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**PITT: Pronuclear Injection-Based Targeted Transgenesis, a Reliable Transgene Expression Method in Mice**

Masato OHTSUKA¹, Hiromi MIURA¹, Masahiro SATO², Minoru KIMURA¹, Hidetoshi INOKO¹, and Channabasavaiah B. GURUMURTHY³

¹Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan  
²Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, 1–21–20 Korimoto, Kagoshima, Kagoshima 890-0065, Japan  
³Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE 68198, USA

**Abstract:** Transgenic (Tg) mice have been extensively used as valuable tools for analyses of gene function and have also served as models for many human diseases. Typically, a transgenic mouse is created by microinjection of DNA into pronuclei in which the DNA gets integrated at random locations in the genome. Frequently however, the random integration of multiple copies of a transgene results in transgene silencing, probably because of a positional effect and/or repeat-induced gene silencing. The transgene silencing issue has been overcome by single-copy transgene integration into a predetermined locus through ES cell-mediated transgenesis, despite it being expensive and more time-consuming compared with pronuclear injection (PI)-mediated transgenesis. Recently, several groups have reported novel approaches that employ PI for targeted transgenesis. They are based on site-specific recombination catalyzed by a recombinase or an integrase or homologous recombination enhanced by a zinc-finger nuclease via PI. These next-generation transgenesis methods, which we termed as PI-based Targeted Transgenesis (PITT), are more convenient and faster than ES cell-based transgenesis. Furthermore, the Tg mice generated by these newer methods contain a single-copy transgene and exhibit reliable expression of the transgene. The objective of this review is to present the recent progress in mouse targeted transgenesis.

**Key words:** mouse, PITT, pronuclear injection, targeted transgenesis, transgene expression

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**Introduction**

A little over 3 decades ago, the first transgenic (Tg) mouse harboring foreign DNA in its genome was generated by Gordon and his coworkers using pronuclear injection (PI) of the DNA [26]. Subsequent work showed that the transgenic mice generated through this technology can also pass on the transgene to their offspring. Since then, many researchers have employed this technology successfully to generate transgenic mice of their choice [11, 16, 25, 53, 73, 74]. A wide variety of transgenic mice have been generated that either have a modified mouse sequence, a human sequence, a sequence from another species such as a fluorescent protein (GFP) from jellyfish, or an enzyme (Cre) from a virus. Currently, Tg mice generated through this method are...
widely used for analysis of in vivo gene function and greatly contribute to the field of biological and medical sciences. Whereas transgenic mice expressing a particular gene are useful in studying the function of that gene and could also be used as a model of a specific human disease [40, 59], some mice that carry reporter transgenes such as a fluorescent protein (e.g., GFP) or a recombinase enzyme (e.g., Cre) are useful for general purposes [29, 39, 48, 61]. Another category of transgenic mice that has been generated contains a partial genomic DNA fragment and a reporter gene for analyses of cis-regulatory elements [5, 41].

Despite the generation of a number of Tg mice via PI-based transgenesis that have been effectively utilized for over three decades, the method itself still has some limitations. These include uncontrollability of (i) transgene integration site and (ii) transgene copy number, both of which have a profound effect on transgene expression in mice (Fig. 1A). Regarding the integration site, the randomly integrated transgene is susceptible to “position effect variegation (PEV)” that often causes impaired transgene expression and in worst cases, complete gene silencing [2, 19]. PEV is caused by the presence of cis-regulatory sequences and/or the local chromatin configuration at the integration site [75]. This limitation can be partially overcome by including insulator sequences in the construct [24, 56] or by using large DNA constructs such as bacterial artificial chromosome (BAC) clones [23]. Although the transgene expression is somewhat assured in these cases, there still remains a risk of disruption of an endogenous gene by random transgene integration.

The second drawback of this method is that it frequently results in integration of a multiple-copy transgene as tandem arrays [9, 52] that often affect the extent of transgene expression. Although increased copy number of transgenes sometimes leads to increased transgene expression, this is not always the case. In some cases, low or silenced transgene expression is reported in transgenic mice with high-copy-number arrays of transgene, and a reduction in the copy number in these mice resulted in a significant increase in transgene expression [22]. This phenomenon, termed “repeat-induced gene silencing (RIGS),” involves DNA methylation and heterochromatin formation [6].

In order to obtain reliable results from Tg mice generated via PI, an investigator needs to analyze multiple Tg lines derived from independent founders (Fig. 1A). Such a task demands excessive cost, time, and space to maintain and analyze many Tg mice lines. These fundamental drawbacks of the PI-based Tg techniques forced many technology developers to either improve the method or to develop newer methods that allow precise control of the integration site and copy number of a transgene. In the sections below, we review two aspects of targeted transgenesis in mice: 1) general and novel approaches to achieve targeted transgenesis and 2) the options of genomic loci available for transgene targeting. In addition, we discuss the applications and future prospects of new technology in transgenic research.

**General Approaches of Targeted Transgenesis**

As discussed in the previous section, to overcome PEV and RIGS, targeted insertion of a single-copy transgene into a predetermined genomic locus, where the spatial and time-dependent activity of the promoter/enhancer is well defined, would be an ideal strategy. The general approaches that have been employed to achieve this over the years are detailed below.

**Traditional PI-based transgenesis**

The pioneering work of targeted transgenesis through PI in zygotes was performed in late 1980’s by Brinster and his coworkers [10]. They examined whether homologous recombination-based targeted insertion occurs in zygotes after PI and found that its integration efficiency was very low (0.05% [1/1841]), indicating that this approach appeared to be difficult from a practical point of view.

**ES cell targeting by homologous recombination**

Albeit a tedious and time-consuming approach, many groups have sought ES cell targeting by homologous recombination—a method that was established in late 1980’s for generating knockout (KO) mice—to create targeted Tg mice (Fig. 1B). In this approach, the targeting construct containing “DNA of interest (DOI)” flanked by homologous sequences is introduced into ES cells, and chimeric mice are created from the genetically modified ES cells. Some specific genomic loci have been frequently used in this method. Hprt (hypoxanthine-guanine phosphoribosyltransferase) and Rosa26 are two such loci, and many studies have been performed to drive constitutive, tissue-specific or inducible transgene expression using several promoter/enhancer combinations.
Fig. 1. Schematic of steps involved in three methods of mouse transgenesis. (A) Traditional PI-based transgenesis: In this method, the transgene is integrated randomly into the genome, and there is no control over the number of copies integrated. (B) ES cell-based targeted transgenesis: A single copy of transgene is integrated into the predetermined genomic locus. This method involves additional steps and typically takes about 6 to 8 months from ES cell targeting to obtain a transgenic mouse. (C) PI-based targeted transgenesis (PITT): A single copy of transgene is integrated into the predetermined genomic locus. This targeted transgenesis method is much quicker than the ES cell-based approach that yields faithful and reliable founders almost all the time. Please see the text for additional details.
in these loci [1, 18, 20, 51, 63, 72, 77]. Since expression of the targeted transgene from these loci is highly reproducible, they are frequently used for rigorous analysis of transgene expression [13, 38, 55, 65, 77]. Details of these two genetic loci along with other commonly used loci are described later.

Site-specific recombinase/integrase approach

In addition to the homologous recombination-based strategy, targeted integration has also been attempted using site-specific recombination systems (e.g., Cre-loxP, FLP-FRT and PhiC31). The “recombinase-mediated cassette exchange (RMCE)” strategy enables efficient targeted integration or exchange of DOI into ES cells containing specific docking sites (recombinase recognition sequences) [50]. The RMCE approach has been widely applied in many loci including Hprt and Rosa26 and utilized for many kinds of studies [15, 58, 68].

Although these newer targeted transgenesis approaches promise stable and reproducible transgene expression, creation of Tg mice with these methods (except for the study by Brinster et al. [10]) has always been coupled with ES cell-based genetic modification, which is more laborious and time-consuming compared with PI-based transgenesis.

### Table 1. Overview of PITT experiments in mice

<table>
<thead>
<tr>
<th>Recombinase/Integrase/ZFN</th>
<th>Sequence tags employed</th>
<th>Target loci</th>
<th>Targeted integration efficiencies and additional comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-Cre fusion (plasmid DNA)</td>
<td>loxP and lox511 (same orientation)</td>
<td>NGF</td>
<td>9.9% (21/213) in 2- or 4-cell stage embryos - Half of the injected zygotes were hemizygotes for the tagged site - Incomplete exclusiveness of loxP combination</td>
<td>[43]</td>
</tr>
<tr>
<td>MBP-Cre fusion (protein)</td>
<td>loxP and lox511 (inverted orientation)</td>
<td>β-actin</td>
<td>2.3% (7/311) in embryos and newborns - Half of the injected zygotes were hemizygotes for the tagged site - Incomplete exclusiveness of loxP combination (orientation uncontrollable)</td>
<td>[62]</td>
</tr>
<tr>
<td>Cre (plasmid DNA)</td>
<td>JT15/JTZ17 and lox2272 (same orientation)</td>
<td>Rosa26</td>
<td>4.3% (21/492) in ref. 47 and 5.4% (5/93) in ref. 46 - All of the injected zygotes were hemizygotes for the tagged site - Donor vectors 13.6–14.8 and 9.8–13.9 kb in size were used in refs. 47 and 46, respectively</td>
<td>[46, 47]</td>
</tr>
<tr>
<td>Cre (plasmid DNA)</td>
<td>Endogenous lox-like site (Ψloxm5)</td>
<td>Ψloxm5</td>
<td>13.6% (3/22) - B6C3F1 mice were used</td>
<td>[35]</td>
</tr>
<tr>
<td>PhiC31 (mRNA)</td>
<td>attB/attP (both tandem and single sites were used)</td>
<td>Rosa26</td>
<td>10.8% (45/415) - All of the injected zygotes were homozygotes for the tagged site - 3–6 kb of DNA was used as the donor vector except in one trial using 14 kb of DNA</td>
<td>[67]</td>
</tr>
<tr>
<td>ZFN* (mRNA)</td>
<td>No tagged site</td>
<td>Rosa26</td>
<td>1.7% (1/58) and 4.5% (1/22) - F1 hybrid (FVB and C57BL/6N) was used</td>
<td>[44]</td>
</tr>
<tr>
<td>ZFN* (mRNA)</td>
<td>No tagged site</td>
<td>Mdr1a</td>
<td>25% (1/4) and 5% (2/40) - FVB strain was used</td>
<td>[17]</td>
</tr>
</tbody>
</table>

* These approaches can also be used for knockout or knock-in mouse generation.

In the last two to three years, a few reports of PI-mediated targeted transgenesis have been published (Fig. 1C, Table 1). In these systems (that we termed as PITT-Pronuclear Injection-based Targeted Transgenesis), a DOI can be inserted into a predetermined genomic locus in zygotes without steps needed for ES cell targeting and subsequent generation of chimeras. The PITT approach requires an ES cell targeting step only at the stage of establishing the so-called “seed” mice that contain suitable docking sites. Once such mice are created, they can be used for generating targeted transgenes in those loci for any DOI. The highly promising result of these studies is that targeted integration of Tg DNA directly in zygotes proceeds in a non-selectable way. This method appears to be more feasible for targeted integration of
DOI in zygotes than the traditional PI-mediated transgenesis, which integrates DOI randomly. Various recombinases and integrases used in the PITT approach are described below and outlined in Fig. 2.

**Cre-LoxP-based PITT (Cre-PITT)**

The Cre recombinase is an enzyme isolated from bacteriophage P1 that binds to a 34 bp loxP sequence and mediates recombination between two loxP sites. The Cre enzyme has been widely used for modification of the mammalian genome through deletion or inversion of a target DNA molecule flanked by the loxP sequences [80]. It can also catalyze the integration reaction of a target DNA via intermolecular recombination. The integration efficiency is very low whenever wild-type loxP sites are used. This is because the integrated DNA segment is easily excised as a result of an intramolecular recombination [42]. However, the use of two inverted loxP sites or a pair of mutant loxPs can increase the integration efficiency of target DNA. This system, called RMCE, enables exchange of a preexisting DNA cassette flanked by mutant loxPs with the DOI flanked by compatible loxPs [7, 76]. Since the integration efficiency in RMCE has been proven to be much higher than that in homologous recombination-based targeting, targeting of DOIs with RMCE is now frequently performed using ES cells containing loxP-tagged sites. It should be kept in mind that performance of RMCE requires the placement of loxP tags into the predetermined locus through homologous recombination-based targeting.

Targeting of DOIs using Cre-PITT has been reported recently. Lauth et al., [43] inserted a DNA fragment containing wild-type loxP and mutant lox511 (spacer variant) arranged in the same orientation. They chose the promoter region of the nerve growth factor (NGF) gene to create a mouse with docking sites for Cre-PITT (Table 1). The donor vector containing the same combination of loxP sites was co-injected with a plasmid DNA containing a GFP-Cre fusion construct into pronuclei of zygotes derived from mating of a heterozygous seed male with a wild-type female. Nested PCR of genomic DNA was used to confirm targeted integration in two- to four-cell stage embryos. The integration efficiency of the donor vector was almost 10% (number of embryos showing correct integration/number of embryos analyzed). However, integration did not seem to be stable for a long

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**Fig. 2.** Schematic of the currently available PI-based targeted transgenesis strategies. All these strategies will enable targeted integration of DOI not only in cultured cells but also in zygotes. (A) Cre-loxP site-specific recombination-based strategy: When a donor vector containing DOI with a combination of mutant loxPs (JT15/JT17 and lox2272) is introduced into zygotes together with a Cre recombinase expressing plasmid, site-specific recombination occurs between mutant loxPs located in the donor vector and the previously engineered genome [46, 47]. (B) PhiC31 integrase-based strategy: Like the Cre-based system, a donor vector containing DOI is introduced into zygotes together with PhiC31 integrase that catalyze site-specific integration between attP and attB. For simplicity, a single attP site tagged in the genome is shown in here, although three tandem attP sites were used by Tasic et al. (2011) [67]. (C) ZFN-based strategy: A pair of ZFNs (ZFN1 and 2) is injected into a zygote together with a targeting vector containing DOI flanked by homology arms. ZFN introduces double-strand breaks (DSBs) into the target site, and DOI can be integrated into this site via a homologous recombination-based repair system. Cre, PhiC31, and ZFN can be supplied as DNA, mRNA, or protein.
time; the integrated DNA was lost by embryonic days (E) 8 and 10. The integrated sequence was continuously subjected to excision due to incomplete exclusiveness between loxP and lox511 and prolonged expression of Cre recombinase. The phenomenon in which the targeted integration event is not stable is hereafter termed “incomplete exclusiveness of the loxP combination.”

Shmerling et al., created loxP and lox511 docking sites at the β-actin locus following a similar strategy as that of Lauth and coworkers [43], except that they placed the sequences in an inverted orientation to prevent excision of the integrated cassette [62]. They injected the loxP set-containing donor vector together with a recombinant maltose binding protein (MBP)-Cre fusion protein into the pronuclei of zygotes derived from mating of a heterozygous seed male with a wild-type female. They obtained correctly recombined E12 embryos and newborns with an efficiency of ~2.3% (Table 1). Due to the random integration rate, however, a mating test demonstrated that more than 90% of the resulting founder mice had transmitted the transgene to the next generation. We also confirmed that the Tg offspring generated by Cre-PITT exhibited faithful transgene expression. Furthermore, we used this method to create knockdown mice (described in the “Applications of PITT” section).

Recently, another Cre-PITT approach was reported by Ito et al. [35] in which they used a pseudo-loxP site that exists in the mouse genome. They tried to integrate a DOI into the endogenous lox-like site (Ψloxm5) by co-injecting a circular donor vector and pCAG/NCre into the pronuclei of zygotes collected from B6C3F1 mice. The targeted integration efficiency in the resulting newborn pups and fetuses was 13.6% (3/22) (Table 1). The study did not mention if germline transmission was achieved with the transgenic founders. Since this method does not require engineering of artificial docking sites to create seed mice, it could, in theory, be readily accessible for Cre-PITT in any mouse strain.

**PhiC31 integrase-based PITT (PhiC31-PITT)**

The *Streptomyces* phage PhiC31 is an integrase that belongs to the serine recombinase family of site-specific recombinases. It catalyzes recombination between attachment sites, attB and attP, without the assistance of any cofactors to generate hybrid sites, attL and attR, that are not targets to regenerate attB and attP in the absence of the excisionase/resolvase protein [64]. Therefore, an integration reaction using PhiC31 is unidirectional. Like the Cre-loxP system, the PhiC31 integrase system has been used for RMCE in mammalian cells [8, 31].

The PhiC31 integrase system for targeted integration through pronuclear injection in mice was recently reported by Tasic et al. [67]. They used a single full-length attP or three shortened tandem attP sites [27] and placed them into the Rosa26 and Hipp1 (H11) loci. PhiC31-PITT (Fig. 2B) was performed by injecting PhiC31 integrase mRNA together with a circular donor vector containing one or two attB sites into the zygotes harboring attP docking sites homozgyously (Fig. 2B). As a result, they obtained correctly integrated embryos and pups with high efficiencies (3.0–40.0%) (Table 1) [67]. The random integration rate, however, was also high (0–20.0%) compared with the Cre-PITT that we reported. This may be attributed to the integration of donor vector into pseudo-attP sites in the mammalian genome.
Despite the higher random integration rate, simultaneous occurrence of both site-specific integration and random integration was rare in their study. As compared here, Cre-PITT and PhiC31-PITT follow similar experimental approaches except that they use different recombinases and their respective recognition sequences as docking sites engineered in the mouse genome (Fig. 2A and 2B). The authors commented that the PhiC31-PITT may be preferred over Cre-PITT, since the former can be used in conjunction with transgenic cassettes that contain a “loxP-STOP-loxP” sequence, which is difficult in Cre-PITT. They also confirmed the ability of the founder mice for germline transmission and for faithful expression of the transgene.

**Zinc finger nuclease-based PITT (ZFN-PITT)**

Zinc finger nuclease (ZFN) is an engineered chimeric protein consisting of a custom-made zinc finger domain and a nuclease domain derived from FokI restriction endonuclease. The zinc finger domains can be designed to bind the target DNA sequence of interest. Upon binding, two such custom-made ZFNs bind to two adjacent targeted sites, and the dimerized nuclease domain causes a double-strand break (DSB) in the region between the targeted sites. These DSB sites can be repaired either by nonhomologous end-joining (NHEJ) (which can lead to the introduction of insertion/deletion mutations) or homology-directed repair (HDR). Therefore, once the DSB is generated by ZFN, HDR can induce high-frequency homologous recombinations allowing targeted integration of the DOI into the genome. This technique has been successfully tested in various cell types including human and mouse cells [32, 45, 54, 78] (Fig. 2C).

Targeted gene integration by ZFN through PI (ZFN-PITT, Fig. 2C) in mice was first reported by Meyer et al. [44]. A targeting vector consisting of 1-kb 5′ and 4-kb 3′ homology arms for the Rosa26 locus was co-injected into the pronuclei of zygotes of FVB mice together with ZFN mRNA. As a result, 25% (1/4) and 5% (2/40) of the fetuses obtained contained the correctly integrated allele, suggesting the applicability of ZFN-PITT for targeted transgenesis in mice (Table 1). Notably, two of the three founders also contained a deletion allele generated by NHEJ in a mosaic fashion.

Although germline transmission of founder mice was not examined in these two reports, ZFN-PITT is advantageous as i) it is not necessary to generate seed mice first as needed in Cre-PITT and PhiC31-PITT systems and ii) it can be performed in any mouse strain.

**Where Should the Transgene Be Targeted in the Genome?**

With the use of ES cell-based targeted transgenesis, it is now evident in the literature that certain loci in the mouse genome have become popular for targeted transgenesis, as they have been used repeatedly. The PITT methods also utilized these well-studied genetic loci for targeted transgenesis. These common genetic loci and some newer ones used in targeted transgenesis are described below.

**Rosa26**

Rosa26 is located on chromosome 6 and was originally identified by retroviral gene trapping experiments [21, 79]. Three mRNAs are transcribed from this locus; two are from the plus strand that does not encode an open reading frame, and the third is from the minus strand that encodes a 505-amino acid putative protein [79]. The function of these transcripts is still unclear, and disruption of Rosa26 transcripts does not cause any detrimental effects on the individual’s life [14]. This locus offers an open chromatin configuration, and mice in which βgeo, the first reporter gene that was integrated into this locus, exhibit ubiquitous and constitutive expression of the reporter gene [79]. Based on these features, in addition to the high degree of gene targeting efficiency at this locus using the ES cell approach, Rosa26 is now one of the most frequently used loci for targeted integration of genes and shRNAs [1, 14, 31, 63].

In all of the PITT-related technologies, the Rosa26 locus has been the first choice to test the feasibility of the method [44, 46, 47, 67], and in fact, targeted Tg mice
at this locus have been readily obtained with these methods. Thus, the Rosa26 locus can confer efficient targeted integration of DOI in ES cells as well as zygotes. The integrated transgenes were expressed strongly and ubiquitously when they were transcribed from the CAG (cytomegalovirus early enhancer + chicken β-actin promoter) promoter, though promoter activity may be affected by the orientation of DOI integrated into the Rosa26 locus [65]. In our PITT trials, all transgenes were integrated in the opposite orientation with respect to the endogenous Rosa26 promoter [47]. The versatility of this locus in the PITT system is further enhanced when a tissue-specific, conditional and/or inducible expression cassette is used.

Hipp11 (H11)

The H11 locus, named by Hippenmeyer et al., is located within an intergenic region between the Eif4enif1 and Drg1 genes that are mapped close to the centromere of chromosome 11 [30]. Since the expressions of Eif4enif1 and Drg1 genes are spatially and temporally broad, the H11 region is expected to allow global expression of a ubiquitous promoter-driven transgene integrated into this locus. Hippenmeyer et al. integrated a cassette for “mosaic analysis with double markers (MADM)” into the H11 locus for genetic mosaic analyses to examine the roles of Lis1 (Lissencephaly-1) and Ndel1 (nuclear distribution gene E-like homolog 1) in neuronal cell migration by knocking out these genes in a sparse subpopulation of neurons.

Tasic et al. [67] utilized the H11 locus in the PhiC31-based PITT system because they expected that H11 might allow better access to PhiC31 integrase than Rosa26 based on their previous study using MADM cassettes (containing CAG promoter-driven transgenes, loxP, and FRT sites) integrated into the H11 locus, which showed a high level of global transgene expression and a higher rate of interchromosomal recombination compared with those integrated into the Rosa26 locus [30, 67]. In fact Tasic et al. successfully integrated a single-copy transgene into the H11 locus using a PhiC31-PITT system with high efficiency (up to 40%) [67]. Therefore, H11, like Rosa26, is thought to be one of the promising genomic loci candidates for PITT.

H2-Tw3

H2-Tw3 is a gene on chromosome 17 in mouse strains with the H2bc haplotype (e.g., 129 strain), and encodes a nonclassical major histocompatibility complex (MHC) antigen. This is similar to the H2-T3 gene present in H2b haplotype mouse strains (e.g., C57BL/6) but absent in H2b haplotype mouse strains [66]. Its expression is restricted to some tissues such as the thymus and intestine. Although the function of H2-Tw3 is unknown, we hypothesize that H2-Tw3 is functionally redundant with H2-T3, which is known to be involved in mucosal immune responses in the intestine [49], based on the similarity in the nucleotide sequence and gene expression pattern between them. H2-Tw3-deficient mice are viable and develop normally with normal reproductive ability [47].

We introduced a cassette containing a CAG promoter-driven eGFP gene into the H2-Tw3 locus using a Cre-based PITT system, and a targeted Tg line was successfully obtained [47]. The integration efficiency at this locus (5.0% [2/40]) was comparable to the Rosa26 locus (4.4% [24/545]) [46, 47]. The resultant H2-Tw3CAG::eGFP mouse showed ubiquitous eGFP fluorescence, although the eGFP expression level in this line was lower than that of the Rosa26CAG::eGFP mouse. This locus, therefore, can serve as an additional site suitable for PITT.

Hprt

The Hprt gene is located on the X chromosome and encodes an enzyme required for the salvage pathway of purine biosynthesis. Cells deficient in Hprt gene are selectable in medium containing 6-thioguanine, and cells reconstituted with Hprt can be grown in hypoxanthine-aminopterin-thymidine (HAT) medium. Since the Hprt gene is expressed essentially in all tissues and at virtually all developmental stages, this locus is thought to exhibit a relaxed chromatin configuration that is transcriptionally favorable [13, 51]. Mice deficient in the Hprt gene are viable and fertile, whereas in humans, mutations in its counterpart gene causes Lesch-Nyhan syndrome, which is characterized by mental retardation and self-mutilation [34]. Notably, mosaic expression of a transgene integrated into the Hprt locus occurs in the Tg females, since one of the two X chromosomes is always inactivated by X-inactivation (lyonization). This feature sometimes is advantageous in mosaic analyses of a transgene (e.g., mosaic complementation study) [58]. Mosaic expression of a transgene might also improve the survival of Tg mice that might otherwise die. Moreover, female offspring derived from Tg males are always Tg, since the transgene is present on the X chro-
mosome, which enables omission of the genotyping step [77].

To our knowledge, no PITT trials have been performed with integration at the Hprr locus so far. However, this locus could be a potential site for PITT, since plenty of studies indicate that a single-copy transgene introduced into the Hprr locus through gene targeting in ES cells exhibits ubiquitous or tissue-specific expression [18, 20, 28, 37, 51, 72].

**Other loci**

As described above, two other loci (NGF and β-actin) have been used for Cre-PITT trials [43, 62]. Targeted Tg founder mice were successfully obtained at the β-actin locus, although the correct integration efficiency was low (0 to 2.4%). The disadvantage in using these loci for PITT is that homozygously tagged mice are not viable, and thus only about half of the fertilized eggs contain a tagged allele, since zygotes are only available from mating between heterozygous males and wild-type females. In addition, the heterozygous KO mice in which the β-actin locus on one allele had been destroyed exhibited abnormal phenotypes [36, 62].

The loci containing endogenous pseudo-docking sites present in the mouse genome (e.g., Ψloxm5) can be candidate sites for PITT. With these sites, PITT could be performed without the need for generating “seed mice” that contain engineered docking sites. To date, several cryptic sites (both pseudo-loxP and pseudo-attP) have been reported in mice [70, 71]. It will be interesting to test whether these sites could work efficiently as docking sites for PITT.

**Faithful Transgene Expression in PITT Mice**

One of the advantages of the PITT system is controllability of the integration site and the copy-number of the transgene. These favorable features guarantee stable, faithful transgene expression not affected by PEV and RIGS.

Among the PITT studies performed so far, our group and Tasic et al. have examined the expression pattern of fluorescent transgenes in PITT mice in greater detail [47, 67]. In our study, we observed that a single-copy CAG promoter-driven fluorescent gene integrated into the Rosa26 or H2-Tw3 locus was expressed ubiquitously, strongly, and stably in organs such as the liver, kidney, and small intestine, which was in contrast with the previous fluorescent mice generated using the random integration-based method that exhibited a variegated pattern of fluorescence. Notably, the intensity of fluorescence in the PITT mice was higher when the transgene was integrated into the Rosa26 locus than when the same construct was present in the H2-Tw3 locus. Furthermore, stable expression was accomplished for both loci only after the removal of extra sequences containing the vector backbone and the hygromycin resistance gene (the latter of which was transcribed from the CAG promoter). Very weak and mosaic transgene expression or no transgene expression was detectable with these extra sequences. We also demonstrated that this is true in ES cells [47].

Similar results were reported by Tasic et al., who used the H11 locus for PhiC31-PITT [67]. They found that not only the bacterial backbone but also the VASA promoter and/or other elements including the neomycin resistance gene could affect proper transgene expression, and such variegated transgene expression was remarkable in the liver. However, removal of these extra sequences resulted in reproducible, high, and uniform expression of the transgene.

From these two independent studies that used three genomic loci, it is evident that Tg mice exhibiting faithful transgene expression could be reproducibly obtained by using the PITT method. These features make PITT superior to the traditional random integration-based transgenesis (Fig. 1A). Once PITT founder Tg mice are obtained, it is enough to maintain only one germline transmissible line for further analyses (Fig. 1C). However, care should be taken to ensure that unwanted sequences including those derived from bacteria are excluded from the transgene as much as possible for satisfactory expression of the transgene.

**Applications of PITT**

**Expression of foreign (or modified) genes**

The primary goal of generating a transgenic mouse is the expression of a foreign gene or a mutant gene through stable integration of the DNA in the mouse genome. Majority of Tg mice generated so far fall into this category. PITT can be effectively used for generating transgenic mice wherever traditional PI-based transgenesis is used.
Gene knockdown

KO mice generated through ES cell targeting have been widely used as a tool for loss-of-function studies in mice. RNA interference (RNAi)-mediated gene silencing is also a useful technique for this purpose, since knockdown mice carrying an RNAi expression construct can be produced faster and are less expensive than KO mice. However, as indicated by Premsrirut et al. [57], reliable generation of RNAi-expressing Tg mice has remained a technical challenge because those generated by a traditional PI method often exhibited a high degree of variability in knockdown efficiency. According to their study, such disadvantages could be overcome by establishing a rapid and scalable system for production of short hairpin RNA (shRNA) Tg mice [57]. However, for production of such Tg mice, ES cell-mediated transgenesis is required, which includes ES cell manipulation and production of chimeras.

We have successfully demonstrated that knockdown mice can be generated using PITT [47]. In this new system, we chose the *Tyrosinase* gene involved in melanin synthesis as a target to be knocked down because the phenotypic alterations caused by reduced *Tyrosinase* gene function can be easily monitored by the coat color. For *in vivo* knockdown, artificial microRNA against *Tyrosinase* with a miR-155 backbone was designed to express along with an eGFP fluorescent reporter under the transcriptional control of a ubiquitous CAG promoter. The resultant construct was subjected to PITT, and as a result, hemizygous microRNA-overexpressing Tg mice with a light coat color were successfully obtained. As expected, the amounts of *Tyrosinase* mRNAs in the ear and eye of microRNA Tg mice were reduced to 10–20% of those of wild-type mice, indicating successful *in vivo* RNAi-mediated gene silencing. We also have generated eGFP knockdown mice using this method, which indicates that microRNA-based *in vivo* knockdown of eGFP expression is efficient and reproducible (manuscript in preparation).

Tissue-specific expression

Most of the PITT attempts have adopted ubiquitous promoters (e.g., CAG promoter) to evaluate the mode of transgene expression in PITT. It would be much more versatile if tissue-specific expression of DOI is possible with PITT. Tasic et al. integrated a cassette containing a 9-kb *Hb9* (homeobox gene transcription factor, a selective marker of motor neurons in the developing spinal cord [4]) promoter and GFP cDNA into the *H11* locus of embryos at the zygotic stage [67]. The resulting Tg mice expressed GFP in the motor neurons of the ventral spinal cord and tail tip with an expression pattern that was similar to that reported previously [4]. More studies are needed to evaluate the suitability of PITT to generate tissue-specific transgenic mice.

Conclusions and Future Perspectives

The PITT strategies reviewed here have distinct advantages over traditional random integration-based transgenesis methods. Unlike the Tg mice generated using traditional PI-based methods, the Tg mice generated by PITT are less susceptible to gene silencing caused by PEV and/or RIGS and therefore exhibit faithful and stable transgene expression. The reproducibility of transgene expression in PITT-derived Tg mice would allow researchers to perform detailed and meaningful comparisons among a series of alleles or mutations associated with various diseases. With the PITT method, analysis of only one Tg mouse line/construct can be sufficient (Fig. 1C). This leads to a reduction in cost, space, and labor required to maintain multiple mice lines as needed in traditional transgenesis. Furthermore, PITT is less expensive and rapid compared with ES cell targeting-based transgenesis.

At present, there are only a few reports related to PITT technology, each of which showed successful targeted transgenesis although with different efficiencies. The integration efficiencies in each strategy cannot be directly compared at the moment because each study employed different mouse strains, sizes of donor vectors, recombination enzyme systems (DNA, mRNA or protein), target sites, and zygosities (i.e., hemizygote or homozygote) (Table 1). Regarding the size of the donor vectors, Tasic et al. suggested that smaller donor vectors (3–6 kb) tend to be integrated more efficiently (4.8–40.0%) than larger ones (14 kb; 3.0%) into the target site [67]. In our study, integration efficiencies ranged from 2.4 to 9.7% (4.3% on average) when donor vectors of 13.6–14.8 kb in size were used [47]. Tasic et al. injected mRNA of PhiC31 integrase into homozygous zygotes [67], whereas we injected Cre expression plasmid DNA into hemizygous zygotes [47]. Further experiments and detailed comparisons will be required for improvement of the PITT method. In addition, participation of many researchers in developing/improving the PITT method
will enhance its utility and availability.

So far, only a limited number of loci have been used for PITT. Exploration of additional and alternative loci to be targeted would be important and urgent to respond to future demands (generation of double and triple Tg mice or Tg mice with various KO mouse backgrounds). Although the transgene expression pattern in the offspring of PITT mice has been extensively explored, there have still been very few attempts to create PITT mice carrying a cassette containing a tissue-specific promoter or a conditional expression cassette.

We do not know at this moment which PITT method is the best (Fig. 2), and as commented by Rossant et al. [60], it depends on the researcher’s needs, local institutional support, and cost. In the near future, each method could be expected to be improved with respect to its efficiency, ease of handling, and cost-effectiveness. We are currently making some modifications to our original PITT method (e.g., improvement of integration efficiency, generation of a new seed mouse with inbred genetic background). Considering the distinct advantages of PITT, we anticipate that the random integration-based traditional transgenesis will be gradually replaced by the PITT method as a “next-generation transgenesis method.”

### Abbreviations

Tg, transgenic; ES cell, embryonic stem cell; PI, pronuclear injection; PITT, pronuclear injection-based targeted transgenesis; PEV, position effect variegation; BAC, bacterial artificial chromosome; RIGS, repeat-induced gene silencing; KO, mice, knockout mice; DOI, DNA of interest; Hprt, hypoxanthine-guanine phosphoribosyltransferase; RMCE, recombinase-mediated cassette exchange; Cre-PITT, Cre-loxP-based PITT; NGF, nerve growth factor; MBP, maltose binding protein; PhiC31-PITT, PhiC31 integrase-based PITT; H11, Hipp1; ZFN-PITT, zinc finger nuclease-based PITT; ZFN, zinc finger nuclease; DSB, double-strand break; NHEJ, nonhomologous end-joining; HDR, homology-directed repair; MADM, mosaic analysis with double markers; Lis1, Lissencephaly-1; Ndel1, nuclear distribution gene E-like homolog 1; MHC, major histocompatibility complex; HAT, hypoxanthine-aminopterin-thymidine; RNAi, RNA interference; shRNA, short hairpin RNA

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### References


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42. Lauth, M. 2004. The family is growing: old and new members of the family of site-specific recombinases and their application to genome engineering. Curr. Pharmacoceonometrics 2: 267–276. [CrossRef]


64. Srivastava, V. and Gidoni, D. 2010. Site-specific gene in-

65. Strathdee, D., Ibbotson, H., and Grant, S.G. 2006. Expression of transgenes targeted to the Gi(ROSA)26Sor locus is orientation dependent. *PLoS ONE* 1: e4. [Medline] [CrossRef]


