Application of Transposon Systems in The Transgenesis of Bovine Somatic And Germ Cells

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Abstract

Background: Several DNA transposons, PiggyBac (PB), Sleeping beauty (SB) and Tol2 have been applied as effective means for transgenesis in many species. Cattle are not typical experimental animals, and relatively little verification has been studied in this species. Thus, the goal of this study was the applicability of three transposon systems in somatic and embryo cells in cattle, while also determining which of the three systems is appropriate for each type of cell. To conduct the experiment, green fluorescent protein (GFP)-expressing transposon systems were used for electroporation and microinjection in the somatic cells and embryo stage, respectively. After transfection, GFP-positive cells or blastocysts were observed through a fluorescent microscope and transfection efficiency was calculated by FACS.

Results: In the bovine somatic cells experiment, the PB (63.97 ± 11.56) showed higher efficiency as compared to the other two systems (SB: 50.74 ± 13.02 and Tol2: 16.55 ± 5.96). Unlike the results of the somatic cells, Tol2 (75.00%) and SB (70.00%) in the embryo were more efficient as compared to PB (42.86 %).

Conclusions: These results demonstrate that all three transposon systems can be used in bovine somatic cells and embryos as a gene engineering experimental method and which type of transposon system is appropriate to apply depending on the cell type.

Highlights

• Non-viral methods for genome integration of gene-of-interest.

• Potential of Tol2 transposon system for bovine somatic cell and embryo transfection.

• PiggyBac transposon system for electroporation-mediated transfection in somatic cell.

• Sleeping Beauty and Tol2 systems for microinjection-mediated transfection in embryo.

• Applicability of three transposon systems in transgenesis of bovine cells and embryo.

Background

Genomic engineering approaches, such as transgenesis are largely divided into viral and non-viral vector methods. Although viral methods have the advantage of high transfection efficiency, disadvantages include immunogenic and oncogenic side effects as well as limited vector capacities. On the other hand, non-viral methods can provide a relatively low immune response and allow for the introduction of a moderately larger exogeneous DNA, although they exhibit lower transfection efficiency compared to viral methods (1, 2). DNA transposons used in these non-viral vector methods are mobile jumping genes that account for a large portion of the genome of mice and human, as well as rats, E. coli, plants like maize, and zebra fish (3–5). Transposons have been continuously used as non-viral gene-editing methods for

integration into the genome by recognizing specific sequences by corresponding transposases according to the type of transposon. In this DNA transposon integration step, transposases bind to Terminal Inverted Repeats (TIRs) and cut-and-paste to specific target sequences (6). The transposon method also allows stable expression of the introduced exogenous genes by causing random site preferred integration in genomes and with stability in favor of low-risk chromosomal intron integration (6, 7).

Among the transposon systems, PiggyBac (PB), Sleeping beauty (SB) and Tol2 have been used as three main transposons in vertebrates (8–12). These three transposon methods have been validated in several species, such as rodents, zebra fish, and human cells, and have been mainly used in studies related to overexpression and therapeutic approaches (8, 13–15). However, even though the cows have 80% genome consistency and they can be used as a potential alternative model for human disease research and unlike rodents, cattle show low Alternative Splicing (AS) which is a critical process for changing the genomic instruction into functional proteins, application of those transposon systems have not been well investigated in large animals (16). Accordingly, in this study, we investigated which transposon could effectively deliver and integrate transgenes to bovine somatic cells or embryos. As such, these methods may be applied to produce genetically modified cattle models in the future.

Results

3.1. Delivery of transposon systems to bovine somatic cells by electroporation

To know the initial and integration transfection efficiency of the three transposons, GFP expression ratio was analyzed by FACS on Day 3 and Day 10 after transfection, respectively. FACS analysis on Day 3 after transfection showed significant differences in PB as compared to SB and Tol2 systems showing high transfection efficiency (Fig. 2B, PB: 98.37 ± 1.29, SB: 63.43 ± 13.84, and Tol2: 61.57 ± 5.68, $p < 0.05$) and even in the re-sorting results on Day 10 after transfection, integration efficiency showed higher efficiency in order of PB, SB and Tol2 (Fig. 2C, PB: 63.97 ± 11.56, SB: 50.74 ± 13.02 and Tol2: 16.55 ± 5.96, $p < 0.05$).

3.2. Application of transposon systems in bovine embryo cytoplasmic microinjection

After microinjection of two different DNA concentrations (50 ng/μL vs 25 ng/μL), there were no significant differences in developmental competence at 8-cells and blastocysts formation (Table 1). In terms of total cell number in all the blastocysts derived from microinjection, no differences were found (Table. 1). However, the ratio of GFP expression in blastocysts at 50 ng/μL of SB microinjection group (66.70%) was higher than the other two transposons (Tol2: 47.80%, PB: 35.30%) although there was no significant difference. In the 25 ng/μL microinjection trial, SB (70.00%) and Tol2 (75.00%) showed higher efficiency than PB (42.86%) (Table 1).

Table 1
Cytoplasmic microinjection efficiency of transposon systems in bovine embryos

Concentration	IVM	Microinjection	IVC			
High	No. COCs	Condition	No. 8-cells (%)	Total Blastocysts (%)	GFP Expressing Blastocysts (%)	Total Cell Number
	122	Wild type	79 (64.8)	41 (33.6)	0 (0)	92.64 ± 23.69
	130	PiggyBac	60 (46.2)	17 (13.1)	6 (35.3)	89.20 ± 14.82
	130	Sleeping beauty	51 (39.2)	15 (11.5)	10 (66.7)	86.40 ± 23.52
	130	Tol2	67 (51.5)	23 (17.7)	11 (47.8)	96.00 ± 18.58
Low	No. COCs	Condition	No. 8-cells (%)	Total Blastocysts (%)	GFP Expressing Blastocysts (%)	Total Cell Number
	78	Wild type	51 (65.38)	21 (26.92)	0 (0)	100.00 ± 33.08
	77	PiggyBac	31 (40.26)	14 (18.18)	6 (42.86)	95.50 ± 25.03
	77	Sleeping beauty	30 (38.96)	10 (12.99)	7 (70.00)	102.00 ± 28.58
	77	Tol2	31 (40.26)	15 (15.58)	9 (75.00)	100.78 ± 24.99

* High concentration : 50 ng/μL of Transposon and transposase, Low concentration : 25 ng/μL of Transposon and transposase.

DNA concentrations were described as High and Low (High : 50 ng/μL, Low : 25 ng/μL). The percentage of GFP expressing blastocyst was calculated as the number of GFP expressing blastocysts out of the total number of blastocysts. IVM: In vitro maturation.

IVM = In vitro maturation; IVC = In vitro culture; COC = cumulus-oocyte complex

Discussion

In this study, electroporation was used with three transposon systems to determine whether each could be used as a stable gene engineering tool in bovine somatic cells. After verification in the somatic cell, embryo microinjection was performed to verify that these systems worked reliably in germline cells. First, in the bovine somatic cell transfection experiment, it was difficult to guarantee high efficiency and survival using primary cultured somatic cells, and not immortalized cell lines (17). For this reason, methods of introducing viruses such as adeno-associated virus (AAV), retrovirus, and lentivirus were

implemented. However, this viral method had a major disadvantage of causing side effects such as triggering an immune response and tumor formation, as well as difficulty in accompanying large plasmids (18). Our study shows that each transposon system could be applied in the introduction of genes using electroporation methods as an alternative to the viral methods. In addition, PB-based transfection showed high gene transfection and integration efficiency despite the delivery of genes in primary cells.

In the embryo experiment, cytoplasmic microinjection was utilized to deliver exogenous transposon system plasmids. Because cytoplasmic microinjection is easy to perform and shows higher embryo viability than pronuclear microinjection, direct damage to the nucleus is avoided. Moreover, most livestock animal zygotes such as pig, sheep and cattle are composed of a high composition of fatty acids that makes the cytoplasm dark, so makes it difficult to find pronuclear (19, 20). Here, the injection concentration was divided into high and low concentrations to assess the transposon systems at germ cell stage. The injection efficiency changed greatly depending on the concentration. In follow-up experiments, optimization of the concentration will be performed, according to each transposon system when conducting the bovine cytoplasmic injection. Contrary to the results in the somatic cell, SB and Tol2 showed a higher frequency of GFP expression than the PB system, likely due to the fact that the somatic cell and germ line cell are distinctly different cells, as well as the different methods of transfection - electroporation of the somatic cell and microinjection of the germ cell (21). Furthermore, it was difficult to determine significant differences between the embryo microinjection tests due to the quality of ovaries received for each experiment.

The vectors applied in the transposon experiments in our study did not exceed 10 kb. In further studies, it will be necessary to investigate whether transposon vectors longer than 10 kb affect the efficiency in each transposon system (22). Moreover, the transposon is also a method of introducing exogenous coding sequences to randomly integrate into genomes. Therefore, future research requires the study of the number of copies into external genes, introduced genetic loci, stability, and changes in genes associated with cellular and embryonic development as well as gene introduction and integration efficiency.

Furthermore, each transposon recognizes a particular target sequence during random integration into the genome. In the case of PB, SB, and Tol2, the three transposon systems used in this study, integration occurs by recognizing TTAA, TA and heterogenic sequences of 8 bp in length, respectively (6). As such, various characteristics are divided according to the type of transposon. Previous in vivo experiments in our laboratory have produced transgenic cattle using PB and SB and have shown germ line transmission for more than 6 years without health problems (23). The embryo results of this experiment showed that not only PB and SB, but also Tol2, are highly efficient and Tol2 can be used as an alternative to PB and SB.

Double or sandwich transposon methods were discovered to make more efficient and powerful transposon systems. In further studies, development of three fusion transposon systems could be a key

factor in efficient DNA delivery, particularly the PB-SB-Tol2 fused form of transposon, which could compensate for the shortcomings of each transposon alone (24, 25). In conclusion, these data demonstrated that all three transposon (PB, SB, and Tol2) showed stable expression of exogenous genes without silence in long-term culture. On a practical level, we suggest that PB is preferred for gene delivery and SB for embryonic levels for bovine genomic studies.

Conclusion

As cow showing 80% genome competency with human and low Alternative Splicing (AS), It is suitable as human disease and transgenesis model. In this paper, mainly used three transposon systems (PB, SB and Tol2) in other species were applied for non-viral genome integration method in bovine somatic and germ cells. In conclusion, our findings indicate all systems have possibility of application not only bovine somatic but germ cells and which transposon is the appropriate method depending on cell type.

Methods

2.1. DNA construction

In previous studies, the transposon system vectors for SB (pCMV(CAT)T7-SB100X) and PB (pCy43 and PB-CA) were purchased from Addgene (http://www.addgene.org, Plasmid #34879 and Plasmid #20960) and/or provided by the Sanger Institute (Hinxton, UK) for the PB and SB systems (26, 27). To newly establish the Tol2 system, transposon and transposase were purchased from Addgene (http://www.addgene.org, Plasmid #97151 and Plasmid #31823). To avoid the backbone sequences effect in application of the transposon systems, all transposon element sequences were amplified by PCR and then PCR products were run for 15 min and extracted by using a Qiagen Gel extraction Kit (Cat No. 28704). Extracted PCR products were cloned with a Qiagen TA cloning kit (Cat No. 231124) (Fig. 1A). All reconstructed vectors were sequenced fully.

2.2. Primary cell culture

For preparation of bovine somatic cells, three types of ear skin were isolated from newborn calves using biopsy punch. After isolation, tissues were collected directly to 50 mL conical tube containing 5 mL of 10% penicillin in PBS to prevent contamination and moved to bench in 3 hours. The tissues were washed 3 times with 10% penicillin-PBS again and minced on 100 mm petri dishes (Falcon, Cat No. 351029) for 5 min and collected in 15 mL conical tubes(SPL, Cat No. 50015) with 5 mL of 10% penicillin-PBS. After centrifugation at 13,000 rpm for 3 min, the supernatant was suctioned off and then the pellet was washed with 5 mL of 10% penicillin-PBS. After 3 times of the centrifugation to washing step, the pellet was suspended with 10 mL of HBSS containing 1% collagenase and incubated at 37°C, 5% CO2 for 17 hrs. After that, samples were centrifuged at 13,000 rpm for 3 min and the pellet was suspended with DMEM (HyClone, Cat No. SH30243.01, USA) containing 20% FBS (Gibco, Cat No. 26140079,USA). The resuspended samples were seeded on 60 mm dish for further cell culture.

2.3. Cell transfection (Electroporation) and FACS sorting

Three types of primary cells were used for electroporation-mediated DNA transfection by using Neon® Transfection system (Invitrogen Cat No. MPK5000). Cell Countess II Automated Cell Counter (Thermo Fisher Scientific) was used to count 3 × 105 cells for transfection. Transfection was replicated 3 times per cell type and transfection conditions were optimization no.16 (1400 V, 20 ms and 2 pulses). Post transfection, transfected cells were seeded directly on incubated 6-well plates containing 3 mL of DMEM. Seventeen hours later, culture media was changed with fresh media to remove dead cells (Fig. 1B).

FACS analysis was conducted to measure the transfection efficiency and integration. To measure the transfection efficiency, sorting was conducted through FACS after 3 days of transfection (Fig. 2A. [a]) and for integration efficiency, sorted cells on day 3 were sub-cultured for 7 days more and re-sorted on day 10 (Fig. 2A. [b]). To conduct FACS sorting, transfected cells were suspended with 500 µL of PBS and the samples were analyzed on BD Bioscience FACS Aria II, installed at the National Center for Inter-university Research Facilities (NCIRF) at Seoul National University.

2.4. In vitro maturation of bovine immature oocytes

Ovaries were collected from a local slaughterhouse within 2-3 h and carried in saline solution 0.9% at 30-35°C during transport to the laboratory. Cumulus–oocyte complexes (COCs) from follicles 2 to 8 mm in diameter were aspirated using an 18-gauge needle, selected, and collected in a 10-cm petri dish. The residue was washed three times with HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM NaHCO3 (Sigma–Aldrich Corp., St. Louis, MO, USA), 10% FBS and 1% penicillin–streptomycin (v/v). For in vitro maturation, COCs were cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton-Dickinson Ltd., Plymouth, UK) for 22–24 h in 450 µL TCM-199 supplemented with 0.005 AU/mL FSH (Sigma–Aldrich), 10% FBS, 1 µg/mL 17β-estradiol (Sigma–Aldrich) and 100 µM Cysteamine (Sigma-Aldrich) at 39°C under 5% CO2.

2.5. Sperm preparation, in vitro fertilization (IVF) and in vitro culture of embryos (IVC)

The Percoll gradient method for the separation and purification of motile spermatozoa has been described in detail elsewhere (28). In brief, spermatozoa were refined from thawed semen straws by density-gradient centrifugation on a Percoll discontinuous gradient (45–90%) at 1680 rpm for 15 min. For the Percoll density gradient, the 45% Percoll solution was prepared with 1 mL of 90% Percoll (Nutricell, Campinas, SP, Brazil) and 1 mL of capacitation-TALP (Nutricell) (29). and then 1 mL of 45% Percoll solution was added onto 1 mL of 90% Percoll solution in a 15 mL conical tube. The thawed semen was layered onto the top of the Percoll gradient solution and the tube was centrifuged. The pellet was washed twice with 3 mL of TALP by pipetting briefly and centrifugation for 5 min at 1680 rpm. The active, motile spermatozoa from the pellet were added to droplets containing matured oocytes. Oocytes were inseminated on (day 0) with 1–2 × 106 spermatozoa/mL for 17 h in IVF-TALP medium (Nutricell) under NidOil (Nidacon). The fertilized zygotes were denuded and cultured in two-step defined culture medium (4 days in D1 medium before transfer to D2 medium) at 39°C in an atmosphere of 5% O2, 5% CO2 and 90%

N2 (30). Cleavage rates were recorded on Day 4 and embryonic development was monitored according to the stages of the International Embryo Transfer Society (IETS).

2.6. Microinjection

Microinjection experiments were conducted through three different transposon systems to see whether the stable transposon systems in somatic cells could be used at the bovine embryo stage. After 17 h in IVF, denuded zygotes were used for microinjection (Femtojet®, Eppendorf, Germany). To find out the optimal microinjection conditions, 2 different DNA concentrations were assessed (High: 50 ng/μL, Low: 25 ng/μL). Six days later, GFP-expressing blastocysts were observed with a fluorescent microscope and stained with Hoechst 33342 (Sigma Aldrich) to count total cell number in blastocysts (Fig. 1C, Fig. 3, and Table 1).

2.7. Statistical Analysis

Data obtained from each of three replicated experiment results were statistically analyzed using One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com, The results were considered statistically significant when the p value was equal to or lower than 0.05.

Abbreviations

PB : PiggyBac

SB : Sleeping beauty

GFP : green fluorescent protein

FACS : Fluorescence-activated cell sorting

TIRs : Terminal Inverted Repeats

AS : Alternative Splicing

AAV : adeno-associated virus

PBS : phosphate-buffered saline

DMEM : dulbecco's modified eagle's medium

COCs : Cumulus–oocyte complexes

IVM = In vitro maturation

IVF : in vitro fertilization

IVC : in vitro culture

Declarations

- **Ethics approval and consent to participate**

All animal care and experiments were approved by the Institutional Animal Care and Use Committee(#SNU-180403-1) and performed under the guideline of Seoul National University.

- **Consent for publication**

‘Not Applicable’

- **Availability of data and materials**

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

- **Competing interests**

This study was financially supported by the National Research Foundation of Korea (2017R1A2B3004972), the Research Institute of Veterinary Science, and the BK21 PLUS Program for Creative Veterinary Science Research, and a SNU-Grant (#550–2020005).

- **Funding**

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- **Authors' contributions**

D.H.K. carried out the main experiments i.e. DNA construction, cell transfection and Microinjection. G.M.G., K.H.E. and J.H.L. helped in the collection of samples and experiment assistantce. i.e. primary cell culture, oocyte collection, hormones and culture media preparation. G.J. attended and supervised all procedures, participated in the design of the study, drafted the manuscript, and gave final approval of the version to be published. All authors read and approved the final manuscript.

- **Acknowledgements**

‘Not Applicable’

- **Authors' information (optional)**

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Figures

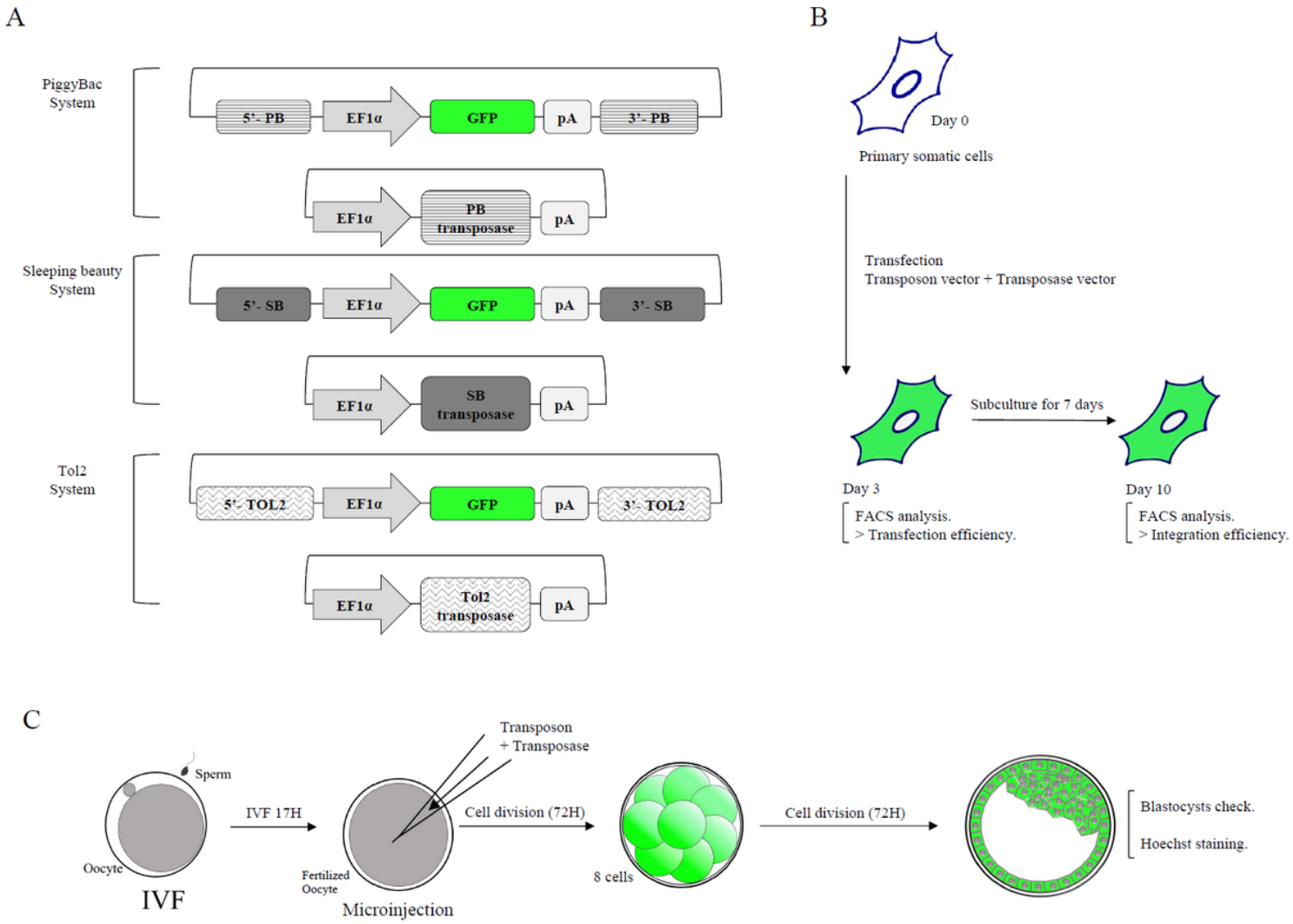

Figure 1

Schematic design for application of transposon systems (PB, SB, and Tol2) in bovine somatic and germ cells. A) Illustration of transposon DNA composition including Ef1α promoter and GFP reporter gene, B) Transfection and analysis method for somatic cell and C) germ cell.

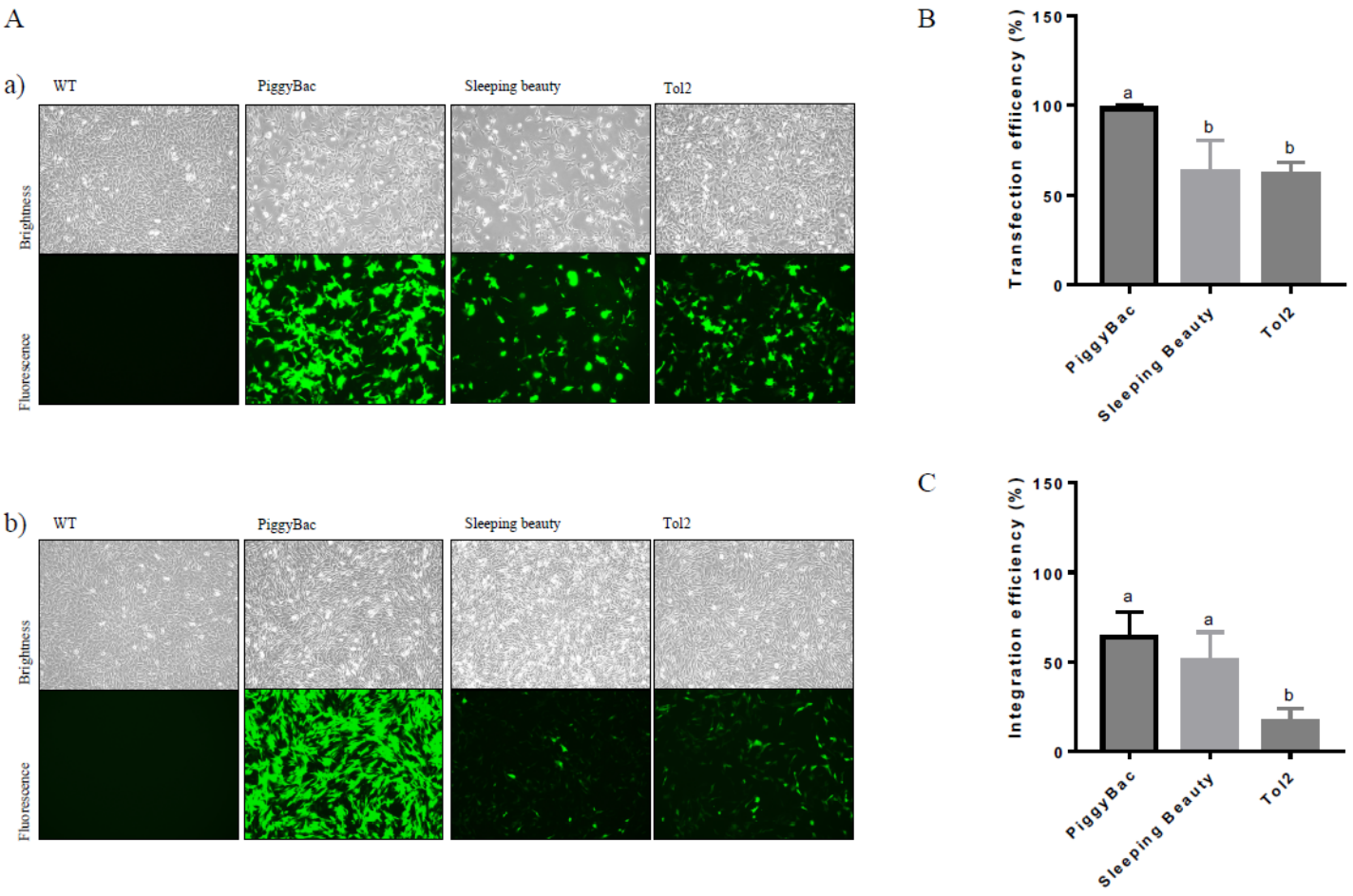

Figure 2

Transposon system mediated bovine somatic cell transfection using electroporation and FACS analysis to examine transfection and integration efficiencies. All transfections were replicated 3 times with 3 types of primary cells A) Representative bright and GFP fluorescent field of each transposon systems, A-a and B) Representative picture and FACS result for transfection efficiency on Day 3 and A-b and C) for integration efficiency on Day 10 after transfection.

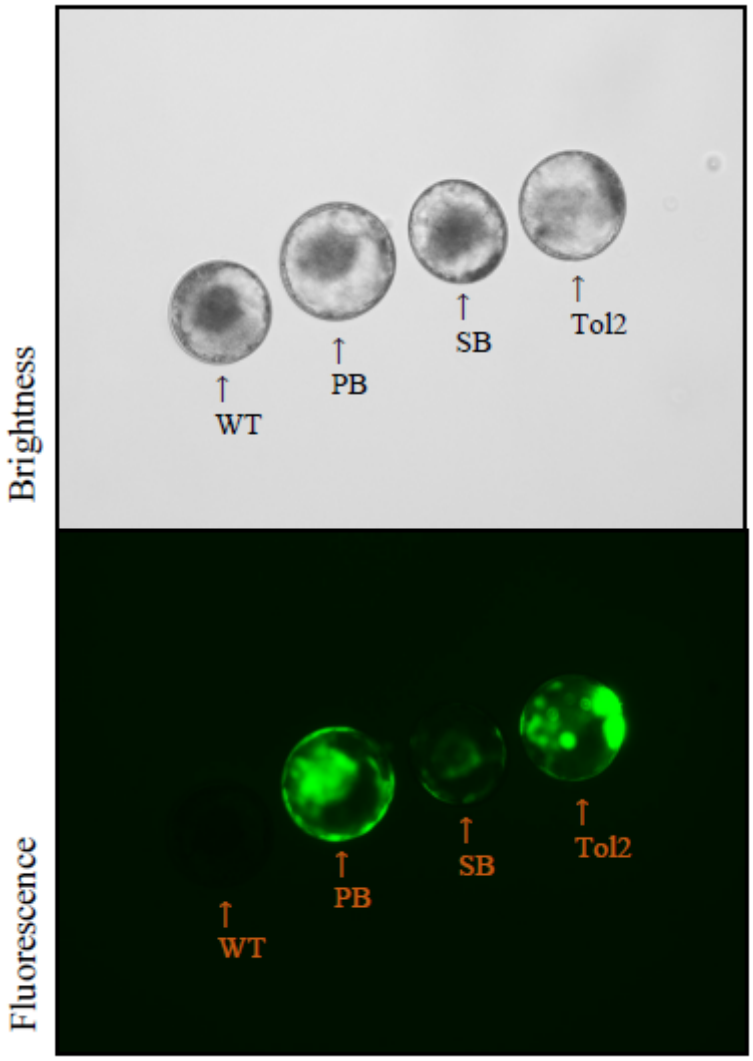

Figure 3

Representative bright and GFP fluorescent field of each transposon system in bovine blastocysts and Hoechst staining to compare total cell numbers in the blastocysts. A) Blastocysts on Day 7 were observed through fluorescent microscope. B) Total cell number of stained blastocysts with Hoechst 33342 were manually counted through Image J software (NIH).

Supplementary Files

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