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Establishment of cell-based transposon-mediated transgenesis in cattle

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ABSTRACT

Transposon-mediated transgenesis is a well-established tool for genome modification in small animal models. However, translation of this active transgenic method to large animals warrants further investigations. Here, the piggyBac (PB) and sleeping beauty (SB) transposon systems were assessed for stable gene transfer into the cattle genome. Bovine fibroblasts were transfected either with a helper-independent PB system or a binary SB system. Both transposons were highly active in bovine cells increasing the efficiency of DNA integration up to 88 times over basal nonfacilitated integrations in a colony formation assay. SB transposase catalyzed multiplex transgene integrations in fibroblast cells transfected with the helper vector and two donor vectors carrying different transgenes (fluorophore and neomycin resistance). Stably transfected fibroblasts were used for SCNT and on *in vitro* embryo culture, morphologically normal blastocysts that expressed the fluorophore were obtained with both transposon systems. The data indicate that transposition is a feasible approach for genetic engineering in the cattle genome.

1. Introduction

The advent of high throughput DNA sequencing methods and comprehensive annotated genome maps concomitantly with advanced active transgenic techniques promise to revolutionize the field of animal biotechnology. In particular, areas like disease modeling, biopharming, and basic research will benefit enormously by introducing precise genetic engineering tools to manipulate livestock genomes. Initial transgenic methods relayed on passive (nonfacilitated) genomic integration of transgenes at sites of spontaneously arising double-strand breaks of chromosomes after direct injection of naked DNA into zygotes (pronuclear injection) or transfection of cultured cells followed by SCNT. Homologous recombination in somatic cells of livestock is an extremely rare event, and only a few

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genes were targeted in recent years [1–7]. These inefficient and unreproducible approaches were gradually superseded by a new generation of active methods in which genomic insertion of heterologous DNA molecules is catalyzed by exogenously provided enzymes (reviewed in [8,9]).

For gain-of-function approaches in farm animals, the engineered transposon systems Sleeping Beauty (SB) and piggyBac (PB) gained increasing interests in recent years [10,11]. Transposon-based transgenic methods, derived from naturally occurring DNA transposable elements, are nonviral gene delivery systems capable of efficient enzyme-mediated genomic insertion of DNA segments into the genome. During transposition, a single copy of the sequence of interest framed by inverted terminal repeats (ITRs) is integrated into the genome through a precise, transposase-catalyzed mechanism, providing long-term expression of the gene of interest in cells [12]. Bicomponent transposon-based transgenic systems comprise a donor vector containing the transgene flanked by transposase-specific ITRs and the transposase enzyme provided as protein, mRNA, or most commonly as a helper DNA vector. In addition, systems that combine both components in a single vector, known as helper-independent transposons, have been developed and validated in cells and animals [13–16]. Transposase catalyzes both the excision of the transgene from the donor vector and its integration into a genomic target site. Integration occurs at short consensus sequences, for example Tc1/mariner transposases, such as SB, recognize TA dinucleotides [17], and PB transposase recognizes TTAA tetranucleotides [18]. Through this mechanism one monomeric copy of a transposon is integrated in the genome, leaving the empty backbone of the donor plasmid, which is eventually degraded [12,19,20] or, rarely, randomly integrated [14,21]. Expression units introduced by transposition are less prone to epigenetic silencing and show long-term expression of the transgene [12], suggesting that transposons have a tendency to land in genomic regions that are transcriptionally permissive [22].

PiggyBac and SB transposons have been extensively studied for transgenesis in mice, rats, and rabbits [13,23–26]. Both *in vivo* (intracytoplasmic injection of zygotes) and *in vitro* (SCNT) approaches have been exploited to generate transposon–transgenic pig models [12,20,27–31]. Zygote microinjection with SB transposon components has resulted in single-copy integration units into the pig preimplantation embryo genome [20], into born F0 animals and successful transmission to F1 generation [12]. Garrels et al. [12] demonstrated segregation of individual transposons in the F1 offspring, copy number–dependent expression of reporter protein over a prolonged time with no evidence of gene silencing. Similarly, transgenic pigs generated by microinjection of a helper-independent, self-inactivating PB transposon had monogenic and often single transgene genomic integration and the absence of concatemers or variegated transgene expression [28].

Alternatively, genetic modification of somatic cells by PB or SB transposition followed by SCNT is an avenue to generate transgenic livestock. Here, we assessed the suitability of the PB and SB transposon systems for the genetic modification of bovine fibroblasts, which were subsequently used in SCNT.

To this end, cultured fibroblast cells were transfected or electroporated with both transposon systems, respectively, and on selection or enrichment of transgenic cells, they were used as nuclear donor in SCNT. The use of transposons is associated with an enhanced proportion of stably transfected cells, as it has been documented for established immortalized and primary porcine cells [32] transfected with SB, PB, Tol2, or passport transposon systems [20,32] as well as for primary bovine fibroblasts transfected with PB [33]. These promising results warrant more research that extends and adapts transposon-based methods to functional transgene products and to economically important livestock species such as cattle.

2. Materials and methods

2.1. Experimental design

The experimental design is summarized in the Figure 1. Fibroblast cultures were established from fetuses collected at a local abattoir. Fibroblasts were transfected with either SB transposon components or PB helper-independent plasmids followed by a 3-week antibiotic selection period.

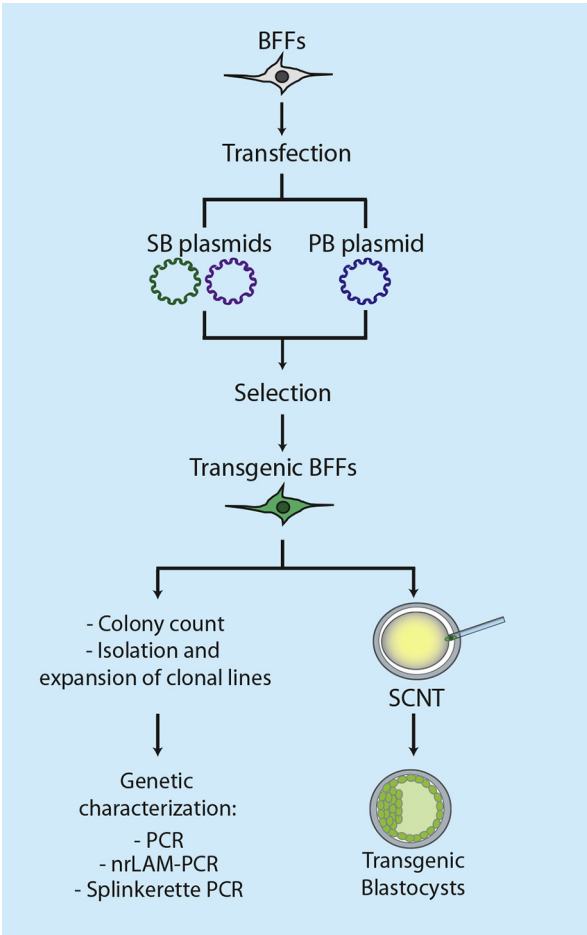

Fig. 1. Schematic representation of the experimental design.

The number of resistant colonies was counted and representative colonies were picked and expanded to obtain genomic DNA (gDNA) for molecular analysis of transposon genomic integration (polymerase chain reaction [PCR], nrLAM-PCR, and Splinkerette PCR). Transgenic polyclonal cell cultures generated from PB or SB were used as nuclear donors in SCNT experiments to assess their ability to support early embryo development.

2.2. Vectors

2.2.1. PiggyBac plasmids

The experiments were carried out using p*m*GENIE-3–based plasmids. p*m*GENIE-3 combines in a single plasmid the transposon and the transposase coding sequence (helper-independent system). Another feature of this plasmid is that a portion of the transposase coding sequence is deleted on transposition (self-inactivation) [14].

p*m*GENIE-3: this plasmid carries enhanced green fluorescent protein (EGFP) coding sequence which is under of control of CMV early enhancer/chicken β actin promoter. In addition, the plasmid harbors the hygromycin B resistance gene.

p*mhy*GENIE-3: differs from p*m*GENIE-3 in that it contains a sequence that codes a hyperactive version of the PB transposase.

p*m*GENIE-2/ΔPB: it is a control plasmid that codes for a nonfunctional truncated transposase. We will refer to this plasmid as p*m*GENIE-control.

p*mhy*GENIE-3, p*m*GENIE-3, and p*m*GENIE-control were previously described [14].

2.2.2. Sleeping Beauty plasmids

pCMV(CAT)T7-SB100X: this plasmid contains an eukaryotic expression cassette for SB transposase under the control of CMV promoter. The SB transposase expressed by this construct is an optimized version (SB100X) with enhanced transposition activity [10].

pT2RMCEVenus: this plasmid contains a eukaryotic expression cassette for Venus fluorescent protein under the control of CAGGS promoter [34]. The expression cassette is flanked by 5′and 3′ ITRs of SB transposon.

pT2/SV40-Neo: this plasmid contains an expression cassette for neomycin phosphotransferase, which confers resistance to the antibiotic geneticin (G418), flanked by SB ITRs. SV40 promoter drives expression of the antibiotic resistance gene [35].

pBSII-ISceI-skA: this plasmid is used as control in experiments with SB system (kindly provided by Dr. Jochen Wittbrodt, Developmental Biology Programme, EMBL-Heidelberg, Heidelberg, Germany).

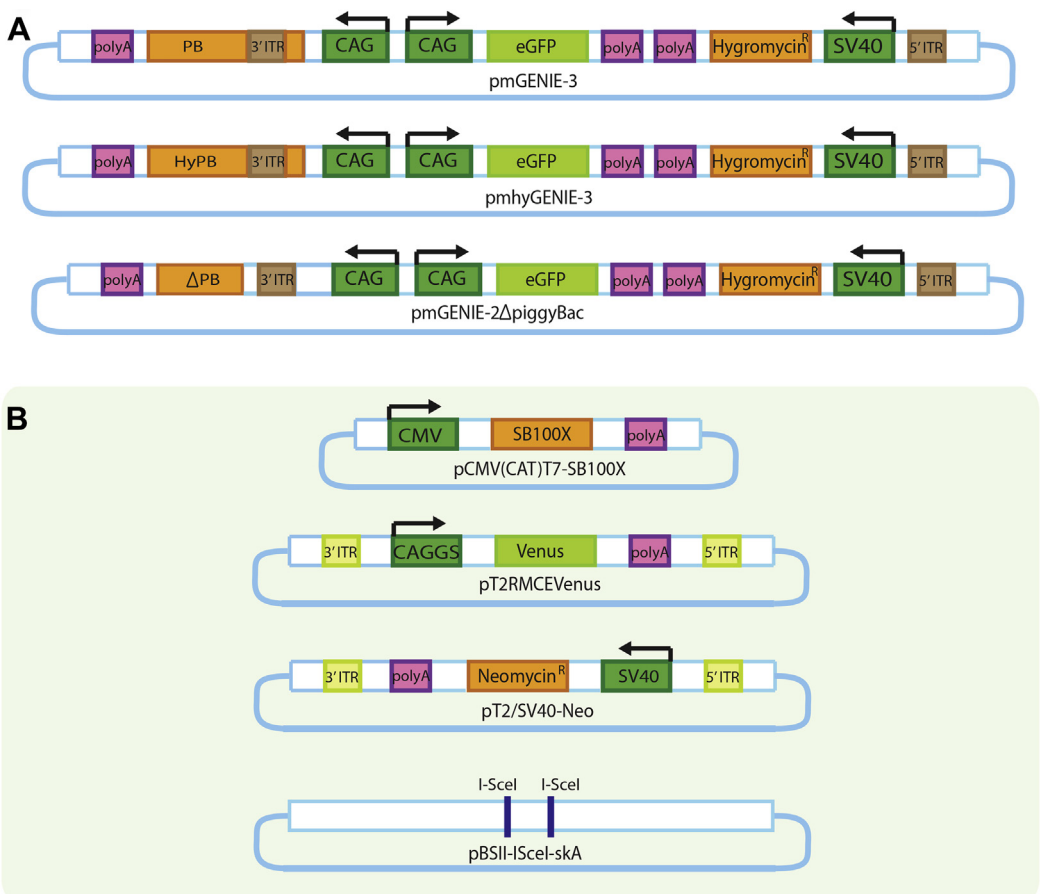

Fig. 2. Diagrams of piggyBac (A) and Sleeping Beauty (B) plasmid constructs used in the experiments showing the major components of each vector.

Schematic representations of all plasmids are depicted in Figure 2.

2.3. Culture of primary cells

Primary bovine fetal fibroblasts (BFFs) were obtained from slaughterhouse fetuses of 90 to 150 days of gestation. Fetuses were transported to the laboratory where they were processed in a laminar-flow cabinet. A piece of subdermal tissue (about 1 cm^2) was removed from the flank of the fetus and sectioned with a scalpel blade into smaller pieces. Explants were placed in cell culture plates (three–four explants per 100 mm plate) in 6 mL of cell culture medium (Dulbecco's Modified Eagle Medium [DMEM], 1x antibiotic/antimycotic, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Natocor, Cordoba, Rep. Argentina) and cultured for approximately 10 days in an atmosphere of 5% CO_2 in air and high humidity at 38.5 °C. When the cells reached 70% to 80% confluence, they were trypsinized (trypsin 0.5%, Sigma–Aldrich Co., St. Louis, MO, USA) and passaged to a T75 culture flask for cell propagation. Fibroblasts were frozen in DMEM containing 20% FBS and 10% DMSO (Sigma–Aldrich Co., St. Louis, MO, USA) and kept in liquid nitrogen until use.

2.4. Cell transfection with transposon vectors

Bovine fetal fibroblasts were seeded at 0.5×10^5 cells per well of a 24-well plate. When the cells reached 80% confluence (12–24 hours), cultures were transfected with a polyethylenimine-based transfection reagent (JetPRIME; Polyplus-transfection SA, Illkirch, France) and 1 μg of plasmid DNA according to the manufacturer's instructions. Forty-eight hours posttransfection, expression of reporter fluorescent protein was assessed in a microscope equipped with epifluorescence (excitation filter 450–490 nm and an emission filter 530 nm). Images of transfected cells were captured with a Nikon DS-Qi1Mc camera using the same acquisition settings for all pictures taken. Subsequently, cells from each well were trypsinized and transferred into a 100-mm culture plate with 8 mL DMEM supplemented with 10% FBS and selected for 14 days with the appropriate antibiotic, depending on the particular plasmid used (p*m*GENIE-3: hygromycin B; SB system: G418).

For PB system, fibroblasts were transfected with p*m*GENIE-3–based plasmids (p*mhy*GENIE-3, p*m*GENIE-3, or p*m*GENIE-Control) followed by 14-day selection with 50 μg/mL hygromycin B (Invitrogen; Van Allen Way Carlsbad, California, USA). The media were replaced every other day.

In the first SB transfection experiment, fibroblast cultures were cotransfected with pT2RMCEVenus (donor plasmid) and pCMV(CAT)T7-SB100X (helper plasmid). In the second experiment, fibroblasts were cotransfected with pT2RMCEVenus, pT2/SV40-Neo, and pCMV(CAT)T7-SB100X. In both experiments, we included a treatment in which the pCMV(CAT)T7-SB100X was replaced by the same amount of pBSII-ISceI-skA which lacks a SB transposase as a negative control, thus background, (nonfacilitated) transgene integration could be estimated. No antibiotic selection was applied for cells transfected with pT2RMCEVenus because this vector is devoid of antibiotic selection cassette, and the number of fluorescent colonies was determined 12 to 14 days after transfection. In experiments of cotransfection in which pT2/SV40-Neo was included, G418 selection (250 μg/mL, Invitrogen; Van Allen Way Carlsbad, California, USA) was implemented for two weeks with media replacement every other day.

2.5. Colony formation assay and derivation of monoclonal transgenic cell lines

Transfected cells were kept under antibiotic selection for two weeks and the numbers of fluorescent colonies were counted in an inverted microscope (Nikon Corp., Tokyo, Japan) with epifluorescence equipment. To assess the transposition activity of the different plasmids, we calculated the relative fold change, which was obtained by dividing the number of antibiotic resistant colonies counted in cultures transfected with p*mhy*GENIE-3 or p*m*GENIE-3 plasmids by the number of colonies in those transfected with p*m*GENIE-control plasmid.

In the SB experiment in which fibroblasts were cotransfected with two plasmids (pT2RMCEVenus and pCMV(CAT)T7-SB100X), the number of Venus positive colonies formed after two-week culture was compared with those in the control treatment. When cell cultures were cotransfected with three plasmids (pT2RMCEVenus, pT2/SV40-Neo, and pCMV(CAT)T7-SB100X), the number of colonies originated under antibiotic selection (G418) was determined after 14 days.

For clonal expansion, individual cell colonies were recovered from 100 mm plates using cloning rings, small plastic cylinders of 7 mm of diameter that were placed over each individual colony and fixed and sealed with agarose [36]. Cells inside the cloning ring were trypsinized and cultivated in 24-well plates up to 80% to 90% confluence. Cell lines were sequentially passaged into larger sized culture plates as the number of cells increased. Once they reached 80% to 90% of confluence in a T75 culture flask each cell line was processed to obtain gDNA for molecular characterization of transgene integration.

2.6. Molecular characterization of transgene genomic integrations

Polymerase chain reaction: genomic DNA was isolated from transgenic cell lines using Quick-gDNA MiniPrep (Zymo Research Corporation, Irvine, USA) according to the manufacturer's protocol. We designed a PCR strategy (four reactions) to characterize PB transgene genomic integrations (nonfacilitated or transposase catalyzed). Polymerase chain reaction1: amplification of a 187-bp fragment of the EGFP gene with primers pair PB-GFP-F/PB-GFP-R. The presence of a correct size PCR product confirmed the presence of the transgene (Fig. 3G). Polymerase chain reaction2: amplified a 537-bp fragment extending from the 3′-ITR into the backbone of the plasmid using primers pair PB-ITR-F/PB-ITR-R. Amplification of the correct size product would indicate nontranspositional transgene integration (Fig. 3G). Polymerase chain reaction3: this reaction was included to determinate if nontranspositional integration of the recircularized

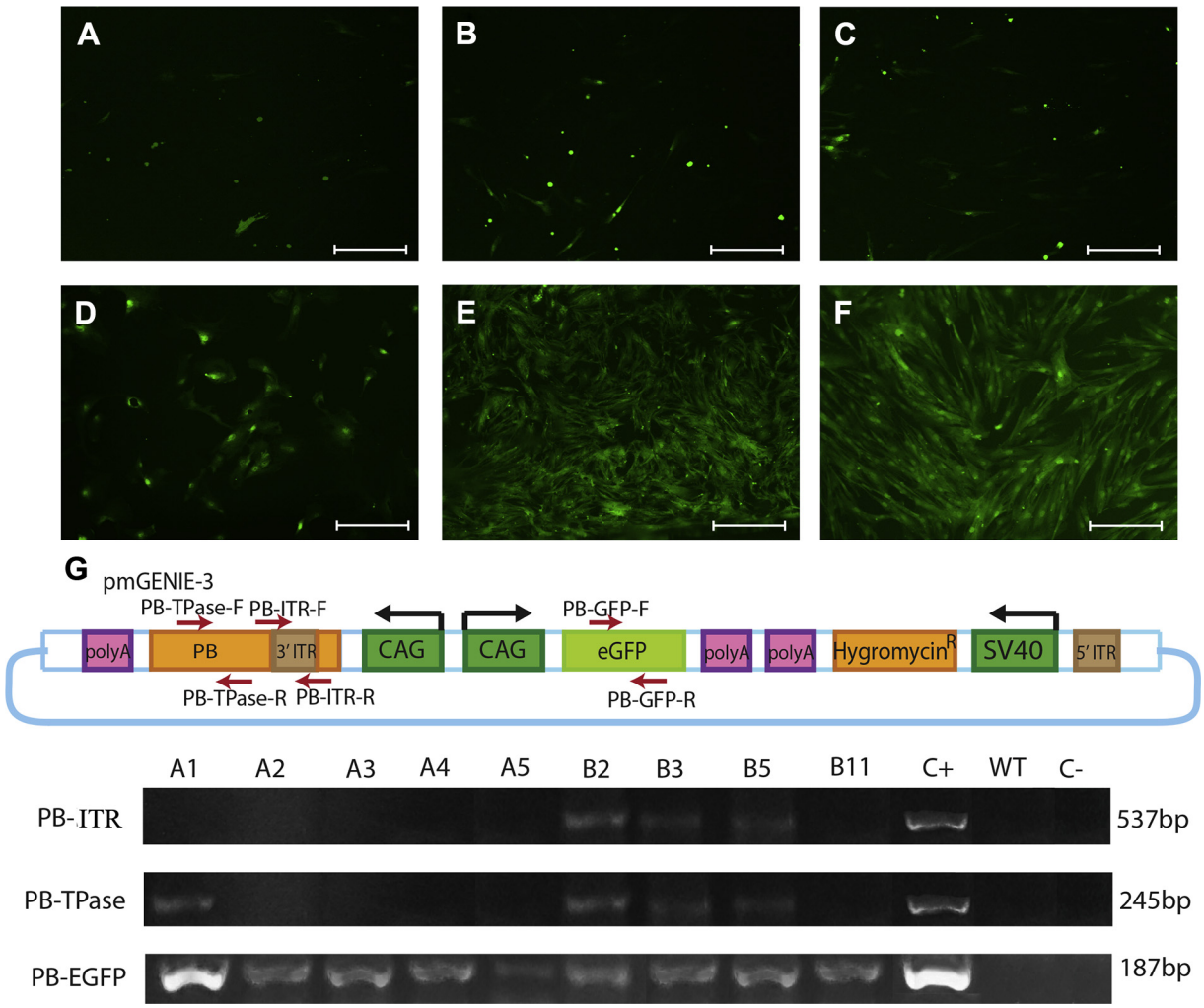

Fig. 3. Microphotographs of fibroblast cells two days (A, B, and C) and 16 days (D, E, and F) after transfection with p*m*GENIE-2/ΔpiggyBac (PB; A and D), p*m*GENIE-3 (B and E), or p*mhy*GENIE-3 (C and F; bars = 100 μm). After 14 days of antibiotic selection, discrete colonies of different sizes were evident (D, E, and F). (G) Schematic representation of p*m*GENIE-3 plasmid showing position of three pairs of primers (PB-TPase-F/PB-TPase-R, PB-ITR-F/PB-ITR-R, and PB-GFP-F/PB-GFP-R) used for PCR analysis of genomic DNA (gDNA) from p*m*GENIE-3 (A1–A5) and p*mhy*GENIE-3 (B2, B3, B5, and B11) transgenic cell lines. A 187-bp fragment of EGFP gene (GFP) was amplified from gDNA from all transgenic cell lines and positive control, but it was not amplified from wild type gDNA and negative control (C−). A PCR product corresponding to a 537-pb fragment extending from the 3′-ITR into the backbone of the plasmid was present in B2, B3, and B5 and absent in A1, A2, A3, A4, A5, B11, and wild type samples. A 245-bp product generated from PB transposase sequence was present in cell lines A1, B2, B3, and B5.

backbone of the plasmid after transposition had occurred, as it was reported in mice [14]. Polymerase chain reaction was performed using PB-RL-F/PB-RL-R primer pair to amplify a 442-pb backbone fragment (Fig. 4A). Identity of PCR products was confirmed by sequencing. Polymerase chain reaction4: To determinate integration of PB transposase sequences into the bovine genome primer pair PB-TPase-F/PB-TPase-R was used (Fig. 3G). A PCR amplicon of 245 bp was expected in cell lines that contained at least one integrated copy of the PB transposase coding sequence.

Multiplex PCRs were run using the following parameters: initial denaturalization at 94 °C for 9 minutes followed by 30 cycles of 20 seconds denaturalization at 94 °C, 30 seconds annealing at 60 °C, and 30 seconds elongation at 72 °C, with a final elongation of 5 minutes.

Genomic DNA isolated from six SB monoclonal transgenic cell lines was used as template in a series of four reactions of PCRs to characterize SB transgene integrations. Polymerase chain reaction1: amplification of a 280-bp fragment from the Venus gene with primers pair SB-Venus-F/SB-Venus-R (Fig. 5E). The presence of a correct size PCR product confirmed the presence of the transgene. Polymerase chain reaction2: amplification of a 408-bp fragment extending from the 3′-ITR into the backbone of the plasmid using primers pair SB-ITR-F/SB-ITR-R (Fig. 5E). Amplification of the correct size product indicated nontranspositional transgene integration. Polymerase chain reaction3: amplification of a 493-bp fragment of the plasmid backbone with primer pair SB-BB-F/SB-BB-R (Fig. 5E). The presence of correct sized product indicated genomic integration of backbone

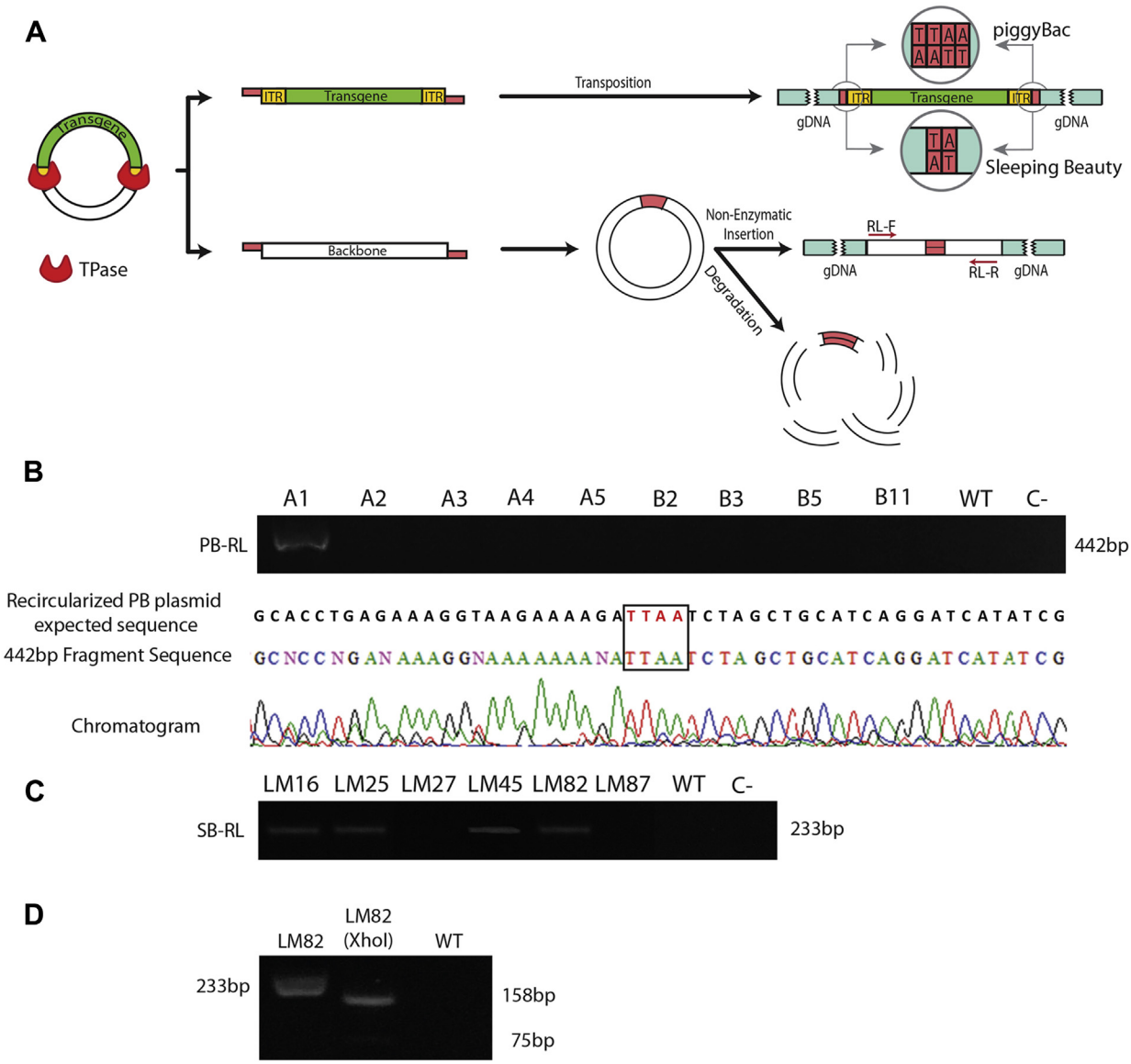

Fig. 4. (A) Schematic drawing that shows the possible fates of the transgene and the recircularized backbone after transposition for SB and PB transgenic systems. Position of primers RL-F and RL-R is depicted in the figure. (B) Agarose gel showing a product of 442 bp amplified from the PB religated vector backbone inserted into the genome of BFF line A1. PCRs with gDNA from the remaining cell lines generated no amplification product. A negative control (C−) was included. Alignment between the expected religated backbone sequence and the actual sequence obtained by Sanger sequencing of the purified PCR product is shown in B. Boxed is the reconstituted TTAA tetranuclotide generated after transposition. (C) Agarose gel showing a PCR product of 233 bp amplified from the SB religated vector backbone inserted into the genome of four BFF lines (LM16, LM25, LM45, and LM82). Digestion of PCR product from LM82 with XhoI rendered the expected two products of 75 and 158 bp. (D) Lane one nondigested PCR product, lane two digested PCR product, and lane three wild type control. BFF, bovine fetal fibroblast; gDNA, genomic DNA; PB, piggyBac; PCR, polymerase chain reaction; SB, Sleeping Beauty.

sequences by nontranspositional mechanisms. Polymerase chain reaction4: this reaction was conducted with primer pair SB-RL-F/SB-RL-R (Fig. 4A). The presence of a 233-bp amplicon indicated genomic integration of religated backbone after transposition had occurred. Polymerase chain reaction product was identified by restriction enzyme digestion (XhoI). On digestion, the expected product would render two fragments of 75 bp and 158 bp. Polymerase chain reaction5: this reaction was run with primer pair SB-TPase-F/SB-TPase-R (Fig. 5E). The presence of a 643-bp fragment indicated genomic nonfacilitated insertion of the helper SBX100 plasmid.

All PCRs were run with the following parameters: initial denaturalization at 95 °C for 5 minutes followed by 35 cycles of 15 seconds denaturalization at 95 °C, 15 seconds annealing at 63 °C, and 45 seconds elongation at 72 °C, with a final elongation of 10 minutes.

All primer sequences are shown in Supplementary Table 1.

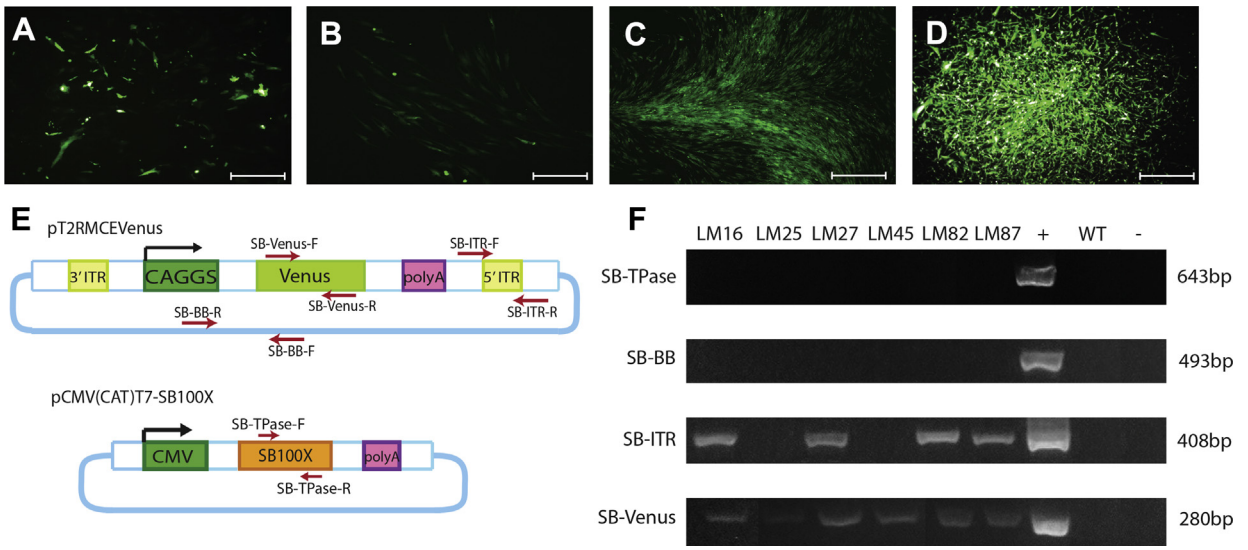

Fig. 5. Microphotographs of transient and stable expression of the Venus reporter, two days after transfection (A), 4 days (B), 11 days (C), and 15 days (D) after G418 antibiotic selection of BFF transfected with the SB plasmids. Note colony formation in C and D panels (bar = 100 μm). (E) Schematic of pT2RMCEVenus and pCMV-SB100X plasmids showing the hybridization sites of SB-Venus, SB-ITR, SB-BB, and SB-TPase pairs of primers used in PCRs. (F) Polymerase chain reaction analysis of gDNA from six SB monoclonal cell lines. Genomic DNA from all transgenic cell lines generated an expected PCR product of 280 bp corresponding to the Venus gene. A PCR product corresponding to a 408-bp fragment extending from the 3′-ITR into the backbone of the plasmid was present in LM16, LM27, LM82, and LM87 and absent in LM25 and LM45. None of the cell lines gDNAs generated a PCR product with primers SB-BB (amplification of part of plasmid backbone) or SB-TPase (amplification from pCMV(CAT)T7-SB100X). Positive control (C+) and negative controls (C−) were included. BFF, bovine fetal fibroblast; gDNA, genomic DNA; ITR, inverted terminal repeat; PCR, polymerase chain reaction; SB, Sleeping Beauty.

2.7. Real-time quantitative polymerase chain reaction

The number of EGFP copies per clonal cell line (n = 9) was determined by real-time PCR as described previously by Lee et al. [37]. Briefly, 20 ng of gDNA was used as template in real-time quantitative PCR (qPCR) using KAPA SYBR FAST qPCR kit (Kapa Biosystem Inc., Boston, MA, USA) and Applied Biosystems 7500 instrument (Applied Biosystems, Waltham, MA, USA). Primer set used to amplify EGFP transgene was PB-GFP-F-PB-GFP-R (Supplementary Table 1). Quantitative PCR was run using the following parameters: initial denaturalization at 94 °C for 10 minutes followed by 40 cycles of 10 seconds denaturalization at 95 °C, 10 seconds annealing at 61 °C, and 10 seconds elongation at 72 °C. The fluorescent signal was measured at the end of each elongation step at 72 °C. After amplification, a melting curve was performed by increasing the temperature from 70 °C to 95 °C at a rate of 0.1 °C/s to confirm the identity of PCR product. The number of copies was calculated from a standard curve which was generated using 10-fold serial dilution p*m*GENIE-3 (5.93×10^2–5.93×10^6 copies/μL).

2.8. Measurement of cell line fluorescence intensity

To establish a putative association between transgene copy number and fluorescence intensity of transgenic cell lines (n = 9), we measured fluorescence in individual cells using ImageJ software (V1.49, NHI) and corrected total cell fluorescence (CTCF) using the following equation: CTCF = integrated density − (area of selected cell × mean fluorescence of background readings) as previously described [38]. Linear regression analysis was performed using CTCF as an independent variable and transgene copy number as a dependent variable.

2.9. Nonrestrictive linear amplification–mediated polymerase chain reaction

Identification of transpositionally generated sites of insertion was achieved by nonrestrictive linear amplification–mediated PCR (nrLAM-PCR) according to published protocols [39,40]. Genomic DNA from p*m*GENIE-3 transgenic cell lines was used as a template for linear amplification, and nested PCR was implemented to amplify the sequence flanking the PB insertions. Polymerase chain reaction products were cloned into a vector and sequenced. DNA sequences directly flanking the transposon were mapped to the bovine genome by doing a BLAST search at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.10. Splinkerette PCR

Sleeping Beauty insertions in six Venus+/Neo monoclonal cell lines were characterized by a splinkerette PCR method as previously described [41]. Transgene flanking sequences were used to map insertion sites to the publicly available bovine genome (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.11. Somatic cell nuclear transfer

Oocyte collection and *in vitro* maturation: bovine ovaries were collected from abattoirs and transported to the laboratory at 25 °C to 30 °C. Cumulus-oocyte complexes were aspirated with 21-gauge needles from follicles with a

diameter of 2 to 5 mm into HEPES-buffered Tyrode's albumin lactate pyruvate (HEPES-TALP). Oocytes covered with at least three layers of granulosa cells were selected for IVM. The maturation medium was bicarbonate-buffered tissue culture medium 199 (TCM-199) (31100-035; Gibco, Grand Island, NY, USA), containing 10% FBS (013/07; Internegocios, Buenos Aires, Argentina), 10 μg/mL FSH (NIH-FSH-P1, Folltropin; Bioniche, Caufield North, Victoria, Australia), 0.3-mM sodium pyruvate (Sigma, P2256), 100 μM cysteamine (Sigma, M9768), and 2% antibiotic-antimycotic (15240-096; Gibco). The oocytes were incubated for 21 hours in 100 μL droplets under mineral oil (Sigma, M8410), in 6.5% CO_2 in humidified air at 39 °C.

Enucleation procedure: after 21 hours of IVM, metaphae II oocytes were subjected to hyaluronidase treatment followed by incubation in 1.5 mg/mL pronase to remove the zona pellucida (ZP). Zona-free oocytes were stained with 1 μg/mL of Hoechst 33342 for 10 minutes and afterward transferred into 50 μL microdroplets of HEPES-TALP supplemented with 0.3 g/mL BSA, under mineral oil, in 100 × 20 mm tissue culture dishes (430167; Corning, Horseheads, NY, USA). The stained oocytes were mechanically enucleated using a Narishige hydraulic micromanipulator (Narishige Sci., Tokyo, Japan) mounted on a Nikon Eclipse E-300 microscope (Nikon, Tokyo, Japan). Enucleation was performed using a blunt 20 μm internal diameter pipette. Zona-free oocytes were supported for enucleation with a closed holding pipette (100–150 μm outer diameter, perpendicular break, closed fire-polished tip). Metaphase chromosomes were visualized under ultraviolet light (<10 seconds) and aspirated into the blunt pipette with a minimal volume of oocyte cytoplasm. Chromosome removal was confirmed by the presence of stained metaphase II chromosomes inside the pipette.

Donor cell preparation and fusion to enucleated oocytes: cells transfected with p*mhy*GENIE-3 or SB plasmids, selected during 20 days, were used as donors for cloning. Briefly, the ZP-free enucleated oocytes were individually transferred to a drop of 1 mg/mL phytohemagglutinin (Sigma, L8754) dissolved in TCM-199 without serum, where they remained for a few seconds. After this, they were quickly dropped over a single cell resting on the bottom of a 100 μL TALP-H drop. After attachment, the ZP-free enucleated oocyte and/or cell pair was picked up, transferred to fusion medium (0.3-M mannitol, 0.1-mM $MgSO_4$, 0.05-mM $CaCl_2$, 1 mg/mL polyvinyl alcohol), for 2 to 3 minutes and then to a fusion chamber (BTX Instrument Division; Harvard Apparatus, Holliston, MA, USA) containing 2 mL of the same warm medium. Fusion was performed with a double direct current pulse of 75 V, each pulse for 30 ms, 0.1 second apart. The reconstructed zygotes were then carefully transferred to synthetic oviductal fluid culture droplets for 2 hours to allow for nuclear reprogramming.

Chemical oocyte activation: embryos produced by SCNT were activated with 5-μM ionomycin (I24222, Invitrogen; Van Allen Way Carlsbad, California, USA) in HEPES-TALP for 4 minutes and subsequently transferred individually to 1.9-mM 4-(Dimethylamino)pyridine (Sigma, D2629) in SOF droplets for 3 hours. Embryos were then washed three times in HEPES-TALP to remove the inhibitor and cultured as described below.

In vitro embryo culture: reconstructed SCNT embryos were cultured in SOF medium in a system similar to the well of the well (WOW) [42], whereby microwells were produced using a heated glass capillary slightly pressed to the bottom of a culture dish and then covered with a 100 μL microdrop of SOF medium (20–30 WOW in each microdrop, one embryo per WOW). During nuclear transfer embryo culture, the medium was 50% replaced on Day 2 and supplemented with 7.5% FBS on Day 5. Cleavage was evaluated on Day 2, morula formation at Day 5, and blastocyst formation on Day 7 after fusion.

Evaluation of fluorophore expression in embryos: cloned embryos were briefly exposed to blue light using specific excitation (460–498 nm) and an emission filter (510–540 nm) to determine EGFP or Venus expression on Days 5 and 7 after chemical activation. Images were captured with a Nikon DS-Qi1Mc camera using the same acquisition settings for all pictures taken.

2.12. Embryo vitrification, thawing, and cell counting

Transgene expressing cloned blastocysts were vitrified as previously reported [43,44]. Briefly, embryos were immersed sequentially in a series of glycerol and ethylene glycol solutions at room temperature (25 °C) as follows: 10% glycerol for 5 minutes followed by 10% glycerol + 20% ethylene glycol for 5 minutes and finally 25% ethylene glycol + 25% glycerol for 30 seconds. The embryos were aspirated into 1-μL tips and cryotubes immersed in liquid nitrogen. For devitrification, immediately after recovery of embryos from liquid nitrogen, they were placed for 5 minutes in a solution of 12.5% ethylene glycol + 12.5% glycerol + 0.5-M sucrose in PBS with 20% FBS. Afterward, they were placed in 0.5-M sucrose solution and 0.25-M sucrose solution (5 minutes in each solution). Finally, the embryos were washed two times in PBS supplemented with 1% serum. After thawing, embryos were placed back in synthetic oviductal fluid (SOF) medium with amino acids, and cell numbers of blastocysts that reexpanded were determined by staining with 1 μg/mL of Hoechst 33342 (B-2261; Sigma–Aldrich Co., St. Louis, MO, USA) for 10 minutes. Nuclei were visualized and counted using ultravoilet light in a microscope equipped with epifluorescence.

2.13. Statistical analysis

The number of colonies of each group was analyzed by ANOVA followed by Tukey's test to compare the means of different treatments. Data are expressed as means ± standard error of the meanand different letters in graph bars indicate statistically significant differences ($P < 0.05$). All statistical analyses were performed with Statgraphics software (Statpoint Technologies, Inc. Warrenton, Virginia).

3. Results

3.1. Assessment of PB transposon activity in bovine fetal fibroblasts

The experimental design is summarized in Figure 1, and the used plasmids are depicted in Figure 2. To assess the

activity of PB transposase to mediated gene transfer into the bovine genome, BFFs were transfected with 1 μg of p*m*GENIE-3 or p*mhy*GENIE-3 (self-inactivating hyperactive PB transposase-based plasmid). As control for random non-transpositional integration, BFFs were transfected in parallel with the control p*m*GENIE-2/ΔPB plasmid (Fig. 3A–F). The mean number of hygromycin B-resistant colonies was markedly different among treatments (ANOVA, $P < 0.0001$; Fig. 6A). In cell cultures transfected with PB constructs containing a functional sequence of PB transposase (i.e., p*m*GENIE-3 and p*mhy*GENIE-3) the number of resistant clones was higher compared with that in cultures transfected with the control plasmid in which only a few colonies developed (range 0–4 resistant colonies). More active at delivering DNA into the genome was the hyperactive version of the PB transposase, as p*mhy*GENIE-3–transfected group developed 3.24 times more colonies compared with that in the p*m*GENIE-3 group and 85-fold above of those in the p*m*GENIE-Control group (Fig. 6A).

3.2. Molecular characterization of transgene integration sites

Molecular analyses by PCR were performed on gDNA isolated from a total of nine transgenic monoclonal cell lines. p*m*GENIE-3 and p*mhy*GENIE-3 plasmid DNA were used as positive controls. As expected, we obtained EGFP amplicons from all gDNA samples from p*m*GENIE-3 transgenic group, demonstrating that analyzed cell lines carried the EGFP transgene (Fig. 3G), conversely the same PCR product was absent when gDNA from wild type cells was used as a template. Furthermore, no amplification product was obtained from gDNA isolated from six individual cell clones subjected to PCR to amplify a region extending from the 3′-ITR into the backbone of the plasmid (Fig. 3G). These data support a transposase-mediated transgene integration mechanism and the absence of random, noncatalyzed whole plasmid integration events in six of nine analyzed cell lines. In the remaining three transgenic cell lines (B2, B3 and B5; Fig. 3G), PCR products of the correct size were obtained in PCR2 and PCR3 what indicates random, non-facilitated genomic integration of full-length plasmids. Results from PCR4 revealed that one of nine p*m*GENIE transgenic cell lines (A1) analyzed carried at least a religated plasmid backbone incorporated randomly into the genome (Fig. 4B) but no evidence of full-length plasmid insertion (Fig. 3G). Religated plasmid backbone integration was confirmed by sequencing the 442-bp PCR product (Fig. 4B).

To identify the exact integration sites of the PB transposons in the bovine genome, we performed a nonrestrictive linear amplification–mediated PCR as described previously [39,40]. Cloning and sequencing of 17 integration sites from five monoclonal transgenic cell lines transfected with p*m*GENIE-3 confirmed specific transposase-mediated integration events at the PB TTAA tetranucleotide consensus sites. Fourteen of the 17 analyzed integration sites could be assigned to specific positions in the bovine genome. Eleven of these were located in intergenic regions and three in intronic regions of genes (Supplementary Table 2).

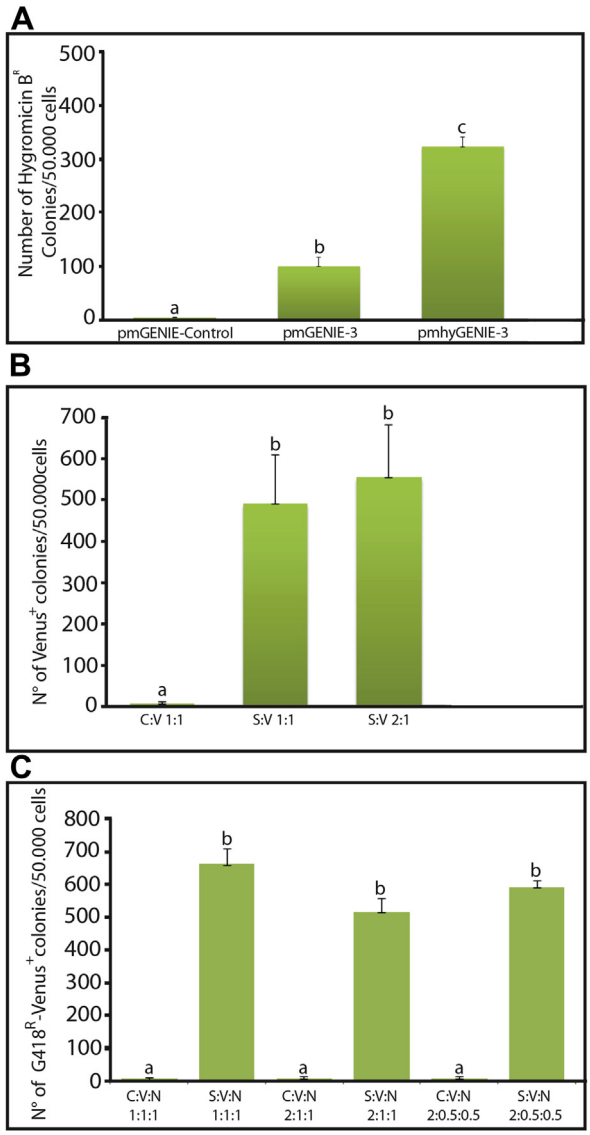

Fig. 6. Results of the colony formation assay obtained from cells transfected with PB (A) or SB transposon systems (B and C). Transposition activity was measured by counting EGFP-positive and hygromycin-resistant colonies after two-week selection period. (A) The colony counts assay was performed in triplicated and the data were expressed as mean of no. of hygromycin B resistant colonies ± standard error of the mean ($n = 4–7$). (B) Transposition activity in cultures transfected with different ratios of helper to donor plasmids was measured by counting Venus-positive colonies after a two-week culture period. (C) Number of Venus-positive and G418-resistant colonies in BFF cultures transfected with different ratios of helper-to-donor plasmids (pT2RMCEVenus and pT2/SV40-Neo) after two-week antibiotic selection. Different letters indicate statistically significant differences ($P < 0.05$). C, pBSII-ISceI-skA (control plasmid); S, pCMV(CAT)T7-SB100X; V, pT2RMCEVenus; N, pT2/SV40-Neo. BFF, bovine fetal fibroblast; PB, piggyBac; SB, Sleeping Beauty.

3.3. Transgene copy number and cell line fluorescence intensity

The number of transgene copies per genome in p*m*GENIE-3 and p*mhy*GENIE-3 transgenic cell clones ranged from

A

Cell Lines	Copy number/Genome
A1	13
A2	2
A3	1
A4	2
A5	9
B2	2
B3	3
B5	2
B11	2

B

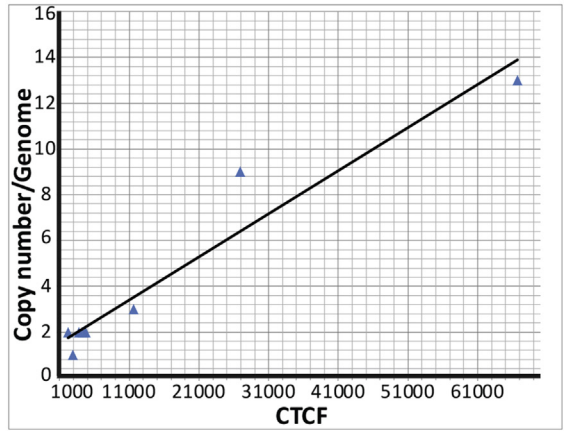

Fig. 7. Number of copies of transgene as determined by RT-qPCR (A) and relationship between fluorescence intensity (CTCF) and transgene copy number (B). Linear regression demonstrated an association between the two variables ($R^2 = 0.93$; $P < 0.05$). Cell lines A1 to A5 correspond to cell lines transfected with p*m*GENIE-3, whereas cell lines B2, B3, B5, and B11 were transfected with p*mhy*GENIE-3.

one to 13 copies. However, most cell lines carried one to three copies per genome (Fig. 7A).

Phenotypic analysis of each PB transgenic cell line reveled different fluorescence intensity among cell lines with larger CTCF values in those cell lines with a higher number of trangene copies per genome. Linear regression analysis demonstrated that fluorescence intensity is associated with transgene copy numbers ($R^2 = 0.93$; $P < 0.05$; Fig. 7B).

3.4. Sleeping Beauty activity in bovine fetal fibroblasts

Two separate experiments were conducted to study SB transposase activity in cultured BFF cells. In the first experiment, BFFs were cotransfected by a chemical method (Polyethylenimine-based transfection reagent) with different ratios of donor (the plasmid carrying the gene for Venus protein) and helper (SB100X) plasmids of the SB system. The presence of the transposase expression vector SBX100 in the transfection mixture boosted the number of colonies compared with those in the control treatment without transposase. We studied the effect of different weight ratios of helper to donor plasmid in the transfection reaction on the number of fluorescent colonies. When vectors were used at ratios of 1:1 and 2:1 (helper:donor), it caused a 78-fold and 88-fold increase in the number of colonies compared with that in the no transposase control and 1:2 ratio treatment respectively ($P < 0.05$; Fig. 6B).

Because the Venus vector lacks an antibiotic selection cassette, we designed an experiment to study SB transposition activity when two donor vectors are present. In this experiment, we cotransfected BFFs with the helper vector plus two donor plasmids; one was carrying the gene for Venus protein and the other an antibiotic resistance gene (*neo*). After transfection, BFFs were cultured for 14 days in media containing G418 for resistant colony formation (Fig. 5A–D). Regardless of the ratio used to transfect the BFF cultures, every time a functional SB transposase vector was included, the number of fluorescent and G418 resistant colonies was markedly higher compared with that in the respective control without transposase ($P < 0.001$; Fig. 6C). Interestingly, all G418 resistant colonies expressed the fluorescent protein.

Selected SB transgenic colonies were propagated for gDNA isolation, PCR analysis, and SCNT.

3.5. Molecular characterization of SB transgene genomic integrations

We designed five PCRs to characterize SB transposon integrations into the genomes of six monoclonal cell lines generated by cotransfection (ratio 2:0.5:0.5). As anticipated gDNA from all cell lines rendered an amplification product (280 bp) from Venus sequence (Fig. 5F). To allow identification of putative nontranspositional genomic integration of the SB plasmid, we designed a PCR in which the forward primer hybridize in the 5′ITR and reverse primer on the vector backbone; therefore, a product (408 bp) is generated if the complete plasmid is integrated by a nonfacilitated mechanism. Polymerase chain reactions using gDNA from cell lines LM16, LM27, LM82, and LM87 generated a 408 bp product. The absence of a 408 bp product in gDNAs from lines LM25 and LM45 (Fig. 5F) and concurrent amplification of Venus sequences from the same gDNA provides strong evidence for SB transposase–mediated transgene integration. None of the gDNA samples from transgenic cell lines generated a PCR product using the set of primers specific for the plasmid backbone (Fig. 5E and F). We found no PCR evidence for genomic integration of SB helper plasmid (pCMV(CAT)T7-SB100X) in any SB transgenic cell lines studied. PCR analysis revealed that four of six cell lines had at least one copy of the religated backbone plasmid inserted in their genomes (Fig. 4C). Identity of PCR product was confirmed in by restriction enzyme digestion (Fig. 4D).

We have applied a splinkerette PCR method to clone and analyze junctions of integrated SB transposons and the bovine genome. We identified 15 SB integrations that were mappable on the bovine genome and showed hallmarks of

SB transposase–mediated integration; i.e., insertion at a TA target site dinucleotide. Ten of these were located in intergenic regions and five in genes (Supplementary Table 3).

3.6. Reprogramming transposon transgenic cells by SCNT

To test the ability of transposon transgenic cells to undergo nuclear reprograming to support early embryo development, we performed SCNT with p*mhy*GENIE-3 or Venus^{+}/G418-resistant transgenic polyclonal cell line. Development to blastocyst stage reached 33.0% and 36.0% for PB and SB transgenic cell lines, respectively (Table 1; representative blastocysts are shown in Fig. 8A and B). Approximately half of the blastocyst homogenously expressed the reporter fluorophore protein (EGFP or Venus) at Day 7 (Fig. 8D and E). Autofluorescence was negligible in nontransgenic blastocysts (Fig. 8C and F). One of three GFP expressing blastocysts from the p*mhy*GENIE group reexpanded upon thawing and culture; this blastocyst had 99 nuclei (Fig. 8G). Two of four nuclear transfer blastocysts generated with SB transgenic cells reexpanded after vitrification/devitrification procedure and consisted of 176 (Fig. 8H) and 81 nuclei, respectively.

4. Discussion

Transposons belong to a new generation of nonviral transgenic tools, known generically as active transgenesis methods, which have in common an enzyme-mediated mechanism of genome modification [8]. Being an enzymatic-based process, active transgenesis is more efficient and precise than traditional techniques in which transgenes are randomly integrated at natural occurring chromosomal double-strand breaks and resolved by nonhomologous end joining.

In this study, we have implemented a transposon-mediated transgenic approach to introduce transgenes into the bovine genome. Both transposons, a helper-independent PB and a bicomponent SB system, catalyzed efficient reporter gene integration into the bovine genome, and these genetically modified cells were amenable to nuclear reprogramming to drive development to morphologically normal blastocysts on SCNT.

It has been demonstrated that PB and SB transposases are functional in cells and embryos from different species including livestock [12,20,28,30,45–49]. Transgenic pigs carrying reporter genes introduced by transposition in cultured cells used for SCNT [20,29] or one-cell embryos [12,28] have been produced. Similarly, transposase-mediated transgene integration has been documented in ruminant cells [33,45,47,50] and zygotes [51]. However, function and efficacy of PB and SB transposons in bovine cells and embryos have not been rigorously examined [52].

Initial experiments were designed to study if a PB helper–independent transgenic system already validated in different species [13,28] and a bicomponent SB transposon [10] could increase the efficiency of DNA integration into bovine fibroblast genome, the type of cell most commonly used as nuclear donor for SCNT.

Transfection of cultured BFFs with p*m*GENIE-3 plasmids was associated with higher numbers of resistant cell colonies, indicating that the PB transposase facilitated genome integration of reporter and antibiotic resistance genes. Transfection of bovine cells with an upgraded version of p*m*GENIE-3 carrying a hyperactive variant of PB transposase [13] enhanced genome integration of reporter genes as indicated by approximately three-fold increase in colony formation over the conventional nonoptimized enzyme and an unprecedented 85 times over those transfected with the control vector. High rates of transgene delivery have been achieved with both binary and single-plasmid PB transposon systems in cultured cells using classic colony formation assay [16]. p*m*GENIE-3 and its hyperactive version p*mhy*GENIE-3 were able to enhance HEK-293T colony formation seven and ten times respectively over those in the control without transposase [13]. Even higher transposition efficiency was observed in an established porcine cell line (28-fold) [32] and primary porcine fibroblasts (30-fold) transfected with the two-component PB system [29]. Under our experimental conditions, p*mhy*GENIE-3 was highly active in bovine cells being able to increment 85 times the number of resistant colonies compared with the control. With 324 ± 17.8 resistant colonies obtained in p*mhy*GENIE-3 transfected cultures per 0.5×10^5 plated fibroblasts, we can calculate a 21.6% of stably transfected cells on he basis of ~3% of transient transfection efficiency. The observed higher gene transfer activity in bovine cells compared with that reported by others in pig [29] and sheep cells [45] may reflect species-specific cell characteristics or conditions that favor transgene transposition, such as the presence or absence of cellular cofactors that affect transposase function, availability of DNA repair enzymes among others factors beyond intrinsic transposase activity. We also looked for nonenzymatic full-length or vector backbone insertion into the host cell genome. We found PCR evidence for nontranspositional genomic insertions of full-length PB vectors in three of nine cell lines analyzed and in four of six SB

Table 1
SCNT embryos produced with piggyBac and Sleeping Beauty transgenic cells.

Treatment	n	Cleaved (%)	Morulae (%)	Blastocysts (%)	Tg+[a]	
					Morulae (%)	Blastocysts (%)
p*mhy*GENIE-3	124	108 (87.0)	43 (34.6)	41 (33.0)	23 (53.4)	23 (56.0)
pT2RMCEVenus	75	66 (88.0)	29 (38.6)	27 (36.0)	15 (51.7)	15 (55.5)
PA control	126	122 (96.8)	76 (60.3)	67 (53.1)	—	—

n: number of reconstituted/fused presumptive embryos that were put in culture. Data from two independent replicates.
Abbreviations: PA, parthenogenetic activation and *in vitro* culture control; Tg+, Venus and/or EGFP expressing morulae and/or blastocyst.
[a] Tg morulae and blastocysts were calculated over total morulae and blastocysts, respectively.

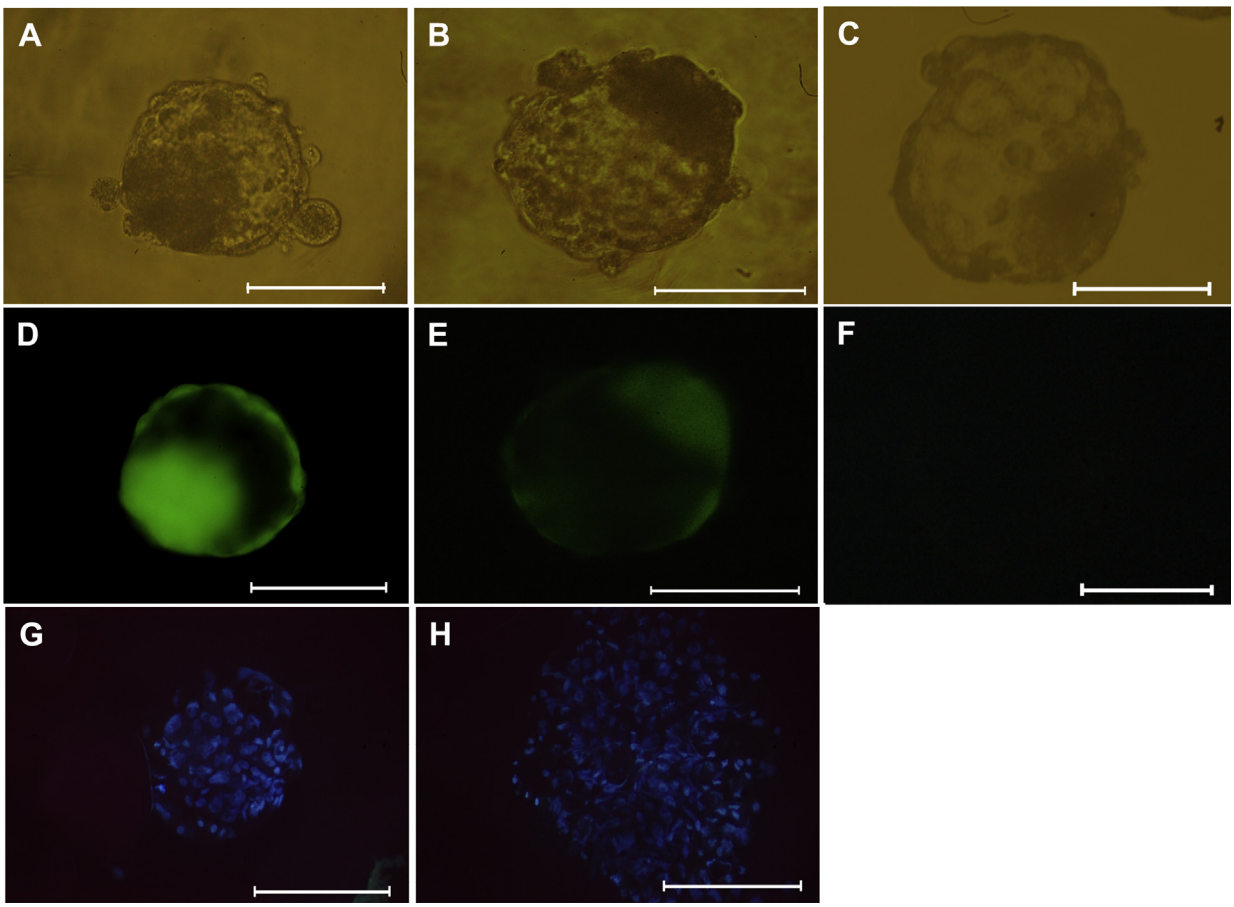

Fig. 8. Representative bright-field (A and B), fluorescent (D, E, G, and H) microphotographs of blastocysts obtained by SCNT with PB (A, D, and G) or SB (B, E, and H) transgenic BFF (bars = 100 μm). Transgenic blastocysts homogenously expressed reporter fluorophore in the inner cell mass and trophoblast cells (D and E). Vitrified/de-vitrified transgenic blastocysts were stained with Hoechst 33342 to determine cell numbers (G and H). Parthenogenic blastocyst (C) displayed negligible autofluorescence (F).BFF, bovine fetal fibroblast; PB, piggyBac; SB, Sleeping Beauty.

transgenic cell lines. In addition, recircularization of the remnant plasmid backbone after transposon excision followed by random genomic insertion has been documented for PB [14,21] and SB transposon systems [53]. PCR analysis of gDNA from nine PB transgenic cell lines revealed that only one harbored plasmid backbone sequences in its genome, whereas four of six SB transgenic cell lines had at least one copy of the religated plasmid backbone. These findings support the idea that besides degradation [54], chromosome integration is a potential fate for the plasmid backbone.

To study the function of SB transposase in bovine cells, we cotransfected primary fibroblast cultures with the plasmids that comprises a binary SB system. Being a bicomponent system, it seemed reasonable to optimize the amounts of helper to donor plasmids to achieve the desired transposition efficiency. Ratios of 1:1 and 2:1 (helper:donor) resulted in 78 and 88 times more colonies respectively compared with the control. These results can be interpreted as SB transposase is highly active in bovine cells and by increasing the proportion of SBX100 helper vector in the transfection mixture it is possible to maximize transgene integration into the cell genome without apparent cell toxic effects. Interestingly, within the range of SB transposase studied in our experiment, the well-characterized overproduction inhibition phenomenon described for SB transposons [16,55] was not observed.

In the second SB experiment, we use two donor plasmids, one carrying the Venus expression cassette and the other harboring a *neo* gene which confers resistance to G418. This design allows for selection of transgenic clonal cell lines using the neomycin analog G418. Results from this experiment confirmed the capacity of SB transposase to mediate incorporation of exogenous DNA into the bovine genome very efficiently. Surprisingly, all resistant colonies examined expressed the Venus protein what highlights the ability of SB transposase to simultaneously transpose two transgenes provided in separate donor plasmids. This multiplex feature could be of interest when the objective is to obtain bitransgenic animals. In addition, independent genomic integration of the antibiotic selection cassette from the transgene of interest may be used to segregate the selectable marker by breeding [20] to generate transgenic animals devoid of antibiotic resistance sequences to comply with the recommendations of most regulatory agencies.

Our nrLAM-PCR and splinkerette PCR data demonstrated that transgene integrations in the bovine genome corresponded with transposition-mediated events catalyzed by PB and SB transposase. All detected genomic integrations had on the right side the tetranucleotide TTAA for PB or TA dinucleotide for SB, followed by bovine genomic sequences, structures that agree with the known PB and SB transposition mechanisms [17,56]. Transposition ensures single copy, independent genomic integrations of transgenes thus reducing markedly epigenetic problems, namely silencing and variegated transgene expression as observed with viral vectors [57] and transgenic methods that rely on nonfacilitated transgene integration. The limited number of PB and SB genomic integration sites (17 and 15, respectively) in the bovine genome does not allow us to make conclusive statements about the preferences of PB or SB transposase to direct transgenes to particular domains of the bovine genome. Analysis of 575 PB transposase–mediated integration sites in the human genome showed that there is a slight tendency to incorporate the transposon into transcriptionally active regions or near them [16]. On the other hand, it has been established that SB transposase shows no predilection for transcription units, it rather preferentially recombines at intergenic chromosomal regions [58,59]. In our study, a clear copy number-dependent fluorescence intensity was found in nine PB transgenic cell lines indicating that most transgenes landed in transcriptionally permissive chromatin domains allowing for a faithful transgene expression [60].

We have produced a total of 38 transgenic cloned bovine blastocysts using cells from PB and SB polyclonal lines as nuclear donor for SCNT. Developmental rates to blastocyst were not different for embryos reconstructed with PB or SB transgenic cells (36.0% vs. 33.0%; respectively) and they are comparable to current standards of nuclear transfer blastocyst development rates reached with nontransgenic cells as nuclear donors [61–63]. Our nuclear transfer results reveal that transposon transgenic cells retain the ability to undergo nuclear reprograming to support early embryo development. Of the embryos that reached blastocyst stage, about half expressed the reporter fluorophore as per direct observation under blue light in an inverted microscope. We speculate that epigenetic silencing of transgene sequences could have occurred during initial cleavage divisions of the embryo. However, it cannot be ruled out that low-level reporter gene expression in some blastocysts was below the detection limit. Persistence of fluorophore expression without mosaicism in nuclear transfer blastocyst generated from cells transfected with PB plasmids was reported by Kim et al. [52].

4.1. Conclusions

In conclusion, both transposon systems under study were able to efficiently and precisely transpose monomeric copies of transgenes into bovine cell chromosomes. Transfection of primary bovine cell cultures with a helper-independent PB or the components of SB binary system notably increased (up to 88 times) the efficiency of genomic integration of foreign DNA molecules. Although donor cells are not normally considered a limiting resource for SCNT, improvements in stable gene integration in primary cells can be of value when working with difficult-to-transfect primary cells. Demonstrated multiplexing ability of SB is an asset when more complex genetic manipulations of the bovine genome are sought. These transposon–transgenic cells can be reprogramed on transfer to enucleated mature oocytes to morphologically normal blastocysts that expressed the transgene. Transposon-based transgenic methods alone or combined with other recombinase-based tools will simplify the production of transgenic cattle that consistently express the gene of interest. Incorporation of this active transgenesis method to the bovine transgenesis toolkit will certainly expand the opportunities for directed manipulation of the bovine genome for agricultural and biomedical applications.

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Drs. Alessio and Fili contributed equally.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2015.12.016

References

[1] Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, et al. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol 2002;20:251–5.
[2] McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 2000;405:1066–9.
[3] Ramsoondar JJ, Machaty Z, Costa C, Williams BL, Fodor WL, Bondioli KR. Production of alpha 1,3-galactosyltransferase-knockout cloned pigs expressing human alpha 1,2-fucosylosyltransferase. Biol Reprod 2003;69:437–45.
[4] Mendicino M, Ramsoondar J, Phelps C, Vaught T, Ball S, LeRoith T, et al. Generation of antibody- and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain locus. Transgenic Res 2011;20:625–41.
[5] Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 2008;321:1837–41.
[6] Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, et al. Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet 2004;36:775–80.
[7] Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, et al. Production of cattle lacking prion protein. Nat Biotechnol 2007;25:132–8.
[8] Bosch P, Forcato DO, Alustiza FE, Alessio AP, Fili AE, Olmos Nicotra MF, et al. Exogenous enzymes upgrade transgenesis and genetic engineering of farm animals. Cell Mol Life Sci 2015;72:1907–29.
[9] Laible G, Wei J, Wagner S. Improving livestock for agriculture - technological progress from random transgenesis to precision genome editing heralds a new era. Biotechnol J 2015;10:109–20.

[10] Mátés L, Chuah MK, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, et al. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet 2009;41:753–61.
[11] Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A 2011;108:1531–6.
[12] Garrels W, Mates L, Holler S, Dalda A, Taylor U, Petersen B, et al. Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLoS One 2011;6:e23573.
[13] Marh J, Stoytcheva Z, Urschitz J, Sugawara A, Yamashiro H, Owens JB, et al. Hyperactive self-inactivating piggyBac for transposase-enhanced pronuclear microinjection transgenesis. Proc Natl Acad Sci U S A 2012;109:19184–9.
[14] Urschitz J, Kawasumi M, Owens J, Morozumi K, Yamashiro H, Stoytchev I, et al. Helper-independent piggyBac plasmids for gene delivery approaches: strategies for avoiding potential genotoxic effects. Proc Natl Acad Sci U S A 2010;107:8117–22.
[15] Mikkelsen JG, Yant SR, Meuse L, Huang Z, Xu H, Kay MA. Helper-Independent Sleeping Beauty transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression in vivo. Mol Ther 2003;8:654–65.
[16] Wilson MH, Coates CJ, George Jr AL. PiggyBac transposon-mediated gene transfer in human cells. Mol Ther 2007;15:139–45.
[17] van Luenen HG, Colloms SD, Plasterk RH. The mechanism of transposition of Tc3 in C. elegans. Cell 1994;79:293–301.
[18] Fraser MJ, Cary L, Boonvisudhi K, Wang HG. Assay for movement of Lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. Virology 1995;211:397–407.
[19] Plasterk RH, Izsvák Z, Ivics Z. Resident aliens: the Tc1/mariner superfamily of transposable elements. Trends Genet 1999;15:326–32.
[20] Carlson DF, Garbe JR, Tan W, Martin MJ, Dobrinsky JR, Hackett PB, et al. Strategies for selection marker-free swine transgenesis using the Sleeping Beauty transposon system. Transgenic Res 2011;20:1125–37.
[21] Saha S, Woodard LE, Charron EM, Welch RC, Rooney CM, Wilson MH. Evaluating the potential for undesired genomic effects of the piggyBac transposon system in human cells. Nucleic Acids Res 2015;43:1770–82.
[22] Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. Nat Rev Cancer 2012;12:51–8.
[23] Ivics Z, Hiripi L, Hoffmann OI, Mátés L, Yau TY, Bashir S, et al. Germline transgenesis in rabbits by pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc 2014;9:794–809.
[24] Ivics Z, Mátés L, Yau TY, Landa V, Zidek V, Bashir S, et al. Germline transgenesis in rodents by pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc 2014;9:773–93.
[25] Katter K, Geurts AM, Hoffmann O, Mates L, Landa V, Hiripi L, et al. Transposon-mediated transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. FASEB J 2013;27:930–41.
[26] Jang CW, Behringer RR. Transposon-mediated transgenesis in rats. CSH Protoc 2007;2007. pdb.prot4866.
[27] Staunstrup NH, Madsen J, Primo MN, Li J, Liu Y, Kragh PM, et al. Development of transgenic cloned pig models of skin inflammation by DNA transposon-directed ectopic expression of human beta1 and alpha2 integrin. PLoS One 2012;7:e36658.
[28] Li Z, Zeng F, Meng F, Xu Z, Zhang X, Huang X, et al. Generation of transgenic pigs by cytoplasmic injection of piggyBac transposase-based pmGENIE-3 plasmids. Biol Reprod 2014;90:1–10.
[29] Wu Z, Xu Z, Zou X, Zeng F, Shi J, Liu D, et al. Pig transgenesis by piggyBac transposition in combination with somatic cell nuclear transfer. Transgenic Res 2013;22:1107–18.
[30] Al-Mashhadi RH, Sorensen CB, Kragh PM, Christoffersen C, Mortensen MB, Tolbod LP, et al. Familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of a human PCSK9 gain-of-function mutant. Sci Transl Med 2013;5:166ra1.
[31] Ivics Z, Garrels W, Mátés L, Yau TY, Bashir S, Zidek V, et al. Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. Nat Protoc 2014;9:810–27.
[32] Clark KJ, Carlson DF, Foster LK, Kong BW, Foster DN, Fahrenkrug SC. Enzymatic engineering of the porcine genome with transposons and recombinases. BMC Biotechnol 2007;7:42.
[33] Alessio A, Fili A, Forcato D, Olmos-Nicotra F, Alustiza F, Rodriguez N, et al. Efficient piggyBac transposon-mediated transgene integration into bovine fetal fibroblast genome. Reprod Domest Anim 2014;49:8.
[34] Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:193–9.
[35] Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell 1997;91:501–10.
[36] Mathupala S, Sloan AA. An agarose-based cloning-ring anchoring method for isolation of viable cell clones. Biotechniques 2009;46:305–7.
[37] Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. J Biotechnol 2006;123:273–80.
[38] McCloy RA, Rogers S, Caldon CE, Lorca T, Castro A, Burgess A. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. Cell Cycle 2014;13:1400–12.
[39] Owens JB, Urschitz J, Stoytchev I, Dang NC, Stoytcheva Z, Belcaid M, et al. Chimeric piggyBac transposases for genomic targeting in human cells. Nucleic Acids Res 2012;40:6978–91.
[40] Paruzynski A, Arens A, Gabriel R, Bartholomae CC, Scholz S, Wang W, et al. Genome-wide high-throughput integrome analyses by nrLAM-PCR and next-generation sequencing. Nat Protoc 2010;5:1379–95.
[41] Ivics Z, Izsvák Z, Medrano G, Chapman KM, Hamra FK. Sleeping Beauty transposon mutagenesis in rat spermatogonial stem cells. Nat Protoc 2011;6:1521–35.
[42] Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, et al. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. Mol Reprod Dev 2000;55:256–64.
[43] Martínez AG, Valcárcel A, Furnus CC, de Matos DG, Iorio G, de las Heras MA. Cryopreservation of in vitro-produced ovine embryos. Small Rumin Res 2006;63:288–96.
[44] Traldi AS, Leboeuf B, Cognié Y, Poulin N, Mermillod P. Comparative results of in vitro and in vivo survival of vitrified in vitro produced goat and sheep embryos. Theriogenology 1999;51:175.
[45] Hu S, Ni W, Sai W, Zhang H, Cao X, Qiao J, et al. Sleeping Beauty-mediated knockdown of sheep myostatin by RNA interference. Biotechnol Lett 2011;33:1949–53.
[46] Jakobsen JE, Li J, Kragh PM, Moldt B, Lin L, Liu Y, et al. Pig transgenesis by Sleeping Beauty DNA transposition. Transgenic Res 2011;20:533–45.
[47] Bai DP, Yang MM, Chen YL. PiggyBac transposon-mediated gene transfer in Cashmere goat fetal fibroblast cells. Biosci Biotechnol Biochem 2012;76:933–7.
[48] Liu X, Li N, Hu X, Zhang R, Li Q, Cao D, et al. Efficient production of transgenic chickens based on piggyBac. Transgenic Res 2013;22:417–23.
[49] Park TS, Han JY. piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. Proc Natl Acad Sci U S A 2012;109:9337–41.
[50] Jiang BC, Kaleri HA, Zhang HX, Chen J, Liu HL. Application of the Sleeping Beauty system in Saanen goat fibroblast cells for establishing persistent transgene expression. Genet Mol Res 2011;10:3347–55.
[51] Garrels W, Talluri TR, Bevacqua RC, Alessio A, Fili A, Forcato D, et al. Sleeping Beauty transgenesis in cattle. Reprod Fertil Dev 2015;27:266.
[52] Kim S, Saadeldin IM, Choi WJ, Lee SJ, Lee WW, Kim BH, et al. Production of transgenic bovine cloned embryos using piggybac transposition. J Vet Med Sci 2011;73:1453–7.
[53] Bell JB, Aronovich EL, Schreifels JM, Beadnell TC, Hackett PB. Duration of expression and activity of Sleeping Beauty transposase in mouse liver following hydrodynamic DNA delivery. Mol Ther 2010;18:1796–802.
[54] Wang X, Sarkar DP, Mani P, Steer CJ, Chen Y, Guha C, et al. Long-term reduction of jaundice in Gunn rats by nonviral liver-targeted delivery of Sleeping Beauty transposon. Hepatology 2009;50:815–24.
[55] Geurts AM, Yang Y, Clark KJ, Liu G, Cui Z, Dupuy AJ, et al. Gene transfer into genomes of human cells by the sleeping beauty transposon system. Mol Ther 2003;8:108–17.
[56] Mitra R, Fain-Thornton J, Craig NL. piggyBac can bypass DNA synthesis during cut and paste transposition. EMBO J 2008;27:1097–109.
[57] Ellis J. Silencing and variegation of gammaretrovirus and lentivirus vectors. Hum Gene Ther 2005;16:1241–6.
[58] Ikeda R, Kokubu C, Yusa K, Keng VW, Horie K, Takeda J. Sleeping beauty transposase has an affinity for heterochromatin conformation. Mol Cell Biol 2007;27:1665–76.

[59] Yant SR, Wu X, Huang Y, Garrison B, Burgess SM, Kay MA. High-resolution genome-wide mapping of transposon integration in mammals. Mol Cell Biol 2005;25:2085–94.
[60] Grabundzija I, Irgang M, Mátés L, Belay E, Matrai J, Gogol-Döring A, et al. Comparative analysis of transposable element vector systems in human cells. Mol Ther 2010;18:1200–9.
[61] Canel N, Bevacqua R, Fernández-Martín R, Salamone DF. Activation with ionomycin followed by dehydroleucodine and cytochalasin B for the production of parthenogenetic and cloned bovine embryos. Cell Reprogram 2010;12:491–9.
[62] Li X, Amarnath D, Kato Y, Tsunoda Y. Analysis of development-related gene expression in cloned bovine blastocysts with different developmental potential. Cloning Stem Cells 2006;8:41–50.
[63] Iager AE, Ragina NP, Ross PJ, Beyhan Z, Cunniff K, Rodriguez RM, et al. Trichostatin A improves histone acetylation in bovine somatic cell nuclear transfer early embryos. Cloning Stem Cells 2008;10:371–9.

Supplementary Table 1
Primers name and sequences.

Primer name	Sequence
PB-GFP-F	ACGTAAACGGCCACAAGTTC
PB-GFP-R	AAGTCGTGCTGCTTCATGTG
PB-ITR-F	GATCTCGTCGGTGAAGAACAG
PB-ITR-R	ACTCCCAGACATGCCCAAGT
PB-TPase-R	AGATCCTGGACGAGCAGAAC
PB-TPase-F	GATCTCGTCGGTGAAGAACAG
PB-RL-F	GGTGCTCTTGGAGGTGGAC
PB-RL-R	CCGCGACGAACTGGTATC
SB-Venus-F	TAGCCCAGGGTGGTCACCAG
SB-Venus-R	TGTGACCGGCGGCTCTAGAG
SB-ITR-F	CTCGAGAAGCTTGTGGAAGG
SB-ITR-R	TGACCATGATTACGCCAAGC
SB-BB-F	GCTCTTGATCCGGCAAACAA
SB-BB-R	GAGGCGGATAAAGTTGCAGG
SB-RL-F	GGGGATGTGCTGCAAGGC
SB-RLR	TGACCATGATTACGCCAAGC
SB-TPase-F	CAGCAAGGAAGAAGCCACTG
SB-TPase-R	ACTTGGGTCAAACGTTTCGG

Supplementary Table 2
Bovine genomic insertion sites for piggyBac transposons.

Insertion number	Chromosome	Location	Gene	ITR TTAA genomic sequence
1	ch15	19428292	Intergenic	cagactatctttctagggTTAACATATGAACTTGAGAGGG
2	chrUn_JH122283	39105	unknown	cagactatctttctagggTTAAGGGATTTGAGTGAATTTG
3	ch21	25472450	Intergenic	cagactatctttctagggTTAAGACCCC ATGGACTGCAGC
4	chrUn_AAFC03095622	1018	unknown	cagactatctttctagggTTAAGACCCCATGGACTGCAGC
5	ch5	79123143	*RBFOX2*, intron	cagactatctttctagggTTAATCTCCTTTGCAGAAAGGA
6	ch6	37957543	Intergenic	cagactatctttctagggTTAAGGAGTTTAACAGGAATCA
7	ch7	105149760	Intergenic	cagactatctttctagggTTAAAGTCAGATCCACCAGTCT
8	chrUn_JH121295	390708	unknown	cagactatctttctagggTTAATCTGAAGAAAAGTGAAAA
9	ch15	141258670	Intergenic	cagactatctttctagggTTAATGTACTGACAAAGCTCCA
10	chX	529630	Intergenic	cagactatctttctagggTTAAGACAAATGTGCTCTTAAA
11	ch9	82826492	Intergenic	cagactatctttctagggTTAAGCCACAAAAGCGGGGGTC
12	ch26	18051846	Intergenic	cagactatctttctagggTTAAAAAGGCAGTTTCCGCCAT
13	ch2	8600480	*GULP1*, intron	cagactatctttctagggTTAAGATTAAGTGTGCCCCATC
14	ch16	19235265	Intergenic	cagactatctttctagggTTAAAAGATGTTGAATTAAGTT
15	ch27	45374270	*UBE2E2*, intron	cagactatctttctagggTTAAAGATGAAAAATCCCCAGT
16	ch27	10507265	Intergenic	cagactatctttctagggTTAAAAAAGGTAGCCAGTTGCA
17	ch7	49826129	Intergenic	cagactatctttctagggTTAAAGTGGAGGAGACTTTTTT

Supplementary Table 3
Bovine genomic insertion sites for SB transposon.

Insertion	Chomosome	Location	Gene	Genomic sequence TA SB-ITR
C5.1	13	78983	Prion Protein (*PRNP*)	TTCTGTCATAAGGGTGGTGTCATATGCATACAGTTGAAGT
C5.2	X	1	*ZFX* intron	TCCCATGGGCCAAACACTGATCTAAGTATACAGTTGAAGT
C5.4	25	3016	Sorting nexin 29 (*SNX29*)	CCCTGGTGGGGCGTATGCGGAAAGGAACTACAGTTGAAGT
C5a.2	26	2080521	Intergenic	AANTGGCCAACAAAACAATATAGTGACCTACAGTTGAAGT
C5a.5	12	27360511	Intergenic	TTTTTAGTGCAGGTTTGAGGGCTGGAATTACAGTTGAAGT
C6.1	mtDNA	26009	Mitochondrial acetyl-Coenzyme A acyltransferase 2 (*ACAA2*), complete cds	CTGACACTGTTTCCACTGTTTCCCCATCTACAGTTGAAGT
C6a.3	19	44952926	Intergenic	GCCAGGCTGGGAGGATCTGGACCATATACAGTTGAAGT
C6a.5	25	36298502	Intergenic	TGAATGGATAAAGAAAATATGGCATGTACAGTTGAAGT
C7.2	13	35994	Intergenic	GACACTGTTTCCACTGTTTCCCCATCTACAGTTGAAGT
C7.3	22	24703993	Intergenic	TTAAATAGATCTGAATTCTAAGCATTCATACAGTTGAAGT
C7a.1	5	Unknown	Intergenic	TTTTAAGTGTTACATTTATTTCCAGCTACAGTTGAAGT
C8a.2	18	Unknown	Intergenic	TTGATTACATAATGGATTATCTGGGATCTACAGTTGAAGT
11.1	Y	465464	Y-linked ubiquitin-specific protease 9 (*USP9Y*) gene	CTAAGGATTTTTAAAAATTAAATAAATATACAGTTGAAGT
11.3	22	15535315	Intergenic	GAACTGAAGTTGGAGAGACACAACTGTACAGTTGAAGT
31.1	26	24931481	Intergenic	AAGGATTTTTAAAAATTAAATAAATATACAGTTGAAGT