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| Title | piggyBac transposon-mediated long-term gene expression in mice. |
| Author(s) | Nakanishi, Hideyuki; Higuchi, Yuriko; Kawakami, Shigeru; Yamashita, Fumiyoshi; Hashida, Mitsuru |
| Citation | Molecular therapy (2010), 18(4): 707-714 |
| Issue Date | 2010-01-26 |
| URL | http://hdl.handle.net/2433/129434 |
| Right | © 2010 American Society of Gene & Cell Therapy |
| Type | Journal Article |
| Textversion | author |

# *PiggyBac* Transposon Mediated Long-term Gene Expression in Mice

Hideyuki Nakanishi[1], Yuriko Higuchi[2], Shigeru Kawakami[1], Fumiyoshi Yamashita[1] Mitsuru Hashida[1, 3]

[1]Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

[2]Institute for Innovative NanoBio Drug Discovery and Development, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

[3]Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, 69 Yoshida Ushinomiya-cho, Sakyo-ku, Kyoto 606-8501, Japan

Correspondence should be addressed to M. Hashida (hashidam@pharm.kyoto-u.ac.jp)

Kyoto, Japan

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Tel: +81-75-753-4525; Fax: +81-75-753-4575

E-mail address: hashidam@pharm.kyoto-u.ac.jp

*piggyBac*-mediated long-term gene expression

**ABSTRACT**

Transposons are promising systems for somatic gene integration because they can not only integrate exogenous genes efficiently, but also be delivered to a variety of organs using a range of transfection methods. *piggyBac* transposon has a high transposability in mammalian cells *in vitro*, and has been used for genetic and preclinical studies. However, the transposability of *piggyBac* in mammalian somatic cells *in vivo* has not been demonstrated yet. Here, we demonstrated *piggyBac*-mediated sustained gene expression in adult mice. We constructed *piggyBac*-based plasmid DNA containing reporter (firefly and *Gaussia* luciferase) genes. Mice were transfected by injection of these plasmid DNA using a hydrodynamics-based procedure, and the conditions for high level sustained gene expression were examined. Consequently, gene expressions were sustained over two months. Our results suggest that *piggyBac* is useful for organ-selective somatic integration and sustained gene expression in mammals, and will contribute to basic genetic studies and gene therapies.

## INTRODUCTION

Non-viral vectors for gene transfer are promising tools for genetic studies and therapies because of their high productivity and high safety [1, 2]. Because conventional plasmid DNA (pDNA)-based non-viral vectors have no tendency for chromosomal integration, gene expression from these vectors is transient. However, some diseases such as hereditary or chronic diseases need sustained therapeutic gene expression.

One of the approaches to overcome this limitation is utilization of transposons [3]. Transposons are mobile genetic elements that transpose between or within vectors and chromosomes. In this transposition, transposase recognizes transposon-specific inverted terminal repeat sequences (IRs) located on both ends of transposons, and removed from their original sites and integrated into other sites. Because of this feature, transposons containing genes of interest between their two IRs are able to carry the genes from vectors to chromosomes.

The transposability of a few transposons has been demonstrated in mammalian cells. After molecular reconstruction of *Sleeping Beauty* (*SB*) transposon [4], *SB* has been widely used for mammalian genetic [5, 6] and preclinical studies [7] because of its high transposability in mammalian cells. Recently, *piggyBac* (*PB)*, a transposon derived from cabbage looper moth *Trichoplusia ni* [8], was shown to transpose more efficiently than other transposons including *Tol2* [9, 10], *passport* [10] and two hyperactive versions of *SB*

[9, 10, 11] in mammalian cells. In addition, *PB* can integrate up to 9.1kb of foreign sequence without significant reduction in transposition efficiency [12], whereas the transposition efficiency of *SB* is reduced size-dependent manner (about 50% when the size of transposon reaches 6 kb) [13]. Because of its high cargo capacity and high transposition efficiency in mammalian cells, *PB* is regarded as a promising tool for basic genetic studies and gene therapies. *PB* has been used for chromosomal integration in mammalian germ lines [12], embryonic stem cells [14], and tumor xenograft [15]. In addition, *PB* has also been used for induction of pluripotency [16-18]. However, the transposability of *PB* in mammalian somatic cells *in vivo* has not been demonstrated yet. An *in vivo* transposition investigation of *PB* is needed for *in vivo* genetic applications, such as preclinical studies of gene therapies or organ-specific tumor model establishment [19, 20].

In the present study, we investigated and demonstrated *PB*-mediated sustained gene expression in adult mice *in vivo*. At first, we constructed *PB*-based pDNA containing reporter (firefly and *Gaussia* luciferase) genes. Mice were transfected by injection of these pDNA using a hydrodynamics-based procedure, and the conditions for sustained gene expression were optimized. Consequently, gene expressions were sustained over two months. Our results suggest that *PB* is useful for organ-selective somatic integration and sustained gene expression in mammals, and will contribute to basic genetic studies and gene therapies.

## RESULTS

### Transposition in human hepatocyte-derived cell lines

Initially, we created two pDNAs. One contains an expression cassette of *PB* transposase (pFerH-PBTP) while the other contains expression cassettes of firefly luciferase (Fluc) and neomycin resistance genes flanked with *PB* IRs and internal sequences necessary for efficient chromosomal integration [21] (pIR-CMVluc) (**Fig. 1**). To examine the transposition activity by the constructed pDNA, we transfected these pDNA to human hepatocellular liver carcinoma cell lines, HepG2 and Hep3B. We selected these cells because the liver is the major target organ of hydrodynamics-based transfection procedure [22, 23]. To investigate chromosomal integration and sustained expression of neomycin resistance gene, transfected cells were incubated in G418-containing medium for two weeks. The transposase groups (pIR-CMVluc and pFerH-PBTP) of HepG2 and Hep3B formed 147-fold and 71-fold more colonies than the control groups (pIR-CMVluc and negative control pDNA; pFerH-mcs; **Fig. 1**), respectively (**Fig. 2a, b, c**). In addition, these colonies showed luciferase luminescence (**Fig. 2d**). These results indicated that both Fluc and neomycin resistance genes were integrated into chromosomes by the constructed pDNA in mammalian hepatocyte-derived cells.

### Prolonged firefly luciferase expression *in vivo*

We next transfected these pDNAs to adult mice by a hydrodynamics-based procedure to determine the transposability of *PB in vivo*. Because expression by this procedure in liver is much higher (>1000-fold) than that in other organs [22], we measured Fluc expression in livers. The Fluc activity of the transposase group (pIR-CMVluc and pFerH-PBTP) did not decrease from 5 to 8 days after transfection, whereas that of the control group (pIR-CMVluc and pFerH-mcs) decreased to about 1/4 during the same time period (**Fig. 3a**). *PB* transposase did not increase expression from conventional pDNA under these experimental conditions both 1 and 8 days after transfection (**Fig. 3b**).

**Prolonged secreted protein expression**

For a longer investigation of *PB*-mediated sustained exogenous gene expression, we next created another pDNA containing the *Gaussia* luciferase (Gluc) [24] expression cassette flanked with *PB* IRs and the same internal sequences as pIR-CMVluc (pIR-CMVGluc) (**Fig. 1**). We selected Gluc because it is secreted in blood and enables continuous measurement of the expression level in the same mice [25], and because it can be expressed without being compromised by neutralizing antibodies at least 3 weeks [26]. In addition, because the half-life of Gluc in blood is about 20 min [25], Gluc activity in serum correlates well with the real-time expression. In the transposase group (pIR-CMVGluc and pFerH-PBTP), the Gluc expression decreased rapidly until 1 day after transfection, but the rate of decrease became slow and Gluc expression was still detected at 80 days after transfection. In

contrast, in the control group (pIR-CMVGluc and pFerH-mcs), Gluc expression decreased rapidly until 7 days after transfection by which time the Gluc expression had reached background level (**Fig. 4a**). As in the case of Fluc, *PB* transposase did not affect Gluc expression from conventional pDNA (pCMV-Gluc; **Fig. 1**) (**Fig. 4b**). These results indicated that expression from pDNA containing *PB* IRs is prolonged over two month by *PB* transposase in mice.

**Effect of promoters on gene expression *in vivo***

Although Gluc expression from pIR-CMVGluc was prolonged when pIR-CMVGluc was cotransfected with pFerH-PBTP, Gluc expression decreased gradually. We assumed that the gradual decrease in Gluc expression resulted from post-integrative gene silencing because the CMV promoter is susceptible to gene silencing [27]. A previous study about the post-integrative gene silencing of *SB* showed that the EF1 promoter was less susceptible to post-integrative gene silencing [28]. Therefore, we created a new pDNA (pIR-EF1Gluc; **Fig. 1**) by exchanging the CMV promoter of pIR-CMVGluc for the human EF1 promoter. In the transposase group (pIR-EF1Gluc and pFerH-PBTP), Gluc expression decreased until 10 days after transfection, but no apparent decrease was observed from 10 to 55 days after transfection. In contrast, in the control group (pIR-EF1Gluc and pFerH-mcs), Gluc expression resulted in a near background level at 14 days after transfection (**Fig. 4c**).

**Molecular confirmation of transposition**

To confirm chromosomal integration was resulted from transposition and not from recombination, we performed plasmid excision assay using PCR. In transposition, *PB* transposon is excised from donor plasmid before integration. Therefore, if transposition occurred, the short version of the donor plasmid should be produced (**Fig. 5a**). The excision-dependent PCR products were detected only in the transposase groups both in Hep3B, HepG2 (**Fig. 5b**) and mouse livers (**Fig. 5c**). These results suggested that chromosomal integration was resulted from transposition.

For further confirmation of transposition, we examined the sequence of integration sites by plasmid rescue. In accord with previous studies [9-12], *PB* were integrated into only TTAA sequences (**Table 1**).

**Effect of the amount of transposase on transposition *in vitro***

We next investigated the effect of the amount of pFerH-PBTP with regard to the transposition efficiency *in vitro*. The number of G418 resistant colonies increased in a pFerH-PBTP-dependent manner over the range 0 to 250 ng, but a further increase in pFerH-PBTP to 500 ng resulted in a decrease in the number of G418-resistant colonies in both $1\times10^5$ HepG2 and Hep3B (**Fig. 6a, b**). To examine whether the cytotoxicity of transposase contributed to this decrease, we investigated the cell viability with a constant amount of pIR-CMVluc and an increasing amount of pFerH-PBTP. The cell viability decreased in a pFerH-PBTP- dependent manner (**Fig. 6c**). In addition, we also examined

whether the cytotoxicity depends on transposase itself or transposition catalyzed by transposase. When pFerH-PBTP was cotransfected with conventional pDNA (pCMV-luc), the cell viability slightly decreased, but no statistical significance was observed. In contrast, when pFerH-PBTP was cotransfected with pIR-CMVluc, the cell viability decreased with statistical significance. These results suggest that the cytotoxicity may be partially caused by transposase itself, but mainly caused by transposition.

**Effect of the amount of transposase on transposition *in vivo***

To investigate the ideal amount of pFerH-PBTP *in vivo*, we cotransfected mice with a constant amount of 25 μg pIR-EF1Gluc and a variable amount of pFerH-PBTP from 1 to 50 μg using the hydrodynamics-based transfection procedure. The sustained Gluc expression level increased in a pFerH-PBTP-dependent manner over the range 1 to 25 μg, but a further increase in pFerH-PBTP to 50 μg resulted in a reduction of the sustained Gluc expression level (**Fig. 7**).

## DISCUSSION

Here, we demonstrated that *PB* can prolong gene expression from pDNA *in vivo*. Exogenous gene expression was prolonged by *PB* transposase *in vivo* only when pDNA contains *PB* IRs (**Fig. 3, 4**). When we injected *PB*-based pDNA containing Gluc gene under EF1 promoter control, expression levels did not apparently decrease from 10 days after

injection (**Fig. 4b**). These results support a previous report showing that an *SB*-based pDNA containing human factor IX under EF1 promoter control succeeded in producing long-lasting expression without apparent decrease in the expression level [29].

It was previously reported that gene expression from conventional pDNA containing mammalian promoters persists longer than pDNA containing viral promoters [26]. In our study, although expression from *PB*-based pDNA containing Gluc gene under CMV promoter control was prolonged, Gluc expression decreased gradually. In addition, when pDNA containing *PB* IRs was injected without pFerH-PBTP, Gluc expression by EF1 promoter was detected 10 days after injection, while that by CMV promoter had reached background level 7 days after injection (**Figs. 4a, b**). These results and previous studies [27, 28] suggest that mammalian promoters (ubiquitin C and EF1) are less susceptible to gene silencing than viral promoters [28], and tend to express longer than viral promoters (CMV, RSV, and SV40) [27, 28] not only in the episomal state [27] but also when integrated by transposons (**Fig. 4**) [28]. Therefore, mammalian promoters could be suitable for sustained gene expression regardless of whether the vectors are integrative or not.

Sustained Gluc expression ranged from 0.8 to 5.3 % of the initial Gluc expression (**Fig. 7b**). However, the initial Gluc expression may not reflect the actual amount of the expression cassette, because the hydrodynamics-based procedure activates transcription [30]. Therefore, the initial expression may be lower if transcription was not highly

activated by the hydrodynamics-based procedure, and the actual percentage of integration may be higher than the percentage shown in Fig. 3e. Interestingly, Gluc expression became stable earlier in higher pFerH-PBTP groups (**Fig. 7a**). The initial decrease in Gluc expression may be partially explained by the decrease in the remaining episomal expression cassettes. Therefore, this earlier stabilization of Gluc expression in higher pFerH-PBTP groups may be due to fewer remaining episomal expression cassettes. In addition, the peak expression was also higher when more pFerH-PBTP was transfected (**Fig. 7a**). This may result from more efficient expression from chromosomes than from pDNA.

Although IRs of *PB* possesses promoter or enhancer effects [31, 32], the initial expressions from pIR-CMVluc and pIR-CMVGluc were lower than those from pCMV-luc and pCMV-Gluc, respectively (**Figs. 3a, b and Figs. 4a, c**). These differences may be explained by the differences of plasmid backbones. For example, the number of CG motif, which may cause gene silencing, in pIR-CMVluc is about one hundred more than that in pCMV-luc. Because the construct outside of the transposon is not necessary for transposition, transferring the transposon into other constructs could solve this lower expression. While *SB* transposase was reported to increase expression from conventional pDNA [33], *PB* transposase did not affect expression from conventional pDNA in our experimental conditions (**Fig. 3b and Fig. 4c**).

In the case of some transposons including *SB*, transposition efficiency decreases in the

presence of an excess of transposase [9, 11, 29, 31]. This decrease is called "overproduction inhibition". A decrease in transpositon efficiency could result in a low-level of sustained transgene expression [29] and therapeutic effect. Avoiding "overproduction inhibition" and identifying the ideal amount of transposase expressing pDNA may be necessary to achieve high-level sustained transgene expression. In our study, both the numbers of colonies *in vitro* (**Figs. 6a, b**) and sustained gene expression levels *in vivo* (**Figs. 7a, b**) decreased in the presence of an excess of pFerH-PBTP. In addition, cell viability decreased when the amount of tranfected pFerH-PBTP increased (**Fig. 6c**). Although it is still unclear whether other factors such as transposase-transposase interaction can contribute the decrease of the numbers of colonies and sustained expression level, this result suggests that the cytotoxicity of *PB* transposase can partially contribute the decrease of sustained gene expression level. From this viewpoint, high transfection efficiency may increase the amount of *PB* transposase and decrease sustained gene expression levels. Moreover, the promoter strength in transfected cells may also affect sustained gene expression level. Because transcription factor expression may differ among cell types, cell types may affect not only the transfection efficiency but also the transcription activity of promoters controlling transposase expression. In a previous study showing "overproduction inhibition" of *PB* [9], the maximal number of colonies was about 5000 when the amount of transposase was increased. On the other hand, in two other

studies showing no “overproduction inhibition” of *PB* [11, 31], the maximal number of colonies was about 400 and 100, respectively, when the amount of transposase was increased. This difference also suggests that a higher transfection efficiency could induce “overproduction inhibition”. The transfection efficiency is affected by both the transfection methods and the cell types. Therefore, adjustment of the optimum amount of transposase for each transfection method and cell type may be needed to achieve high transposition efficiency.

Transposon-based vectors still have problems for therapeutic applications because transposons can integrate into or nearby the coding region and affect endogenous gene expression. In the case of integrative viral vectors, cancer produced by insertional mutagenesis has been reported [34]. Although *PB* has a lower tendency to integrate into or nearby genes than lentivirus, the tendency of *PB* to integrate into or nearby genes is higher than random integration and *SB* [11], and the risk of insertional mutagenesis remains. Site-specific integration using sequence-specific DNA binding proteins is one approach to avoid insertional mutagenesis [35-37]. *PB* is suitable for site-specific integration, because DNA binding protein-transposase chimera is active as native transposase, while the chimeric transposase of *SB* and *Tol2* is inactive [9] or low active [36, 37]. Moreover, the chimeric *PB* transposase was reported to integrate 67% of *PB* transposons into a single target site on a pDNA in mosquito embryos [35].

During preparing this manuscript, a new hyperactive *SB* transposase named *SB100X*, which has higher transposability than *PB*, has been reported [38]. Because use of *PB* for mammals are relatively new, such hyperactive versions of *PB* transposase has not been reported yet. However, *PB* may be improved as use of *PB* increases.

In the present study, we used a hydrodynamics-based procedure to introduce transposon-based pDNA into mice. Systemic injection of such a high volume solution is not suitable for clinical applications. However, the organ-restricted hydrodynamics-based procedure that injects solution into a specific vein of an expandable organ using a balloon catheter may be suitable for clinical treatment [2]. In contrast to viral vectors, transposon-based pDNA can be transfected by various conventional non-viral methods such as lipoplex, polyplex, electroporation, and mechanical massage [1, 2]. Therefore, transposon-based pDNA can easily be adapted to a variety of organs such as the lung [39, 40], liver [28], kidney [41, 42] or spleen [43] using a suitable transfection method for each target, whereas the targets of viral vectors may be limited by the nature of each virus. In addition, when the target organs are susceptible to some viral vectors, the transposon can also be loaded on viral vectors such as adenovirus [44], herpes simplex virus [45] or integrase-defective lentivirus [46, 47].

In conclusion, we succeeded in prolonged gene expression by co-transfection of pDNA containing the *PB* transposase expression cassette and pDNA containing *PB* IRs. The

present study is an initial report that demonstrates *PB*-mediated sustained gene expression *in vivo*, and provides evidence that *PB* is a promising tool for various *in vivo* genetic applications such as gene therapies. In addition, the present study showed "overproduction inhibition" of *PB in vivo*, suggesting that optimization of the amount of *PB* transposase is necessary for high level sustained gene expression. Improvement of both transposon systems and gene delivery methods will develop new therapy to overcome refractory diseases. We believe that the present study will encourage the development of *PB*-based vectors, and contribute to future basic genetic studies and studies of gene therapies.

## MATERIALS AND METHODS

### Animals

Female ICR mice (5-week-old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All the animals were housed with free access to food and water. The light (dark/light cycle was 2/12h), temperature, and humidity were kept constant throughout the experiments. All protocols for animal experiments were carried out with the approval of the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

### plasmid DNA construction

To create pFerH-mcs, the portion of pVIVO2-mcs (Invivogen, San Diego, CA, USA) from

the pMB1 replication origin to EF1polyA was amplified by PCR using primer1 and primer2 (primer sequences are listed below). The PCR fragment was purified, and self-ligated using Mighty cloning kit [blunt end] (Takara Bio Inc., Shiga, Japan). To create pFerH-PBTP, *PB* transposase ORF was amplified using p3E1.2 (gift from Prof. Hajime Mori, Kyoto Institute of Technology, Kyoto, Japan) as a template and primer3 and primer4. The PCR product was cloned into pFerH-mcs using In-Fusion PCR cloning kit (Takara Bio Inc.). The PCR product including *PB* transposon IRs (p3EIR) was created using p3E1.2 as a template, primer5, and primer6. The expression cassette including the firefly luciferase gene under CMV promoter control and neomycin resistance gene under SV40 promoter control was amplified by PCR using pCMV-luc as a template, primer7, and primer8. To create pIR-CMVluc, these two PCR products were ligated using Mighty cloning kit [blunt end]. The expression cassette including *Gaussia* luciferase (Gluc) gene under CMV promoter control was amplified by PCR using pCMV-Gluc Control Plasmid (New England BioLabs Japan Inc., Tokyo, Japan) as a template, primer9, and primer10. This PCR product was ligated with p3EIR to create pIR-CMVGluc. hEF1 promoter was amplified by PCR using pBLAST49-hHGF as a template, primer11, and primer12. The *PB* transposon including the Gluc expression cassette without CMV promoter was created by PCR using pIR-CMVGluc as a template, primer13, and primer14. To create pIR-hEF1Gluc, this PCR product was ligated with hEF1 promoter PCR product. To create pIR-blastHGF, pBLAST49-hHGF

(Invivogen) was digested by restriction enzyme SgfI and ligated with p3EIR using Mighty cloning kit [blunt end]. KOD-FX or KOD-plus ver.2 (Toyobo Co., Ltd., Osaka, Japan) were used for all PCR, and High Pure PCR Product Purification Kit (Roche Diagnostics K. K., Tokyo, Japan) or gel indicator DNA extraction kit (Biodynamics Laboratory Inc, Tokyo, Japan) were used for purification of PCR products. All pDNAs were amplified in the *E. coli* strain DH5α, isolated and purified using QIAprep Spin Miniprep Kit (Qiagen K.K., Tokyo, Japan) or JETSTAR2.0 Plasmid Giga Kit (Genomed GmbH., Lohne, Germany)

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|---|---|---|---|
| Primer1 | TCTACAAATGTGGTATGGAAATGTTAAT | Primer2 | CAGCTTTCTATGCAACCCAAGGA |
| Primer3 | TTCAAAGCAATCATGATGGGTAGTTCTTTAGACGATGAGCA | Primer4 | TAGGGATAATCCTAGTCAGAAACAACTTTGGCACATATCA |
| Primer5 | AGAACTACCCATTTTATTATATATTAGTCACGA | Primer6 | AATACAACATGACTGTTTTTAAAGTACAAAAT |
| Primer7 | TATTCGTCTTCCTACTGCAGCAGGCTTTACACTTTATGCTTCC | Primer8 | GAACATTGTCAGATCTCGATGTACGGGCCAGATATA |
| Primer9 | CGATGTACGGGCCAGATATACGC | Primer10 | CAGAAAAGCATCTTACTTGGCATGA |
| Primer11 | ACATTTCTCTATCGAAGGATCTGC | Primer12 | CCGGTGATCTCAGGTAGGC |
| Primer13 | ATGGGAGTCAAAGTTCTGTTTGC | Primer14 | AATACAACATGACTGTTTTTAAAGTACAAAAT |
| Primer15 | TCGCGCGTTTCGGTGATG | Primer16 | GACCCCGTAGAAAAGATCAAAGGA |

**Cell culture**

HepG2 was maintained in Dulbecco's modified Eagle essential medium containing 10% FBS. Hep3B was maintained in Eagle's minimum essential medium containing 2 mM Glutamine, 1% Non Essential Amino Acids, 1 mM sodium pyruvate, and 10% FBS.

***In vitro* transposition study**

The indicated numbers of cells were seeded into individual wells of 6 or 12 well plates 18

hr before transfection. Cells were transfected with the indicated amount of pDNA by FuGENE6 (Roche Diagnostics K. K.). Two days after transfection, cells were harvested, and 1/10～1/100$^{th}$ of the cells were transferred to 100 mm plates or 6 well plates and maintained in medium containing 800 μg/ml G418 (Nacalai Tesque Inc., Kyoto, Japan) for two weeks. For luciferase imaging, 0.3 mM D-luciferin (Promega K. K., Tokyo, Japan) in PBS were added to the cells, and then luminescence was captured for 5 min using a Night Owl NC320 Molecular Light Imager (Berthold Technologies GmbH, Bad Wildbad, Germany). To count G418-resistant colonies, cells were fixed with 4% paraformaldehyde in PBS (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 10 min and stained with 0.2% methylene blue (Wako Pure Chemical Industries Ltd.) in PBS. The numbers of colonies were corrected by the dilution ratio.

**Cell viability assay**

Hep3B ($1\times10^4$) cells were seeded into individual wells of 96 well plates 18 hr before transfection. Cells were transfected with the indicated amount of pDNA by FuGENE6. Two days after transfection, viability was determined using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

**Plasmid excision assay *in vitro***

Hep3B and HepG2 ($2\times10^5$) cells were seeded into individual wells of 6 well plates 18 hr before transfection. Cells were transfected with 0.67 μg pIR-CMVluc and 0.33 μg

pFerH-PBTP of pFerH-mcs. Two days after transfection, cells were harvested and DNA was isolated using Genelute mammalian genomic DNA extraction kit (Sigma-aldrich Japan, Tokyo, Japan). PCR amplification was performed using the isolated DNA as templates, primer15, primer16, and PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.)

**Analysis of transposon-chromosome junctions via plasmid rescue**

Hep3B ($2\times10^5$) cells were seeded into individual wells of 6 well plates 18 hr before transfection. Cells were transfected with 0.67 μg pIR-blastHGF and 0.33 μg pFerH-PBTP. Two days after transfection, cells were harvested and transferred to 100 mm plates and propagated in medium containing 3 μg/ml blasticidinS (Invivogen). DNA was isolated from these cells using Genelute mammalian genomic DNA extraction kit (Sigma-aldrich Japan), and digested by restriction enzyme BglII (Takara Bio Inc.) and BamHI (Toyobo Co., Ltd.). After digestion by restriction enzymes, DNA was purified using High Pure PCR Product Purification Kit (Roche Diagnostics K. K.) and ligated using Ligation-Convenience Kit (Nippon Gene Co., Ltd.). The ligation products were used to transform *E. coli* Competent Quick DH5α (Toyobo Co., Ltd.) or *E. coli* HST08 Premium Competent Cells (Takara Bio Inc.). pDNA was isolated and purified using QIAprep Spin Miniprep Kit (Qiagen K.K.). Nucleotide sequences of the pDNA were sequenced by Fasmac sequencing service (Fasmac Co., Ltd., Kanagawa, Japan). UC Santa Cruz BLAT was used to map *piggyBac* integration sites.

**Assay of firefly luciferase activity in liver**

Mice were injected intravenously via the tail vein with 1.6 ml saline containing the indicated amount of pDNA. At indicated time points, mice were killed, and livers were harvested. The livers were homogenized by adding lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH7.8). The homogenate was centrifuged at 16060 xg for 10 min at 4 °C. The firefly luciferase activity of the supernatant was measured using Picagene luciferase substrate (Toyo-Ink Mfg., Co., Ltd., Tokyo, Japan) and Lumat LB 9507 (EG & G Berthold, Bad Wild-bad, Germany).

**Assay of *Gaussia* luciferase activity in serum**

Mice were injected intravenously via the tail vein with 1.6 ml saline containing the indicated amount of pDNA. At indicated time points, blood was collected via the tail vein. The blood samples were put on ice for 30 min, and centrifuged at 16060 xg for 10 min at 4 °C. The Gluc activity of the supernatant was measured using *Gaussia* luciferase assay kit (New England BioLabs Japan Inc) and Lumat LB 9507.

**Plasmid excision assay *in vivo***

Mice were injected intravenously via the tail vein with 1.6 ml saline containing the 25 μg pIR-EF1Gluc and 25 μg pFerH-PBTP or pFerH-mcs. Three days after injection, livers were harvested and DNA was isolated using Genelute mammalian genomic DNA extraction kit (Sigma-aldrich Japan). PCR amplification was performed using the isolated DNA as

templates, primer15, primer16, and PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.)

**ACKNOWLEDGEMENT**

We would like to thank Prof. Hajime Mori (Kyoto Institute of Technology, Kyoto, Japan) for p3E1.2 (a plasmid DNA containing the *piggyBac* original fragment).

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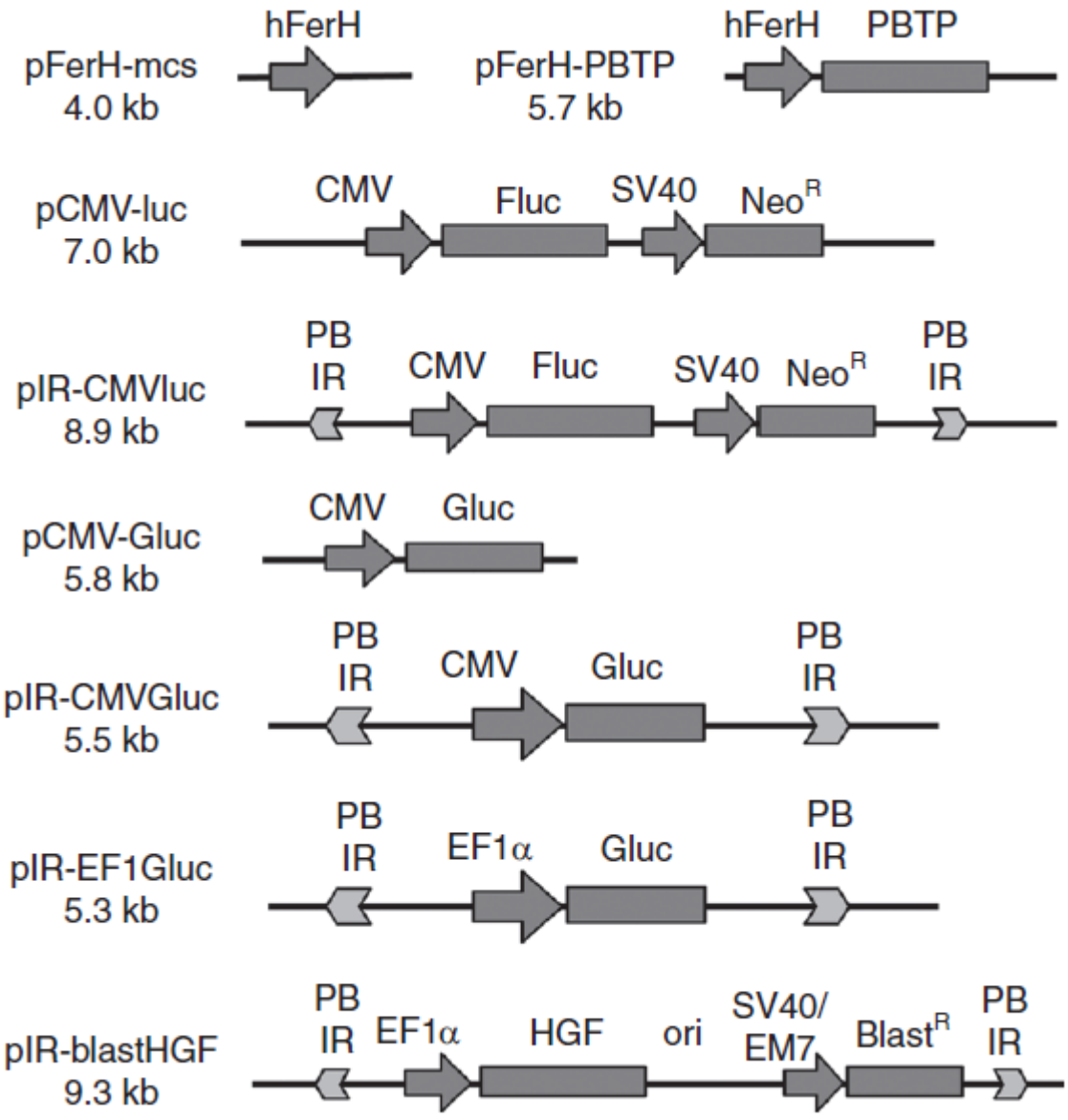


**Figure 1** plasmid DNA construction. hFerH , human ferritin heaby chain promoter; PBTP, *piggyBac* transposase gene; PBIR; *piggyBac* terminal inverted repeat sequence; CMV, cytomegalovirus promoter; Fluc, firefly luciferase gene; SV40, simian virus 40 promoter; $Neo^R$, neomycin resistance gene; Gluc, *Gaussia* luciferase gene; EF1α, human elongation factor 1α promoter; HGF, human hepatocyte growth factor gene; ori, E. coli origin of replication; EM7, bacterial EM7 promoter; $Blast^R$, blasticidin resistance gene.

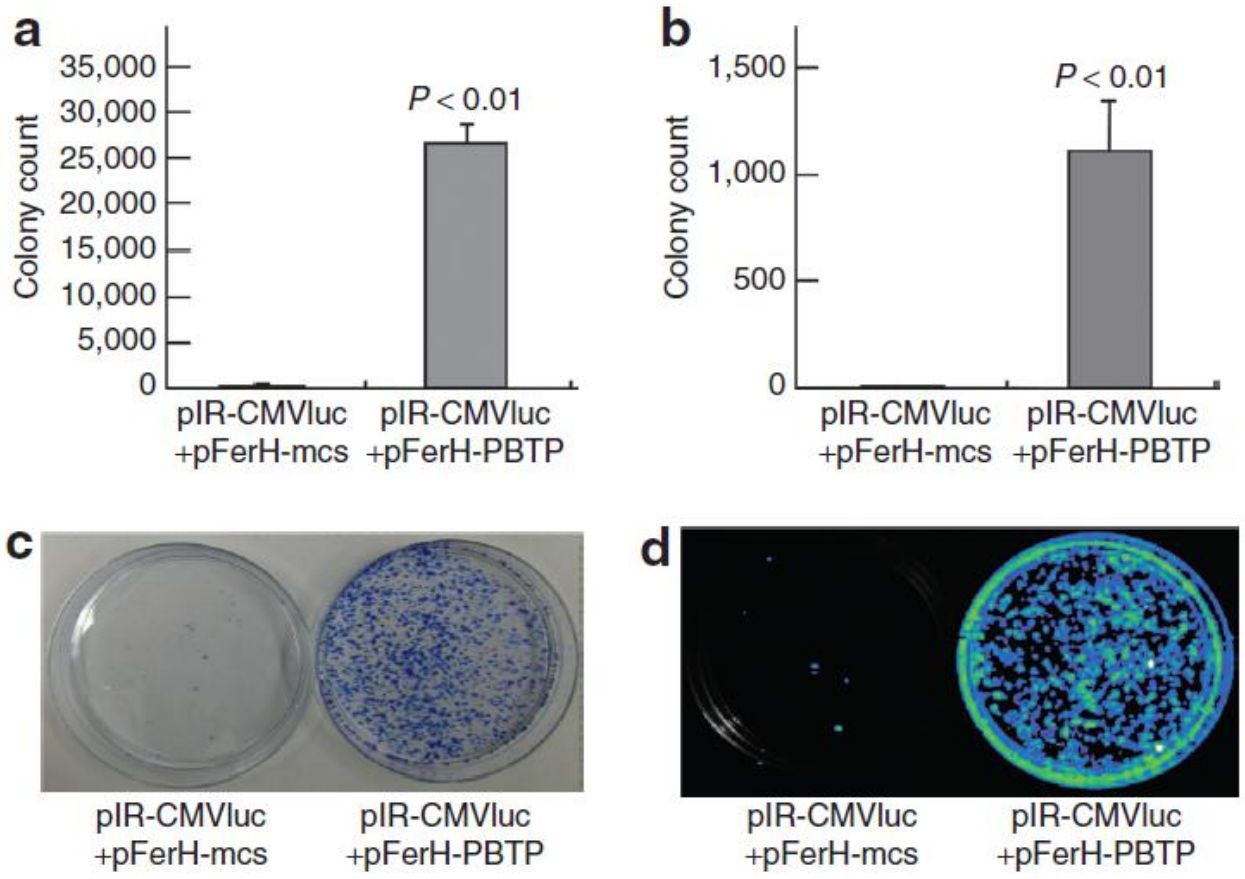


**Figure 2** Sustained gene expression *in vitro.* (**a, b**) *In vitro* transposition study. Hep3B (**a**) and HepG2 (**b**) cells ($2\times10^5$ cells/well) were transfected with 0.67 μg pIR-CMVluc and 0.33 μg pFerH-mcs (left bar) or pFerH-PBTP (right bar). The number of colonies was counted by methylene-blue staining after two weeks selection with G418. Each value represents the mean + S.D (n = 4). (**c, d**) An image of Hep3B colonies. The colonies were stained with methylene-blue (**c**). 0.3 mM D-luciferin was added to the colonies (**d**). Both images were captured after two weeks selection with G418.

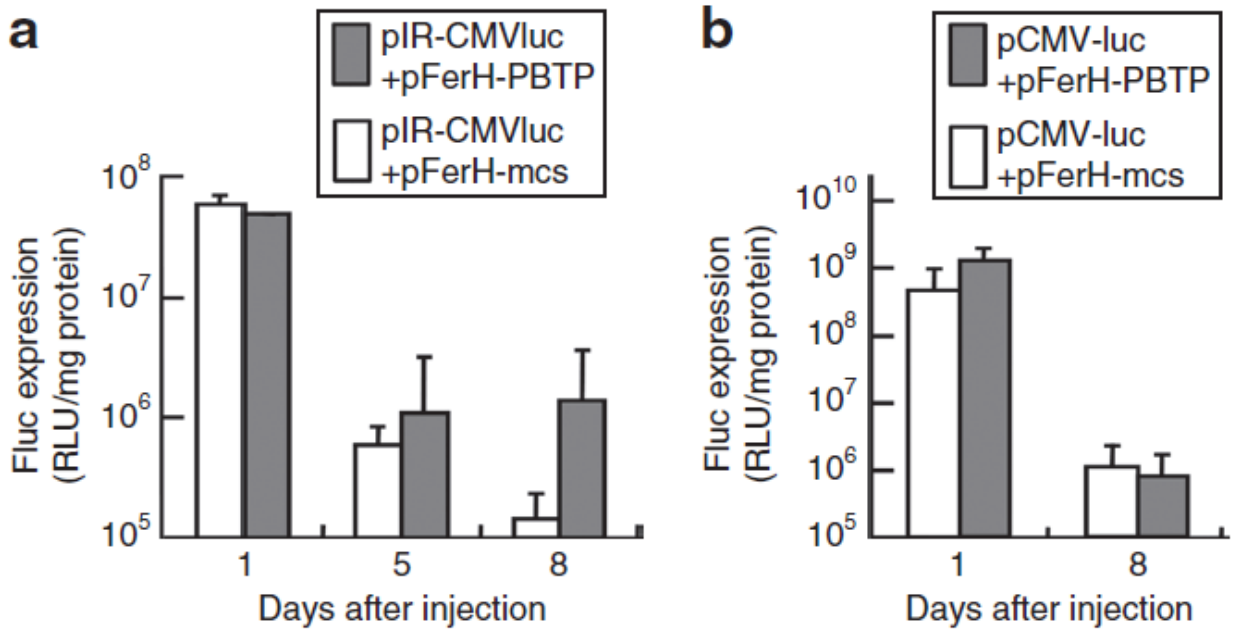


**Figure 3** Sustained Fluc expression *in vivo.* Expression time course of Fluc from *PB*-based (**a**) or conventional (**b**) pDNA. 25 μg pIR-CMVluc and 1μg pFerH-PBTP (gray bar) or pFerH-mcs (white bar) were injected (**a**). 25 μg pCMV-luc and 1 μg pFerH-PBTP (gray bar) or pFerH-mcs (white bar) were injected (**b**). After pDNA injection, livers were collected at indicated time points, and Fluc activities were measured. Each value represents the mean + S.D (n = 4-8). (**d, e, f**)

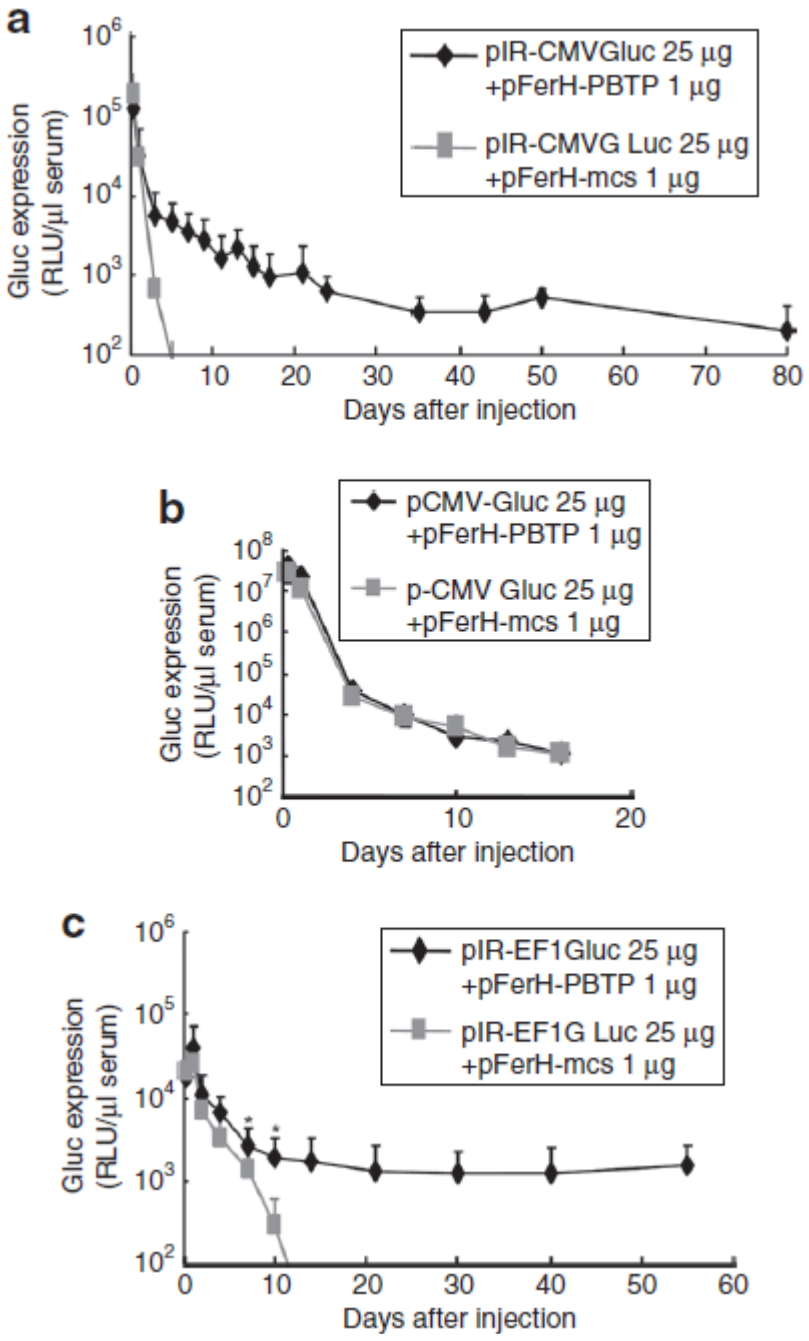


**Figure 4** Sustained Gluc expression *in vivo*. Expression time course of Gluc from *PB*-based (**a,** **c**) or conventional (**b**) pDNA. 25 µg pIR-CMVGluc and 1 µg pFerH-PBTP (closed rhombus ◆) or pFerH-mcs (closed square ■) were injected (**a**). 25 µg pCMV-Gluc and 1 µg pFerH-PBTP (closed rhombus ◆) or pFerH-mcs (closed square ■) were injected (**b**). 25 µg pIR-EF1Gluc and 1 µg pFerH-PBTP (closed rhombus ◆) or pFerH-mcs (closed square ■) were injected (**c**). Blood samples were collected at indicated time points, and Gluc activities in serum were measured. Each value represents the mean + S.D (n = 4-6).

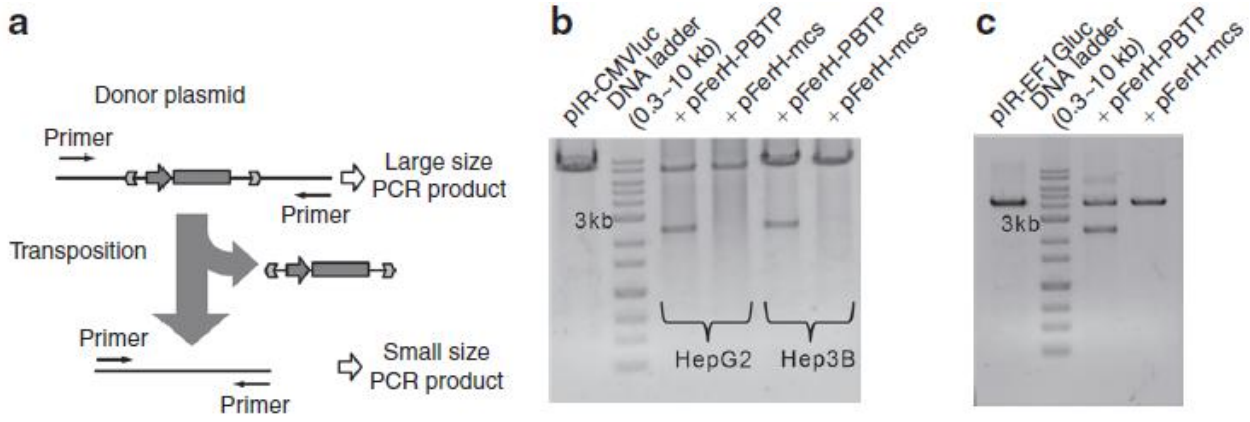


**Figure 5** molecular confirmations of the excisions of donor plasmids. (**a**) Schematic diagram of plasmid excision assay. (**b**) pIR-CMVluc (lane 1), plasmid DNA isolated from HepG2 cells transfected with pIR-CMVluc and pFerH-PBTP (lane 3) or pFerH-mcs (lane 4), Hep3B cells transfected with pIR-CMV luc and pFerH-PBTP (lane 5) or pFerH-mcs (lane 6) were used as template of PCR respectively. (**c**) pIR-EF1Gluc (lane 1), plasmid DNA isolated from livers of mouse transfected with pIR-EF1Gluc and pFerH-PBTP (lane 3) or pFerH-mcs (lane 4) were used as template of PCR respectively.

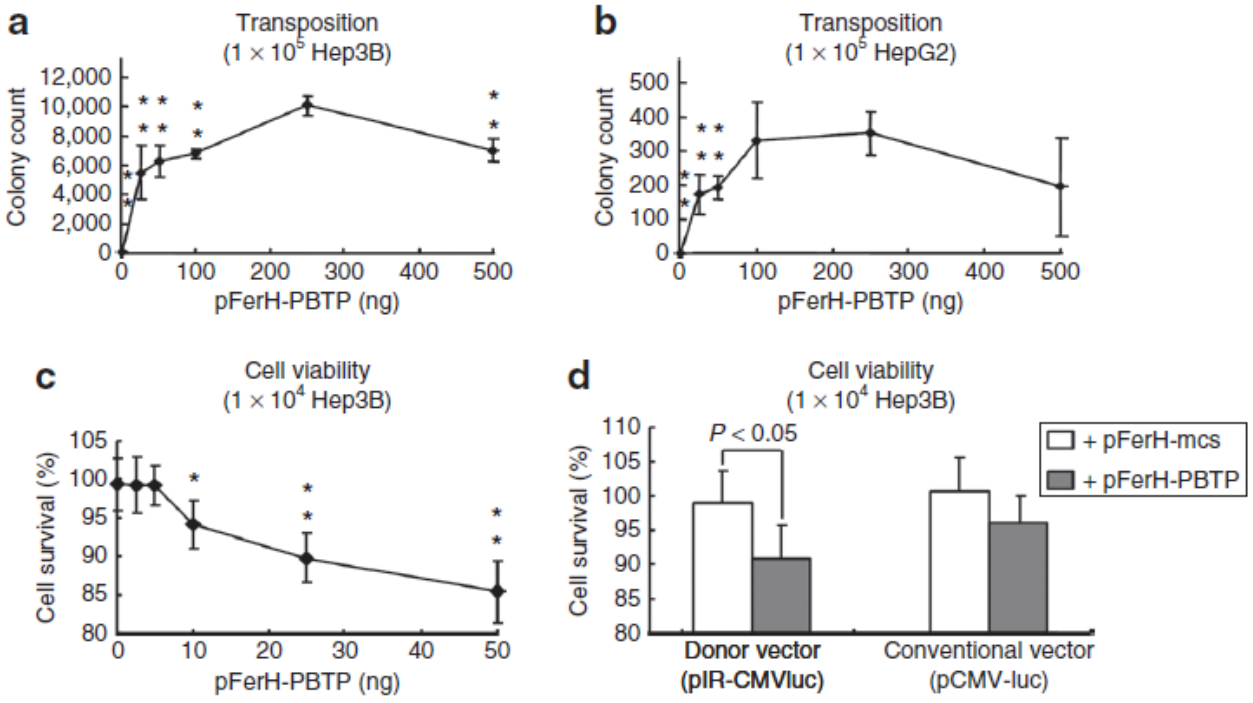


**Figure 6** Effect of the amount of transposase. (**a**, **b**) Effect of the amount of transposase versus the transposition *in vitro*. Hep3B (**a**) and HepG2 (**b**) cells ($1\times10^5$ cells/well) were transfected with 100 ng pIR-CMVluc and indicated amount of pFerH-PBTP respectively. Total DNA amount was adjusted by adding pFerH-mcs. The number of colonies was counted by methylene-blue staining after two weeks selection with G418. (**c**) Effect of the amount of transposase versus the cell viability *in vitro*. Hep3B cells ($1\times10^4$ cells/well) were transfected with 10 ng pIR-CMVluc and indicated amount of pFerH-PBTP. Cell viability was determined at two days after transfection, and represented by % of cells without transfection. Each value represents the mean ±S.D (n = 4-6). Asterisks (* and **) indicate t-test statistical different (P < 0.05 and P < 0.01 respectively) from the peak point of each graphs (i. e., 250 ng of pFerH-PBTP in (**a**) and (**b**), 0 ng of pFerH-PBTP in (**c**)). (**d**)

Effect of tranposase versus cell viability *in vitro*. Hep3B cells ($1 \times 10^4$ cells/well) were transfected with 10 ng pIR-CMVluc (left) or pCMV-luc (right) and 50 ng pFerH-mcs (white bar) or pFerH-PBTP (gray bar) respectively. Cell viability was determined at two days after transfection, and represented by % of cells without transfection. Each value represents the mean +S.D (n = 6).

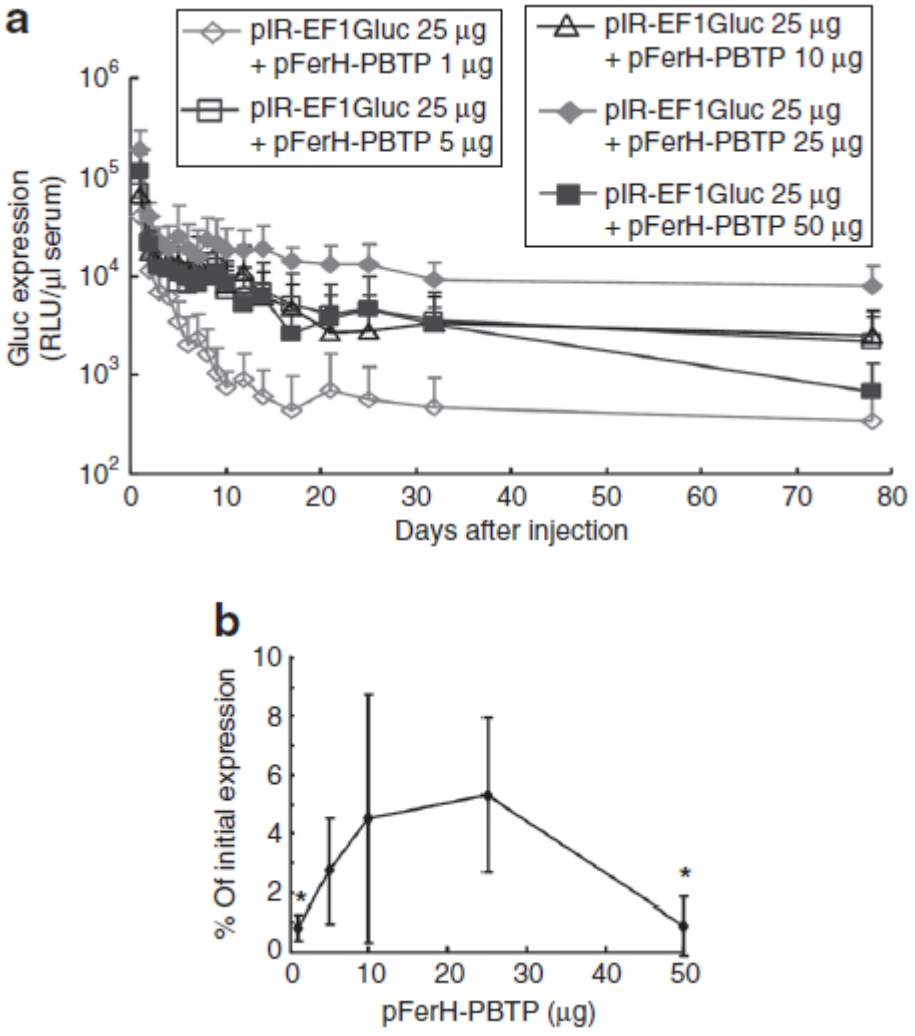


**Figure 7** Effect of the amount of transposase versus the transposition *in vivo*. (**a**) Expression time course. Mice were injected with 25 µg pIR-EF1Gluc and 1 (open rhombus ◇), 5 (open square □), 10 (open triangle △), 25 (closed rhombus ◆), 50 (closed square ■) µg pFerH-PBTP, respectively. Blood was collected at indicated time points, and Gaussia luciferase activity in serum was measured. Each value represents the mean + S.D (n = 4-6). (**b**) Percentage of sustained gene expression. *Gaussia* luciferase activities 78 days after injection were divided by those 1day after injection, and multiplied by 100. Each value represents the mean ± S.D (n = 4-6). Asterisks (*) indicate t-test statistical different ($P < 0.05$) from the peak point (i. e., 25 µg of pFerH-PBTP).

**Table 1 *piggyBac* integration sites in Hep3B cells**

| Location | Sequence | RefSeq Gene |
|---|---|---|
| Chr9 (p13, 3) | **TTAA**AAGGGTAGGAATAAGCAGTCTAATTCAGACATACTTTGTATAGGGG… | RUSC2 |
| Chr18 (q21, 33) | **TTAA**ACATTATATATCCTTAGGGAGTTTCAAATTAAGACAACACTGAGAT… | --- |
| Chr2 (p16, 1) | **TTAA**ATAAATTTGCACGCTTTTCTCTTATTAATCTGTCTTTTCTTATAAGGG… | --- |
| Chr21 (q21, 1) | **TTAA**ACAATTACTAGCTGTCAAAATCTGTGCTTGGGACATTTATATTTCAA… | --- |
| Chr10 (q23, 31) | **TTAA**TGAAGCTTATAAATGGCAAAAAGCAAAGTAAGTACAGTAAATGCT… | PANK1 |
| Chr15 (q25, 2) | **TTAA**ACAGATATTTCTCAAAAGAAGGCATAGAAATGCCCAACAGTATATG… | --- |
| Chr6 (p22, 3) | **TTAA**ACGCTCAACGAGTAACAGGGTATGTCGATGAATTCTGATTTTTTTTC… | --- |
| Chr8 (p22) | **TTAA**TATTGTATAAATGTTGGAATTCTTGTTTATGCCAAGGTGGACAACAAC… | MTUS1 |
| Chr1 (q31, 2) | **TTAA**CAAAGGTACGTATTATACATTGTCATACTATTTCTCAAAAACATTATT… | --- |

Colum 1, 2, 3 shows chromosome numbers and locations, sequences, and RefSeq genes of integration sites respectively. All sequences were determined by plasmid rescue method.