

# Adjusting the *attB* Site in Donor Plasmid Improves the Efficiency of $\Phi$ C31 Integrase System

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$\Phi$ C31 integrase, a site-specific recombinase, can catalyze integration of circular DNA bearing *attB* site into pseudo *attP* sites in mammalian genomes. However, the integration efficiency mediated by integrase is relatively low. Our study centered on the investigation of the impact of the position, orientation, and number of *attBs* in the donor plasmid on the efficiency of  $\Phi$ C31 integrase system. Donor plasmids bearing various types of *attBs* (including forward and reverse directions, tandem, and intersperse) and reporter enhanced green fluorescent protein (EGFP) were constructed. The plasmids plus helper plasmid encoding integrase were co-transfected into HeLa cells. After G418 selection, the resistant cell colonies were counted for calculating chromosomal integration frequency. EGFP expression was detected by fluorescence-activated cell sorter and enzyme-linked immunosorbent assay analysis. The results showed that efficiency of integration mediated by integrase accounted for  $70\% \pm 7.1\%$  of total integration events in the transfected HeLa cells. Compared with a forward orientation of *attB* in donor plasmid, a reverse direction of *attB* or interspersed *attBs* showed 1.5- or 2.8-fold increase in integration efficiency, respectively, while tandem *attBs* in donor plasmids caused a decreased efficiency of integration. We conclude that the adjustment of *attB* sites in donor plasmids may be of value for gene therapy and routine genetic engineering by using  $\Phi$ C31 integrase system.

## Introduction

$\Phi$ C31 INTEGRASE, a member of a serine-catalyzed superfamily, was first described as a recombinase of 613 amino acids (Kuhstoss and Rao, 1991).  $\Phi$ C31 integrase mediates recombination between the 34-bp bacterial attachment site *attB* and the 39-bp phage attachment site *attP* (Groth *et al.*, 2000), allowing integration of the phage genome into the bacterial host chromosome. Co-transfecting cells with donor plasmid bearing *attB* site and helper plasmid encoding  $\Phi$ C31 integrase results in integration at preintegrated wild-type *attP* or native cellular pseudo *attP* sites in the mammalian genomes. Pseudo *attP* sites share partial sequence identity to the wild-type *attP* sequence. Previous studies found that integration of target genes at some pseudo *attPs* was predominant (Groth *et al.*, 2000; Ou *et al.*, 2008). In addition, many pseudo *attP* sites resided in open chromatins that might lead to a high expression of the integrated gene at these sites mediated with integrase (Chalberg *et al.*, 2006). These features make  $\Phi$ C31 integrase a useful tool for genetic engineering, and gene therapy.

However,  $\Phi$ C31 integrase system showed relatively low efficiency compared with other nonviral system such as transposon system (Franz *et al.*, 2011). Improved versions of  $\Phi$ C31 integrase system were recently developed. Hyperactive integrase was made by synthesizing host codon-optimized  $\Phi$ C31 integrase, or screening mutants for more efficient recombination (Raymond and Soriano, 2007; Keravala *et al.*, 2009). Recently, Tasic *et al.* (2011) found that the efficiency of precise cassette exchange between *attP* and *attB* was significantly improved by inserting *attP* sites into a novel locus, H11 of the mouse genome.

The *attB* sequence is an important element in  $\Phi$ C31 integrase system. We wonder whether the integration efficiency of  $\Phi$ C31 integrase system can be improved by adjusting *attB* site in donor plasmid. The previous study showed that mutagenesis at the distal end of *attB* or close to the crossover site inhibits DNA-binding affinity and cleavage, indicating that sequences in *attB* affect the ability of  $\Phi$ C31 integrase to synapse and to activate DNA cleavage (Gupta *et al.*, 2007).

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Accordingly, we investigated the impact of the direction, position, and number of *attB* upon the efficiency of  $\Phi$ C31 integrase system. Our results demonstrated that manipulating the orientation and number of *attB* in the donor plasmid could provide an easy, rapid means for improving the efficiency of integration mediated by integrase.

## Materials and Methods

### Plasmid construction

All restriction enzymes were purchased from New England Biolabs (Ipswich, MA) unless otherwise stated. The plasmids of pCMV-Int, pBCPB, and pInt were kindly provided by Dr. M.P. Calos, Stanford University. The construction of the donor plasmids is briefly described in the following procedures. The *attB* site was amplified using the primers attBPF and attBPR (Table 1), and the resulting polymerase chain reaction (PCR) fragments were inserted into the *Pci*I sites of pEGFP-N1 (Clontech, Mountain View, CA). The plasmids of pBtta-EGFP-N1 (reverse direction for *attB*), pattB-EGFP-N1 (forward direction of *attB*), and p2attB-EGFP-N1 (tandem with two *attBs*) were then constructed (Fig. 1). The plasmid p3attB-EGFP-N1 (tandem with three *attBs*) was also generated, in which the other two tandem *attBs* from p2attB-EGFP-N1 were amplified using the primers attBAF and attBAR (Table 1) and the PCR fragment was inserted into the *Ase*I site of pattB-EGFP-N1 (Fig. 1). For construction of the plasmid pEGFP-Btta-N1 (reverse direction of *attB* in downstream), *attB* was released from pEGFP-attB-N1 (Ma *et al.*, 2006) by *Afl*II digestion and then inserted into the same site of pEGFP-N1. For pattB-EGFP-attB-N1 (interspersed with two *attBs*), *attB* fragment was amplified using primers attBEF and attBER (Table 1), and the resulting fragment was inserted into the *Eco*O109I site of pEGFP-attB-N1. For pattB-attB-EGFP-attB-N1 (interspersed with three *attBs*), the amplification of *attB* was directed by primers attBAPF and attBAPR (Table 1), and the product was inserted into the *Ase*I and *Pci*I sites of pattB-EGFP-attB-N1. For pBCP2B and pBCP3B, a 318-bp fragment was amplified from pEGFP-attB-N1 using primers attBHF and attBHR (Table 1), and the amplified fragment was then ligated into the *Hind*III sites of pBCPB. For pBCP, pBCPB was cut by *Xho*I to remove *attB* and self-ligated afterward.

TABLE 1. PRIMERS USED IN THIS STUDY

Primers	Sequences 5' to 3'
attBPF	AGTAACATGTTTCGAGGTCGACGATGTAGGT
attBPR	AGTAACATGTGAGGGGCCCAAGCTT
attBAF	AGTACTTAAGAACCGTATTACCGCCATGCAT
attBAR	AGTACTTAAGACCCC GTAATTGACTACTAT
attBEF	AAGGACCTTCGAGGTCGACGATGTAGGT
attBER	AAGGACCTGAGGGGCCCAAGCTTATC
attBAPF	AGTAATTAATTTCGAGGTCGACGATGTAGGT
attBAPR	AGTAACATGTGAGGGGCCCAAGCTTATC
attBHF	ATGAAGCTTTTCGAGGTCGACGATGTAGGT
attBHR	ATGAAGCTTTCGAGGGGCCCAAGCTTATC
attBRDF	CCCCTGAACCTGAAACAT
attBRDR	CAACACTCAACCTATCTCG
attB8A1F	GTGGTTTGTCCAAACTCATC
attBA1R1	ACATAAAATGAATGCAATTG
attBA1R2	TTGCATGGCCTCATTCCGTC

### Cell culture and integration assays

HeLa cells (ATCC, Cat. No. CCL-2) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Clontech). For integration assays, donor plasmids plus helper plasmids (encoding the integrase) were co-transfected into HeLa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h of transfection, cells were seeded on 100-mm dishes at a 1:20 dilution in 400  $\mu\text{g}\cdot\text{mL}^{-1}$  G418 for 2 weeks. G418-resistant colonies were stained for 10 min with 1% methylene blue in 70% ethanol, washed by distilled water, and air-dried. Only colonies >0.3 mm in diameter were counted by the Tanon Colon Counter system (Tanon Technology, Shanghai, China).

### Detection of genomic integration at pseudo *attP* sites

HeLa cells with 50%–80% confluency in 60-mm plates were co-transfected with 50 ng pEGFP-attB-N1 and 250 or 500 ng pCMV-Int or 200 ng pCDNA3.1/Zeo (+) using Lipofectamine 2000. Cells were harvested for DNA extraction 72 h after transfection. Integration at the pseudo *attP* sites in human genome was identified by PCR using primers attBRDF and attBRDR (Table 1) with the following conditions: 94°C for 5 min; 31 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 45 s; and final extension at 72°C for 8 min. If integration was inserted at pseudo *attP* sites, no 630 bp of amplified fragment would be achieved due to the break at the "TT" core of *attB* site. Additionally, the most prevalent pseudo *attP* site ( $\psi$ A) in the chromosome 8 (Thyagarajan *et al.*, 2001) was also identified by nested PCR using primers attB8A1F and attBA1R1 from the vector sequence, and attBA1R2 (Table 1) from human genome. PCR was conducted as follows: 94°C for 5 min; 31 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 30 s; and final extension at 72°C for 8 min. A 320 bp of amplified fragment could be visualized as site-specific integration occurred at  $\psi$ A. The integration at this site was further confirmed by sequencing analysis.

### Enhanced green fluorescent protein expression assays in transfected cells

To measure enhanced green fluorescent protein (EGFP) expression, HeLa cells with 50%–80% confluency in 60-mm plates were co-transfected with 50 ng donor plasmid plus 500 ng pCMV-Int (ratio 1:10) or control plasmid pCDNA3.1/Zeo (+) (400 ng) using Lipofectamine 2000. Cells were harvested 24 h after transfection. Two-thirds of cells were analyzed by flow cytometry (BD FACS Calibur, Franklin Lakes, NJ). The remaining cells were seeded on 60-mm plates at an appropriate dilution. After 24 h, proteins were extracted from two-thirds of the left cells using a Cytosol Protein Extraction Kit (Beyotime, Haimen, China), and then stored at  $-80^{\circ}\text{C}$ . The remaining cells were again plated on 60-mm plates. The process was repeated every 2–4 days, depending on the confluency of the cells. A sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure EGFP expression in the protein extracts as described previously (Ou *et al.*, 2008).

### Statistical analysis

All experiments were performed at least twice, and all samples were tested in triplicate. Data from each group were

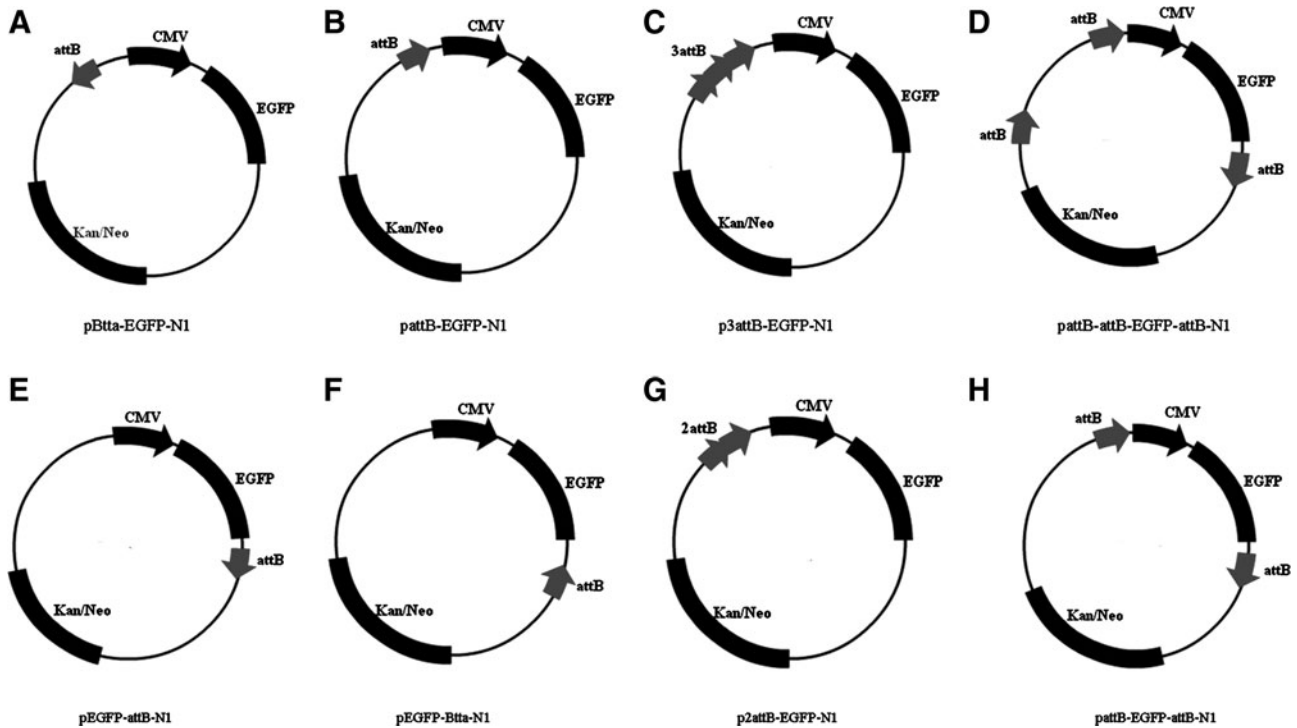


FIG. 1. Schematic diagram of eight plasmids used to investigate the relationship between *attB* sites and the efficiency of recombination in the ΦC31 integrase system. (A, B, E, and F) The four plasmids used for assessing *attB* position/orientation effects; (B, C, and G) plasmids with tandem *attB*s; (B, D, and H) plasmids with interspersed *attB*s.

expressed as mean ± standard error or raw numbers with percentages. The Student's *t*-test was used for statistical analysis.

**Results**

*Detection of integration at pseudo attP sites*

PCR results from the DNA of the HeLa cells co-transfected by donor plasmid (pEGFP-attB-N1) and helper plasmid (pCMV-Int) showed that efficiency of integration mediated by integrase accounted for 70% ± 7.1% of total integration events.

A prevalent integration site ( $\psi$ A) in the human chromosome 8 was analyzed by nested PCR. About 320 bp of amplified fragment was present, indicating that the site-specific recombination occurred at this site. The sequencing result of the region containing the genome-vector junction confirmed site-specific integration at the  $\psi$ A site. However, the recombination events were slightly imprecise, accompanied by small deletion (Fig. 2). In addition, cells that were transfected with 50 ng pEGFP-attB-N1 and 500 ng pCMV-Int showed a greater amount of PCR product than cells transfected with 50 ng pEGFP-attB-N1 and 250 ng pCMV-Int (Fig. 2A), which may indicate a higher

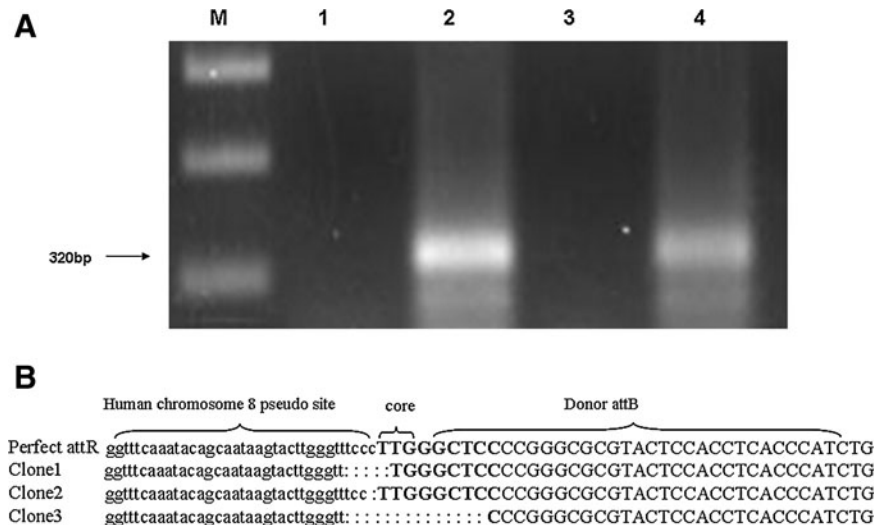
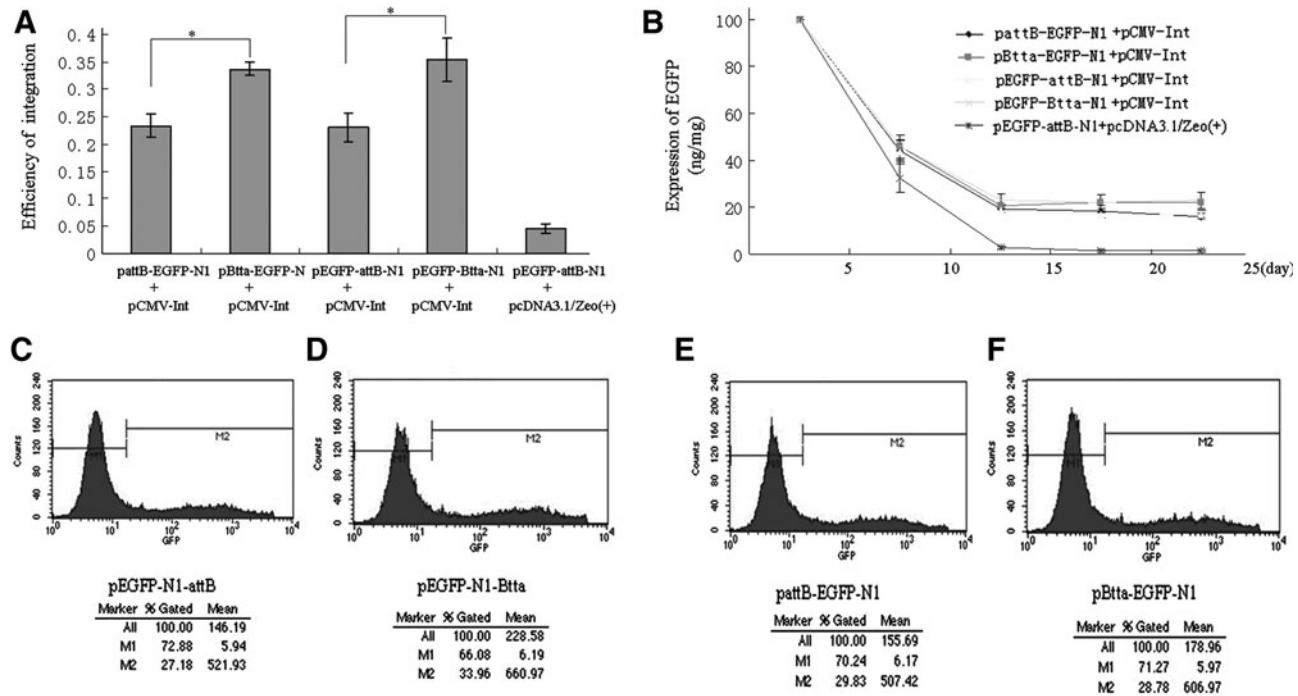


FIG. 2. Analysis of integration at the  $\psi$ A site in the human chromosome 8 by nested polymerase chain reaction (PCR). (A) PCR analysis for site-specific integration at the  $\psi$ A site. Lane M: 1 kb marker; lane 1: 50 ng pEGFP-N1 and 400 ng pCDNA3.1/Zeo (+); lane 2: 50 ng pEGFP-attB-N1 and 500 ng pCMV-Int; lane 3: 50 ng pEGFP-attB-N1 and 200 ng pCDNA3.1/Zeo (+); lane 4: 50 ng pEGFP-attB-N1 and 250 ng pCMV-Int. (B) Sequencing of PCR fragments reveals imprecise recombination between pseudo *attP* and *attB*, with a loss of 1–13 bp.



**FIG. 3.** Effects of *attB* position and orientation on integration efficiency and enhanced green fluorescent protein (EGFP) expression. **(A)** Integration efficiency for the indicated plasmids. \*Significant difference at  $p < 0.05$ . **(B)** EGFP expression measured by enzyme-linked immunosorbent assay after transfecting the indicated plasmids into cells. **(C–F)** Time course of EGFP expression measured by flow cytometry 24 h after transfection with the indicated plasmids.

efficiency of site-specific integration with greater amounts of integrase-encoded plasmid transfected into cells.

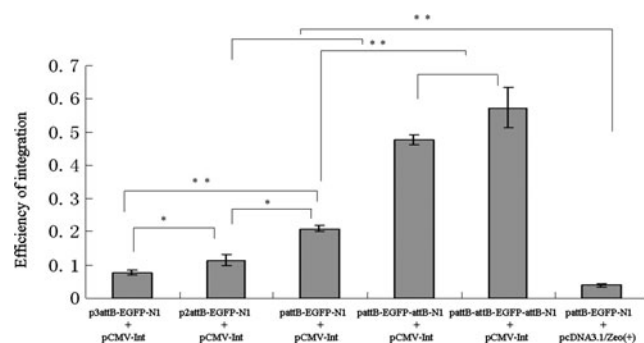
#### The orientation of *attB*, but not position, affects integration efficiency and EGFP expression

To evaluate whether position or orientation of the *attB* sites in donor plasmids influences the efficiency of integration as well as the expression of transgenes, four plasmids bearing different types of *attB* and EGFP reporter gene were constructed (Fig. 1A, B, E, F), followed by transfection into HeLa cells. After G418 selection, the resistant cell colonies were counted for calculating chromosomal integration rate and EGFP expression was detected by fluorescence-activated cell sorter (FACS) and ELISA analysis. The results showed that integration efficiency was not affected by the position of *attB* in relation to EGFP, while influenced by the orientation of *attB* (Fig. 3A). The number of colonies from cells transfected with reverse *attB* was 1.5-fold higher than that from cells transfected with forward *attB* (Fig. 3A;  $p < 0.05$ ). Additionally, four donor plasmids were, respectively, co-transfected into HeLa cells with pCMV-Int. FACS analysis demonstrated that cells receiving the reverse-oriented *attB* had stronger transient expression of EGFP than those with forward *attB* (Fig. 3C–F). ELISA results showed a stability of EGFP expression over 4-week time course, compared with its rapid extinction in the control group (Fig. 3B), suggesting that EGFP may integrate in the human genome. EGFP expression was comparable no matter whether the *attB* site was upstream or downstream of EGFP. However, the reverse-oriented *attB* site resulted in a 1.4-fold higher expression compared with the forward *attB* (Fig. 3B,  $p < 0.05$ ). Moreover, transient or stable expression of

EGFP determined by FACS or ELISA showed that the direction rather than position of *attB* significantly influenced the expression efficiency of the target gene.

#### Tandem *attBs* decrease site-specific integration efficiency in HeLa cells

In the Cre system, multiple LoxP sites can increase the frequency of recombination events compared with a single allelic LoxP site (Liu *et al.*, 2002). In the present study,



**FIG. 4.** Effects of multiple tandem or interspersed *attBs* on integration efficiency. HeLa cells were transfected with the plasmid combinations indicated. After 2 weeks of selection, the number of G418-resistant colonies stained with methylene blue was used to calculate the integration efficiency. The data are indicated as mean  $\pm$  standard error from three independent experiments. Significant difference at \* $p < 0.05$  and \*\* $p < 0.01$ .

plasmids containing two or three tandem *attBs* were investigated to determine their effect on integration efficiency in HeLa cells. Surprisingly, adjacent duplicate or triplicate *attBs* (Fig. 1C, G) decreased the efficiency of site-specific recombination (Fig. 4). Integration assay showed that plasmids containing one *attB* produced higher frequency of integration than those bearing two or three *attBs* in tandem (1.8- vs. 2.7-fold, respectively,  $p < 0.05$ ).

#### *Interspersed attBs increase site-specific integration efficiency*

In addition to donor plasmids bearing *attBs* in tandem, vectors containing two or three interspersed *attBs* were also constructed for evaluating their influence on integration efficiency (Fig. 1D, H). The results showed that these plasmids had 2.2- and 2.8-fold increase in integration efficiency, respectively, as compared with the plasmid bearing one *attB* ( $p < 0.01$ , Fig. 4). The integration efficiency of all experimental groups (tandem *attBs* and interspersed *attBs*) was significantly higher than that of the pcDNA3.1/Zeo (+) control group ( $p < 0.01$ , Fig. 4).

#### Discussion

Although the efficacy and specificity shown by the wild-type  $\Phi$ C31 integrase system appears successful in gene therapy (Calos, 2006), additional improvements to some parameters regarding this system are likely to make it even more powerful for gene delivery. The current study focused on the investigation of the effect of the orientation, position, and number of *attB* on the integration efficiency and expression of the integrated gene (reporter *EGFP*). The results demonstrated that the donor plasmids we constructed could specifically integrate into pseudo *attP* sites of human genome and accounted for  $70\% \pm 7.1\%$  of total integration events. Particularly, the reverse *attB* significantly enhanced the integration efficiency as well as the expression of reporter *EGFP*. The mechanism of difference in directions of *attBs* of the donor plasmids is unknown. A previous study assumed that a topological difference in a DNA sequence can affect the expression of a target gene (Li and Emery, 2008). In another experiment, we found that site-specific recombination mediated by integrase was undertaken efficiently between pseudo *attP* and reverse *attB* (data not shown). These observations made us speculate that, compared with forward *attB*, reverse *attB* might form more stable synapsis with *attP* mediated by integrase, which might account for the topological difference in integration efficiency. Watanabe *et al.* (2010) found that the reverse *attB* in donor plasmid could increase the expression of reporter luciferase in cells, suggesting that side effect may account for the topological difference. In spite of these probable explanations, the mechanism by which reverse *attB* improved the gene expression needs to be further investigated.

In this study, the number of *attB* in donor plasmids was also evaluated the effect on integration efficiency. As expected, interspersed *attBs* significantly improved the efficiency of integration (Fig. 4). It was possibly due to an increased opportunity for the binding of integrase with the independent *attB* sites within one plasmid. Effective use of this strategy would require further optimization of the distance between *attB* sites. However, tandem *attBs* significantly

inhibited integrase-mediated integration in HeLa cells. The precise mechanism for this inhibition is unclear.  $\Phi$ C31 integrase interacts with intracellular proteins, such as TTRAP (TRAF and TNF receptor-associated protein) and DAXX (death-associated protein 6), in eukaryotic cells (Chen *et al.*, 2006; Wang *et al.*, 2010). We suspected that the interaction between some intracellular proteins and integrase might block the formation of functional synapses between tandem *attB* sites and integrase, which may inhibit the efficiency of integration.

In summary, the efficacy of integration and expression of a target gene mediated by integrase may be effectively improved by adjusting the orientation, position, and number of *attB* in the donor plasmids. The donor plasmids bearing one reverse-oriented *attB* or interspersed rather than tandem *attBs* promoted the efficiency of site-specific integration. We expect that the improvements of *attB* sites described in this study would provide valuable information as well as another potential tool for transgenesis application and therapeutic gene transfer by using  $\Phi$ C31 integrase system.

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#### Disclosure Statement

The authors declare that there are no conflicts of interest.

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