Efficient Production of Fluorescent Transgenic Rats using the piggyBac Transposon

Tianda Li¹, Ling Shuai¹,², Junjie Mao¹,³, Xuepeng Wang¹, Mei Wang¹, Xinxin Zhang¹,4, Leyun Wang¹,4, Yanni Li², Wei Li¹ & Qi Zhou¹

Rats with fluorescent markers are of great value for studies that trace lineage-specific development, particularly those assessing the differentiation potential of embryonic stem cells (ESCs). The piggyBac (PB) transposon is widely used for the efficient introduction of genetic modifications into genomes, and has already been successfully used to produce transgenic mice and rats. Here, we generated transgenic rats carrying either the desRed fluorescent protein (RFP) gene or the enhanced green fluorescent protein (eGFP) gene by injecting pronuclei with PB plasmids. We showed that the transgenic rats expressed the RFP or eGFP gene in many organs and had the capability to transmit the marker gene to the next generation through germline integration. In addition, rat embryonic stem cells (ESCs) carrying an RFP reporter gene can be derived from the blastocysts of the transgenic rats. Moreover, the RFP gene can be detected in chimeras derived from RFP ESCs via blastocyst injection. This work suggests that PB-mediated transgenesis is a powerful tool to generate transgenic rats expressing fluorescent proteins with high efficiency, and this technique can be used to derive rat ESCs expressing a reporter protein.

PiggyBac (PB) is a DNA transposon element that was first isolated from Trichoplusia ni and has shown an efficient transposition ability in many species. Compared with other transposons (e.g., sleeping beauty, Tol2 and MosI), PB can insert with higher transposition activity into mammalian genomes, which makes it an ideal tool for research on the genetic traits of mammalian cells. Therefore, PB has been extensively utilized in studies of genetic function and pathway regulation, such as the reprogramming of somatic cells into pluripotent stem cells, the discovery of novel regulators that govern the pluripotency of stem cells and the generation of a mutation library for genetic screening.

The rat is an important and useful laboratory species for modeling clinical diseases. Although the rat genome has been sequenced and shared, little work has been performed to predict genetic functions compared with the mouse. To date, several gene manipulation strategies (such as N-ethyl-N-nitrosourea (ENU) and zinc-finger nucleases (ZFNs) and transcription activator-like (TAL) effector nucleases (TALENs)) have been used to produce genetically modified rats. Meanwhile, embryo manipulation techniques, including traditional pronuclear injection and haploid ESC intracytoplasmic injection, have been developed for the production of transgenic rats. However, these methods are difficult to perform and are time-consuming. To date, compared with the thousands of types of transgenic mice, rats carrying fluorescent markers are still lacking in biological research, thus hindering the study of the developmental potential of rats during embryogenesis and differentiation.

Here, we have developed a simple and efficient strategy to generate transgenic rats that carry fluorescent marker genes. We microinjected a modified PB vector carrying either the desRed fluorescent protein (RFP) gene or the enhanced green fluorescent protein (eGFP) gene with a PBase plasmid into the pronuclei of rat zygotes. The RFP and eGFP genes were able to insert into many sites of the rat genome and were expressed in various organs. This stable insertion by the PB system was passed to the next generation through germline transmission. In summary, this study provided a novel method to produce fluorescent rats that can be used as a fluorescent ESC platform to target differentiation potential.

¹State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China. ²State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin 300350, China. ³Graduate School of Chinese Academy of Sciences, Beijing 100049, China. ⁴College of Life Science, Northeast Agricultural University of China, Harbin 150030, China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Q.Z. (email: zhouqi@ioz.ac.cn)
Results

Generation of RFP and eGFP rats with the PB transposon. As a fluorescent marker, the RFP gene has been frequently used in cell and molecular biology. We designed a system to insert a PB vector containing the RFP or eGFP gene into rat genomes using the traditional pronuclear injection method. As shown in Fig. 1A, donor transgene (which was abbreviated TG) DNA (RFP or eGFP) was inserted into the PB vector adjacent to the EF1α promoter (Fig. 1A). For each injection, 30 ng/μl PB plasmid and 10 ng/μl PBase plasmid were co-injected into the pronuclei of male zygotes to test the efficiency of the PB integration (Fig. 1B). The zygotes were obtained from Sprague Dawley (SD) and Dark Agouti (DA) strain rats. Two hundred twenty-three zygotes were manipulated via plasmid injection. One hundred ninety-seven embryos survived (88.3%) and developed to the blastocyst stage (Fig. 1C, Table 1). All blastocysts were transferred back to the uteri of pseudo-pregnant rats, and 98 of them developed to term. Of the generated pups, 44 founder pups (F0) carried the fluorescent markers, among which 26 were SD strain rats (white coat) and 18 were DA strain rats (agouti coat) (Table 1). These pups showed...
strong red or green fluorescence, as detected by a stereo fluorescence microscope (S165, Leica, Germany). The emission wavelength peaks were detected between 510 nm and 600 nm using in vivo imaging instruments (IVIS, PerkinElmer, USA), indicating that the marker genes had been integrated into the genomes by transposition (Fig. 1D, supplementary Fig. S1A,S1B).

We sacrificed one RFP rat to assess whether the PB transposon had integrated into different organs. All eight organs assessed (the stomach, heart, liver, lung, intestine, kidney, brain and spleen) showed strong red fluorescence (Fig. 2A), indicating that the PB transposon had been introduced efficiently. A PCR analysis of the RFP gene in the eight organs further confirmed this result (Fig. 2B). These data showed that RFP and eGFP (see supplementary Fig. S2A) rats can be efficiently generated using genomic transposition.

### Germline transmission of RFP and eGFP

We assessed the gametes of the founder rats to determine whether the RFP and eGFP marker genes can be transmitted to the progeny through the germline. The data revealed that testes and germ cells from male rats were RFP-positive under a fluorescence microscope (Fig. 2C,D). However, mature spermatids without a cytoplasm were RFP-negative, indicating that RFP was located inside the cytoplasm (Fig. 2E). Furthermore, we performed a fluorescence-activated cell sorting (FACS) analysis to determine the percentage of RFP-positive cells in haploid germ cells (round spermatids and mature spermatids).

In the RFP testes group, 34.4% of the haploid peak gated cells were RFP-positive, whereas no RFP-positive cells were observed in the wild type (WT) control haploid cells (see supplementary Fig. S2B). These data showed that male RFP rats can generate gametes with the RFP modification. In a parallel experiment, female RFP rats were assessed for the formation of RFP germ cells. The data showed that ovaries and germ cells at the mature MII oocyte and germinal vesicle (GV) stages (Fig. 2E,F and supplementary Fig. S2C) were RFP-positive, which implied that female RFP rats can also form gametes with the RFP modification by PB transposon. Furthermore, the RFP-positive rats grew to adulthood, and full-term pups (F1) carrying RFP expression were obtained after crosses with WT rats (Fig. 2H and supplementary Table S1). In this assay, the fluorescent eGFP gene is also an ideal genetic marker for rats (data not shown). In conclusion, the PB-introduced RFP or eGFP marker gene can be stably inherited by the next generation through germline transmission.

### Integration of the PB transposon in the rat genome

We investigated the insertion sites of the PB transposon in the genomes of the RFP and eGFP rats. Among the 10 insertion sites, 5 were inserted into an intron and 5 were inserted into intergenic sites (Fig. 3A). The five genes with PB insertion in an intron were Macf1, Tyw1, Ptpn3, Dact1 and Rad51b, which are located on chromosomes 5, 12, 5, 6 and 6, respectively (Fig. 3B). Some of the rats had a single copy insertion, but many others had multiple copy insertions (Fig. 3C). We explored the genomes of 21 RFP rats and 24 eGFP rats by inverse PCR to evaluate the efficiency of the transgene transfer (see supplementary Fig. S3A). Our data showed that the PB transposon could efficiently deliver the exogenous genes into the rat genome (see supplementary Fig. S3B, S3C). A Southern blot assay was performed to identify the number of PB transposon copies. Our data showed that the PB transposon could integrate into the genome at multiple sites (Fig. 3D), which is consistent with the inverse PCR result (see supplementary Fig. S3B). Furthermore, we explored the entire genome to test whether the PBase vector integrated. Although PBase integration occurred in both RFP- and eGFP-positive rats, some of the offspring were PBase-free, with stable integration of the marker genes (Fig. 3E and supplementary Fig. S3D).

### RFP-positive ESCs derived from the PB-integrated rats

We next performed rat ESC derivation experiments using RFP blastocysts harvested from the PB-integrated DA strain rats. RFP was stably expressed in both E4.5 blastocysts and derivative outgrowths after 5 days of culture in N2B27 medium supplemented with “2i” (PD0325901 and CHIR99021) and leukemia inhibitory factor (LIF)^20,21 (Fig. 4A). Standard rat ESC cell lines that expressed RFP were established and passaged in vitro for long periods. The expression of alkaline phosphatase (Fig. 4B) and pluripotent marker genes (Fig. 4C) indicated that the PB insertion did not affect the core pathways regulating pluripotency. We assessed whether RFP would be silenced during the ESCs culture procedure using FACS analysis. The results indicated that the percentage of RFP-positive cells at passage five was 98.7% (Fig. 4D) and was 97.8% after passage 12, with no subsequent significant decrease (Fig. 4E). Hence, PB integration can stably introduce RFP genes into the genome of the derivative ESCs, and the expression remained constant.

We produced chimeras by injecting blastocysts to examine the differentiation potential of these RFP-positive rat ESCs (see supplementary Fig. S4A). In two independent donor cell lines, 39 offspring were derived from 86 reconstructed and transferred embryos (Table S2). Ten chimeric rats were generated, with the contributions distinguished by coat color (Fig. 4F) and fluorescence detection (Fig. 4G). In summary, genomically integrated,

| Injected PBase vectors | Rat strain | No. of embryos | No. of pups (%) | GFP or RFP pups |
|------------------------|------------|----------------|----------------|-----------------|
| PB-GFP                 | SD         | 75             | 72             | 38(52.8) 18(25.0) |
|                        | DA         | 54             | 45             | 26(57.8) 12(26.7) |
| PB-RFP                 | SD         | 42             | 39             | 18(46.1) 8(20.1)  |
|                        | DA         | 52             | 41             | 16(39.0) 6(14.6)  |

Table 1. Summary of GFP and RFP pups generated via PB transposition. The percentages of pups that expressed GFP or RFP were calculated based on the numbers of embryos transferred.
pluripotent RFP-positive ESCs could be derived from PB-integrated RFP rats. This technique enables the generation of a supply of fluorescently labeled rat ESCs for future research.

In this study, we developed a simple and efficient method to generate transgenic rats that carry fluorescent marker genes. Our results showed that RFP and eGFP rats can be produced by microinjection of PB vectors carrying RFP or eGFP genes into the pronuclei of zygotes. We further showed that the PB system and fluorescence genes can synergistically form a powerful tool for genetic research in mammals.
Discussion

Sleeping beauty was the first DNA-based transposon system used for genomic engineering in mammalian cells. However, because PB was proven to efficiently deliver genes in mice in 2005, it has been widely used in gene modification. Without requiring DNA synthesis, the PB transposon can excise itself by forming a hairpin structure, which provides a seamless excision with no “footprint”. Recently, the combination of CRISPR/Cas9 and the PB transposase system was used to produce a model to track and transform neocortical progenitors and provided a new strategy to study genetic function. The protocol of co-injecting PB and PBase-encoding mRNA into pronuclei was specifically developed to improve the efficiency of producing transgenic animals and prevent re-transposition events, and can obtain average transformation frequencies of 80%. Cytoplasmic microinjections of hyPBase mRNA and pPB-CAG-TagRFP DNA showed that 94.4% of blastocysts were TagRFP-positive. However, it is a leap from the cellular level to an individual animal, and the injection of the PBase mRNA has great potential as an easier and highly effective method to generate transgenic animals. In this study, PBase did not show active state in the generated eGFP/RFP transgenic rats, which might relate to powerful reproductive ability of rodents. But application of this technique in larger farm animals warrants more investigations. To date, the PB system has proven itself as a versatile genetic tool for various applications, such as mutagenesis and transgenesis. Compared with sleeping beauty and other conventional viral vectors, such as retrovirus AAV and adenovirus, the PB system has a larger cargo size and a protein domain fusion that can flexibly modify the transposase to achieve site-directed integration.

The PB transposon is preferentially biased toward transcription units, particularly in regions that contain genes. We found that out of 10 randomly selected rats, five had insertion sites in introns and five had insertion sites in intergenic regions. This system produced fluorescent rats with no deficiencies. In addition, some rats had a single copy insertion, whereas others had multiple insertions. As PB is prone to integrate near or within coding units, we have not clearly determined the reason why the rats did not carry insertion sites in exons or promoter regions.

Our study provided a new method to generate genetically modified rats expressing a fluorescent protein in different organs. RFP pups were produced by mating RFP rats with WT rats, which indicated that the fluorescent...
marker genes can be stably inherited by the next generation through germline transmission. This technique will enable the production of a supply of fluorescently labeled rat ESCs for further studies.

Methods

Rats. Sprague-Dawley (SD), Fisher 344 (F344) and Dark Agouti (DA) strain rats and CF-1 strain mice were purchased from the Beijing Vital River Laboratory Animal Center. All experiments involving animals were conducted according to the Guidelines for the Care and Use of Laboratory Animals established by the Beijing Association for Laboratory Animal Science and approved under the Animal Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences (1 Beichen West Road, Chaoyang District, Beijing, P. R. China).

Construction of the vectors. Basic PB (PB533-A) and transpose vectors were purchased from SBI System Biosciences and then modified. The desRed (RFP) or enhanced green fluorescent protein (eGFP) genes were inserted into the PB533A-1 vector at the EcoRI and BamHI sites.

Pronuclear microinjection. The PB plasmid (30 ng/μl) carrying the RFP or eGFP genes and the PBase vector (10 ng/μl) were co-injected into pronuclei of zygotes harvested from 0.5 dpc (days post-coitus) SD and

Figure 4. Derivation of ESCs from the RFP rats. (A) RFP-positive ESCs derived from E4.5 blastocysts of RFP rats. Top panel: rat blastocysts; middle panel: outgrowth (day 5); and bottom panel: established rat ESCs; scale bar = 50 μm. (B) Alkaline phosphatase (AP) staining of RFP-rat ESC colonies; scale bar = 50 μm. (C) RT-PCR analysis of pluripotent marker gene expression in the derived RFP-ESCs. (D) Percentages of RFP-positive cells (PBES1-1) at the initial passage (P5, 98.7%) were detected by FACS analysis; scale bar = 50 μm. (E) Percentages of RFP-positive cells in PBES1-1 cells after 12 passages (P17, 97.8%); scale bar = 50 μm. (F) Chimeric rat pups derived from the RFP-rat ESCs. The chimeric rats showed a high contribution of RFP-rat ESCs, according to the coat color. (G) Expression of the desRed gene in the chimeric pups. The chimeric pups showed a high level of RFP expression compared with the negative control.
DA strain rats to produce transgenic embryos. The reconstructed embryos were transferred to the oviducts of pseudo-pregnant female rats.

**Inverse PCR and genotyping.** The schematic of the inverse PCR was illustrated in Fig. S3A. Briefly, the genomic DNA from each sample was extracted using the MicroElute Genomic DNA kit (Omega, USA) and digested with BstYI for 16 hours at 37 °C. The BstYI enzyme was then inactivated at 80 °C for 30 min. The ligase reaction conditions were 4 °C for 16 hours after the direct addition of T4 DNA Ligase (NEB, USA) into the inactivated digestion reaction. The PCR experiments (primers are listed in the supplementary information; see supplementary Table S3) were performed under the following conditions: 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 2 min, and 72 °C for 10 min for terminal replication (first round); and 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 90 sec, and 72 °C for 10 min for terminal replication (second round). The products of the inverse PCR were cloned into the pMD™ 18-T vector (TaKaRa, China) and then sequenced by Sanger sequencing with the M13F primer. The sequence files were analyzed and aligned using the BLAST tool (NCBI, USA) on the official website.

The fluorescent cassettes in the transgenic rats were amplified in a PCR assay under the following conditions: 95 °C for 5 min, 32 cycles of 95 °C for 30 sec, 59 °C for 30 sec, and 72 °C for 1 min, followed by 72 °C for 10 min.

A pair of primers (supplementary Table S3) was designed to amplify the PBase DNA in the genome, under the following conditions: 95 °C for 5 min, 32 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1 min, followed by 72 °C for 10 min.

**Southern blot.** Genomic DNA from the transgenic rats was digested with BamHI and EcoRV at 37 °C for 16 hours, and then separated on 0.8% agarose gels prior to Southern analysis. The probe was synthesized using the Prime-a-Gene Labeling kit (Promega, lot:0000182475), which used the Neo cassette as the template, and the genomic DNA from each sample was extracted using the MicroElute Genomic DNA kit (Omega, USA) and digested with BstYI for 16 hours. The BstYI enzyme was then inactivated at 80 °C for 30 min. The ligation products were amplified in a PCR assay under the following conditions: 95 °C for 5 min, 32 cycles of 95 °C for 30 sec, 59 °C for 30 sec, and 72 °C for 1 min, followed by 72 °C for 10 min.

**ESC derivation.** Blastocysts labeled with RFP or GFP were seeded on mitomycin-C-treated mouse embryonic fibroblasts (MEFs) and cultured in the previously reported standard “2i” rat ESC medium, which was N2B27 supplemented with “2i” (PD0325901 and CHIR99021) and leukemia inhibitory factor (LIF)27. Outgrowths were picked manually and trypsinized into single cells with 0.05% trypsin-EDTA. The cells from each outgrowth (one cell line) were cultured in a new well of the plate in rat ESC medium on feeder cells. The medium was changed daily for each cell line, and the cell lines were passaged every other day.

**AP staining and karyotype analysis.** AP staining was performed according to the standard manufacturer’s instructions using the alkaline phosphatase kit. The results were observed under an inverted microscope (DMi-8, Leica, Germany). Karyotype analysis was performed according to standard methods28.

**Chimera production.** Rat blastocysts were collected from 4.5 dpc F344 strain female rats and injected with 10 to 12 RFP- or GFP-labeled ESCs. The reconstructed embryos were cultured in mR1ECM (246 mOsM)29 in a 37 °C incubator with 5% CO2 for 30 min. Images of the reconstructed blastocysts were captured on an inverted microscope (DMi-8, Leica, Germany). The chimeric embryos were transferred to the uteri of pseudo-pregnant female SD rats. The chimeric rats were identified by coat color or expression of the fluorescent proteins.

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Author Contributions
T.L., W.L. and Q.Z. designed the study; T.L., L.S., J.M., X.W., M.W., L.W. and Y.L. collected and analyzed the data; T.L., L.S. and W.L. wrote the paper. All authors revised and edited the manuscript.

Additional Information
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