A hyperactive piggyBac transposon system is an easy-to-implement method for introducing foreign genes into mouse preimplantation embryos

Shinnosuke SUZUKI¹, Tomoyuki TSUKIYAMA², Takehito KANEKO³, Hiroshi IMAI¹ and Naojiro MINAMI¹

¹) Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
²) Research Center for Animal Life Science, Shiga University of Medical Science, Shiga 520-2192, Japan
³) Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan

Abstract. Transgenic mice are important tools for genetic analysis. A current prominent method for producing transgenic mice involves pronuclear microinjection into 1-cell embryos. However, the total transgenic efficiency obtained using this method is less than 10%. Here, we demonstrate that highly efficient transgenesis in mice can be achieved by cytoplasmic microinjection using a hyperactive piggyBac system. In embryos in which hyPBase mRNA and pPB-CAG-TagRFP DNA were co-injected into the cytoplasm, TagRFP fluorescence was observed after the 2-cell stage; when 30 ng/µl pPB-CAG-TagRFP DNA and 30 ng/µl hyPBase mRNA were co-injected, 94.4% of blastocysts were TagRFP positive. Furthermore, a high concentration of hyPBase mRNA resulted in creation of mosaic embryos in which the TagRFP signals partially disappeared. However, suitable concentrations of injected DNA and hyPBase mRNA produced embryos in which almost all blastomeres were TagRFP positive. Thus, the hyperactive piggyBac transposon system is an easy-to-implement and highly effective method that can contribute to production of transgenic mice.

Key words: Cytoplasmic injection, Hyperactive piggyBac transposase, Transgenic mice

Genetic analysis in mice is essential for biology and medicine. Transgenic mice are important tools for genetic analysis. A current general method for producing transgenic mice is pronuclear microinjection of linear DNA into 1-cell embryos. However, pronuclear microinjection is technically challenging, and the survival percentage after pronuclear microinjection is very low [1]. Consequently, the final transgenesis efficiency (i.e., the rate at which individual animals are produced from manipulated embryos) obtained using this method is less than 10% (% of pups) [2]. To improve transgenesis efficiency, DNA transposons such as Sleeping beauty, Tol2 and piggyBac have been used to produce transgenic mice [3–6]. The transposon system has several advantages: the transposase recognizes DNA regions flanked by terminal repeat sequences, so inserted DNAs tend to contain full sequences; in addition, the system is nonviral and thus avoids the risk of insertional mutagenesis. In the Tol2 transposon system, a transgene donor plasmid and a Tol2 transposase mRNA are microinjected into the cytoplasm of 1-cell embryos [3]; this manipulation is much easier than pronuclear microinjection. Moreover, the total transgenesis efficiency of the Tol2 transposon system is more than 60% (% of pups). Recent studies showed that a hyperactive (mutant) piggyBac transposase (hyPBase) had higher activity in both excision and integration assays than other types of transposases [7, 8]; consequently, this system has often been used to generate induced pluripotent stem (iPS) cells [9, 10] and to introduce transgenes in mouse iPS cells [11]. However, this method has not yet been used to generate transgenic mice. In this study, we designed a method using the hyperactive piggyBac transposon system to produce transgenic mice. As shown in Fig. 1, after microinjection of a donor transgene DNA (pPB-CAG-TagRFP) containing the inverted terminal repeats (ITRs; hyPBase recognition sequences) and the hyPBase mRNA, ITRs of the donor transgene DNA are cut, transported from the cytoplasm into the nucleus [12] and integrated into genomic DNA by the hyPBase protein. Therefore, the presence of TagRFP fluorescence in embryos indicates that the target transgene has been successfully integrated into genomic DNA by the hyperactive piggyBac transposon system.

To develop our transgenic method, we examined several conditions by testing different concentrations of the hyPBase mRNA. In addition, by comparing the signal observed in embryos not subjected to hyPBase mRNA injection, we confirmed that the TagRFP fluorescence observed in embryos microinjected with hyPBase mRNA originated from the integrated transgene. In embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/µl) and hyPBase mRNA (30 ng/µl), the percentage of TagRFP-positive blastocysts was 94.4%, while the percentage was only 2.7% in embryos injected without hyPBase mRNA (Fig. 2A and Table 1), suggesting that the hyPBase system is a very effective method. However, since a small amount of embryos injected without hyPBase mRNA exhibited the TagRFP signals (2.7%), we cannot...
exclude the possibility that part of the TagRFP signals are derived from donor plasmid DNA. Furthermore, we determined when TagRFP fluorescence starts to emerge. Weak TagRFP fluorescence was already observed in 2-cell embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/µl) and hyPBase mRNA (10, 30, 50 and 100 ng/µl), whereas it was scarcely observed in embryos injected without hyPBase mRNA (Fig. 2B). However, in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/µl) and low concentrations of hyPBase mRNA (10 and 30 ng/µl), TagRFP signals were observed in almost all blastomeres at the blastocyst stage; by contrast, in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/µl) and high concentrations of hyPBase mRNA (50 and 100 ng/µl), the TagRFP signals partially disappeared (Fig. 3). Thus, it is probable that excess hyPBase proteins act to remove integrated DNAs from the genome, resulting in the production of mosaic embryos. Because a second generation of transgenic mice could be obtained from mosaic embryos only when cells containing the target gene contributed to the germline, even mosaic embryos could be utilized to produce transgenic progeny. Additionally, TagRFP fluorescence in embryos injected with pPB-CAG-TagRFP DNA (30 ng/µl) and hyPBase mRNA (10 ng/µl) was stronger than that in embryos injected with pPB-CAG-TagRFP DNA (30 ng/µl) and hyPBase mRNA (10 ng/µl) (Fig. 2A and 3), suggesting that the copy number of donor DNA integrated into the genomic DNA depends on the concentration of hyPBase mRNA.

On the basis of these findings, we conclude that the hyperactive piggyBac transposon system is an easy and highly effective method for contributing to production of transgenic mice.

**Methods**

**Superovulation and embryo collection**

Eight- to ten-week-old ICR female mice (Japan SLC, Hamamatsu, Japan) were superovulated by injection of 5 IU of equine chorionic gonadotropin (eCG; ASUKA Pharmaceutical, Tokyo, Japan), followed by 5 IU of human chorionic gonadotropin (hCG; ASUKA Pharmaceutical) 48 h later. Unfertilized eggs were harvested 14 h after hCG injection and placed in a 90-µl droplet of HTF supplemented with 4 mg/ml BSA (A3311; Sigma-Aldrich, St. Louis, MO, USA) [13]. Spermatozoa were collected from the cauda epididymis of 11- to 15-week-old ICR male mice (Japan SLC) and cultured for 2 h in 100-µl droplets of HTF supplemented with 4 mg/ml BSA. After preincubation, sperm were introduced into fertilization droplets at a final concentration of 1 × 10⁶ cells/ml. After a 3-h incubation, fertilized 1-cell embryos were collected and washed 3 times in KSOM supplemented with amino acids [14] and 4 mg/ml BSA and then used for microinjection [15].
In vitro transcription, microinjection, embryo culture and observation

For construction of a hyPBase expression vector, the hyPBase ORF was amplified from pCMV-hyPBase [8] by PCR using specific primers (5'-GGGACCGGTTAATACGACTCACTA TAGGGAATTCCGCGACCACATGGGC-3', 5'-GGGGGTACCGAAACAGCTCTGGCACATGT-3'), and the SV40 polyadenylation signal was added to the amplicon. The resultant DNA fragment was used as a template for in vitro transcription. RNA synthesis and poly(A) tailing were performed with a MEGAscript T7 kit (Invitrogen, Carlsbad, CA, USA). Approximately 5–10 pl of 0, 10, 30, 50 and 100 ng/µl hyPBase mRNA and 30 ng/µl pPB-CAG-TagRFP [10] in DEPC water (Invitrogen) were microinjected into the cytoplasm of 1-cell embryos between 3 and 4 h after insemination. After injection, the embryos were cultured in KSOM medium supplemented with amino acids [14] and 4 mg/ml BSA under mineral oil (Sigma-Aldrich).
Statistical analysis

at 37 C in an atmosphere containing 5% CO2. To examine TagRFP fluorescence, embryos were observed at 38 and 108 h after insemination. At 108 h after insemination, embryos were collected and fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After washing three times in PBS, nuclei were stained in PBS containing 10 µg/ml Hoechst 33342 (Sigma-Aldrich) for 10 min. Stained embryos were mounted on slides in 50% glycerol/PBS, and fluorescent signals were detected using a fluorescence microscope (BX50, Olympus, Tokyo, Japan).

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis of the data was performed by analysis of variance (ANOVA) with the Student’s t-test. P values < 0.05 were considered to be statistically significant.

Ethical approval for the use of animals

All animal experiments were approved by the Animal Research Committee of Kyoto University (permit number: 24–17) and performed in accordance with the guidelines of the committee.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (no. 23380164 to NM) from the Japan Society for the Promotion of Science.

References

1. Nakanoishi T, Kuroiwa A, Yamada S, Isotani A, Yamashita A, Tairaka A, Hayashi T, Takagi T, Iwao M, Matsuda Y, Okabe M. FISH analysis of 142 EGFP transgene integration sites into the mouse genome. *Genomics* 2002; 80: 564–574. [Medline] [CrossRef]
2. Ittner LM, Götz J. Pronuclear injection for the production of transgenic mice. *Nat Protoc* 2007; 2: 1206–1215. [Medline] [CrossRef]
3. Sumiyama K, Kawakami K, Yagita K. A simple and highly efficient transgenesis method in mice with the Tol2 transposon system and cytoplasmic microinjection. *Genomics* 2010; 95: 306–311. [Medline] [CrossRef]
4. Takada J, Keng VW, Horie K. Germline mutagenesis mediated by Sleeping Beauty transposon system in mice. *Genome Biol* 2007; 8(Suppl 1): S14. [Medline] [CrossRef]
5. Marj J, Stoytecheva Z, Urschitz J, Sugawara A, Yamashiro H, Owens JB, Stoytechev I, Pelczar P, Yanagimachi R, Moisyadi S. Hyperactive self-inactivating piggyBac for transposase-enhanced pronuclear microinjection transgenesis. *Proc Natl Acad Sci USA* 2012; 109: 19184–19189. [Medline] [CrossRef]
6. Urschitz J, Kasumori M, Owens J, Moruzumi K, Yamashiro H, Stoytechev I, Marj J, Dee JA, Kawamoto K, Coates CJ, Kaminski JM, Pelczar P, Yanagimachi R, Moisyadi S. Helper-independent piggyBac plasmids for gene delivery approaches: strategies for avoiding potential genotoxic effects. *Proc Natl Acad Sci USA* 2010; 107: 8117–8122. [Medline] [CrossRef]
7. Wu SC, Mei YJ, Coates CJ, Handler AM, Pelczar P, Moisyadi S, Kaminski JM. PiggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. *Proc Natl Acad Sci USA* 2006; 103: 15008–15013. [Medline] [CrossRef]
8. Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci USA* 2011; 108: 1531–1536. [Medline] [CrossRef]
9. Tsuchiyama T, Kato-Itoh M, Kanauchi H, Ohinata Y. A comprehensive system for generation and evaluation of induced pluripotent stem cells using piggyBac transposition. *PLoS ONE* 2014; 9: e92973. [Medline] [CrossRef]
10. Tsuchiyama T, Ohinata Y. A modified EpiSC culture condition containing a GSK3 inhibitor can support germline-competent pluripotency in mice. *PLoS ONE* 2014; 9: e95329. [Medline] [CrossRef]
11. Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PJ, Paschon DE, Miranda E, Orford A, Hannan NR, Rouhani FJ, Darche S, Alexander G, Marciniak SJ, Fusaki N, Hasegawa M, Holmes MC, Di Santo JP, Lomas DA, Bradley A, Vallier L. Targeted gene correction of Z-fetuin deficiency in induced pluripotent stem cells. *Nature* 2017; 478: 391–394. [Medline] [CrossRef]
12. Crabb BS, de Koning-Ward TF, Gilson PR. Toward forward genetic screens in malaria-causing parasites using the piggyBac transposon. In: BMC Biol, vol. 9. England; 2011: 21.
13. Minami N, Sasaki K, Aizawa A, Miymamoto M, Imai H. Analysis of gene expression in mouse 2-cell embryos using fluorescent differential display: comparison of culture environments. *Biol Reprod* 2001; 64: 30–35. [Medline] [CrossRef]
14. Hu Y, Wigglesworth K, Eppig JJ, Schulte RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995; 41: 232–238. [Medline] [CrossRef]
15. Tsukamoto S, Haru T, Yamamoto A, Ohita Y, Wada A, Ishida Y, Kito S, Nishikawa T, Minami N, Sato K, Kokubo T. Functional analysis of lysosomes during mouse preimplantation embryo development. *J Reprod Dev* 2013; 59: 33–39. [Medline]