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Research Article

Cytoplasmic Microinjection of *piggyBac* Transposase mRNA and Transposon Vectors for Efficient *In Vitro* Production of Transgenic Porcine Parthenotes

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Abstract

The efficient production of transgenic (Tg) piglets has remained a challenge in the field of domestic animal studies. Unlike mice, the pronuclei of pig zygotes cannot be easily studied because of the abundance of lipid droplets. Therefore, the zygotes must be briefly centrifuged before pronuclear injection (PNI) to move the lipid droplets to the periphery of the zygote for PNI-mediated production of Tg piglets. However, this procedure is temporal because lipid droplets return to the original space during PNI, hampering the consecutive PNI. Cytoplasmic injection (CPI) of nucleic acids is comparatively simple than PNI because CPI does not require such pre-centrifugation. Unfortunately, CPI using purified DNA fragments is inadequate for creating Tg piglets because it is challenging to integrate nucleic acids into the host genome. *PiggyBac* (PB), one of the transposons, is a valuable tool enabling efficient chromosomal integration of a transgene. The PB-mediated gene transfer requires two components, namely, transposase and transposons harboring the gene of interest (GOI) flanked by PB acceptor sites. We speculate that the CPI of transposase mRNA and transposons could accelerate the chromosomal integration of the GOI in pig zygotes. To prove this hypothesis, we performed CPI using transposase mRNA (super PB transposase mRNA) + transposon DNA carrying the enhanced green fluorescent protein (*EGFP*) cDNA (referred to as "pT-EGFP"), transposase expression plasmid DNA (referred to as "pTrans") + pT-EGFP, pT-EGFP alone, or non-transposon EGFP expression plasmid DNA using porcine parthenotes. Consequently, 50% (2/4 tested) of green-fluorescent embryos exhibited chromosomal integration of GOI. In contrast, green-fluorescent embryos derived from CPI with pTrans + pT-EGFP or pT-EGFP alone did not show chromosomal integration. We used mRNA for super PB transposase, which is an engineered hyperactive version of the wild-type PB transposase. To conclude, the PB system, based on the CPI of super PB transposase mRNA + transposon DNA, could be useful for producing Tg porcine parthenotes.

Keywords

Cytoplasmic injection; *piggyBac*; transposase mRNA; transgenic pigs; EGFP; parthenotes; Splinkerette-PCR; gene of interest; transposon; chromosomal integration; pronuclear injection; nucleic acids

1. Introduction

Among domestic animals, pigs have long been used as edible livestock and have the potential to generate high economic value. In addition, pigs have the potential to be applied in the biomedical field because they are similar to humans from physiological and anatomical perspectives; therefore, they have been used as animal models for human diseases and as an alternative for organ transplantation [1, 2].

The creation of genetically modified (GM) animals (as exemplified by transgenic [Tg] animals) is vital to elucidate the mechanism underlying human diseases, develop therapeutic drugs, and analyze gene functions *in vivo* [3]. The first success in Tg animal production was reported by Gordon et al. [4], who performed pronuclear injection (PNI) of nucleic acids (linearized plasmid DNA) into

the male pronuclei of mouse zygotes using a micromanipulator system. Since then, this approach has been widely employed as a standard technique to produce Tg mice. Furthermore, this approach has been applied to produce larger Tg animals such as rabbits, sheep, and pigs [5]. However, it is highly challenging to perform PNI on zygotes derived from these larger animals because they always contain lipid droplets in their cytoplasm, which often hinders the visualization of pronuclei. To circumvent this difficulty, zygotes were briefly centrifuged to visualize pronuclei before PNI [6]. However, this procedure often damages the embryos and is temporal because the lipid droplets that have moved to the marginal region of the zygote return to the original location during PNI, thereby hampering consecutive PNI [5]. This background has motivated several researchers to explore other PNI-independent approaches that are less damaging to embryos and more convenient to produce Tg animals. For example, GM pigs were produced using somatic cell nuclear transfer (SCNT) from GM fibroblasts as the SCNT donor [7-10]. Tg mice were produced through lentiviral infection of preimplantation embryos [11, 12] and by cytoplasmic injection (CPI) of DNA-associated sperm previously incubated with a nucleic acid-containing solution (which was later called "intracytoplasmic sperm injection [ICSI] using sperm associated with the transgene [TransICSI]") [13-17]. These procedures have fewer advantages than disadvantages. In addition, compared to PNI, they still appear to be laborious and often time-consuming.

CPI-based transgenesis is an attractive method to deliver nucleic acids to zygotes. It is simpler and more convenient than PNI-based transgenesis because CPI does not require centrifugation to remove lipid droplets temporally. Furthermore, it can be performed at any site in a zygote between the nucleus and the egg plasma membrane. Chromosomal integration of transgenes is a prerequisite to producing Tg pigs. CPI of nucleic acids, including plasmid DNA and DNA fragments, is not superior to PNI of nucleic acids, considering the efficient chromosomal integration of transgenes [18]. However, transient expression (at least up to the blastocyst stage) of the exogenous DNA introduced in murine zygotes after CPI has been reported [19]. These findings suggest that nucleic acids administered to the cytoplasm are transferred to the nucleus, following which the mRNA is transcribed from the transgene. However, the exogenous nucleic acids transferred within the nucleus may not have been integrated into the host genome, and therefore, their expression remains transient. Therefore, CPI is a tool that allows nucleic acids to be efficiently integrated into the host genome, thus, improving the susceptibility of the gene of interest (GOI) to chromosomal integration, leading to successful transgenesis.

Recently, transposons such as Sleeping Beauty (SB), Tol2 (a transposon derived from fishes), and *piggyBac* (PB) are valuable tools, allowing the efficient chromosomal integration of GOI into the host genome. Moreover, these are considered a powerful alternative method to create GM animals/cells (reviewed by Sato et al. [20]). Among these transposons, PB is commercially available as a transposon-based gene delivery system and has been used for several applications, including PNI-based production of Tg animals [21-23], generation of induced pluripotent stem cells (iPSCs) [24-26], production of stably transfected human cells [27, 28], and *in vivo* gene transfer into experimental animals [29, 30]. PB-based gene delivery is extremely simple because it involves only two types of nucleic acids, namely, a PB transposase expression vector and a transposon vector carrying a GOI flanked by two inverted terminal repeat (ITR) sequences. Inside a cell, transposase binds to the ITR, allowing the GOI to be individually integrated into the host chromosomal sites containing the TTAA sequence, two copies of which are present on the two flanks of the integrated fragment [31, 32]. Thus, the CPI of transposase mRNA (or protein) and transposon (plasmid DNA)

more efficiently facilitates the chromosomal integration of GOI than that of the transposase expression plasmid and transposon because a time-lag exists when mRNA synthesis initiates from a plasmid DNA introduced into a nucleus.

We examined whether transposase mRNA can accelerate the chromosomal integration of the GOI (included in the co-injected transposon) when porcine parthenotes are subjected to CPI.

2. Materials and Methods

The experiments described here were performed according to the guidelines of the *Kagoshima University Committee on Recombinant DNA Security*. The study was approved by the *Animal Care and Experimentation Committee of Kagoshima University* (no. S28003; May 16, 2016).

2.1 Preparation of Vectors and Transposase mRNA

All PB-related vectors (pTrans and pT-EGFP) were the same as those reported in our previous study [33]. Briefly, pTrans is a plasmid vector carrying a PB transposase gene whose expression is controlled by a chicken β-actin promoter-based promoter system known as CAG [34] (Figure 1A). pT-EGFP is a transposon vector, in which the expression of enhanced green fluorescent protein (*EGFP*) cDNA is controlled by CAG, and its expression unit is flanked by ITRs (Figure 1A). pCE-29 is a non-transposon EGFP expression plasmid [35] and is similar to pT-EGFP as it uses the same promoter and the second intron of the rabbit β-globin gene (Figure 1A). However, pCE-29 uses SV40-derived poly(A) sites, whereas pT-EGFP uses poly(A) site present in the 3' untranslated region of the rabbit β-globin gene. The plasmid backbone of pT-EGFP is a PB vector pPB (pPB-MCS-P5), and that of pCE-29 is pBluescript SK(-).

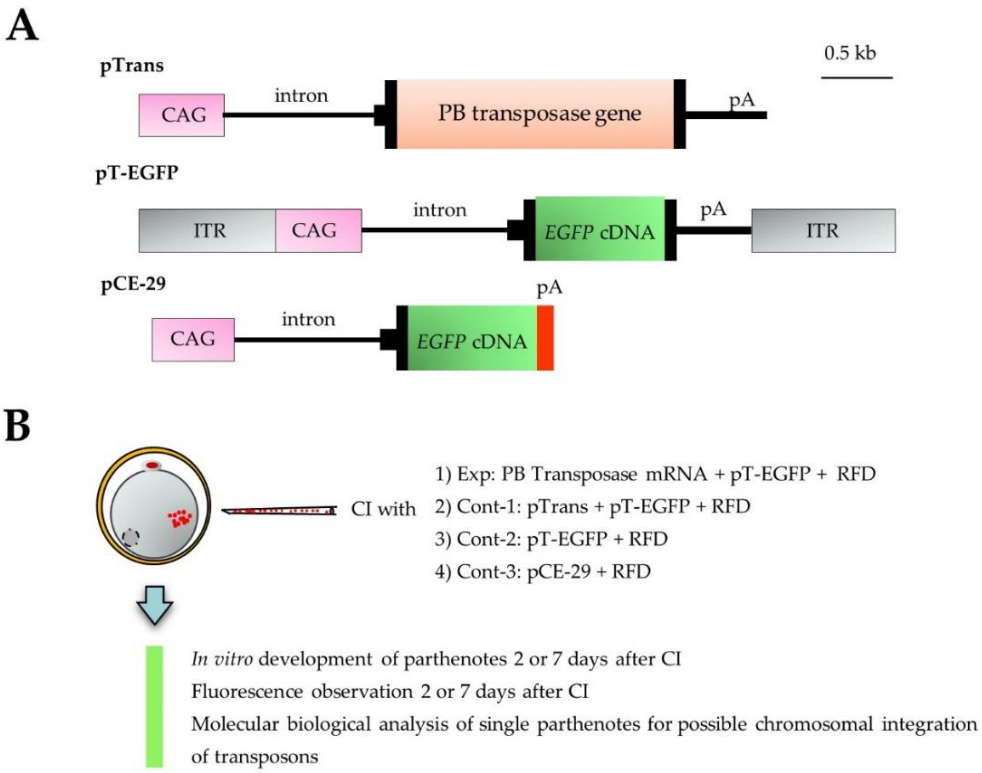

Figure 1 Cytoplasmic injection (CPI) of *piggyBac* (PB) transposase mRNA and PB transposon(s) for efficient production of transgenic (Tg) porcine parthenotes. **A.** Structure of plasmids used in the study. pTrans is a plasmid conferring expression of the PB transposase gene. pT-EGFP is a PB transposon, in which an enhanced green fluorescent protein (EGFP) expression unit is flanked by inverted terminal repeats (ITRs). pCE-29 is a non-transposon plasmid conferring the expression of *EGFP* cDNA. CAG, a chicken β-actin-based promoter; pA, poly(A) sites. **B.** Schema for CPI-based production of Tg porcine parthenotes. Oocytes, after electric activation, were subjected to CPI using solutions containing several reagents derived from the Exp, Cont-1, Cont-2, or Cont-3 groups. After CPI, these treated embryos were cultured up to the morula/blastocyst stages. During these periods, the fluorescence was inspected. The developing blastocysts were finally subjected to molecular biological analysis to examine the possible chromosomal integration of transposons.

All vectors were amplified in the *Escherichia coli* DH5α strain and purified using the MACHEREY-NAGEL plasmid purification kit (Takara Bio Inc., Shiga, Japan), following the method described by Sato et al. [36]. Transposase mRNA was purchased from Transposagen (now Hera BioLabs, Lexington, KY, USA) as Super PB transposase mRNA (#sPBo-m).

2.2 Isolation of Porcine Oocytes, In Vitro *Maturation, and Electric Activation*

Porcine oocytes were prepared as previously described by Himaki et al. [37]. Briefly, the ovaries were collected from the prepubertal gilts at a local slaughterhouse and transported to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from 2 to 5 mm diameter antral follicles using an 18-gauge needle (Terumo; Tokyo, Japan) and fixed to a 5 mL disposable syringe

(Nipro; Osaka, Japan). The COCs were washed twice with HEPES (Nacalai Tesque; Kyoto, Japan)-buffered Tyrode-lactate-pyruvate-polyvinyl alcohol (HEPES-TLP-PVA) (PVA; Sigma-Aldrich Co., St. Louis, MO, USA), and 40 to 50 COCs were transferred to 200 µL of the maturation medium in a 35-mm dish (Becton Dickinson, Franklin Lakes, NJ, USA) and pre-equilibrated overnight at 38.5 °C in a 5% CO_2 atmosphere. The maturation medium contained 90% (v/v) TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY, USA), supplemented with 0.91 mM sodium pyruvate (Sigma-Aldrich Co.), 3.05 mM D-glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.57 mM cysteine hydrochloride hydrate (Sigma-Aldrich Co.), 10 ng/mL epidermal growth factor (Sigma-Aldrich Co.), 10 IU/mL eCG (Aska Pharmaceutical Co., Tokyo, Japan), 10 IU/mL hCG (Aska Pharmaceutical Co.), 100 µg/mL amikacin sulfate (Meiji Seika, Tokyo, Japan), 0.1% (w/v) PVA, and 10% (v/v) pig follicular fluid covered with paraffin oil (Nacalai Tesque). After 42 to 44 h of maturation culturing, cumulus cells were removed by pipetting with 0.1% (w/v) hyaluronidase (Sigma-Aldrich Co.). Oocytes with a polar body were selected for experiments.

For parthenogenetically activated oocyte production, denuded oocytes (20–40) were placed between two wire electrodes 1 mm apart in an activation medium containing 250.3 mM sorbitol, 0.5 mM calcium acetate, 0.5 mM magnesium acetate, and 0.1% bovine serum albumin (BSA) [38]. The activation was induced with one direct current pulse of 100 V/mm for 50 µs using an LF 101 Fusion Machine (Nepa Gene Co., Chiba, Japan).

2.3 CPI

For CPI-based experiments, we used parthenogenetically activated porcine oocytes (also called "parthenotes") instead of using porcine zygotes because the former embryos are known to mimic embryogenesis at least up to blastocysts, similar to normal zygotes [39]. Furthermore, porcine parthenotes can be easily generated through the *in vitro* activation of oocytes collected from ovaries obtained at a slaughterhouse. In our past studies, parthenotes have been used for SCNT as recipient eggs [40] and the CPI of *EGFP* mRNA [41].

About 6 h after electrical activation, the activated oocytes were transferred to HEPES-TLP-PVA and subjected to a single 2 pL CPI of Opti-MEM (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing several reagents divided into four groups (Exp, Cont-1, Cont-2, and Cont-3) (Table 1). Slight swelling of the oocyte cytoplasm and the presence of red fluorescent tetramethyl rhodamine-dextran 3 KDa (RFD) in the cytoplasm confirmed the successful injection. After CPI, treated parthenotes were cultured in a drop (50 µL) of modified PZM-3 (mPZM-3) medium [42] under a 5% CO_2/5% O_2/90% N_2 atmosphere at 38.5 °C up to the blastocyst stage. During this period, the development rates of the two-cell and blastocyst stages and fluorescence in the injected oocytes were recorded for each group. Each blastocyst showing both EGFP-derived green and RFD-derived red fluorescence was fixed for 5 min at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4. Afterward, it was transferred to a small tube for Splinkerette-PCR analysis, as detailed below.

Table 1 Reagents used for cytoplasmic injection (CPI)[1] in porcine parthenotes.

Groups	Reagents dissolved in Opti-MEM
Exp	66 ng/μL of transposase mRNA, 20 ng/μL pT-EGFP, and 2 μg/μL RFD
Cont-1	20 ng/μL of pTrans, 20 ng/μL pT-EGFP, and 2 μg/μL RFD
Cont-2	20 ng/μL pT-EGFP and 2 μg/μL RFD
Cont-3	20 ng/μL pCE-29 and 2 μg/μL RFD

[1]CPI was performed on porcine parthenotes 6 h after the activation. Samples were divided into four groups (Exp, Cont-1, Cont-2, and Cont-3). In each group, a solution containing different components listed in the table was subjected to CPI. Abbreviations: Cont-1, Control group-1; Cont-2, Control group-2; Cont-3, Control group-3; Exp, Experimental group; pCE-29, enhanced fluorescent protein (EGFP)-expressing non-transposon plasmid (shown in Figure 1A); pT-EGFP, EGFP-expressing transposon (shown in Figure 1A); pTrans, transposase-expressing plasmid (shown in Figure 1A); RFD, red fluorescent tetramethyl rhodamine-dextran 3 KDa (Thermo Fisher Scientific Inc.).

2.4 Genomic DNA Isolation and Whole Genome Amplification (WGA) for Genomic Integration Site Analysis Using Splinkerette-PCR

The fixed single blastocyst was transferred to a PBS drop (1 μL) in a 0.5 mL PCR tube (Axygen Scientific, Inc., Union City, CA, USA) using a mouth-piece-controlled micropipette. The genomic DNA was extracted and purified as described previously [43]. Briefly, blastocysts were lysed in a solution containing proteolytic enzymes (Proteinase K and Pronase E) and extracted with phenol/chloroform. Finally, the DNA was ethanol-precipitated. The precipitated DNA was dissolved in 20 μL of sterile water. We then performed whole-genome amplification (WGA) was subsequently performed to increase the amount of whole genomic DNA using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Health Care Japan, Tokyo, Japan), as previously described [44].

Splinkerette-PCR was performed to map the PB integration sites according to the method of Potter and Luo [45] and based on the manufacturer's protocol (Splinkerette Protocol; http://www.cmhd.ca/protocols/genetrap_pdf/Splinkerette%20Protocol%20Single%20Clone.pdf#search =' splinkerette'). In addition, we have previously described the procedure in detail [33, 46]. Briefly, the WGA-treated genomic DNA (~2 μg) was first digested with *Sau* 3AI and subsequently ligated to a splinkerette adapter (generated by annealing HMSpAa and HMSpBb). The junctional fragments between genomic DNA and the adapter sequence were PCR-amplified using the first primer set (HMSp1 and PB-R-Sp1). Nested PCR was performed on the first PCR products using a nested primer set (HMSp2 and PB-L-Sp2). The resulting PCR products were cloned into the TA-cloning vector pCR2.1 (Invitrogen Co., Carlsbad, CA, USA). Next, the recombinant colonies were subjected to PCR using universal primers, M2 and RV, as described by Inada et al. [33]. The resulting PCR products (1 μL) were electrophoresed in a 2% agarose gel to estimate the size of each PCR product. The PCR products of over 300 bp were subjected to sequencing using the M2 or RV primer. Direct sequencing of PCR products was performed using a custom DNA sequencing service (Eurofins Genomics K.K., Tokyo, Japan). True PB integration sites were considered if the genomic sequences started immediately after the terminal TTAA at the end of the 3'-ITR sequence and matched at the

genomic locus with over 80% identity. Sequence similarity was analyzed using the BLASTN program (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE =BlastSearch).

2.5 Fluorescence Detection

A fluorescence microscope (BX60; Olympus, Tokyo, Japan) using DM505 (BP460–490 and BA510IF; Olympus) and DM600 filters (BP545–580 and BA6101F; Olympus) was used to detect EGFP-derived green fluorescence and RFD-derived red fluorescence, respectively, in CPI-treated parthenotes. Micrographs were acquired using a digital camera (FUJIX HC-300/OL; Fuji Film, Tokyo, Japan) attached to the fluorescence microscope. The images were printed using the Mitsubishi digital color printer (CP700DSA; Mitsubishi, Tokyo, Japan).

2.6 Statistical Evaluation

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test. A probability of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Preliminary Experiment for Testing the Fidelity of CPI in Porcine Parthenotes

To confirm the proper introduction into the egg cytoplasm *via* CPI, porcine parthenotes (6 h after electrical activation) were first subjected to CPI with a solution containing 2 μg/μL of RFD (Figure 2A). If CPI is performed appropriately, the injected eggs should exhibit RFD-derived red fluorescence in their cytoplasm. Parthenotes lacking fluorescence suggest failed CPI. Furthermore, possible mechanical damage to embryos during the process of CPI can be evaluated.

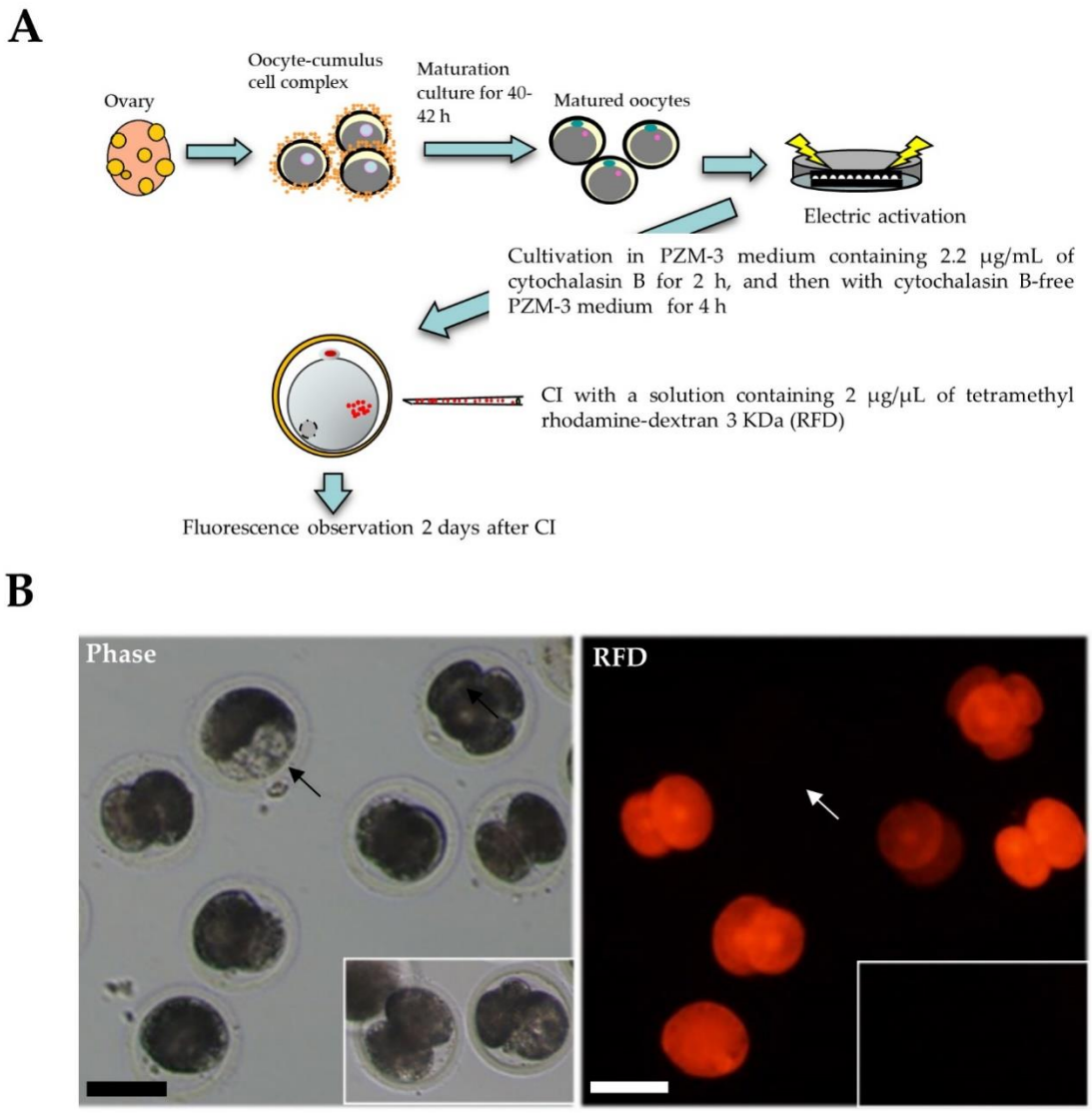

Figure 2 Cytoplasmic injection (CPI) of red fluorescent tetramethyl rhodamine-labeled dextran 3 kDa (RFD) as a valuable tool to evaluate the success of CPI. **A.** Schema for CPI using porcine parthenotes. Ovaries isolated from a slaughterhouse were subjected to isolation of the cumulus–oocyte complex (COC) and further processed to obtain mature oocytes. After electric activation, these oocytes (parthenotes) were subjected to CPI. The treated parthenotes were cultured for 2 days until the 2- to 4-cell stage in a drop (PZM-3 medium) under paraffin oil. The developing embryos were inspected for fluorescence under a fluorescence microscope. **B.** Fluorescence in the 2 to 4-cell stage embryos after CPI of RFD. Almost all embryos exhibited RFD-derived red fluorescence, indicating successful CPI. Certain embryos failed to show fluorescence (indicated by arrows), thereby indicating failure of CPI. Uninjected parthenotes exhibiting no fluorescence are shown in a box in the bottom-right corner of each image. Phase, a photograph acquired under light; RFD, an image captured under UV illumination. Bar: 100 μm.

When the evaluation was performed 2 days after CPI, approximately 32% (10/31) of parthenotes developed to the 2-cell stage. This was in contrast with the case of intact parthenotes, which were

81.2 ±5.3% on average ("Intact" in Table S1). A portion of the data is shown in Figure 2B. Ninety percent (9/10) of parthenotes exhibited RFD fluorescence; however, one parthenote failed to show RFD fluorescence (arrows in Figure 2B). The results indicate the feasibility of CPI and RFD as valuable marker. Thus, we decided to include RFD in the solution used for CPI in the following experiments.

3.2 CPI with pT-EGFP + Transposase mRNA + RFD as Exp

Before the experiment, appropriate concentrations of plasmid DNA and mRNA used for CPI were determined. In this case, we referred to conditions reported by Wang et al. [47] and Yu et al. [48], who used 20 to 125 ng/μL mRNA and approximately 20 ng/μL plasmid DNA for CPI in porcine embryos. Based on their reports, we used a solution containing 20 ng/μL plasmid-based vectors and 2 μg/μL RFD (in some cases, 66 ng/μL transposase mRNA) for CPI, as described in Table 1.

In the Exp group, CPI was performed to examine whether transposase mRNA could facilitate the expression (or chromosomal integration) of GOI (EGFP) from a PB transposon (pT-EGFP; Figure 1A) in the porcine parthenotes 6 h following the electrical activation as Exp (Figure 1B; Table 1). Next, the injected embryos were cultivated in the mPZM-3 medium under paraffin oil at 36.5 °C for 7 days (up to the morula/blastocyst stages). This experiment was repeated four times each day. When the evaluation was performed 2 days after CPI, certain RFD-positive cleaved embryos (33.8 ±44.9%) exhibited a faint green fluorescence (Figure 3; Table S1; Figure 4A-a–d). When assessed 7 days after CPI, the developmental rate to the morula/blastocyst stages was 15.7 ±1.6%, which was significantly lower than 40.9 ±22.7% in the case of intact parthenotes (Exp *vs*. Intact in Table S1). Of the RFD-positive morulae/blastocysts examined, 71.9 ±32.9% exhibited distinct green fluorescence (arrows in Figure 4B-a–d; Figure 3; Table S1). Certain developmentally arrested red fluorescent embryos exhibited no green fluorescence (arrowheads in Figure 4B-a–d).

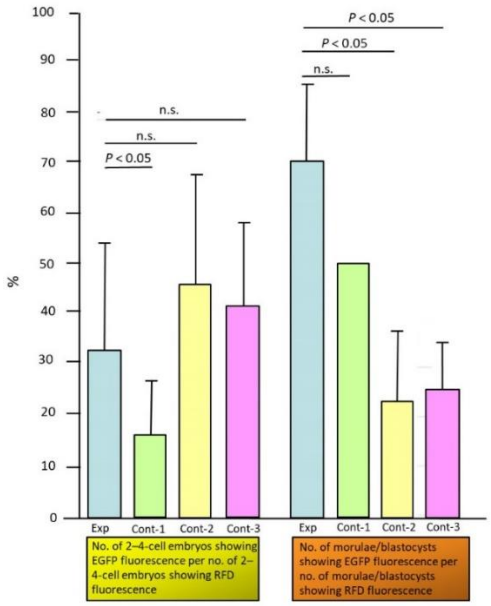

Figure 3 Summary of fluorescence performance after cytoplasmic injection (CPI). CPI was performed on porcine parthenotes 6 h following the activation and grouped into four groups (Exp, Cont-1, Cont-2, and Cont-3), as shown in Table 1. The rates (%) of green embryos per red embryos at the 2 to 4-cell or morula/blastocyst stage in each group are indicated in the bar graph. n.s., not significant.

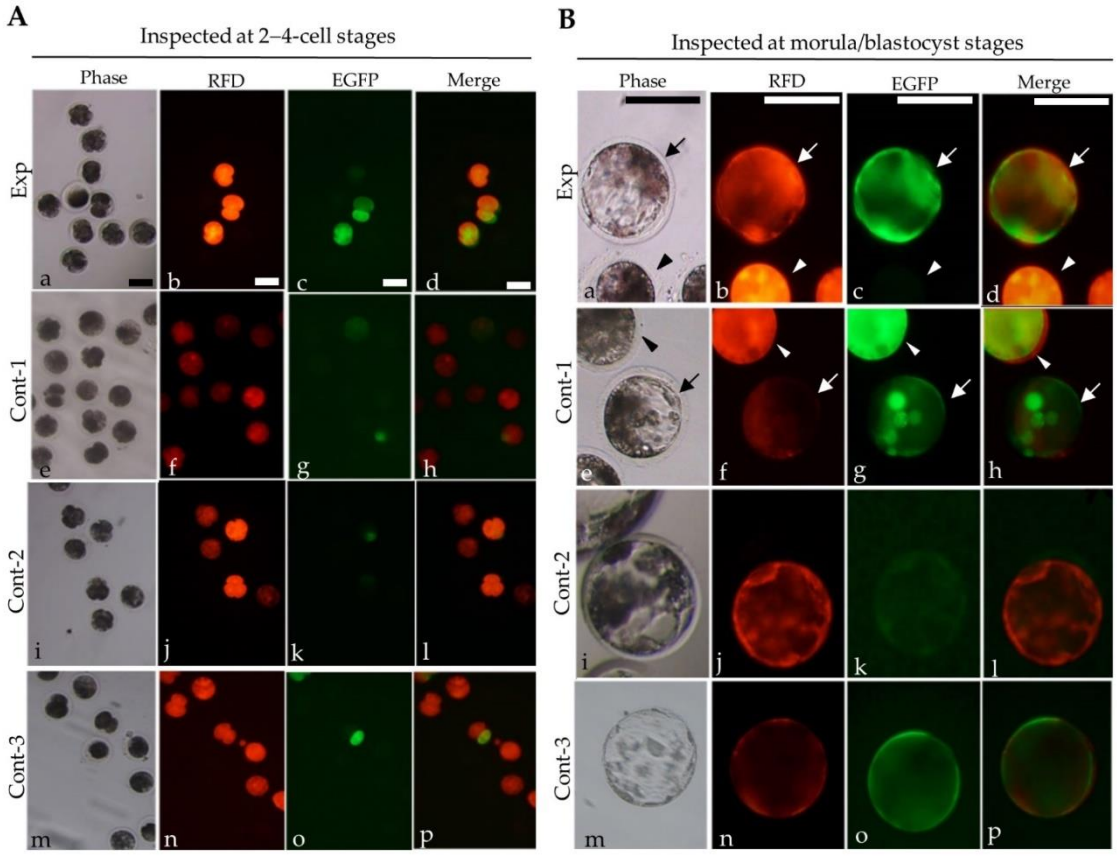

Figure 4 Fluorescence detection in porcine parthenotes after cytoplasmic injection (CPI) of reagents derived from the Exp, Cont-1, Cont-2, or Cont-3 groups shown in Table 1 and Figure 1B. **A.** Detection of fluorescence at the 2–4-cell stage. Note that there are embryos showing both types of fluorescence, derived from tetramethyl rhodamine-labeled dextran 3 kDa (RFD) and enhanced green fluorescent protein (EGFP), suggesting the occurrence of EGFP expression at earlier stages. Merge, both images from RFD and EGFP fluorescence were overlayed. **B.** Detection of fluorescence at the morula/blastocyst stages. Note that embryos showing both RFD and EGFP fluorescence are seen in each group; however, the rate is higher in the Exp group than in other groups (Figure 3, Table S1). Phase, photographs captured under the light; merge, both images from RFD and EGFP fluorescence were overlayed. Bar: 100 µm.

3.3 CPI with pT-EGFP + pTrans + RFD as Cont-1

Next, we examined whether similar events occurred when a PB transposase expression plasmid (pTrans) (Figure 1A) was cytoplasmically introduced together with pT-EGFP as Cont-1 (Figure 1B; Table 1). The injected embryos were treated using the same method detailed for the Exp group. The experiment was repeated four times each day. When inspected 2 days after CPI, certain RFD-positive embryos exhibited a faint green fluorescence (Figure 4A-e–h), similar to the Exp group. When inspected 7 days after CPI, 50.0 ±0.0% of the RFD-positive morulae/blastocysts exhibited a distinct green fluorescence (arrows in Figure 4B-e–h; Figure 3 and Table S1). This rate was lower than the rate (71.9 ±32.9%) observed in the Exp group; however, no statistical differences were

observed (Figure 3, Table S1). Certain developmentally arrested red fluorescent embryos in the Exp group exhibited a strong green fluorescence (arrowheads in Figure 4B-e–h).

3.4 CPI with pT-EGFP + RFD as Cont-2 or with pCE-29 + RFD as Cont-3

In Cont-1, 50% of the RFD-positive morulae/blastocysts exhibited EGFP fluorescence after CPI with pTrans + pT-EGFP + RFD, which could be attributed to two reasons. First, pTrans introduced cytoplasmically is transferred to the nucleus, following which the transposase mRNA is synthesized from the vector. Subsequently, the resulting mRNA is transferred to the cytoplasm and translated into a protein, which binds to pT-EGFP (still present in the cytoplasm) to form a complex. This complex is next transferred to the nucleus, whereby *EGFP* mRNA is synthesized. Second, *EGFP* mRNA is produced due to the direct transfer of pT-EGFP to the nucleus.

To check these probabilities, we performed CPI of the pT-EGFP alone as Cont-2 (Figure 1B; Table 1). The injected embryos were treated using the same method as that detailed for the Exp group. The experiment was repeated four times each day. When inspected 2 days after CPI, certain RFD-positive embryos exhibited faint green fluorescence (Figure 4A-i–l), as in the case of the Exp and Con-1 groups. Fluorescence inspection 7 days after CPI demonstrated that 20.8 ±25.0% of the RFD-positive morulae/blastocysts exhibited green fluorescence (Figure 4B-i–l; Figure 3; Table S1). Because this rate was not significantly different from that obtained for the Cont-1 group (20.8 ±25.0% *vs.* 50.0 ±0.0% in Figure 3 and Table S1), the green fluorescence observed in the RFD-positive morulae/blastocysts of Cont-1 was ascribed to the pT-EGFP-derived *EGFP* mRNA expression that was directly transferred to the nucleus.

Because pT-EGFP used in the Cont-2 group is a transposon plasmid, we next examined whether the CPI of the non-transposon vector pCE-29 (Figure 1A) also generated EGFP-derived fluorescence at the morula/blastocyst stages. CPI was repeated four times each day. When embryos were inspected 2 days after CPI, certain RFD-positive embryos exhibited a faint green fluorescence (Figure 4A-m–p), similar to that observed in the Exp, Con-1, and Cont-2 groups. On day 7 after CPI, 23.8 ±20.6% of the RFD-positive morulae/blastocysts exhibited green fluorescence (Figure 4B-m–p; Figure 3 and Table S1). Because this rate was not significantly different from that obtained for Cont-1 and Cont-2 (Figure 3 and Table S1), we attributed the green fluorescence in the embryos observed in the Cont-3 group to pCE-29-derived *EGFP* mRNA expression that was directly transferred to the nucleus.

3.5 Chromosomal Integration of GOI as Revealed by Splinkerette PCR Analysis

To analyze the possible PB-mediated insertion of pT-EGFP transposons (carrying an EGFP expression unit as GOI) into the porcine genome, we isolated the genomic DNA from a single blastocyst for Splinkerette-PCR, allowing the amplification of the targeted genomic sequences in conjunction with the ITRs on both sides of the transposon [45].

The genomic DNA from eight blastocysts (four for Exp, two for Cont-1, and two for Cont-2) was first subjected to WGA. Next, the amplified genomic DNA (approximately 2 μg) was digested with the *Sau* 3AI enzyme and ligated to a splinkerette adapter. After serial PCR using specific primers, we collected bands of over 300 bp from PCR products containing insertion site fragments of the genomic DNA using 2% agarose gels. After purification, the isolated fragments were sub-cloned into a TA-cloning vector, and blue–white colonies were selected using X-Gal as the substrate. We used

the resulting plasmid DNA to perform PCR using specific primers (M2 and RV) to amplify the insert. Therefore, we obtained 42 clones from four single blastocysts (called Exp-1 to -4) in the Exp group, 25 clones from two single blastocysts (called Cont-1–1 and -1–2) in the Cont-1 group, and two single blastocysts (called Cont-2–1 and -2–2) in the Cont-2 group (Figure 5). Several clones with inserts of different sizes (range 250–400 bp) were identified, which were subjected to direct sequencing using the M2 primer.

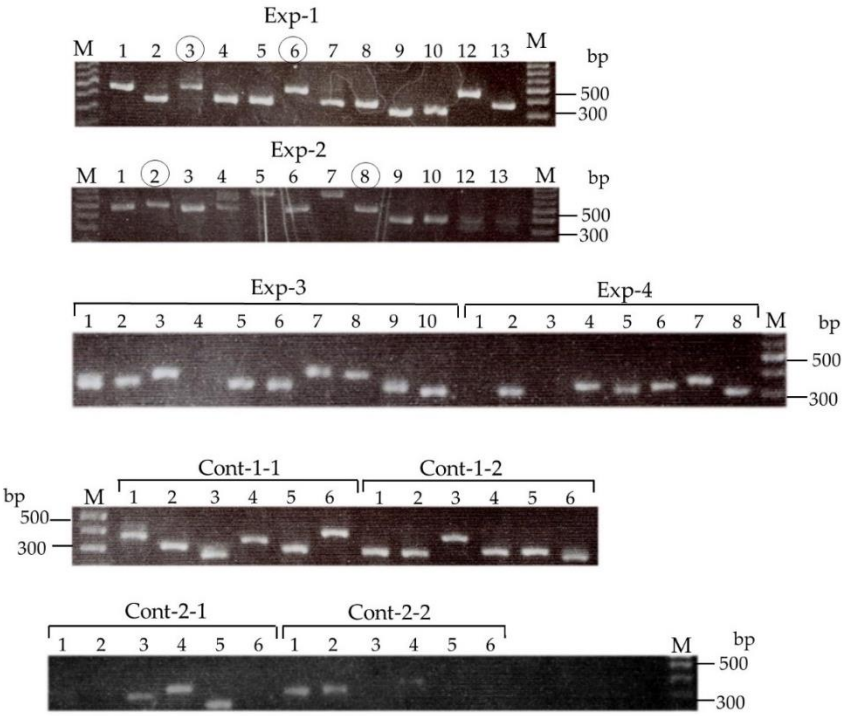

Figure 5 Gel electrophoresis of PCR-amplified products used for Splinkerette-PCR analysis. Genomic DNA isolated from a single blastocyst was first subjected to WGA. Next, the amplified DNA was subjected to Splinkerette-PCR analysis. After digestion with *Sau* 3AI and subsequent ligation to a splinkerette adapter, the first PCR was performed using a primer set (HMSp1 and PB-R-Sp1). Next, nested PCR was performed using the first sample with the nested primer set (HMSp2 and PB-L-Sp2). After insertion of the resulting PCR products into the pCR2.1 vector, the recombinant miniprep DNA was subjected to PCR using the universal primer set (M2/RV) before electrophoresis in 2% gels. After gel electrophoresis, the gels were stained with ethidium bromide. Samples indicated by * in Exp-1 or ** in Exp-2 are identical clones with the junctional sequence between the transposon and porcine genomic sequences. Other clones without any symbols are those with irrelevant sequences. Abbreviations: Exp-1 to -4, single blastocysts from Exp group; Cont-1–1 to -1–2, single blastocysts from the Cont-1 group; Cont-2–1 to -2–2, single blastocysts from Cont-2 group; M, 100-bp ladder markers.

A summary of sequencing results is provided in Table 2. In the Exp group, two clones (called Exp-1–3 and -2–2) from Exp-1 and -2, respectively, contained portions of the porcine genome sequences that had been inserted into the gene coding for dynamin-3-like (for Exp-1–3) on chromosome 9 and a gene coding for GDP-mannose 4,6-dehydratase (for Exp-2–2) on chromosome 7 (Table 2). All clones were identified as authentic clones possessing the desired consensus sequence (TTAA) and

the subsequent host (porcine) chromosomal sequence (Table 2). However, no clones with portions corresponding to the porcine genome sequences were observed when clones from Exp-3 and -4, Cont-1–1 and -1–2, and Cont-2–1 and -2–2 were examined.

Table 2 Splinkerette-PCR analysis.

Clone	Sequence (5'-3')[1]	Known sequences showing similarity[2] to endogenous porcine genome
Exp-1–3	**TAGGG**TTAAACAGATTGTTTATCTTCCTCCAG CGAGCACAAAACGCCATGCCGAAATGGGAAC CAGATTTTTCTACTCAGTGAACTCCGTGTGGTT TCCCCATTTCTCCTGGC	NC_010451.3 *Sus scrofa* breed mixed chromosome 9, Sscrofa10.2; dynamin-3-like; located on chromosome 9; identities = 88/108 (81%)
Exp-2–2	**ACAGT**TTAATGGGGCTTTGAAGTGGCCGGAC AGCTTGAGCGCATCTATAAATTGCCACAAACA GCCCTCTGACTGAGTTAGCTAATTGCTTGCTCT TTGACTTATT	NC_010449.4 *Sus scrofa* breed mixed chromosome 7, Sscrofa10.2; GDP-mannose 4,6-dehydratase; located on chromosome 7; identities = 92/101 (91%)

[1]Sequences that are similar to TTAA are shadowed. Sequences corresponding to the end of ITR are highlighted in bold.

[2]Sequence homology analysis was performed using the BLASTN program (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE =BlastSearch). Sequence similarity is shown below as a percentage (number of nucleotides from the query per number of nucleotides from the known gene) in parentheses.

4. Discussion

The production of Tg animals is challenging after CPI of linear or circular plasmid DNA into a zygote [18]. The production of Tg animals requires the insertion of a transgene into a chromosome. PB-based gene delivery systems enable efficient chromosomal integration of a GOI when a transposase expression vector DNA and transposon DNA (carrying GOI) are co-introduced [31, 32]. In this case, transposase mRNA, rather than a transposase expression vector DNA such as pTrans, could increase the efficiency of chromosomal integration because mRNA can be rapidly translated into protein in the egg cytoplasm (left column of Figure 6). In contrast, the introduction of a transposase expression plasmid DNA causes delayed expression of its protein because the introduced plasmid DNA is first incorporated into the nucleus for mRNA synthesis. Next, the resulting mRNA is transferred to the cytoplasm for protein synthesis (right column of Figure 6). This delay (starting from mRNA synthesis to protein synthesis) could be a major cause of the poor chromosomal integration efficiency of the GOI. To avoid this delay, the use of the PB transposase protein, instead of the PB transposase mRNA, can lead to significantly better results in achieving increased chromosomal integration rates of GOI following CPI because the former more readily catalyzes the transposon than the latter.

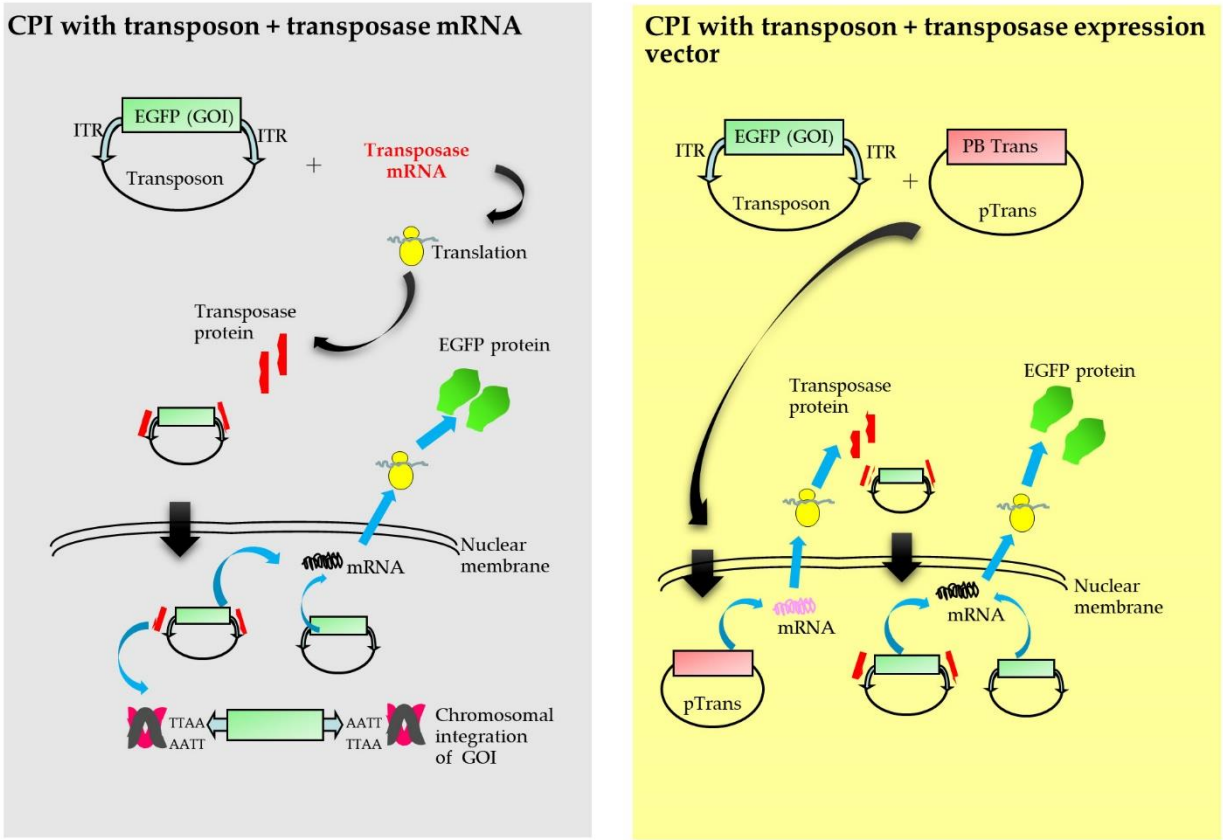

Figure 6 Possible mechanism showing that the chromosomal integration of a gene of interest (GOI) occurs more frequently in embryos injected with *piggyBac* (PB) transposase mRNA + PB transposon (pT-EGFP) than those injected with PB transposase expression plasmid (pTrans) + pT-EGFP. As shown in the left column, the transposase mRNA introduced into the cytoplasm of an oocyte is rapidly translated into protein in the egg cytoplasm, resulting in the rapid formation of a complex between transposase protein and pT-EGFP existing in the cytoplasm. The accumulated complex, in turn, can integrate the GOI (EGFP) into the host chromosome. In contrast, the cytoplasmic introduction of pTrans delays its protein expression because the introduced plasmid must first be incorporated into the nucleus for mRNA synthesis. Next, the resulting mRNA is transferred to the cytoplasm for protein synthesis (as shown in the right column). This delay (starting from mRNA synthesis to protein synthesis) is believed to be a major cause of the poor chromosomal integration efficiency of the GOI.

We used the mRNA for Super PB transposase. This is an engineered hyperactive version of the enzyme that recognizes ITRs and efficiently integrates DNA of any size into the genome at TTAA sites [49]. Increased rates of chromosomal integration of GOI following CPI with transposase mRNA and transposon (but not with a transposase expression vector and transposon) could be partly ascribed to using Super PB transposase mRNA.

Previous studies have successfully conducted transposon-based gene delivery to embryos using SB and Tol2 (a transposon derived from fishes) *via* CPI of a combination of a plasmid DNA (encoding Trans) and a transposon DNA (carrying GOI) to generate Tg animals from bovines [50], pigs [51-53], sheep [50], and mice [54]. For example, Garrels et al. [51] co-injected a plasmid DNA coding for SB-

based hyperactive Trans called SB100X and a transposon plasmid DNA into the cytoplasm of porcine zygotes and obtained Tg fetuses and piglets with high efficiencies (7.5%; 9/120 of transferred embryos). The resulting Tg animals exhibited normal development and persistent expression of the reporter gene for > 12 months. In addition, they demonstrated germ-line transmission, segregation of individual transposons, and copy number-dependent transgene expression in the F1 offspring. Similarly, Li et al. [53] constructed an all-in-one plasmid (called pmGENIE-3) carrying both the PB transposase expression unit and the EGFP transposon and microinjected it into the cytoplasm of porcine parthenotes. They observed that 88.8% (8/9) of the injected embryos (blastocysts) exhibited EGFP-derived fluorescence. Li et al. [53], reported that pmGENIE-3 injected cytoplasmically was first transferred to the nucleus, followed by the production of PB transposase mRNA from the plasmid DNA. The resulting mRNA was later translated into the protein in the cytoplasm, which consequently bound to the ITR of pmGENIE-3 present in the cytoplasm, leading to transposon (pmGENIE-3) excision called "self-inactivation." In contrast, a portion of these transposase/transposon complexes was inserted into the genome of the host embryos *via* the TTAA sequence. Furthermore, Li et al. [53] confirmed that 8% Tg piglets were produced when the fertilized porcine eggs injected with pmGENIE-3 DNA were transferred to the eggs of recipient female pigs. This system appears to be similar to our system as it is based on the CPI of pTrans and pT-EGFP. Unfortunately, in our study, the co-injection of those components into the cytoplasm of porcine parthenotes failed to generate Tg blastocysts because none of the two green blastocysts (in the Cont-1 group) subjected to the Splinkerette-PCR analysis exhibited chromosomal integration of the GOI.

The efficacy of the transposase mRNA to cause chromosomal integration of the GOI included in a transposon has been reported in several other transposon systems such as SB and Tol2 in rabbits [55, 56], mice [55, 57-59], Xenopus [60], and zebrafish [61-66]. For example, Sumiyama et al. [58] injected a foreign DNA cloned in a Tol2-transposon vector and the transposase mRNA into the cytoplasm of fertilized eggs. The foreign DNA was transposed from the plasmid to the genome and was efficiently transmitted to the next generation. The overall Tg efficiency was more than 20%, which contrasts with only approximately 3% when transgenesis using a PNI-based standard protocol was used.

Unlike successful transgenesis using the SB or Tol2-based transposon system, little is known about the production of Tg rats [67] and mice [68] through CPI with PB transposase mRNA and PB transposon DNA. Therefore, we examined the feasibility of using the CPI approach to efficiently generate Tg parthenotes using a PB-based gene delivery system. This is because Suzuki et al. [68] demonstrated that CPI with a hyperactive PB transposase (hyPBase) mRNA and transposon DNA (carrying a gene coding for a monomeric red [orange] fluorescent protein called TagRFP as the GOI) into mouse zygotes resulted in the expression of TagRFP fluorescence in the embryos at the two-cell stage and onward. For instance, 94.4% of blastocysts were TagRFP positive when 30 ng/μL transposon DNA and 30 ng/μL hyPBase mRNA were co-injected. In agreement with the findings of Suzuki et al. [68], we obtained a high expression of GOI in porcine parthenotes when 20 ng/μL transposon DNA (pT-EGFP) and 66 ng/μL of transposase mRNA were subjected to CPI. For example, the rate of the morula/blastocyst stage showing EGFP in the Exp group (co-injection of transposase mRNA + pT-EGFP) was approximately 1.4-fold higher than that in the Cont-1 group (co-injection of pTrans + pT-EGFP) (approximately 72% *vs.* 50%; Figure 3; Table S1). Moreover, we observed successful EGFP expression in RFD-expressing blastocysts with 100% efficiency (3/3 and 3/3) in at least two cases of the Exp group. Similar findings were observed for other control groups

(approximately 72% *vs.* 21% for Cont-2 [injection of pT-EGFP alone] and approximately 72% *vs.* 24% for Cont-3 [injection of pCE-29 alone]) (Figure 3; Table S1).

PB enables the efficient chromosomal integration of GOI into the host genome. To confirm the chromosomal integration of the GOI in morulae/blastocysts showing both RFD- and EGFP-derived fluorescence, we employed Splinkerette-PCR [45], a PCR-based amplification system indicating the junctional site through which the transposon is inserted into the host genome *via* the TTAA consensus sequence. We have previously identified the significance of this system applied to cultured cells [33, 69], porcine parthenotes [46], and *in vivo* tissues (pancreas) [70]. We found that in the Exp group, two of the four (50%) fluorescent blastocysts exhibited successful chromosomal integration of the GOI, whereas blastocysts in Cont-1 and -2 did not. These findings suggest the potential of CPI with PB transposase mRNA and PB transposon DNA for efficient generation of Tg parthenotes. However, the PB-based gene delivery system has certain limitations, such as uncontrolled integration of transgenes into multiple sites of host chromosomes of the oocyte. Furthermore, potential off-target effects occurring during transposase-mediated integration of the GOI in chromosomes could influence the viability of pigs.

We found that in the intact group in which no CPI was performed, the developmental rate of parthenotes into blastocysts was higher than that in the Exp or Cont group (Table S1). This suggests that our CPI procedure was deleterious for the embryonic development of porcine parthenotes. Li et al. [53] reported that developmental rates of cleavage or the blastocyst stage in CPI groups were lower than those in the intact (no injection) group. However, significant differences were not observed between the two groups. Moreover, they successfully generated viable piglets, of which approximately 8% were identified as Tg. This finding encourages us to continue our attempt toward the production of Tg piglets.

5. Conclusions

We assessed whether cytoplasmic co-injection of PB transposase mRNA and PB transposon DNA generated transgenic embryos (morulae/blastocysts) harboring the GOI (EGFP) in their genome. The Splinkerette-PCR analysis demonstrated that GOI was chromosomally integrated into two of the four green fluorescent blastocysts. In contrast, none of the green fluorescent blastocysts in the control groups (co-injection of a transposase expression plasmid + transposon or transposon alone) showed chromosomal integration of GOI. These findings suggest that transposase mRNA could be efficiently employed for PB-based transgenesis in pigs using the cytoplasmic co-injection strategy.

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Author Contributions

M.S. conceived and designed the study and drafted and revised the manuscript. R.M. performed the experiments, especially CPI of nucleic acids into parthenotes, molecular analysis of single morulae/blastocysts, and statistical analysis. H.J. performed the experiments, especially the

acquisition of parthenotes, *in vitro* EP, and embryo cultivation. S.W., E.I., and K.M. critically revised the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Additional Materials

The following additional material is uploaded at the page of this paper.

1. Table S1: Summary of the developmental rates and fluorescence performance after cytoplasmic injection (CPI)[1] in porcine parthenotes.

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