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1 **Revised**

2 # Establishment of cell-based transposon-mediated transgenesis in cattle

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25 **Short title**: Transposon-mediated transgenesis in cattle

1 **Abstract**

2 Transposon-mediated transgenesis is a well-established tool for genome modification in small
3 animal models. However, translation of this active transgenic method to large animals warrants
4 further investigations. Here, the piggyBac (PB) and Sleeping Beauty (SB) transposon systems
5 were assessed for stable gene transfer into the cattle genome. Bovine fibroblasts were transfected
6 either with a helper-independent PB system or a binary SB system. Both transposons were highly
7 active in bovine cells increasing the efficiency of DNA integration up to 88 times over basal
8 non-facilitated integrations in a colony formation assay. SB transposase catalyzed multiplex
9 transgene integrations in fibroblast cells transfected with the helper vector and two donor vectors
10 carrying different transgenes (fluorophore and neomycin resistance). Stably transfected
11 fibroblasts were employed for somatic cell nuclear transfer (SCNT) and upon in vitro embryo
12 culture, morphologically normal blastocysts that expressed the fluorophore were obtained with
13 both transposon systems. The data indicate that transposition is a feasible approach for genetic
14 engineering in the cattle genome.

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1 **1. Introduction**

2 The advent of high throughput DNA sequencing methods and comprehensive annotated genome
3 maps concomitantly with advanced active transgenic techniques promise to revolutionize the
4 field of animal biotechnology. In particular, areas like disease modeling, biopharming, as well as
5 basic research will benefit enormously by introducing precise genetic engineering tools to
6 manipulate livestock genomes. Initial transgenic methods relayed on passive (non-facilitated)
7 genomic integration of transgenes at sites of spontaneously arising double-strand breaks (DSBs)
8 of chromosomes after direct injection of naked DNA into zygotes (pronuclear injection) or
9 transfection of cultured cells followed by somatic cell nuclear transfer (SCNT). Homologous
10 recombination in somatic cells of livestock is an extremely rare event, and only a few genes were
11 targeted in recent years [1-7]. These inefficient and unreproducible approaches were gradually
12 superseded by a new generation of active methods in which genomic insertion of heterologous
13 DNA molecules is catalyzed by exogenously provided enzymes that catalyze DNA
14 recombination [Reviewed in 8, 9].
15 For gain-of-function approaches in farm animals, the engineered transposon systems Sleeping
16 Beauty (SB) and piggyBac (PB) gained increasing interests in recent years [10, 11]. Transposon-
17 based transgenic methods, derived from naturally occurring DNA transposable elements, are
18 non-viral gene delivery systems capable of efficient enzyme-mediated genomic insertion of
19 DNA segments into the genome. During transposition a single-copy of the sequence of interest
20 framed by inverted terminal repeats (ITRs) is integrated into the genome through a precise,
21 transposase-catalyzed mechanism, providing long-term expression of the gene of interest in cells
22 [12]. Bi-component transposon-based transgenic systems comprise a donor vector containing the
23 transgene flanked by transposase-specific ITRs and the transposase enzyme provided as protein,

1 mRNA or most commonly as a helper DNA vector. In addition, systems that combine both
2 components in a single vector, known as helper-independent transposons, have been developed
3 and validated in cells and animals [13-16]. Transposase catalyzes both the excision of the
4 transgene from the donor vector and its integration into a genomic target site. Integration occurs
5 at short consensus sequences, for example Tc1/mariner transposases, like SB, recognize TA
6 dinucleotides [17], and PB transposase recognizes TTAA tetranucleotides [18]. Through this
7 mechanism one monomeric copy of a transposon is integrated in the genome, leaving the empty
8 backbone of the donor plasmid, which is eventually degraded [12, 19, 20] or, rarely, randomly
9 integrated [14, 21]. Expression units introduced by transposition are less prone to epigenetic
10 silencing, and show long-term expression of the transgene [12], suggesting that transposons have
11 a tendency to land in genomic regions that are transcriptionally permissive [22].
12 PB and SB transposons have been extensively studied for transgenesis in mice, rats and rabbits
13 [13, 23-26]. Both in vivo (intracytoplasmic injection of zygotes) and in vitro (somatic cell
14 nuclear transfer, SCNT) approaches have been exploited in order to generate transposon-
15 transgenic pig models [12, 20, 27-31]. Zygote microinjection with SB transposon components
16 has resulted in single-copy integration units into the pig preimplantation embryo genome [20],
17 into born F0 animals and successful transmission to F1 generation [12]. Garrels et al. [12]
18 demonstrated segregation of individual transposons in the F1 offspring, copy-number dependent
19 expression of reporter protein over a prolonged time with no evidence of gene silencing.
20 Similarly, transgenic pigs generated by microinjection of a helper-independent, self-inactivating
21 PB transposon had monogenic and often single transgene genomic integration and absence of
22 concatemers or variegated transgene expression [28].
23 Alternatively, genetic modification of somatic cells by PB or SB transposition followed by
24 SCNT is an avenue to generate transgenic livestock. Here, we assessed the suitability of the PB

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and SB transposon systems for the genetic modification of bovine fibroblasts, which were subsequently employed in SCNT.

To this end, cultured fibroblast cells were transfected or electroporated with both transposon systems, respectively, and upon selection or enrichment of transgenic cells, they were used as nuclear donor in SCNT. The use of transposons is associated with 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 Fig. 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. 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 (PCR, nrLAM-PCR and Splinkerette PCR). Transgenic polyclonal cell cultures generated from PB or SB were used as nuclear donors in SCNT experiments in order to assess their ability to support early embryo development.

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1 *2.2. Vectors*

2 *2.2.1. PiggyBac plasmids:*

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

7 p*m*GENIE-3: this plasmid carries EGFP coding sequence which is under of control of CAG
8 promoter. In addition the plasmid harbors the hygromycin B resistance gene.

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

11 p*m*GENIE-2/Δ piggyBac: it is a control plasmid that codes for a non-functional truncated
12 transposase. We will refer to this plasmid as p*m*GENIE-Control.

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

14 *2.2.2. Sleeping Beauty plasmids:*

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

18 pT2RMCEVenus: this plasmid contains an eukaryotic expression cassette for Venus fluorescent
19 protein under the control of CAGGS promoter. The expression cassette is flanked by 5`and 3´
20 ITRs of SB transposon.

21 pT2/SV40-Neo: this plasmid contains an expression cassette for neomycin phosphotransferase,
22 which confers resistance to the antibiotic geneticin (G418), flanked by SB ITRs. SV40 promoter
23 drives expression of the antibiotic resistance gene [34].

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1 pBSII-ISceI-skA: this plasmid is used as control in experiments with SB system (kindly provided
2 by Dr. Jochen Wittbrodt, Developmental Biology Programme, EMBL-Heidelberg, Heidelberg,
3 Germany).
4 Schematic representations of all plasmids are depicted in Fig. 2.

5 *2.3. Culture of primary cells*

6 Primary bovine fetal fibroblasts (BFF) were obtained from slaughterhouse fetuses of 90-150
7 days of gestation. Fetuses were transported to the laboratory where they were processed in a
8 laminar-flow cabinet. A piece of subdermal tissue (about 1 cm^2) was removed from the flank of
9 the fetus and sectioned with a scalpel blade into smaller pieces. Explants were placed in cell
10 culture plates (3 to 4 explants per 100 mm plate) in 6 ml of cell culture medium (DMEM, 1x
11 antibiotic / antimycotic, Gibco, CA, USA) supplemented with 10% FBS (Natocor, Cordoba,
12 Rep. Argentina) and cultured for approximately 10 days in an atmosphere of 5% CO_2 in air and
13 high humidity at 38.5ºC. When the cells reached 70-80% confluence, they were trypsinized
14 (trypsin 0.5%, Sigma-Aldrich Co., St. Louis, MO, USA) and passaged to a T-75 culture flask for
15 cell propagation. Fibroblasts were frozen in DMEM containing 20% FBS and 10% DMSO
16 (Sigma-Aldrich Co., St. Louis, MO, USA) and kept in liquid nitrogen until use.

17 *2.4. Cell transfection with transposon vectors*

18 Bovine fetal fibroblasts were seeded at $0.5 x 10^5$ cells per well of a 24-well plate. When the cells
19 reached 80% confluence (12-24 h), cultures were transfected with a polyethylenimine-based
20 transfection reagent (JetPRIME®, Polyplus-transfection SA, Illkirch, France) and 1 μg of
21 plasmid DNA according to the manufacturer's instructions. Forty eight hours post-transfection,
22 expression of reporter fluorescent protein was assessed in a microscope equipped with
23 epifluorescence (excitation filter 450-490 nm and an emission filter 530 nm). Images of

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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 piggyBac 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 was replaced every other day.

In the first SB transfection experiment, fibroblast cultures were co-transfected with pT2RMCEVenus (donor plasmid) and pCMV(CAT)T7-SB100X (helper plasmid). In the second experiment, fibroblasts were co-transfected 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, (non-facilitated) transgene integration could be estimated. No antibiotic selection was applied for cells transfected with pT2RMCEVenus since this vector is devoid of antibiotic selection cassette and the number of fluorescent colonies was determined 12-14 days post transfection. In experiments of co-transfection 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.

1 *2.5. Colony formation assay and derivation of monoclonal transgenic cell lines*

2 Transfected cells were kept under antibiotic selection for two weeks and the numbers of
3 fluorescent colonies were counted in an inverted microscope (Nikon Corp., Tokyo, Japan) with
4 epifluorescence equipment. To assess the transposition activity of the different plasmids we
5 calculated the relative fold change, which was obtained by dividing the number of antibiotic
6 resistant colonies counted in cultures transfected with p*mhy*GENIE-3 or p*m*GENIE-3 plasmids
7 by the number of colonies in those transfected with p*m*GENIE-Control plasmid.
8 In the SB experiment in which fibroblasts were co-transfected with two plasmids
9 (pT2RMCEVenus and pCMV(CAT)T7-SB100X), the number of Venus positive colonies
10 formed after two-week culture were compared with those in the control treatment. When cell
11 cultures were co-transfected with three plasmids (pT2RMCEVenus, pT2/SV40-Neo and
12 pCMV(CAT)T7-SB100X), the number of colonies originated under antibiotic selection (G418)
13 were determined after 14 days.
14 For clonal expansion, individual cell colonies were recovered from 100 mm plates using cloning
15 rings, small plastic cylinders of 7 mm of diameter that were placed over each individual colony
16 and fixed and sealed with agarose [35]. Cells inside the cloning ring were trypsinized and
17 cultivated in 24-well-plates up to 80-90% confluence. Cell lines were sequentially passaged into
18 larger-sized culture plates as the number of cells increased. Once they reached 80-90% of
19 confluence in a T75 culture flask each cell line was processed to obtain gDNA for molecular
20 characterization of transgene integration.

21 *2.6. Molecular characterization of transgene genomic integrations*

22 PCR: Genomic DNA was isolated from transgenic cell lines using Quick-gDNA™ MiniPrep
23 (Zymo Research Corporation, Irvine, USA.) according to the manufacturer's protocol. We

1 designed a PCR strategy (four reactions) to characterize PB transgene genomic integrations
2 (non-facilitated or transposase catalyzed). PCR1: amplification of a 187-bp fragment of the
3 EGFP gene with primers pair PB-GFP-F/ PB-GFP-R. Presence of a correct-size PCR product
4 confirmed the presence of the transgene (Fig. 3 g). PCR2: amplified a 537- bp fragment
5 extending from the 3'-ITR into the backbone of the plasmid using primers pair PB-ITR-F/PB-
6 ITR-R. Amplification of the correct size product would indicate non-transpositional transgene
7 integration (Fig. 3 g). PCR3: this reaction was included to determinate if nontranspositional
8 integration of the recircularized backbone of the plasmid after transposition had occurred, as it
9 was reported in mice [14]. PCR was performed using PB-RL-F/PB-RL-R primer pair to amplify
10 a 442-pb backbone fragment (Fig. 5 a). Identity of PCR products was confirmed by sequencing.
11 PCR4: To determinate integration of PB transposase sequences into the bovine genome primer
12 pair PB-TPase-F /PB-TPase-R was used (Fig. 3 g). A PCR amplicon of 245 bp was expected in
13 cell lines that contained at least one integrated copy of the PB transposase coding sequence.
14 Multiplex PCRs were run using the following parameters: initial denaturalization at 94°C for 9
15 min followed by 30 cycles of 20 s denaturalization at 94°C, 30 s annealing at 60°C, and 30 s
16 elongation at 72°C, with a final elongation of 5 min.
17 gDNA isolated from six SB monoclonal transgenic cell lines was used as template in a series of
18 four reactions of PCRs to characterize SB transgene integrations . PCR1: amplification of a 280-
19 bp fragment from the Venus gene with primers pair SB-Venus-F/ SB-Venus-R (Fig. 7 e).
20 Presence of a correct-size PCR product confirmed the presence of the transgene. PCR2:
21 amplification of a 408-bp fragment extending from the 3'-ITR into the backbone of the plasmid
22 using primers pair SB-ITR-F/SB-ITR-R (Fig. 7 e). Amplification of the correct size product
23 indicated non-transpositional transgene integration. PCR3: amplification of a 493-bp fragment of
24 the plasmid backbone with primer pair SB-BB-F/SB-BB-R (Fig. 7 e). Presence of correct sized

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1 product indicated genomic integration of backbone sequences by non-transpositional
2 mechanisms. PCR4: this reaction was conducted with primer pair SB-RL-F/SB-RL-R (Fig. 5 a).
3 Presence of a 233-bp amplicon indicated genomic integration of re-ligated backbone after
4 transposition had occurred. PCR product was identified by restriction enzyme digestion (XhoI).
5 Upon digestion, the expected product would render two fragments of 75 bp and 158 bp. PCR5:
6 this reaction was run with primer pair SB-TPase-F/SB-TPase-R (Fig. 7 e). Presence of a 643 bp
7 fragment indicated genomic non-facilitated insertion of the helper SBX100 plasmid.
8 All PCRs were run with the following parameters: initial denaturalization at 95°C for 5 min
9 followed by 35 cycles of 15 s denaturalization at 95°C, 15 s annealing at 63°C, and 45 s
10 elongation at 72°C, with a final elongation of 10 min.
11 All primer sequences are shown in Supplementary Table 1.

12 *2.7. Real-time quantitative PCR*

13 Number of EGFP copies per clonal cell line (n=9) was determined by real-time PCR as described
14 previously by Lee et al. [36]. Briefly, 20 ng of genomic DNA was used as template in real-time
15 qPCR using KAPA SYBR® FAST qPCR kit (Kapa Biosystem Inc., Boston, MA, USA) and
16 Applied Biosystems 7500 instrument (Applied Biosystems, Waltham, MA USA). Primer set
17 used to amplify EGFP transgene was PB-GFP-F - PB-GFP-R (Supplementary Table 1). qPCR
18 was run using the following parameters: initial denaturalization at 94°C for 10 min followed by
19 40 cycles of 10 s denaturalization at 95°C, 10 s annealing at 61°C and 10 s elongation at 72°C.
20 The fluorescent signal was measured at the end of each elongation step at 72°C. After
21 amplification, a melting curve was performed by increasing the temperature from 70 to 95°C at a
22 rate of 0.1°C / s to confirm the identity of PCR product. Number of copies was calculated from a

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1 standard curve which was generated using 10-fold serial dilution p*m*GENIE-3 (5.93×10^2 to 5.93
2 $\times 10^6$ copies/µl).

3 *2.8. Measurement of cell line fluorescence intensity*

4 To establish a putative association between transgene copy number and fluorescence intensity of
5 transgenic cell lines (n=9), we measured fluorescence in individual cells using ImageJ software
6 (V1.49, NHI) and CTCF using the following equation**:** CTCF = Integrated Density – (Area of
7 selected cell x mean fluorescence of background readings) as previously described [37]**.** Linear
8 regression analysis was performed using CTCF as independent variable and transgene copy
9 number as dependent variable.

10 *2.9. Nonrestrictive Linear Amplification-Mediated PCR (nrLAM-PCR)*

11 Identification of transpositionally generated sites of insertion was achieved by nrLAM-PCR
12 according to published protocols [38, 39]. Genomic DNA from p*m*GENIE-3 transgenic cell lines
13 was used as a template for linear amplification and nested PCR was implemented to amplify the
14 sequence flanking the PB insertions. PCR products were cloned into a vector and sequenced.
15 DNA sequences directly flanking the transposon were mapped to the bovine genome by doing a
16 BLAST search at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

17 *2.10. Splinkerette PCR*

18 SB insertions in six Venus+/Neo^R monoclonal cell lines were characterized by a splinkerette
19 PCR method as previously described [40]. Transgene flanking sequences were used to map
20 insertion sites to the publicly available bovine genome (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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1 *2.11. Somatic cell nuclear transfer*

2 Oocyte collection and in vitro maturation: Bovine ovaries were collected from abattoirs and
3 transported to the laboratory at 25 °C to 30 °C. Cumulus-oocyte complexes were aspirated with
4 21-gauge needles from follicles with a diameter of 2 to 5 mm into HEPES-buffered Tyrode's
5 albumin lactate pyruvate (HEPES-TALP). Oocytes covered with at least three layers of
6 granulosa cells were selected for IVM. The maturation medium was bicarbonate-buffered TCM-
7 199 (31100-035; Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (013/07;
8 Internegocios, Buenos Aires, Argentina), 10 µg/mL follicle stimulating hormone (NIH-FSH-P1;
9 Folltropin; Bioniche, Caufield North, Victoria, Australia), 0.3 mM sodium pyruvate (Sigma,
10 P2256), 100 µM cysteamine (Sigma, M9768), and 2% antibiotic-antimycotic (15240-096;
11 Gibco). The oocytes were incubated for 21 hours in 100 µL droplets under mineral oil (Sigma,
12 M8410), in 6.5% CO_2 in humidified air at 39 °C.

13 Enucleation procedure: After 21 h of IVM, MII oocytes were subjected to hyaluronidase
14 treatment followed by incubation in 1.5 mg/ml pronase to remove the Zona pellucida. Zona-free
15 oocytes were stained with 1 µg/mL of Hoechst 33342 for 10 min. and afterwards transferred into
16 50 µL microdroplets of HEPES-TALP supplemented with 0.3 g/mL BSA, under mineral oil, in
17 100 × 20 mm tissue culture dishes (430167; Corning, Horseheads, NY, USA). The stained
18 oocytes were mechanically enucleated using a Narishige hydraulic micromanipulator (Narishige
19 Sci., Tokyo, Japan) mounted on a Nikon Eclipse E-300 microscope (Nikon, Tokyo, Japan).
20 Enucleation was performed using a blunt 20 µm internal diameter pipette. Zona-free oocytes
21 were supported for enucleation with a closed holding pipette (100-150 µm outer diameter,
22 perpendicular break, closed fire-polished tip). Metaphase chromosomes were visualized under
23 ultraviolet light (<10 s) and aspirated into the blunt pipette with a minimal volume of oocyte

1 cytoplasm. Chromosome removal was confirmed by the presence of stained MII chromosomes
2 inside the pipette.
3 Donor cell preparation and fusion to enucleated oocytes: Cells transfected with p*mhy*GENIE-3 or
4 SB plasmids, selected during 20 days, were used as donors for cloning. Briefly, the ZP-free
5 enucleated oocytes were individually transferred to a drop of 1 mg/ml phytohemagglutinin
6 (Sigma, L8754) dissolved in TCM-199 without serum, where they remained for a few seconds.
7 Following this, they were quickly dropped over a single cell resting on the bottom of a 100 µl
8 TALP-H drop. Following attachment, the ZP-free enucleated oocyte/cell pair was picked up,
9 transferred to fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 1 mg/ml PVA),
10 for 2–3 min and then to a fusion chamber (BTX Instrument Division; Harvard Apparatus,
11 Holliston, MA, USA) containing 2 ml of the same warm medium. Fusion was performed with a
12 double direct current (dc) pulse of 75 V, each pulse for 30 ms, 0.1 s apart. The reconstructed
13 zygotes were then carefully transferred to SOF culture droplets for 2 h to allow for nuclear
14 reprogramming.
15 Chemical oocyte activation: Embryos produced by SCNT were activated with 5 µM ionomycin
16 (I24222; Invitrogen, Van Allen Way Carlsbad, California, USA) in HEPES-TALP for 4 min and
17 subsequently transferred individually to 1.9 mM DMAP (Sigma, D2629) in SOF droplets for 3 h.
18 Embryos were then washed three times in HEPES-TALP to remove the inhibitor, and cultured as
19 described below.
20 In vitro embryo culture: Reconstructed SCNT embryos were cultured in SOF medium in a
21 system similar to the Well of the Well (WOW) [41], whereby microwells were produced using a
22 heated glass capillary slightly pressed to the bottom of a culture dish and then covered with a 100
23 µl microdrop of SOF medium (20 to 30 WOW in each microdrop, 1 embryo per WOW). During
24 nuclear transfer embryo culture, the medium was 50% replaced on day 2 and supplemented with

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1 7.5% FBS on day 5. Cleavage was evaluated on day 2, morula formation at day 5 and blastocyst
2 formation on day 7 post-fusion.
3 Evaluation of fluorophore expression in embryos: Cloned embryos were briefly exposed to blue
4 light using specific excitation (460-498 nm) and an emission filters (510-540 nm) to determine
5 EGFP or Venus expression on days 5 and 7 post-chemical activation. Images were captured with
6 a Nikon DS-Qi1Mc camera using the same acquisition settings for all pictures taken.

7 *2.12. Embryo vitrification, thawing and cell counting*

8 Transgene expressing cloned blastocysts were vitrified as previously reported [42, 43]. Briefly,
9 embryos were immersed sequentially in a series of glycerol and ethylene glycol solutions at
10 room temperature (25 °C) as follows: 10% glycerol for 5 minutes followed by 10% glycerol +
11 20% ethylene glycol for 5 min and finally 25% ethylene glycol + 25% glycerol for 30 sec. The
12 embryos were aspirated into 1 µl tips and cryotubes immersed in liquid nitrogen. For
13 devitrification, immediately after recovery of embryos from liquid nitrogen, they were placed for
14 5 min in a solution of 12.5% ethylene glycol +12.5% glycerol + 0.5 M sucrose in PBS with 20%
15 fetal bovine serum. Afterwards, they were placed in 0.5 M sucrose solution and 0.25 M sucrose
16 solution (5 min in each solution). Finally, the embryos were washed 2 times in PBS
17 supplemented with 1% serum. After thawing, embryos were placed back in SOFaa medium, and
18 cell numbers of blastocysts that re-expanded were determined by staining with 1 µg/mL of
19 Hoechst 33342 (B-2261; Sigma-Aldrich Co., St. Louis, MO, USA) for 10 min. Nuclei were
20 visualized and counted using UV light in a microscope equipped with epifluorescence.

21 *2.13. Statistical analysis*

22 Number of colonies of each group was analyzed by ANOVA followed by Tukey´s test to
23 compare the means of different treatments. Data are expressed as means ± SEM and different

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1 letters in graph bars indicate statistically significant differences ($p < 0.05$). All statistical analyses
2 were performed with Statgraphics software (Statpoint Technologies, Inc. Warrenton, Virginia).

3 **3. Results**

4 *3.1 Assessment of PB transposon activity in bovine fetal fibroblasts*

5 The experimental design is summarized in Fig. 1, and the employed plasmids are depicted in Fig.
6 2. In order to assess the activity of PB transposase to mediated gene transfer into the bovine
7 genome, BFFs were transfected with 1 µg of p*m*GENIE-3 or p*mhy*GENIE-3 (self-inactivating
8 hyperactive piggyBac transposase-based plasmid). As control for random non-transpositional
9 integration, BFFs were transfected in parallel with the control p*m*GENIE-2/ΔpiggyBac plasmid
10 (Fig. 3, a-f). The mean number of hygromycin B-resistant colonies was markedly different
11 among treatments (ANOVA, $p<0.0001$; Fig. 4 a). In cell cultures transfected with PB constructs
12 containing a functional sequence of PB transposase (i.e., p*m*GENIE-3 and p*mhy*GENIE-3) the
13 number of resistant clones was higher compared with that in cultures transfected with the control
14 plasmid in which only a few colonies developed (range 0-4 resistant colonies). More active at
15 delivering DNA into the genome was the hyperactive version of the PB transposase, as
16 p*mhy*GENIE-3-transfected group developed 3.24-times more colonies compared with that in the
17 p*m*GENIE-3 group and 85-fold above of those in the p*m*GENIE-Control group (Fig. 4 a).

18 *3.2. Molecular characterization of transgene integration sites*

19 Molecular analyses by PCR were performed on gDNA isolated from a total of nine transgenic
20 monoclonal cell lines. p*m*GENIE-3 and p*mhy*GENIE-3 plasmid DNA were used as positive
21 controls. As expected, we obtained EGFP amplicons from all gDNA samples from p*m*GENIE-3
22 transgenic group, demonstrating that analyzed cell lines carried the EGFP transgene (Fig. 3 g),
23 conversely the same PCR product was absent when gDNA from wild type cells was used as

1 template. Furthermore, no amplification product was obtained from gDNA isolated from six
2 individual cell clones subjected to PCR to amplify a region extending from the 3'-ITR into the
3 backbone of the plasmid (Fig. 3 g). These data supports a transposase-mediated transgene
4 integration mechanism and absence of random, non-catalyzed whole plasmid integration events
5 in six out of nine analyzed cell lines. In the remaining three transgenic cell lines (B2, B3 and B5;
6 Fig 3 g), PCR products of the correct size were obtained in PCR2 and PCR3 what indicates
7 random, non-facilitated genomic integration of full-length plasmids. Results from PCR4 revealed
8 that one out of nine p*m*GENIE transgenic cell lines (A1) analyzed carried at least a religated
9 plasmid backbone incorporated randomly into the genome (Fig. 5 b), but no evidence of full-
10 length plasmid insertion (Fig. 3 g). Religated plasmid backbone integration was confirmed by
11 sequencing the 442 bp PCR product (Fig. 5 b).
12 In order to identify the exact integration sites of the PB transposons in the bovine genome we
13 performed a non-restrictive linear amplification-mediated PCR as described previously [38, 39].
14 Cloning and sequencing of seventeen integration sites from five monoclonal transgenic cell lines
15 transfected with p*m*GENIE-3 confirmed specific transposase-mediated integration events at the
16 PB TTAA tetranucleotide consensus sites. Fourteen out of the 17 analyzed integration sites could
17 be assigned to specific positions in the bovine genome. Eleven of these were located in
18 intergenic regions and three in intronic regions of genes (Supplementary Table 2).

19 *3.3. Transgene copy number and cell line fluorescence intensity*

20 The number of transgene copies per genome in p*m*GENIE-3 and p*mhy*GENIE-3 transgenic cell
21 clones ranged from 1 to 13 copies. However, the majority of the cell lines carried 1 to 3 copies
22 per genome (Fig. 6 a).

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1 Phenotypic analysis of each PB transgenic cell line reveled different fluorescence intensity
2 among cell lines with larger corrected total cell fluorescence (CTCF) values in those cell lines
3 with a higher number of trangene copies per genome. Linear regression analysis demonstrated
4 that fluorescence intensity is associated with transgene copy numbers ($R^2 = 0.93$; $p < 0.05$; Fig. 6
5 b).

6 *3.4. Sleeping Beauty activity in bovine fetal fibroblasts*

7 Two separate experiments were conducted to study SB transposase activity in cultured BFF
8 cells. In the first experiment BFFs were co-transfected by a chemical method (PEI-based
9 transfection reagent) with different ratios of donor (the plasmid carrying the gene for Venus
10 protein) and helper (SB100X) plasmids of the SB system. Presence of the transposase expression
11 vector SBX100 in the transfection mixture boosted the number of colonies compared with those
12 in the control treatment without transposase. We studied the effect of different weight ratios of
13 helper to donor plasmid in the transfection reaction on the number of fluorescent colonies. When
14 vectors were used at ratios of 1:1 and 2:1 (helper:donor), it caused a 78-fold and 88-fold increase
15 in the number of colonies compared with that in the no transposase control and 1:2 ratio
16 treatment respectively ($p < 0.05$; Fig. 4 b).
17 Since the Venus vector lacks an antibiotic selection cassette, we designed an experiment to study
18 SB transposition activity when two donor vectors are present. In this experiment, we co-
19 transfected BFFs with the helper vector plus two donor plasmids; one was carrying the gene for
20 Venus protein and the other an antibiotic resistance gene (*neo*). After transfection, BFFs were
21 cultured for 14 d in media containing G418 for resistant colony formation (Fig. 7, a, b, c and d).
22 Regardless of the ratio used to transfect the BFF cultures, every time a functional SB transposase
23 vector was included, the number of fluorescent and G418 resistant colonies was markedly higher

1 compared with that in the respective control without transposase (p< 0.001; Fig. 4 c).
2 Interestingly, all G418 resistant colonies expressed the fluorescent protein.
3 Selected SB transgenic colonies were propagated for gDNA isolation, PCR analysis and SCNT.

4 *3.5. Molecular characterization of SB transgene genomic integrations*

5 We designed five PCRs to characterize SB transposon integrations into the genomes of six
6 monoclonal cell lines generated by cotransfection (ratio 2:0.5:0.5). As anticipated gDNA from
7 all cell lines rendered an amplification product (280 bp) from Venus sequence (Fig. 7 f). To
8 allow identification of putative non-transpositional genomic integration of the SB plasmid, we
9 designed a PCR in which the forward primer hybridize in the 5´ITR and reverse primer on the
10 vector backbone, therefore a product (408 bp) is generated if the complete plasmid is integrated
11 by a non-facilitated mechanism. PCRs using gDNA from cell lines LM16, LM27, LM82 and
12 LM87 generated a 408 bp product. Absence of a 408 bp product in gDNAs from lines LM25 and
13 LM45 (Fig. 7 f) and concurrent amplification of Venus sequences from the same gDNA provides
14 strong evidence for SB transposase-mediated transgene integration. None of the gDNA samples
15 from transgenic cell lines generated a PCR product using the set of primers specific for the
16 plasmid backbone (Fig. 7 e, f). We found no PCR evidence for genomic integration of SB helper
17 plasmid (pCMV(CAT)T7-SB100X) in any SB transgenic cell lines studied. PCR analysis
18 revealed that four out of six cell lines had at least one copy of the religated backbone plasmid
19 inserted in their genomes (Fig. 5 c). Identity of PCR product was confirmed in by restriction
20 enzyme digestion (Fig. 5 d).
21 We have applied a splinkerette-PCR method to clone and analyze junctions of integrated SB
22 transposons and the bovine genome. We identified 15 SB integrations that were mappable on the
23 bovine genome and showed hallmarks of SB transposase-mediated integration; i.e., insertion at a

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1 TA target site dinucleotide. Ten of these were located in intergenic regions and 5 in genes
2 (Supplementary Table 3).

3 *3.6. Reprogramming transposon-transgenic cells by SCNT*

4 To test the ability of transposon transgenic cells to undergo nuclear reprograming to support
5 early embryo development we performed SCNT with p*mhy*GENIE-3 or Venus^{+}/G418-resistant
6 transgenic polyclonal cell line. Development to blastocyst stage reached 33.0 and 36.0% for PB
7 and SB transgenic cell lines respectively (Table 1; representative blastocysts are shown in Fig. 8
8 a and b). Approximately half of the blastocyst homogenously expressed the reporter fluorophore
9 protein (EGFP or Venus) at day 7 (Fig. 8 d and e). Autofluorescence was negligible in non-
10 transgenic blastocysts (Fig. 8 c and f). One out of three GFP expressing blastocysts from the
11 p*mhy*GENIE group reexpanded upon thawing and culture; this blastocyst had 99 nuclei (Fig. 8
12 g). Two out of four NT blastocysts generated with SB transgenic cells reexpanded after
13 vitrification/devitrification procedure, and consisted of 176 (Fig. 8 h) and 81 nuclei, respectively.

14 **4. Discussion**

15 Transposons belong to a new generation of non-viral transgenic tools, known generically as
16 active transgenesis methods, which have in common an enzyme-mediated mechanism of genome
17 modification [8]. Being an enzymatic-based process, active transgenesis is more efficient and
18 precise than traditional techniques in which transgenes are randomly integrated at natural
19 occurring chromosomal DSBs and resolved by non-homologous end joining (NHEJ).
20 In this study, we have implemented a transposon-mediated transgenic approach to introduce
21 transgenes into the bovine genome. Both transposons, a helper-independent PB and a bi-
22 component SB system, catalyzed efficient reporter gene integration into the bovine genome and

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1 these genetically modified cells were amenable to nuclear reprogramming to drive development
2 to morphologically normal blastocysts upon SCNT.
3 It has been demonstrated that PB and SB transposases are functional in cells and embryos from
4 different species including livestock [12, 20, 28, 30, 44-48]. Transgenic pigs carrying reporter
5 genes introduced by transposition in cultured cells used for SCNT [20, 29] or one-cell embryos
6 [12, 28] have been produced. Similarly, transposase-mediated transgene integration has been
7 documented in ruminant cells [33, 44, 46, 49] and zygotes [50]. However, function and efficacy
8 of PB and SB transposons in bovine cells and embryos has not been rigorously examined [51].
9 Initial experiments were designed to study if a PB helper-independent transgenic system already
10 validated in different species [13, 28] and a bi-component SB transposon [10] could increase the
11 efficiency of DNA integration into bovine fibroblast genome, the type of cell most commonly
12 used as nuclear donor for SCNT.
13 Transfection of cultured BFFs with p*m*GENIE-3 plasmids was associated with higher numbers
14 of resistant cell colonies, indicating that the PB transposase facilitated genome integration of
15 reporter and antibiotic resistance genes. Transfection of bovine cells with an upgraded version of
16 p*m*GENIE-3 carrying a hyperactive variant of PB transposase [13] enhanced genome integration
17 of reporter genes as indicated by approximately three-fold increase in colony formation over the
18 conventional non-optimized enzyme and an unprecedented 85 times over those transfected with
19 the control vector. High rates of transgene delivery have been achieved with both binary and
20 single-plasmid PB transposon systems in cultured cells using classic colony formation assay
21 [16]. p*m*GENIE-3 and its hyperactive version p*mhy*GENIE-3 were able to enhance HEK-293T
22 colony formation seven and ten times respectively over those in the control without transposase
23 [13]. Even higher transposition efficiency was observed in an established porcine cell line (28-
24 fold) [32] and primary porcine fibroblasts (30-fold) transfected with the two-component PB

1 system [29]. Under our experimental conditions p*mhy*GENIE-3 was highly active in bovine cells
2 being able to increment 85 times the number of resistant colonies compared with the control.
3 With 324±17.8 resistant colonies obtained in p*mhy*GENIE-3 transfected cultures per $0.5x10^5$
4 plated fibroblasts, we can calculate a 21.6% of stably transfected cells based on ~3% of transient
5 transfection efficiency. The observed higher gene transfer activity in bovine cells compared with
6 that reported by others in pig [29] and sheep cells [44] may reflect species-specific cell
7 characteristics or conditions that favor transgene transposition, such as presence or absence of
8 cellular cofactors that affect transposase function, availability of DNA repair enzymes among
9 others factors beyond intrinsic transposase activity. We also looked for non-enzymatic full-
10 length or vector backbone insertion into the host cell genome. We found PCR evidence for
11 nontranspositional genomic insertions of full-length PB vectors in three out of nine cell lines
12 analyzed and in four of six SB transgenic cell lines. In addition, recircularization of the remnant
13 plasmid backbone after transposon excision followed by random genomic insertion has been
14 documented for PB [14, 21] and SB transposon systems [52]. PCR analysis of gDNA from nine
15 PB transgenic cell lines revealed that only one harbored plasmid backbone sequences in its
16 genome, while four out of six SB transgenic cell lines had at least one copy of the religated
17 plasmid backbone. These findings support the idea that besides degradation [53], chromosome
18 integration is a potential fate for the plasmid backbone.
19 To study the function of SB transposase in bovine cells, we co-transfected primary fibroblast
20 cultures with the plasmids that comprises a binary SB system. Being a bi-component system, it
21 seemed reasonable to optimize the amounts of helper to donor plasmids to achieve the desired
22 transposition efficiency. Rations of 1:1 and 2:1 (helper:donor) resulted in 78 and 88 times more
23 colonies respectively compared with the control. These results can be interpreted as SB
24 transposase is highly active in bovine cells and by increasing the proportion of SBX100 helper

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1 vector in the transfection mixture it is possible to maximize transgene integration into the cell
2 genome without apparent cell toxic effects. Interestingly, within the range of SB transposase
3 studied in our experiment, the well-characterized overproduction inhibition phenomenon
4 described for SB transposons [16, 54] was not observed.
5 In the second SB experiment we use two donor plasmids, one carrying the Venus expression
6 cassette and the other harboring a *neo* gene which confers resistance to G418. This design allows
7 for selection of transgenic clonal cells lines using the neomycin analog G418. Results from this
8 experiment confirmed the capacity of SB transposase to mediate incorporation of exogenous
9 DNA into the bovine genome very efficiently. Surprisingly, all resistant colonies examined
10 expressed the Venus protein what highlights the ability of SB transposase to simultaneously
11 transpose two transgenes provided in separate donor plasmids. This multiplex feature could be of
12 interest when the objective is to obtain bi-transgenic animals. In addition, independent genomic
13 integration of the antibiotic selection cassette from the transgene of interest may be used to
14 segregate the selectable marker by breeding [20] to generate transgenic animals devoid of
15 antibiotic resistance sequences to comply with the recommendations of most regulatory
16 agencies.
17 Our nrLAM-PCR and splinkerette PCR data demonstrated that transgene integrations in the
18 bovine genome corresponded with transposition-mediated events catalyzed by PB and SB
19 transposase. All detected genomic integrations had on the right side the tetranucleotide TTAA
20 for PB or TA dinucleotide for SB, followed by bovine genomic sequences, structures that agree
21 with the known PB and SB transposition mechanisms [17, 55]. Transposition ensures single
22 copy, independent genomic integrations of transgenes thus reducing markedly epigenetic
23 problems, namely silencing and variegated transgene expression as observed with viral vectors
24 [56] and transgenic methods that rely on non-facilitated transgene integration. The limited

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1 number of PB and SB genomic integration sites (17 and 15, respectively) in the bovine genome
2 does not allow us to make conclusive statements about the preferences of PB or SB transposase
3 to direct transgenes to particular domains of the bovine genome. Analysis of 575 PB transposase-
4 mediated integration sites in the human genome showed that there is a slight tendency to
5 incorporate the transposon into transcriptionally active regions or near them [16]. On the other
6 hand, it has been stablished that SB transposase shows no predilection for transcription units, it
7 rather preferentially recombines at intergenic chromosomal regions [57, 58]. In our study, a clear
8 copy number-dependent fluorescence intensity was found in nine PB transgenic cell lines
9 indicating that most transgenes landed in transcriptionally permissive chromatin domains
10 allowing for a faithful transgene expression [59].

11 We have produced a total of 38 transgenic cloned bovine blastocysts using cells from PB and SB
12 polyclonal lines as nuclear donor for SCNT. Developmental rates to blastocyst were not different
13 for embryos reconstructed with PB or SB transgenic cells (36.0% vs 33.0%; respectively) and
14 they are comparable to current standards of NT blastocyst development rates reached with non-
15 transgenic cells as nuclear donors [60-62]. Our nuclear transfer results reveal that transposon
16 transgenic cells retain the ability to undergo nuclear reprograming to support early embryo
17 development. Of the embryos that reached blastocyst stage, about half expressed the reporter
18 fluorophore as per direct observation under blue light in an inverted microscope. We speculate
19 that epigenetic silencing of transgene sequences could have occurred during initial cleavage
20 divisions of the embryo. However, it cannot be ruled out that low-level reporter gene expression
21 in some blastocysts was below the detection limit. Persistence of fluorophore expression without
22 mosaicism in nuclear transfer blastocyst generated from cells transfected with PB plasmids was
23 reported by Kim et al. [51].

1 **5. Conclusions**

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

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21 **References**

22 [1] Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, et al. Targeted disruption of the alpha1,3-
23 galactosyltransferase gene in cloned pigs. Nat Biotechnol. 2002;20:251-5.

ACCEPTED MANUSCRIPT

1 [2] McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ. Production of gene-
2 targeted sheep by nuclear transfer from cultured somatic cells. Nature. 2000;405:1066-9.

3 [3] Ramsoondar JJ, Machaty Z, Costa C, Williams BL, Fodor WL, Bondioli KR. Production of alpha 1,3-
4 galactosyltransferase-knockout cloned pigs expressing human alpha 1,2-fucosylosyltransferase. Biol
5 Reprod. 2003;69:437-45.

6 [4] Mendicino M, Ramsoondar J, Phelps C, Vaught T, Ball S, LeRoith T, et al. Generation of antibody-
7 and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain
8 locus. Transgenic Res. 2011;20:625-41.

9 [5] Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, et al. Disruption of the
10 CFTR gene produces a model of cystic fibrosis in newborn pigs. Science. 2008;321:1837-41.

11 [6] Kuroiwa Y, Kasinathan P, Matsushita H, Sathiyaselan J, Sullivan EJ, Kakitani M, et al. Sequential
12 targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. Nat Genet.
13 2004;36:775-80.

14 [7] Richt JA, Kasinathan P, Hamir AN, Castilla J, Sathiyaseelan T, Vargas F, et al. Production of cattle
15 lacking prion protein. Nat Biotechnol. 2007;25:132-8.

16 [8] Bosch P, Forcato DO, Alustiza FE, Alessio AP, Fili AE, Olmos Nicotra MF, et al. Exogenous
17 enzymes upgrade transgenesis and genetic engineering of farm animals. Cell Mol Life Sci.
18 2015;72:1907-29.

19 [9] Laible G, Wei J, Wagner S. Improving livestock for agriculture - technological progress from random
20 transgenesis to precision genome editing heralds a new era. Biotechnol J. 2015;10:109-20.

21 [10] Mátés L, Chuah MK, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, et al. Molecular evolution
22 of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates.
23 Nat Genet. 2009;41:753-61.

24 [11] Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian
25 applications. Proc Natl Acad Sci U S A. 2011;108:1531-6.

26 [12] Garrels W, Mates L, Holler S, Dalda A, Taylor U, Petersen B, et al. Germline transgenic pigs by
27 Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLoS
28 One. 2011;6:e23573.

29 [13] Marh J, Stoytcheva Z, Urschitz J, Sugawara A, Yamashiro H, Owens JB, et al. Hyperactive self-
30 inactivating piggyBac for transposase-enhanced pronuclear microinjection transgenesis. Proc Natl
31 Acad Sci U S A. 2012;109:19184-9.

ACCEPTED MANUSCRIPT

1 [14] Urschitz J, Kawasumi M, Owens J, Morozumi K, Yamashiro H, Stoytchev I, et al. Helper-
2 independent piggyBac plasmids for gene delivery approaches: strategies for avoiding potential
3 genotoxic effects. Proc Natl Acad Sci U S A. 2010;107:8117-22.

4 [15] Mikkelsen JG, Yant SR, Meuse L, Huang Z, Xu H, Kay MA. Helper-Independent Sleeping Beauty
5 transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression in
6 vivo. Mol Ther. 2003;8:654-65.

7 [16] Wilson MH, Coates CJ, George AL, Jr. PiggyBac transposon-mediated gene transfer in human cells.
8 Mol Ther. 2007;15:139-45.

9 [17] van Luenen HG, Colloms SD, Plasterk RH. The mechanism of transposition of Tc3 in C. elegans.
10 Cell. 1994;79:293-301.

11 [18] Fraser MJ, Cary L, Boonvisudhi K, Wang HG. Assay for movement of Lepidopteran transposon
12 IFP2 in insect cells using a baculovirus genome as a target DNA. Virology. 1995;211:397-407.

13 [19] Plasterk RH, Izsvák Z, Ivics Z. Resident aliens: the Tc1/mariner superfamily of transposable
14 elements. Trends Genet. 1999;15:326-32.

15 [20] Carlson DF, Garbe JR, Tan W, Martin MJ, Dobrinsky JR, Hackett PB, et al. Strategies for selection
16 marker-free swine transgenesis using the Sleeping Beauty transposon system. Transgenic Res.
17 2011;20:1125-37.

18 [21] Saha S, Woodard LE, Charron EM, Welch RC, Rooney CM, Wilson MH. Evaluating the potential
19 for undesired genomic effects of the piggyBac transposon system in human cells. Nucleic Acids Res.
20 2015;43:1770-82.

21 [22] Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the
22 human genome. Nat Rev Cancer. 2012;12:51-8.

23 [23] Ivics Z, Hiripi L, Hoffmann OI, Mátés L, Yau TY, Bashir S, et al. Germline transgenesis in rabbits
24 by pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc. 2014;9:794-809.

25 [24] Ivics Z, Mátés L, Yau TY, Landa V, Zidek V, Bashir S, et al. Germline transgenesis in rodents by
26 pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc. 2014;9:773-93.

27 [25] Katter K, Geurts AM, Hoffmann O, Mates L, Landa V, Hiripi L, et al. Transposon-mediated
28 transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. Faseb J.
29 2013;27:930-41.

ACCEPTED MANUSCRIPT

1 [26] Jang CW, Behringer RR. Transposon-mediated transgenesis in rats. CSH Protoc.
2 2007;2007:pdb.prot4866.

3 [27] Staunstrup NH, Madsen J, Primo MN, Li J, Liu Y, Kragh PM, et al. Development of transgenic
4 cloned pig models of skin inflammation by DNA transposon-directed ectopic expression of human
5 beta1 and alpha2 integrin. PLoS One. 2012;7:e36658.

6 [28] Li Z, Zeng F, Meng F, Xu Z, Zhang X, Huang X, et al. Generation of Transgenic Pigs by
7 Cytoplasmic Injection of piggyBac Transposase Based pmGENIE-3 Plasmids. Biol Reprod.
8 2014;90:1-10.

9 [29] Wu Z, Xu Z, Zou X, Zeng F, Shi J, Liu D, et al. Pig transgenesis by piggyBac transposition in
10 combination with somatic cell nuclear transfer. Transgenic Res. 2013.

11 [30] Al-Mashhadi RH, Sorensen CB, Kragh PM, Christoffersen C, Mortensen MB, Tolbod LP, et al.
12 Familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition
13 of a human PCSK9 gain-of-function mutant. Sci Transl Med. 2013;5:166ra1.

14 [31] Ivics Z, Garrels W, Mátés L, Yau TY, Bashir S, Zidek V, et al. Germline transgenesis in pigs by
15 cytoplasmic microinjection of Sleeping Beauty transposons. Nat Protoc. 2014;9:810-27.

16 [32] Clark KJ, Carlson DF, Foster LK, Kong BW, Foster DN, Fahrenkrug SC. Enzymatic engineering of
17 the porcine genome with transposons and recombinases. BMC Biotechnol. 2007;7:42.

18 [33] Alessio A, Fili A, Forcato D, Olmos-Nicotra F, Alustiza F, Rodriguez N, et al. Efficient piggyBac
19 transposon-mediated transgene integration into bovine fetal fibroblast genome. Reprod Dom Anim.
20 2014;49:8.

21 [34] Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like
22 transposon from fish, and its transposition in human cells. Cell. 1997;91:501-10.

23 [35] Mathupala S, Sloan AA. An agarose-based cloning-ring anchoring method for isolation of viable cell
24 clones. Biotechniques. 2009;46:305-7.

25 [36] Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy
26 number in Escherichia coli. J Biotechnol. 2006;123:273-80.

27 [37] McCloy RA, Rogers S, Caldon CE, Lorca T, Castro A, Burgess A. Partial inhibition of Cdk1 in G 2
28 phase overrides the SAC and decouples mitotic events. Cell Cycle. 2014;13:1400-12.

29 [38] Owens JB, Urschitz J, Stoytchev I, Dang NC, Stoytcheva Z, Belcaid M, et al. Chimeric piggyBac
30 transposases for genomic targeting in human cells. Nucleic Acids Res. 2012;40:6978-91.

ACCEPTED MANUSCRIPT

1 [39] Paruzynski A, Arens A, Gabriel R, Bartholomae CC, Scholz S, Wang W, et al. Genome-wide high-
2 throughput integrome analyses by nrLAM-PCR and next-generation sequencing. Nat Protoc.
3 2010;5:1379-95.

4 [40] Ivics Z, Izsvák Z, Medrano G, Chapman KM, Hamra FK. Sleeping Beauty transposon mutagenesis
5 in rat spermatogonial stem cells. Nat Protoc. 2011;6:1521-35.

6 [41] Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, et al. New method for culture of zona-
7 included or zona-free embryos: the Well of the Well (WOW) system. Mol Reprod Dev.
8 2000;55:256-64.

9 [42] Martínez AG, Valcárcel A, Furnus CC, de Matos DG, Iorio G, de las Heras MA. Cryopreservation of
10 in vitro-produced ovine embryos. Small Ruminant Research. 2006;63:288-96.

11 [43] Traldi AS, Leboeuf B, Cognié Y, Poulin N, Mermillod P. Comparative results of in vitro and in vivo
12 survival of vitrified in vitro produced goat and sheep embryos. Theriogenology. 1999;51:175.

13 [44] Hu S, Ni W, Sai W, Zhang H, Cao X, Qiao J, et al. Sleeping Beauty-mediated knockdown of sheep
14 myostatin by RNA interference. Biotechnol Lett. 2011;33:1949-53.

15 [45] Jakobsen JE, Li J, Kragh PM, Moldt B, Lin L, Liu Y, et al. Pig transgenesis by Sleeping Beauty
16 DNA transposition. Transgenic Res. 2011;20:533-45.

17 [46] Bai DP, Yang MM, Chen YL. PiggyBac transposon-mediated gene transfer in Cashmere goat fetal
18 fibroblast cells. Biosci Biotechnol Biochem. 2012;76:933-7.

19 [47] Liu X, Li N, Hu X, Zhang R, Li Q, Cao D, et al. Efficient production of transgenic chickens based on
20 piggyBac. Transgenic Res. 2013;22:417-23.

21 [48] Park TS, Han JY. piggyBac transposition into primordial germ cells is an efficient tool for
22 transgenesis in chickens. Proc Natl Acad Sci U S A. 2012;109:9337-41.

23 [49] Jiang BC, Kaleri HA, Zhang HX, Chen J, Liu HL. Application of the Sleeping Beauty system in
24 Saanen goat fibroblast cells for establishing persistent transgene expression. Genet Mol Res.
25 2011;10:3347-55.

26 [50] Garrels W, Talluri TR, Bevacqua RC, Alessio A, Fili A, Forcato D, et al. Sleeping Beauty
27 Transgenesis in Cattle. Reproduction, Fertility and Development. 2015;27:266.

28 [51] Kim S, Saadeldin IM, Choi WJ, Lee SJ, Lee WW, Kim BH, et al. Production of transgenic bovine
29 cloned embryos using piggybac transposition. J Vet Med Sci. 2011;73:1453-7.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[52] 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.

[53] 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.

[54] 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.

[55] Mitra R, Fain-Thornton J, Craig NL. piggyBac can bypass DNA synthesis during cut and paste transposition. Embo J. 2008;27:1097-109.

[56] Ellis J. Silencing and variegation of gammaretrovirus and lentivirus vectors. Hum Gene Ther. 2005;16:1241-6.

[57] 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.

[58] 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.

[59] 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.

[60] 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.

[61] 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.

[62] 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.

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1 **Figure legends**

2 **Fig. 1**. Schematic representation of the experimental design.

3 **Fig. 2**. Diagrams of the plasmid constructs used in the experiments showing the major
4 components of each vector.

5 **Fig. 3**. Microphotographs of fibroblast cells two days (a, b and c) and 16 days (d, e and f) after
6 transfection with p*m*GENIE-2/ΔpiggyBac (a, d), p*m*GENIE-3 (b, e), or p*mhy*GENIE-3 (c, f)
7 (Bars = 100 µm). After 14 days of antibiotic selection, discrete colonies of different sizes were
8 evident (d, e and f). (g) Schematic representation of p*m*GENIE-3 plasmid showing position of
9 three pair of primers (PB-TPase-F/PB-TPase-R, PB-ITR-F/PB-ITR-R and PB-GFP-F/PB-GFP-
10 R) used for PCR analysis of genomic DNA from p*m*GENIE-3 (A1-A5) and p*mhy*GENIE-3 (B2,
11 B3, B5 and B11) transgenic cell lines. A 187-bp fragment of EGFP gene (GFP) was amplified
12 from gDNA from all transgenic cell lines and positive control, but it was not amplified from wild
13 type gDNA and negative control (C-). A PCR product corresponding to a 537-pb fragment
14 extending from the 3'-ITR into the backbone of the plasmid was present in B2, B3 and B5 and
15 absent in A1, A2, A3 A4, A5 B11 and wild type samples. A 245 bp product generated from PB
16 transposase sequence was present in cell lines A1, B2, B3 and B5.

17 **Fig. 4**. Results of the colony formation assay obtained from cells transfected with PB (a) or SB
18 transposon systems (b and c). Transposition activity was measured by counting EGFP- positive
19 and hygromycin-resistant colonies after two-week selection period. (a) The colony counts assay
20 was performed in triplicated and the data were expressed as mean of No. of hygromycin B
21 resistant colonies ± SEM (n = 4-7). (b) Transposition activity in cultures transfected with
22 different ratios of helper to donor plasmids was measured by counting Venus- positive colonies
23 after a two-week culture period. (c) Number of Venus-positive and G418-resistant colonies in

1 BFF cultures transfected with different ratios of helper to donor plasmids (pT2RMCEVenus and
2 pT2/SV40-Neo) after two-week antibiotic selection. Different letters indicate statistically
3 significant differences ($p<0.05$). C: pBSII-ISceI-skA (control plasmid), S: pCMV(CAT)T7-
4 SB100X, V: pT2RMCEVenus, N: pT2/SV40-Neo.

5 **Fig. 5.** (a) Schematic drawing that shows the possible fates of the transgene and the
6 recircularized backbone after transposition for SB and PB transgenic systems. Position of
7 primers RL-F and RL-R is depicted in the figure. (b) Agarose gel showing a product of 442 bp
8 amplified from the PB religated vector backbone inserted into the genome of BFF line A1. PCRs
9 with gDNA from the remaining cell lines generated no amplification product. A negative control
10 (C-) was included. Alignment between the expected religated backbone sequence and the actual
11 sequence obtained by Sanger sequencing of the purified PCR product is shown in b. Boxed is the
12 reconstituted TTAA tetranuclotide generated after transposition. (c) Agarose gel showing a PCR
13 product of 233 bp amplified from the SB religated vector backbone inserted into the genome of 4
14 BFF lines (LM16, LM25, LM45 and LM82). Digestion of PCR product from LM82 with XhoI
15 rendered the expected two products of 75 and 158 bp (d). Lane 1 non-digested PCR product, lane
16 2 digested PCR product and lane 3 wild type control.

17 **Fig. 6**. Number of copies of transgene as determined by RT-qPCR (a) and relationship between
18 fluorescence intensity (CTCF) and transgene copy number. Linear regression demonstrated an
19 association between the two variables ($R^2 = 0.93$; $p<0.05$). Cell lines A1-A5 correspond to cell
20 lines transfected with p*m*GENIE-3 whereas cell lines B2, B3, B5 and B11 were transfected with
21 p*mhy*GENIE-3.

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1 **Fig. 7**. Microphotographs of transient and stable expression of the Venus reporter, 2 days post-
2 transfection (a) 4 (b), 11 (c) and 15 days (d) after G418 antibiotic selection of BFF transfected
3 with the SB plasmids. Note colony formation in c and d panels (Bar = 100 µm). (e) Schematic of
4 pT2RMCEVenus and pCMV-SB100X plasmids showing the hybridization sites of SB-Venus,
5 SB-ITR, SB-BB and SB-TPase pairs of primers used in PCRs. (f) PCR analysis of gDNA from
6 six SB monoclonal cell lines. gDNA from all transgenic cell lines generated an expected PCR
7 product of 280 bp corresponding to the Venus gene. A PCR product corresponding to a 408-bp
8 fragment extending from the 3'-ITR into the backbone of the plasmid was present in LM16,
9 LM27, LM82 and LM87 and absent in LM25, and LM45. None of the cell lines gDNAs
10 generated a PCR product with primers SB-BB (amplification of part of plasmid backbone) or
11 SB-TPase (amplification from pCMV(CAT)T7-SB100X). Positive control (C+) and negative
12 controls (C-) were included.

13 **Fig. 8.** Representative bright-field (a, b), fluorescent (d, e, g, h) microphotographs of blastocysts
14 obtained by SCNT with PB (a, d, g) or SB (b, e, h) transgenic BFF (Bars = 100 µm). Transgenic
15 blastocysts homogenously expressed reporter fluorophore in the inner cell mass and trophoblast
16 cells (d, e). Vitrified/de-vitrified transgenic blastocysts were stained with Hoechst 33342 to
17 determine cell numbers (e, f). Parthenogenic blastocyst (c) displayed negligible autofluorescence
18 (f).

19 **Table Legend**

20 **Table 1** SCNT embryos produced with PB and SB transgenic cells.

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Table 1 SCNT embryos produced with PB and SB transgenic cells.

Treatment	*n*	Cleavage (%)	Morulae (%)	Blastocysts (%)	Tg+*	
					Morulae (%)	Blastocysts[a] (%)
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. PA: Parthenogenetic activation and in-vitro culture control. Tg+: Venus/EGFP expressing morulae/blastocyst. * Tg morulae and blastocysts were calculated over total morulae and blastocysts respectively.

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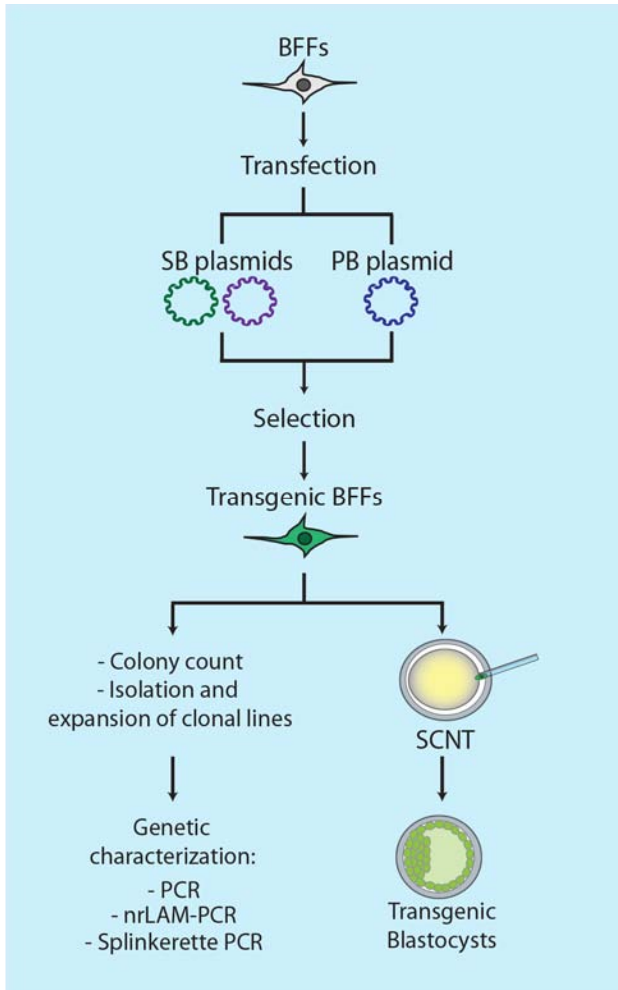

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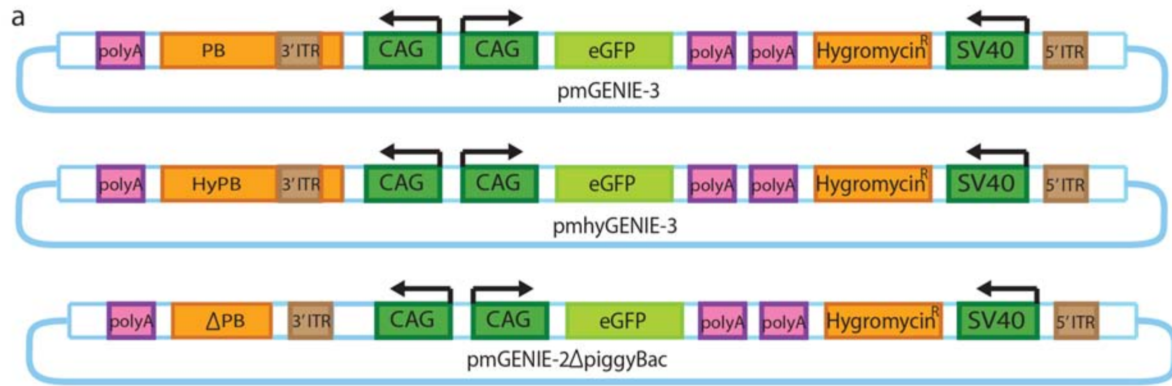

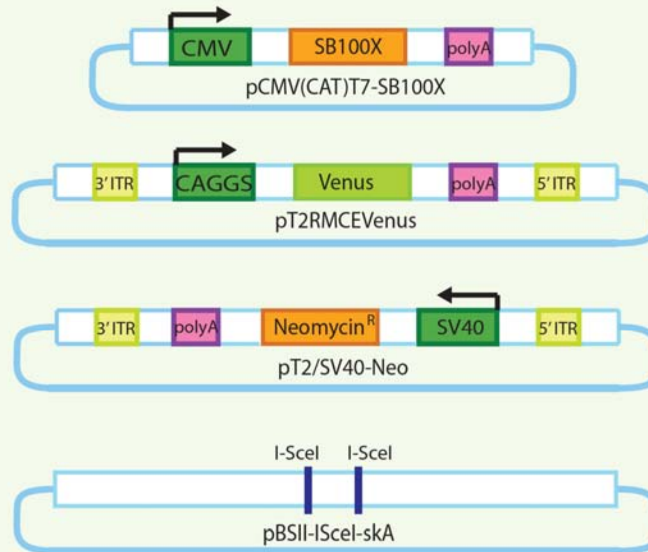

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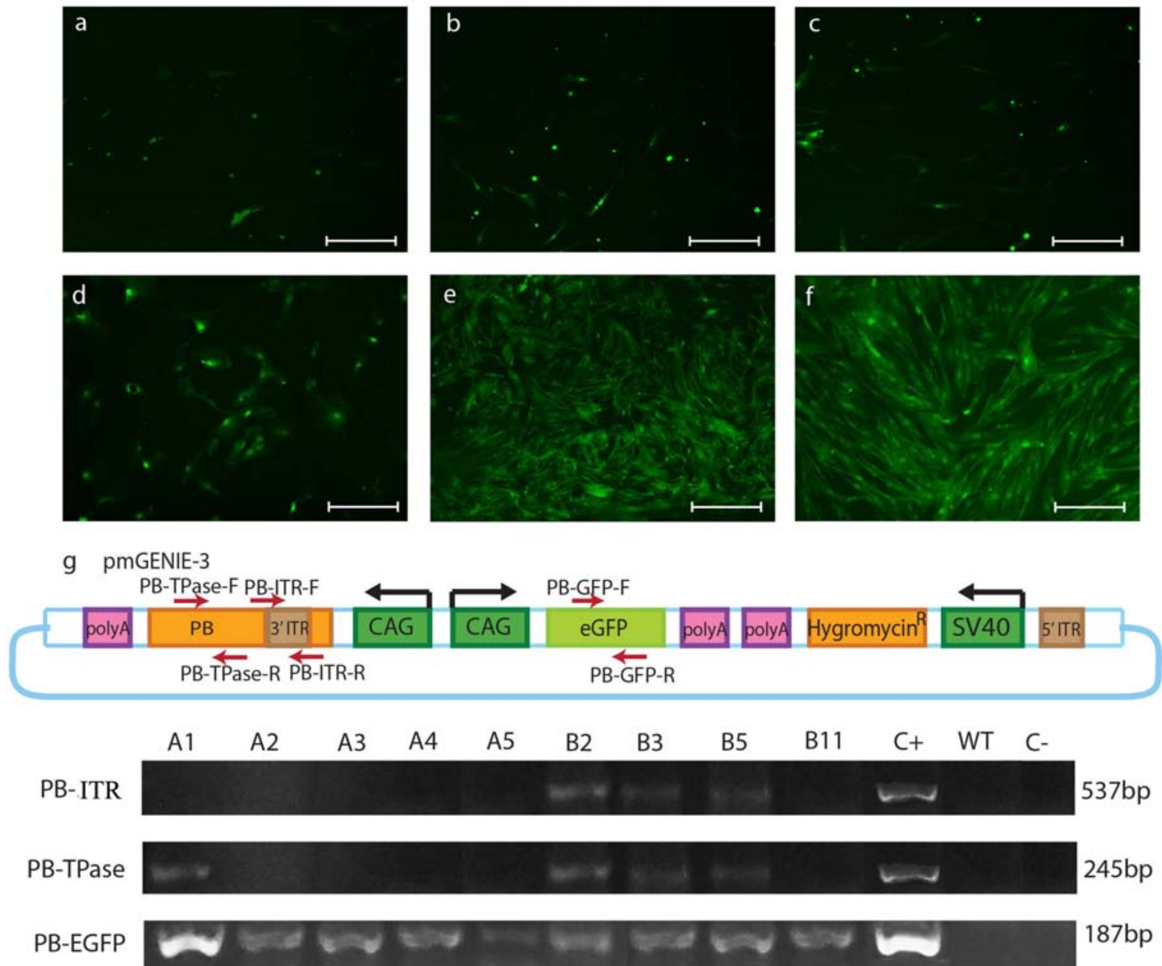

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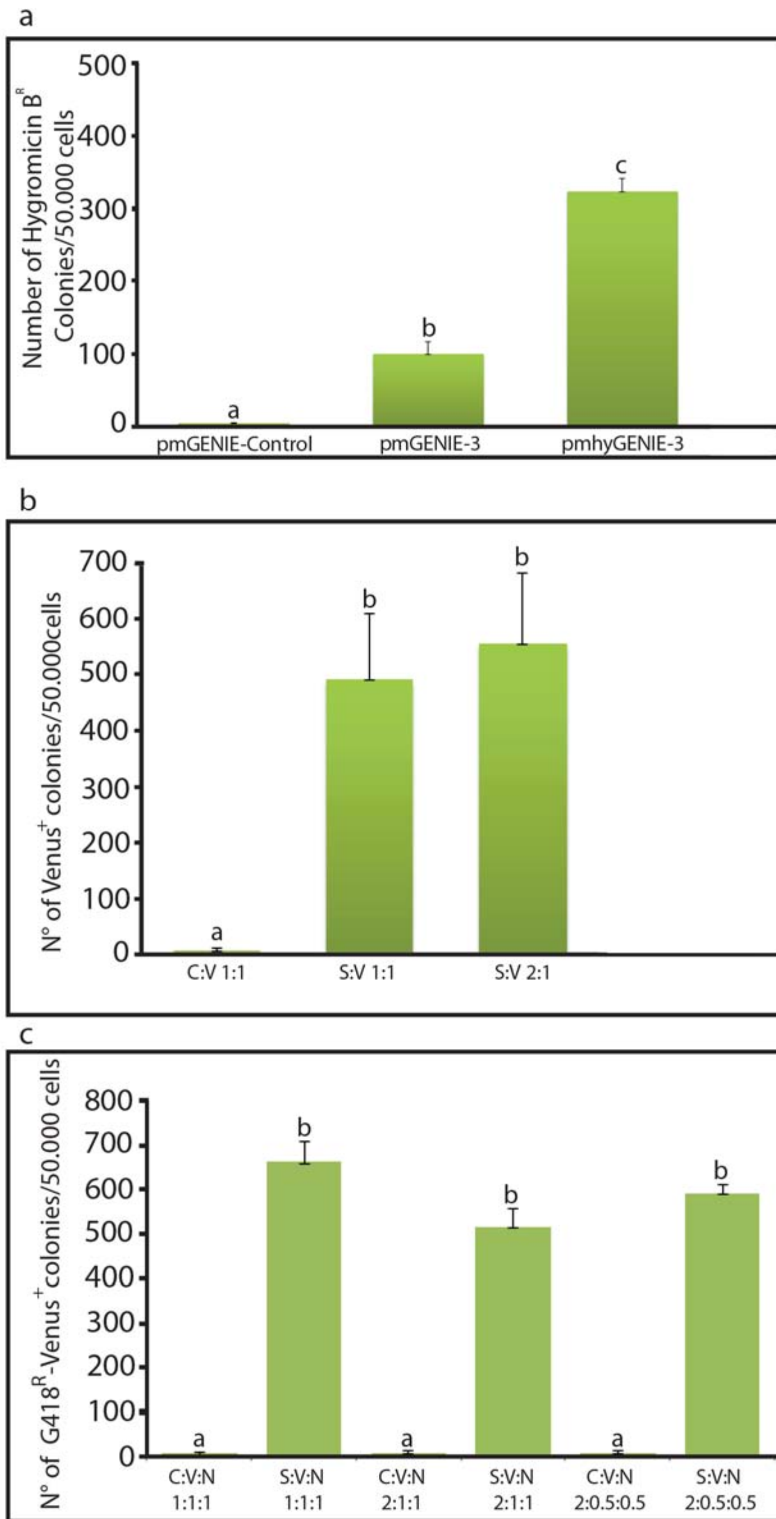

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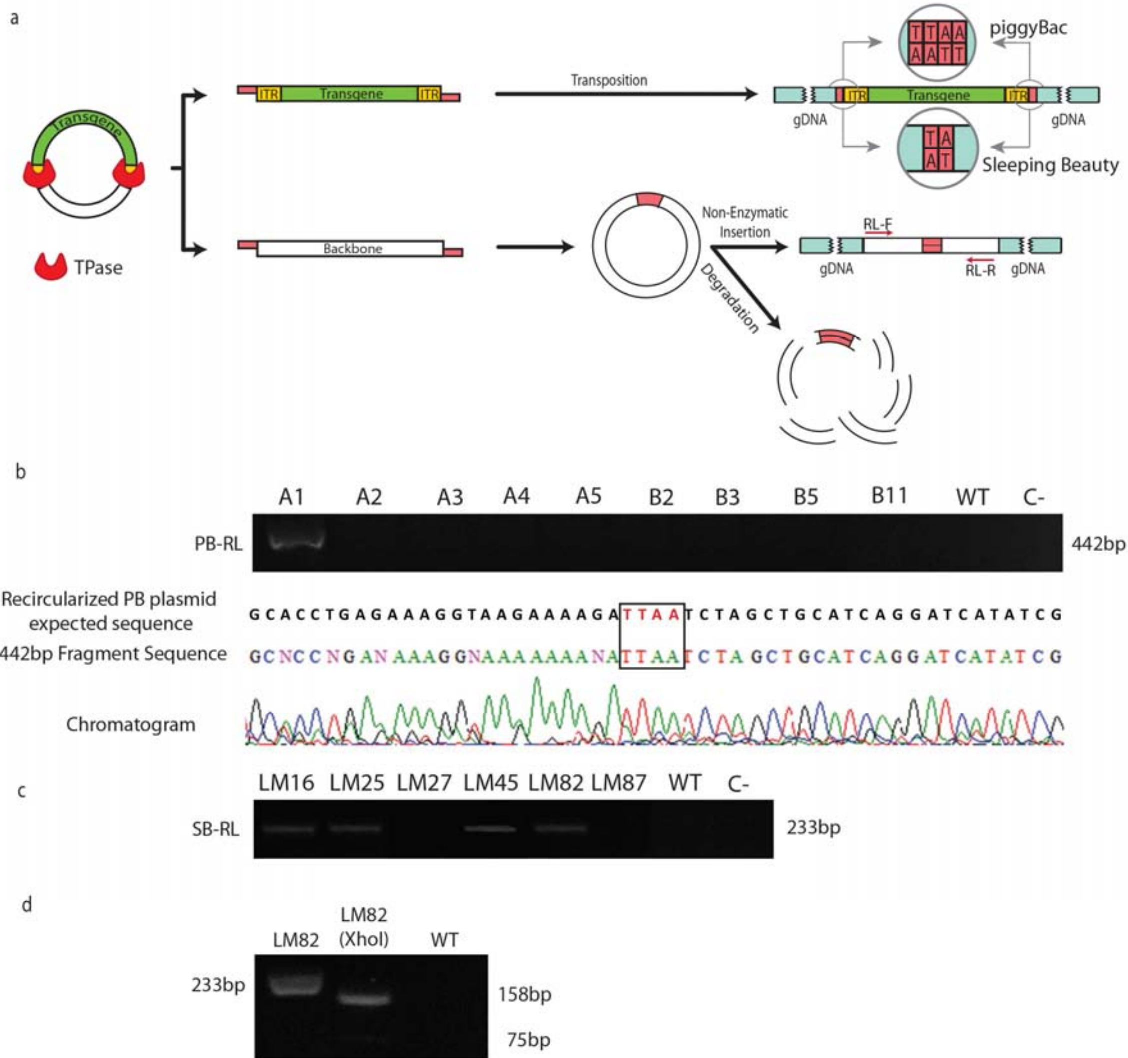

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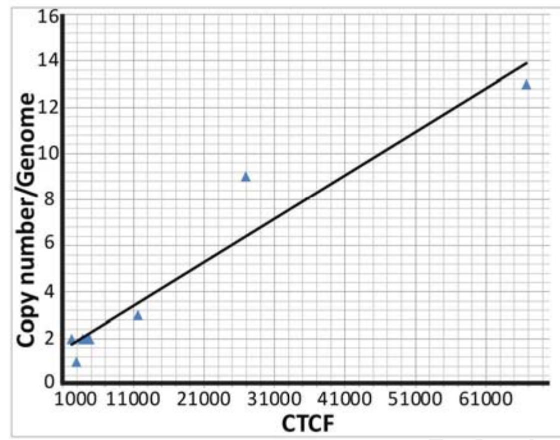
a

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

b



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a b c d

e pT2RMCEVenus

3'ITR CAGGS Venus polyA 5'ITR

SB-Venus-F SB-ITR-F SB-Venus-R SB-ITR-R SB-BB-R SB-BB-F

pCMV(CAT)T7-SB100X

CMV SB100X polyA

SB-TPase-F SB-TPase-R

f LM16 LM25 LM27 LM45 LM82 LM87 + WT -

SB-TPase 643bp

SB-BB 493bp

SB-ITR 408bp

SB-Venus 280bp

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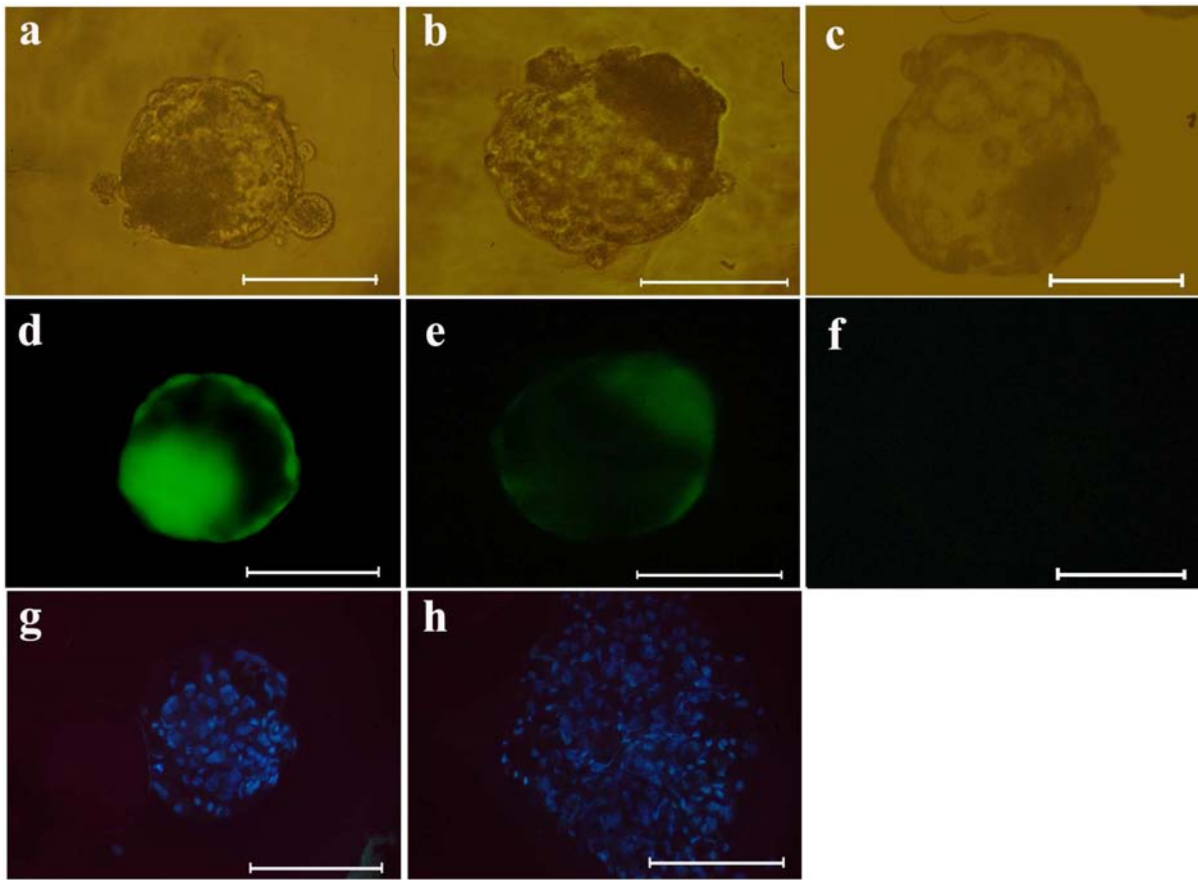

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Highlights

- PiggyBac (PB) and Sleeping Beauty (SB) transposons markedly enhanced transgene transfer into the bovine genome compared with the non-facilitated random integration based transgenic methods.
- Both transposases (PB and SB) catalyzed integration of monomeric copies of the transgene into the bovine genome as determined by PCR based methods.
- Use of SB transposon allows for simultaneously transposing two transgenes provided in separate donor plasmids (multiplex), useful when complex genetic manipulation are sought.
- We determined number of transgene copies in monoclonal cell lines and found copy number-dependent fluorescence intensity, indicating faithful transgene expression.
- Transposon-transgenic cells can be reprogramed upon transfer to enucleated mature oocytes to morphologically normal blastocysts that expressed the transgene

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Supplementary Information

Establishment of cell-based transposon-mediated transgenesis in cattle

Alessio et al.

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

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Table 2 Bovine genomic insertion sites for PB transposons

	Chromosome	Location	Gene	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

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Table 3 Bovine genomic insertion sites for SB transposon

Insertion	**Chomosome**	**Location**	**Gene**	**Sequence**
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

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