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Cytoplasmic injection of murine zygotes with Sleeping Beauty transposon plasmids and minicircles results in the efficient generation of germline transgenic mice

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Transgenesis in the mouse is an essential tool for the understanding of gene function and genome organization. Here, we describe a simplified microinjection protocol for efficient germline transgenesis and sustained transgene expression in the mouse model employing binary Sleeping Beauty transposon constructs of different topology. The protocol is based on co-injection of supercoiled plasmids or minicircles, encoding the Sleeping Beauty transposase and a transposon construct, into the cytoplasm of murine zygotes. Importantly, this simplified injection avoids the mechanical penetration of the vulnerable pronuclear membrane, resulting in higher survival rates of treated embryos and a more rapid pace of injections. Upon translation of the transposase, transposase-catalyzed transposition into the genome results in stable transgenic animals carrying monomeric transgenes. In summary, cytoplasmic injection of binary transposon constructs is a feasible, plasmid-based, and simplified microinjection method to generate genetically modified mice. The modular design of the components allows the multiplexing of different transposons, and the generation of multi-transposon transgenic mice in a single step.

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1 Introduction

The continuous refinement of transgenic technologies for the laboratory mouse made this species the most used mammalian model in basic, biomedical and pharmaceutical research [1]. Since 1976 several methods for mouse transgenesis have been developed, for example retroviral transduction [2], pronuclear DNA injection [3], blastocyst complementation with genetically modified embryonic stem cells [4], intracytoplasmic sperm injection (ICSI)-mediated gene transfer [5] and lentiviral transduction [6]. Transgenesis is a powerful technique for both gain-of-function analysis and for loss-of-function experiments. New tools for targeted mutations and genetic engineering

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Abbreviations: CAGGS, cytomegalovirus enhancer, chicken beta actin promoter; **CPI**, cytoplasmic injection of plasmids; **CRISPR/Cas9,** clustered regularly interspaced short palindromic repeat/CRISPR associated nuclease 9**; ICSI,** intracytoplasmic sperm injection; **ITR**, inverted terminal repeat; **LED**, light emitting diode; **MC**, minicircle; **PB**, piggyBac (transposon system); **PNI**, pronuclear injection; **SB**, Sleeping Beauty (transposon system); **TALEN**, transcription activator-like element nuclease; **VL**, Venus line (of mice)

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include zinc finger nucleases (ZFNs), transcription activator-like element nucleases (TALENs), clustered regularly interspaced short palindromic repeat/CRISPR-associated system (CRISPR/Cas9) [7–13], and DNA transposon-based approaches [14–16].

For gain-of-function approaches, the most routinely applied method is still the pronuclear DNA-injection into murine zygotes [17, 18]. Pronuclear injection (PNI) of transgenes can yield ratios of 5–25% transgenic founders among the offspring [17, 18]. However, standard PNI of a transgene involves the deposition of several hundred copies of linearized DNA molecules into the male pronucleus. Linearized DNA molecules are often recombined to concatemeric arrays before integration into the embryonic genome occurs [19]. Thus the copy number per transgenic locus is stochastic and the multicopy concatemers are prone to undergo transcriptional silencing [20], thereby compromising the production of transgenic lines with predictable patterns of transgene expression. Moreover, integration into a chromosome happens preferentially at sites of double strand breaks caused by physical or chemical mutagens. Due to the stochastic nature of these mechanisms, a certain fraction of the transgene integrations will occur in heterochromatic regions, which are suboptimal for transgene expression [19, 20].

A relative recent innovation for germline transgenesis is the introduction of engineered, non-autonomous DNA transposon systems [14–16, 21, 22]. Engineered transposon systems are commonly applied as bi-component vector systems, in which a gene (sequence) of interest is flanked by transposon-specific inverted terminal repeats (ITRs) on the transposon plasmid, and the transposase is provided as mRNA. In the transposition process, the multi-functional transposase enzyme mediates the excision of the ITR flanked-transgene from its donor plasmid, followed by integration of the transposon into a chromosomal locus in a precise cut and paste manner [13–16]. Transposition occurs preferentially in genomic regions which are permissive for transgene expression, thus transgene silencing is a quite rare phenomenon. The most commonly used DNA transposon systems for mammalian transgenesis, Sleeping Beauty (SB), piggyBac (PB) and Tol2 [16, 21, 22], originate from fish or insect species, and do not have known active orthologous genes in mammalian species. However, the SB belongs to the Tc1-mariner superfamily, which is one of the most widespread transposon families in nature [23]. Hyperactive variants of the SB and PB transposases, called SB100X and hypPB, have been developed by in vitro mutagenesis screens, and have been shown to support efficient active transgenesis in rodents and large farm animals like swine [14–16, 24]. Previously, we showed that the PNI of SB transposase mRNA and a SB transposon is an efficient way for the generation of transgenic mice, rats and rabbits [25, 26]. For the Tol2 transposon system, cytoplasmic co-injection of transposase mRNA and a Tol2 transposon resulted in transgenic mice [22].

Recently, we established a simplified injection protocol for the opaque zygotes of cattle and pig [24, 27], and demonstrated that the cytoplasmic injection (CPI) of SB transposon plasmids resulted in high rates of germline transgenesis in the pig [24, 28, 29], suggesting that the CPI method may be advantageous for mouse transgenesis, too.

Here, we established the CPI of SB transposon plasmids for a highly efficient mouse transgenesis based on the co-injection of plasmid-only SB components. In addition, we showed that SB minicircles (MC) represent an alternative nucleic acid source for transgenesis. MCs are minimal circular expression cassettes devoid of bacterial backbone sequences and therefore significantly reduced in their molecular size [30]. Finally, we demonstrate that multiplexing of SB transposons for the one-step generation of multi-transgenic founders is possible.

2 Material and methods

2.1 Animals and husbandry

Here, outbred NMRI (Naval Medical Research Institute) mice, which have a natural high reproductive outcome, were used. The mice were maintained in an open cage system under controlled standardized conditions of 20°C, 60% relative humidity, and artificial light (12 h light, 12 h dark). The mice received a commercial pelleted diet (Sniff) and water ad libitum. The animal experimentation was in accordance with German laws regulating animal welfare and genetically modified organisms, and approved by an external ethics committee.

2.2 Superovulation of zygote donors and zygote recovery

Superovulation, zygote recovery, embryo culture and embryo transfer were done as described elsewhere [17].

2.3 Cytoplasmic injection of SB transposon components

Microinjection was performed using an inverted microscope equipped with a microinjector (Femtojet, Eppendorf) and two micromanipulators as described before [24, 25, 28]. The embryos were placed in a drop of approximately 500 µL of M2 medium on a siliconized glass plate, and a zygote was fixed by applying gentle sucking of the holding pipette. The holding pipettes are commercial available (BioMedical Instruments, No. 1, bent, small). The injection needles were produced with a capillary containing a filament (No. 100 TF-10, 1 mm, Clark Electromedical Instruments, UK) in a micropuller (Model P-87,

Table 1. Cytoplasmic co-injection approaches of SB transposon components into mouse zygotes

Experiment	Construct(s)	Concentration (ng/µL)	ET (*n*)	Off-spring (*n*)	Transgenic offspring, F0 (*n*)	Transposon number per F0 (*n*)	Germline transmission (n)[a)]	Generation (Name, F, Zygosity)
1	pCMV_SB100X, pT2Venus	5 10	2	3	2 (1 mosaic)	<1–1	1 out of 2	VL1, >F10, ho
2	pCMV_SB100X, pT2Venus	10 20	1	8	3	1–3	2	VL2, F5, ho
3	pCMV_SB100X, pT2Venus	15 30	2	12	7	2–4	2	VL3, F4, ho VL5, F3, he
4	no SB pT2Venus	0 20	5	37	0	na	na	na
5	SB100X mRNA, pT2Venus	0.5 10	2	10	5	3–>10	2	VL4, F5, ho
6	SB100X_MC, SB_Venus_MC	5 10	2	11	2	2–3	2	MC1, F2, he MC2, F2, he
7	pCMV_SB100X pT2TagCFP-mito pT2mCherry pT2CaseinDes	10 10 10 10	1	5	2	3–5	2^{mtg}	TG1, F1, he TG2, F1, he
		Total exp.1–3, 5–7 exp.4(no SB)	10 5	49 37	21 0	67 na	11 out of 12 na	

a) A maximum of two founders were used for testing of germline competence; ET, embryo transfer; he, hemizygous; ho, homozygous; MC, minicircle; na, not applicable; mtg, multi (heteromeric) transposon transgenic founder.

Sutter Instruments und Co.), but are commercially available as well. Approximately 10 pl of the respective plasmid solution was injected in the cytoplasm of a zygote, taking care to avoid penetrating one of the pronuclei.

2.4 Sleeping Beauty constructs

Ultrapure and endotoxin-free plasmids were prepared from transformed XL10 bacteria via commercial anion exchange columns (Qiagen). The pCMV_SB100X (helper plasmid) and the transposon constructs were diluted in different concentrations (Table 1, Fig. 1) in a buffer of 10 mM TrisHCl, pH 7.4 and 0.25 mM EDTA. As control, only a reporter transposon without a SB transposase helper plasmid was injected. In one experimental setting, the helper plasmid was replaced by SB100X mRNA in the injection mixture. Minicircle (MC) constructs of the SB100X and the CAGGS-Venus transposon were provided by Plasmid Factory (Bielefeld) [30], and employed to assess the effects of backbone-free supercoiled constructs. The pCMV_SB100X and pT2Venus plasmids were described previously [16], the pT2mCherry was constructed by subcloning a mCherry-cDNA into the pT2-Venus backbone, replacing the Venus-cDNA. In a similar manner the TagCFP-mito-cDNA from pTagCFP-mito (Evrogen) was subcloned in the transposon backbone. For the pT2CaseinDes, a bovine casein promoter driving a fatty acid desaturase gene (*C. elegans fat-2* gene, GenBank accession number AF240777) cDNA was cloned in the transposon backbone. For the multiplex transgenesis, mixtures of the helper plasmid and different transposons were injected.

2.5 Characterization of mice and breeding schedule

Genotyping by Southern blotting and PCR was performed as described before [24, 28].

2.6 Whole animal fluorescence imaging

Newborn and adult mice were phenotyped by whole animal fluorescence imaging. Therefore free-moving animals were illuminated with colored floodlight LEDs (40W; eurolite, Germany). Blue LEDs were used for the excitation of TagCFP-mito and Venus, and green LEDs for the specific excitation of mCherry. As unspecific excitation source, an orange LED was employed. Images were then recorded with a digital camera (Canon Powershot), fixed on a tripod and equipped with either a yellow (#100, spring yellow, Lee Filter) or red (#106, red primary, Lee Filter) emission filter.

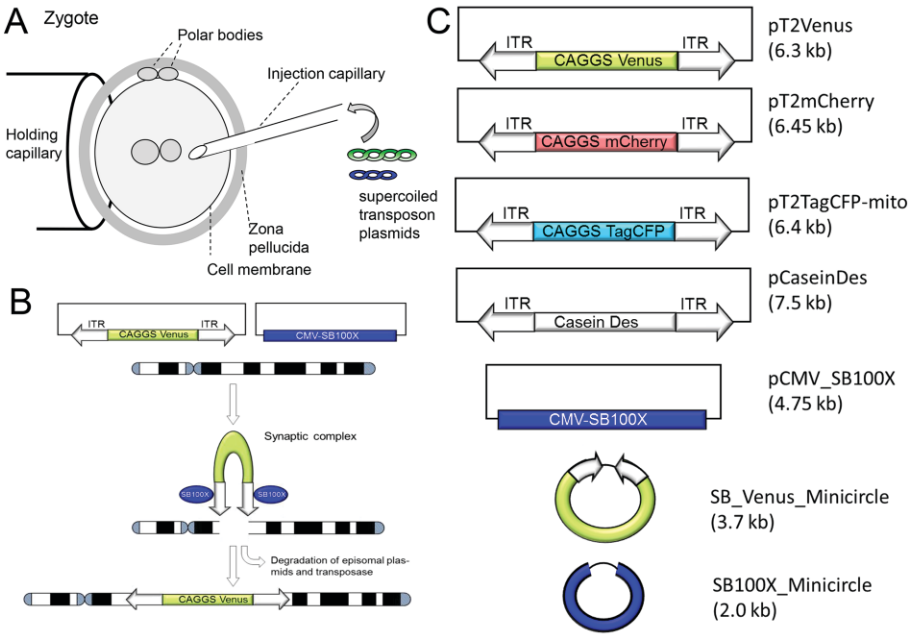

Figure 1. Cytoplasmic injection of SB transposon system into mouse zygotes. (**A**) Schematic depiction of CPI. The mixture of SB plasmids is deposited directly into the cytoplasm of the zygote. (**B**) Mechanism of transposition. Expression of the helper plasmid results in the active transposase protein, which then catalyzes the integration of the ITR-flanked transgene. (**C**) Schematic depiction of the used plasmids and MCs.

3 Results and discussion

3.1 General setup for cytoplasmic injection of transposon plasmids into zygotes

Here we show that the cytoplasmic plasmid injection (CPI) into mouse zygotes with SB based plasmids and MCs is a highly efficient method for the generation of transgenic mouse lines (Fig. 1A). We used a non-autonomous SB system consisting of a helper plasmid carrying the hyperactive SB100X-transposase and different SB transposon plasmids encoding fluorophore reporters driven by the ubiquitously active CAGGS promoter (Fig. 1B and 1C).

The setup of the CPI is identical to the requirements for PNI [25, 31]. In brief, approximately 500 µL of M2 medium were pipetted on a siliconized glass plate, which is positioned under the microscope of the micromanipulation unit. The holding and the injection pipettes are oriented in the microscopic field (10× magnification objective), and five to ten zygotes are transferred into the M2 droplet to the proximity of the holding pipette. Then the first zygote is sucked to the holding needle. The optical focus plane is adjusted to the equator of the zygote (20× magnification objective). With the injection pipette the Zona pellucida and the cell membrane are carefully penetrated at the equatorial plane, and approximately 10 pl of the DNA solution is deposited into the cytoplasm, taking care to avoid contact to the pronuclei (Fig. 1A).

Intact zygotes were transferred into the oviduct of foster mothers. Typically, 10–20 embryos were transferred per surrogate animal. The offspring were born at day 20 (± 2 days) post transfer, and the pups were geno- and phenotyped by PCR, Southern blotting, Western blotting and whole animal fluorescence (Fig. 2; Supporting information, Fig. S1–S3).

In total, 67 transposon insertions were generated and analyzed (Table 1). A maximum of two founders per experiment were employed to test germline transmission, segregation of monomeric transposons, line derivation up to homozygosity, and maintenance for several generations.

3.2 Cytoplasmic injection of different plasmid concentrations allowed to adjust the copy number in transgenic offspring

In the first experimental setting, different concentrations of the helper plasmid and the pT2Venus transposon with a total DNA concentration ranging from 15 ng/µL to 45 ng/µL were injected. These plasmids were used in a quantitative ratio of 1:2 of helper plasmid to Venus transposon (Table 1, experiments 1–3). From a total of five embryo transfers, three pregnancies were established, resulting in 23 offspring, of which 12 were Venus-positive.

As control, the helper plasmid was omitted, and the CPI was performed with the Venus transposon only. In this case, no transgenic offspring were obtained from five pregnancies resulting in a total of 37 offspring (experiment 4). However, in vitro culture of zygotes injected only with the Venus transposon resulted in 40–60% Venus-positive blastocysts (data not shown), confirming previous findings that circular plasmids are transiently maintained and expressed in an episomal manner [27].

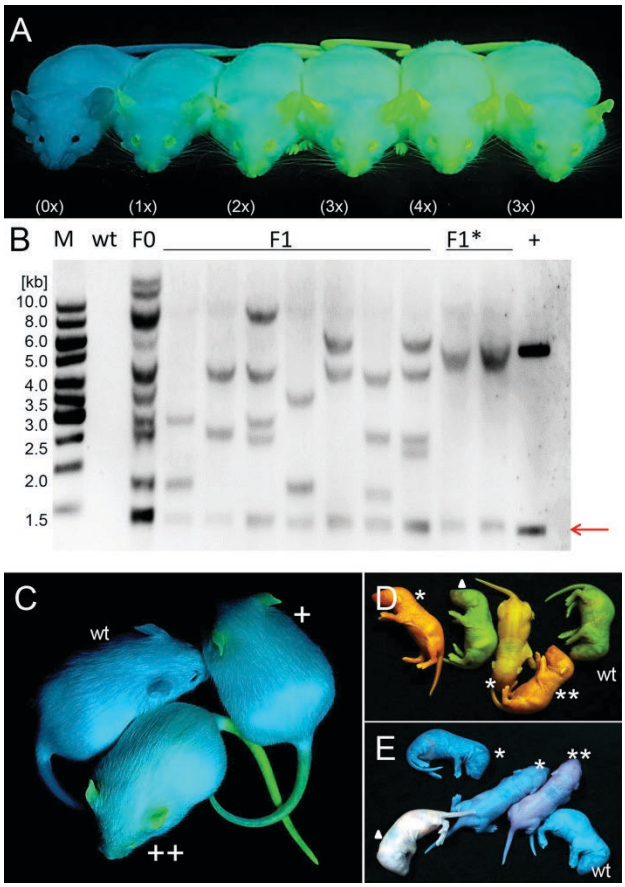

Figure 2. Phenotyping and genotyping of transposon transgenic mice. (**A**) Whole animal fluorescence imaging of F1 animals carrying different monomeric copy numbers of the Venus transposon (0–4). (**B**) Determination of transposon copy number by Southern blotting. The labelled probe hybridized to a constant, internal fragment of ~1.4 kb (red arrow), and to a variable, external fragment per integration site. A founder (F0) with >10 independent integration sites, as well as some of its F1 descendants are shown; wt, wildtype; F1* are one-copy transgenic animals from another founder; +, positive control (transposon plasmid). (**C**) Whole animal fluorescence imaging of pups resulting from SB-MC injections. (**D**) Whole animal fluorescence imaging of pups resulting from the multiplexing experiment (Table 1, experiment 7). Neonatal F1 pups are shown under specific excitation of mCherry. (**E**) Same pups as in (**D**) shown under specific excitation of TagCFT-mito. Legend for (**D**) and (**E**): *, mCherry-positive; **, mCherry and TagCFP-mito positive; triangle, TagCFP-mito positive; wt, non-transgenic. The pups moved their positions during the sequential imaging.

3.3 Cytoplasmic injection of RNA/DNA or minicircle mixtures yielded germline transgenic founders

In experiment 5, a mixture of a synthetic SB100X-mRNA and the Venus transposon plasmid was injected into the cytoplasm. Five of the ten offspring were Venus-positive and carried three to more than ten transposon insertions. The injection of SB transposase as mRNA seems to result in higher numbers of transposon insertions per founder than the injection of plasmid-only mixtures. This may be due to the faster production of the SB enzyme from a mRNA template than from an expression plasmid [15]. However, the elucidation of the molecular mechanisms underlying the efficient active transgenesis will require further research. Fig. 2A shows that the different fluorescence intensities correlated with the copy number of integrated Venus-transposons. Fig. 2B displays the Southern blot analysis of a high-copy founder and segregation of transposons in the F1 generation. In experiment 6, MC topologies of SB100X and the Venus reporter [29] were employed in the injection mixture. Out of the 11 offspring, two Venus-positive pups were identified (Fig. 2C), which were both germline competent. The relatively lower transgenesis frequencies obtained in this experiments suggest that applying MCs in direct embryo microinjections may request further optimization of the injection conditions.

3.4 Cytoplasmic injection of different transposons resulted in multiplex transgenesis

Finally, in a multiplexing approach a mixture of the SB helper plasmid and three different transposons (pT2TagCFP-mito, pT2mCherry and pT2CaseinDes) were co-injected. From one embryo transfer, five offspring were obtained, out of which two were indeed triple transposon-transgenic and germline competent. Fig. 2D and 2E depict a neonatal F1-litter from one of the founders (see also Supporting information, Fig. S2). In a preliminary experiment, the suitability of using a tissue-specific promoter in combination with the transposon CPI was successfully confirmed for lens-cell restricted expression (Supporting information, Fig. S3).

The proof-of-principle approaches, where the cytoplasmic injection was performed with different combinations of the SB system, is shown in Table 1. The important aspect is that in all tested conditions the cytoplasmic injection was sufficient to obtain transgenic offspring. The most routinely applied method for mouse transgenesis is the PNI [3, 17, 31–34]. To establish the CPI method, we designed a proof-of-principle experimental setup, and aimed to avoid unnecessary large animal cohorts. Injection of the SB system as plasmids, MCs or RNA/DNA requested only few embryo transfers to obtain germline competent founders. The success rate of transgenic pups per born pups varied between 15 and 58%. The high variations in the transgenesis rates are partially due to the low numbers of experimental animals, but still favorably compares with the 5–25% transgenesis rate of standard PNI [17]. The injection of the lower dose of SB plasmids (15 ng/μL) resulted in single copy-transgenic founders, the increased doses (30–45 ng/μL) produced founders which carried multiple monomeric integration (copy number 3–5). The founder with the highest trans-

poson copy number (>10) was obtained by co-injection of SB100X-mRNA and pT2Venus (Table 1).

Importantly, cellular mosaicism with reporter expressing and non-expressing cells was the rare exception found only in one out of 21 founders. Typically, two founders per experiment (Table 1) were used for assessing germline transmission. Except of the mosaic animal, all founders were found to be germline competent (11 out of 12 tested founders) and allowed the derivation of single transposon transgenic offspring with robust and ubiquitous expression.

Beside the here employed ITR-flanked constructs, the increasing availability of readymade SB transposons with different promoter, reporter, and selection cassettes [35–37] allows the rapid adaptation to individual demands. A limitation of the described approach is that the insertion sites can not pre-determined. The minimal consensus sequence for a SB-catalyzed insertion is a simple TA-dinucleotide, which implies that a mammalian genome contains several 100 millions of putative targets. The here presented finding that 66 out of 67 different reporter insertions, resulted in robust and reliable phenotypes, however suggests that the SB transposase has a bias for transcriptionally active loci, albeit on a genome scale the integrations sites appear to be randomly distributed in saturation assays [37]. The here found bias for transcriptionally active loci may be due to cell type-specific topologies of the genome, or the availability of supporting cellular factors.

Interestingly, in one of the first systematic studies assessing critical factors affecting the efficiency of mouse transgenesis, the cytoplasmic injection of linear and circular (conventional) constructs resulted in extreme low transgenic rates: "Of 224 fetuses examined after cytoplasmic injection, only two were positive." [34]. Highlightening that the combination of cytoplasmic injection with an exogenous enzyme system (here the SB transposon system), is crucial for highly efficient transgenesis.

4 Concluding remarks

Here, we established a simplified microinjection method for the highly efficient generation of germline transgenic mice. In total, 21 founder animals carrying 67 Venus transposon insertions were genotyped, phenotyped, and employed for the generation of filial generations. Except of one integration site [38], the other 66 Venus transposon insertions resulted in ubiquitous promoter-dependent expression of the reporter. This finding supports the hypothesis that SB transposition in mammalian zygotes preferentially results in the integration in transcriptionally permissive loci. Importantly, the modular mixing of up to three transposons (Table 1, experiment 7) resulted in two multi-transgenic founders that carry all three transposons, thus allowing for the generation of complex genotypes in a one-step procedure. A clear advantage of the CPI procedure is the simplicity of the microinjection process. Thus the combination of CPI with a transposon system is a robust and flexible method to produce transgenic mice. As shown before [24, 28], this approach is equally efficient for the generation of transposon transgenic pig models. It is likely, that the CPI method will also be suitable for the one step genetic engineering of other mammalian species.

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