereyra-Bonnet F, Bevacqua R, La Rosa I, Sipowicz P, Radrizzani M, Fernandez-Martin R, Salamone D, 2011: Novel methods to induce exogenous gene expression in SCNT, IVF and parthenogenic bovine embryos. Transgenic Res **20**, 1379–1388.

Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R, 1999: Mammalian transgenesis by intracytoplasmic sperm injection. Science **284**, 1180–1183.

Sato M, Koriyama M, Watanabe S, Ohtsuka M, Sakurai T, Inada E, Saitoh I, Nakamura S, Miyoshi K, 2015: Direct Injection of CRISPR/Cas9-Related mRNA into Cytoplasm of Parthenogenetically Activated Porcine Oocytes Causes Frequent Mosaicism for Indel Mutations. Int J Mol Sci **16**, 17838–17856.

Shao Y, Guan Y, Wang L, Qiu Z, Liu M, Chen Y, Wu L, Li Y, Ma X, Liu M, Li D, 2014: CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. Nat Protoc **9**, 2493–2512.

Sorek R, Kunin V, Hugenholtz P, 2008: CRISPR–a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat Rev Microbiol **6**, 181–186.

Suganuma R, Pelczar P, Spetz JF, Hohn B, Yanagimachi R, Moisyadi S, 2005: Tn5 transposase-mediated mouse transgenesis. Biol Reprod **73**, 1157–1163.

Tsien RY, 1998: The green fluorescent protein. Annu Rev Biochem **6**, 509–544.

Wall RJ, 2002: New gene transfer methods. Theriogenology **57**, 189–201.

Wang Y, Du Y, Shen B, Zhou X, Li J, Liu Y, Wang J, Zhou J, Hu B, Kang N, Gao J, Yu L, Huang X, Wei H, 2015: Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Sci Rep **5**, 8256.

Westra ER, Buckling A, Fineran PC, 2014: CRISPRCas systems: beyond adaptive immunity. Nat Rev Microbiol **12**, 317–326.

Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O'Gorman C, Walters EM, Murphy CN, Driver J, Mileham A, McLaren D, Wells KD, Prather RS, 2014: Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod **91**, 78.

Whyte JJ, Prather RS, 2011: Genetic modifications of pigs for medicine and agriculture. Mol Reprod Dev **78**, 879–891.

Zhou X, Xin J, Fan N, Zou Q, Huang J, Ouyang Z, Zhao Y, Zhao B, Liu Z, Lai S, Yi X, Guo L, Esteban MA, Zeng Y, Yang H, Lai L, 2015: Generation of CRISPR/Cas9-mediated gene targeted pigs via somatic cell nuclear transfer. Cell Mol Life Sci **72**, 1175–1184.

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