FULL LENGTH ARTICLE

# Sustained high level transgene expression in mammalian cells mediated by the optimized *piggyBac* transposon system

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**Abstract** Sustained, high level transgene expression in mammalian cells is desired in many cases for studying gene functions. Traditionally, stable transgene expression has been accomplished by using retroviral or lentiviral vectors. However, such viral vector-mediated transgene expression is often at low levels and can be reduced over time due to low copy numbers and/or chromatin remodeling repression. The *piggyBac* transposon has emerged as a promising non-viral vector system for efficient gene transfer into mammalian cells. Despite its inherent advantages over lentiviral and retroviral systems, *piggyBac* system has not been widely used, at least in part due to their limited manipulation flexibilities. Here, we seek to optimize *pig-gyBac*-mediated transgene expression and generate a more efficient, user-friendly *piggyBac* system. By engineering a panel of versatile *piggyBac* vectors and constructing recombinant

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adenoviruses expressing *piggyBac* transposase (PBase), we demonstrate that adenovirus-mediated PBase expression significantly enhances the integration efficiency and expression level of transgenes in mesenchymal stem cells and osteosarcoma cells, compared to that obtained from co-transfection of the CMV-PBase plasmid. We further determine the drug selection timeline to achieve optimal stable transgene expression. Moreover, we demonstrate that the transgene copy number of *piggyBac*-mediated integration is approximately 10 times higher than that mediated by retroviral vectors. Using the engineered tandem expression vector, we show that three transgenes can be simultaneously expressed in a single vector with high efficiency. Thus, these results strongly suggest that the optimized *piggyBac* system is a valuable tool for making stable cell lines with sustained, high transgene expression.

## Introduction

Sustained and high level transgene expression may be desired for studying the molecular and cellular functions of a gene of interest, both *in vitro* and *in vivo*. However, it is well known that stable transgene expression in stem cells or progenitor cells is significantly reduced over time, if not silenced.[1,2] For example, in embryonic stem cells (ESCs), control of the gene expression program that establishes and maintains the ESC state is dependent on a small number of master transcription factors, as most of the chromatin is in a repressive state.[3–7] Conventional stable transgene expression approaches usually employ a retroviral or lentiviral vector to generate stable integration in stem cells. However, transgene expression is often low or reduced over time.[1,2] This phenomenon may be caused by either single or low copy numbers of transgenes integrated into the host genome, and/or epigenetic modifications of the constitutive promoters (either viral promoters or non-viral housekeeping gene promoters).[3–5,8,9]

The *piggyBac* transposon has emerged as one of the most promising non-viral vector systems for efficient gene transfer into mammalian cells.[10–15] Transposons are mobile genetic elements that can be used to integrate transgenes into host cell genomes. The *piggyBac* transposon was originally isolated from the cabbage looper moth, Trichoplusiani, and has been recognized as one of the most efficient DNA transposons for manipulating mammalian genomes.[10,16–18] The *piggyBac* transposon system has two major components, a donor plasmid (or transfer vector), carrying the gene of interest flanked by two terminal repeat domains, and a helper plasmid, expressing *piggyBac* transposase (PBase) that catalyzes the movement of the transposon. Although the *piggyBac* transposon has several distinct advantages over the lentiviral and/or retroviral systems, such as large cargo size, multiple copy integration, and leaving no footprint,[10,11] the use of this system has been limited. One factor that may hamper the widespread use of the *piggBac* system is the limited availability of *piggyBac* transfer vectors with high manipulation flexibilities.

In this study, we seek to optimize the *piggyBac*-mediated efficient transgene expression and attempt to generate a more efficient and user-friendly *piggyBac* system. To accomplish this objective, we first engineer a panel of versatile *piggyBac* vectors with different promoters, drug selection markers, and tandem expression cassettes. We further construct recombinant adenoviruses expressing the PBase. Using mouse mesenchymal stem cells (iMEFs) and a human osteosarcoma line (143B), we demonstrate that adenovirus-mediated PBase expression significantly enhances the integration efficiency and expression level of transgenes both *in vitro* and *in vivo*, compared with that obtained from the co-transfection of a PBase expression plasmid. We also determine the drug selection timeline needed to achieve optimal stable transgene expression. Furthermore, we demonstrate that the transgene copy number of *piggyBac*-mediated integration is approximately 10 times higher than that mediated by retroviral vectors. Using the engineered tandem expression vector system, we show that three transgenes can be simultaneously expressed in a single vector with high efficiency. Therefore, our results demonstrate that the optimized *piggyBac* transposon system should be a valuable tool for making stable cell lines with sustained and high transgene expression.

## Materials and methods

### Cell culture and chemicals

HEK-293 and 143B cells were obtained from ATCC (Manassas, VA). iMEFs are mouse embryonic fibroblasts that have been reversibly immortalized as previously described.[19,20] A recently engineered, highly efficient adenovirus packaging and production line 293pTP was used for adenovirus generation and/or amplification.[21] These cell lines were maintained in complete Dulbecco's Modified Eagle Medium (DMEM).[22–26] Unless indicated otherwise, all chemicals were purchased from Sigma—Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA).

### Construction of the versatile *piggyBac* transposon system and establishment of stable cell lines

The parental *piggyBac* vector was purchased from System Biosciences Inc. (Mountain View, CA). The essential components of the *piggyBac* transfer vector, including the *piggyBac* terminal repeats (PB-TRs) and core insulators (CIs),

were subcloned into a spectinomycin resistance-conferring plasmid vector, which contains a large linker with multiple restriction sites. The MPB vector was constructed by sub-cloning the blasticidin S selection marker (BSD) cassette and the constitutive human elongation factor $\alpha$ and HIV enhancer hybrid promoter (hEFH)-driven gene expression cassette. MPB2, 3, and 4 vectors were constructed by cloning 1, 2 or 3 copies of hEFH-SV40Pa cassettes into the MPB vector (Fig. 1A, panel *a*). The MPH vector was con-structed by replacing the BSD selection marker with a

hygromycin cassette (Fig. 1A, panel *b*). PBC series vectors were constructed by replacing the hEFH with the CMV promoter in MPB vectors (Fig. 1A, panel *c*). Similarly, the PNC series vectors were obtained by replacing the BSD se-lection marker with geneticin/G418 marker in the PBC vectors (Fig. 1A, panel *d* ). Representative vector maps are shown in Supplemental Fig. 1. Detailed vector sequence information is available upon request.

For making the MPB-FLuc and MPB-mRFP constructs, the coding regions of firefly luciferase and mRFP were PCR
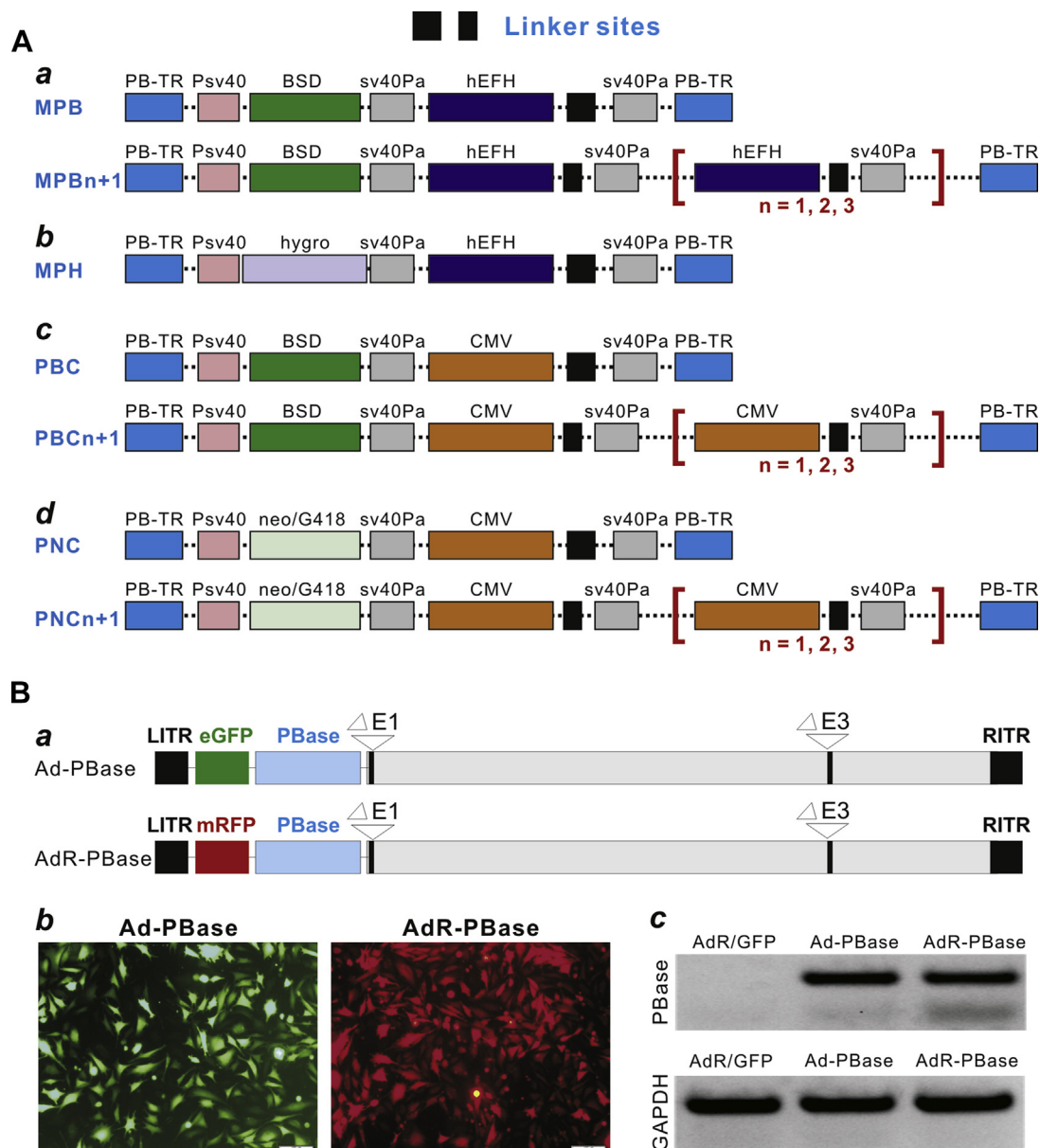


**Fig. 1** Schematic representation of the modified *piggyBac* vectors and the transposase (PBase) expressing adenoviruses. (A) Various *piggyBac* transfer vectors containing different promoters and/or antibiotic selection markers; including hEFH promoter and Blasticidin S marker (BSD) (*a*), hEFH promoter and Hygromycin B marker (hygro) (*b*), CMV promoter and Blasticidin S marker (*c*), and CMV promoter and Geneticin/G418 marker (neo/G418) (*d* ). Representative vector maps are shown in Supplemental Fig. 1. PB, *piggyBac*; PB-TR, *piggyBac* terminal repeats. (B) the generation of recombinant adenoviruses expressing *piggyBac* transposase (PBase). (*a*) The schematic representation of adenoviruses that express PBase, which also co-express eGFP (Ad-PBase) or mRFP (AdR-PBase). (*b*) The adenoviral vectors are shown to transduce iMEF cells effectively. (*c*) Adenovirus-mediated expression of PBase. Subconfluent iMEFs were infected with Ad-PBase, AdR-PBase, or AdR/GFP. Total RNA was isolated at 48 h after infection and subjected to semi-quantitative PCR using primers specific for PBase. The samples were normalized with GAPDH expression level.

amplified and subcloned into MPB vector. For making the MPB-KMR vector, the coding regions of human oncogenic KRAS-G12D, human MDM2, and dominant-negative mouse Runx2 were PCR amplified and subcloned sequentially into the linker sites of the MPB4 vector, resulting in MPB-KMR. These vectors were used to generate stable cell lines by co-transfection with Lipofectamine (Invitrogen, Carlsbad, CA) or transfection/infection with PBase, followed by blastici-din S selection for 5 days. The empty vector was used as a negative control (i.e., iMEF-MPB). All PCR amplified fragments were verified by DNA sequencing. Detailed information regarding vector constructs is available upon request.

## Recombinant adenoviruses expressing *piggyBac* transposase (PBase) and red/green fluorescent proteins (R/GFP)

Recombinant adenoviruses were generated using the AdEasy technology.[23,27−29] Briefly, the coding region of *piggyBac* transposase was PCR amplified and subcloned into the adenoviral shuttle vector pAdTrack-TOX (co-expressing eGFP) or pAdTrace-TOX (co-expressing mRFP), and subsequently used to generate recombinant adenoviruses in HEK-293 or 293pTP cells,[21] resulting in adenoviruses Ad-PBase and AdR-PBase. An analogous control adenovirus AdR/GFP expressing both eGFP and monomeric RFP (mRFP) was used as a control.[30−37] All adenovirus infections were added with 4−8 μg/ml polybrene to increase infection efficiency as recently reported.[38] All PCR amplified fragments were verified by DNA sequencing. Again, detailed information regarding vector constructs is available upon request.

## RNA isolation and semi-quantitative RT-PCR (sqPCR) analysis

Total RNA was isolated using TRIZOL Reagent (Invitrogen) and subjected to reverse transcription reaction with hex-amer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were diluted 10- to 100-fold and used as PCR templates. Semi-quantitative PCR (sqPCR) was carried out as described.[22,26,31,35,39−45] Gene-specific PCR primers were designed by using the *Primer3 Plus* program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify the genes of interest (approximately 150−250 bp). A touchdown PCR program was carried out as follows: 94 °C for 2 min for 1 cycle; 92 °C for 20 s, 68 °C for 30 s, and 72 °C for 12 cycles with a decrease in 1 °C per cycle; and then at 92 °C for 20 s, 57 °C for 30 s, and 72 °C for 20 s for 20−25 cycles, depending on transcript abundancy. The PCR products were confirmed by resolving PCR products on 1.5% agarose gels. All samples were normalized to GAPDH expression levels.

## Genomic DNA isolation and sqPCR analysis of blasticidin gene copy numbers

Stable blasticidin-resistant iMEF lines were established by using the *piggyBac* transposon system and a retroviral vector system, respectively. For generating the retrovirus-mediated stable line, the packaged retroviruses were used to transduce iMEF cells with multiple rounds of infection as previously described.[19,34,42,46−48] Confluent cells in 25 cm$^2$ cell culture flasks were collected and lysed in an alkaline lysis buffer (0.2 M NaOH/1 mM EDTA) at 85 °C for 20 min, followed by phenol-chloroform extraction and ethanol precipitation. The retrieved genomic DNA was diluted and subjected to sqPCR analysis using primers specific for the blasticidin S gene. The sqPCR cycling program was similar to that described above for sqRT-PCR. Primers specific to the mouse Hey1 gene promoter were used to normalize the genomic DNA levels among the samples as previously described.[26,37,49]

## Establishment of stable lines expressing firefly luciferase and firefly luciferase activity assay

To generate stable iMEFs or 143B cells, MPB-FLuc vector was co-transfected with the *piggyBac* transposase expression vector pCMV-PBase or transfected/infected with Ad-PBase into iMEF cells as reported.[20,34,50] Stable cells were selected in the presence of Blasticidin S. For firefly luciferase reporter assay, subconfluent stable cells were lysed at 36 h after plating, and then collected to measure luciferase activity using the Luciferase Assay Kit (Promega, Madison, WI) as described.[51−54] Each assay condition was performed in triplicate.

## Cell viability/proliferation assay (Crystal violet assay)

Subconfluent cells were co-transfected or transfected/infected with MPB-mRFP and pCMV-PBase or Ad-PBase. After blasticidin selection at the indicated time points, the viable cells were subjected to Crystal violet staining as previously reported.[30,55]

## Intramuscular injection of human osteosarcoma cells stably expressing firefly luciferase in nude mice and Xenogen bioluminescence imaging

All animal work was conducted according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC). Intramuscular injection of tumor cells was done as described.[22,30,33,37,55] Briefly, human osteosarcoma 143B cells stably expressing firefly luciferase were collected and resuspended in PBS for intramuscular injection (10$^6$ cells/injection) into the quadriceps of athymic nude (nu/nu) mice (5 per group, 4−6 week old, female, Harlan Laboratories, Indianapolis, IN). At two weeks post injection, animal were anesthetized with isoflurane attached to a nosecone mask within Xenogen IVIS 200 imaging system. For bioluminescence imaging, animals were injected (i.p.) with D-Luciferin sodium salt (Gold BioTechnology) at 100 mg/kg in 0.1 ml sterile saline. Pseudoimages were obtained by superimposing the emitted light over the gray-scale photographs of the animals. Quantitative analysis was done by using Xenogen's Living Image software as described.[37,56,57]

## Statistical analysis

All quantitative experiments were performed in triplicate, or repeated three times. Data were expressed as mean $\pm$ SD. Statistical significance was determined by one-way analysis of variance and the student's $t$ test. A value of $p < 0.05$ was considered statistically significant.

## Results and discussion

### Construction of a panel of user-friendly and versatile *piggyBac* transposon vectors

One of the factors that may hamper the widespread use of the *piggBac* system is the limited availability of *piggyBac* transfer vectors with diverse cloning flexibilities. In this study, we sought to optimize *piggyBac*-mediated efficient transgene expression. A typical *piggyBac* transposon system consists of two essential components, the *piggyBac* transfer vector and a vector to express *piggyBac* transposase (PBase). We attempted to make the current *piggyBac* system more efficient and user-friendly by creating a panel of versatile *piggyBac* vectors with different promoters, drug selection markers, and tandem expression cassettes, and by introducing *piggyBac* transposase using adenoviral vectors with high transduction efficiency.

To achieve these goals, we first subcloned the essential components of the *piggyBac* transfer vector, including the *piggyBac* terminal repeats (PB-TRs) and core insulators (CIs), into an intermediate spectinomycin resistance-conferring plasmid vector, which contains a large linker with multiple restriction sites. The MPB vector was constructed by subcloning the blasticidin S selection maker (BSD) cassette and the constitutive promoter hEFH-driven gene expression cassette. MPB2, 3, and 4 vectors were constructed by cloning 1, 2 or 3 copies of hEFH-SV40Pa cassettes into the MPB vector (Fig. 1A, panel *a*). MPH vector was constructed by replacing BSD selection marker with hygromycin cassette (Fig. 1A, panel *b*). PBC series vectors were constructed by replacing the hEFH with the CMV promoter in MPB vectors (Fig. 1A, panel *c*). Similarly, the PNC series vectors were obtained by replacing the BSD selection marker with geneticin/G418 marker in the PBC vectors (Fig. 1A, panel *d* ). Our recent studies demonstrate that while the CMV promoter drives the strongest gene expression in HEK-293 cells and certain cancer cells, the composite hEFH promoter provides more sustained high level of transgene expression in progenitor cells, such as iMEFs.[9] Taken together, these transfer vectors provide a large of range of selections, which should facilitate the use of *piggyBac* transposon system for stable gene expression (Supplemental Fig. 1).

The traditional *piggyBac* system requires the co-transfection of the transfer vector and the PBase expression vector. However, co-transfection of two or more plasmids usually decreases gene transfer efficiency. Thus, we opted to take advantage of adenovirus-mediated efficient transgene expression[58–60] and generate recombinant adenoviruses that express PBase, namely Ad-PBase and AdR-PBase (Fig. 1B, panel *a*). The generated adenoviruses were shown to transduce mouse mesenchymal stem cells, iMEFs, with high efficiency (Fig. 1B, panel *b*). Semi-quantitative RT-PCR analysis indicates that both Ad-PBase and AdR-PBase mediate a high level of expression of PBase in the infected cells (Fig. 1B, panel *c*). Thus, the use of adenovirus-mediated PBase expression should significantly facilitate the efficient delivery of *piggyBac* transposase into target cells.
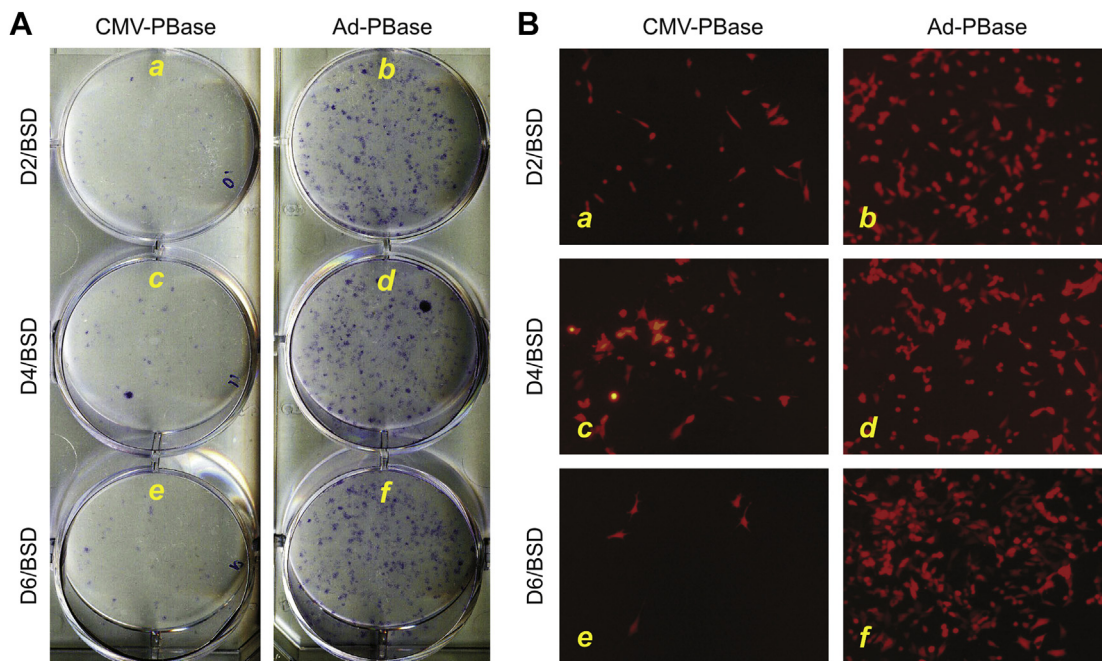
### Adenovirus-mediated PBase expression significantly increases the efficiency of stable gene expression

We next compared the efficiency of *piggyBac* transposon-mediated integration by co-transfection of pCMV-PBase and by infection with Ad-PBase. As the expression of the transposase and ensuing transposase-mediated integration may require a certain amount of time, it is conceivable that non-transposon-mediated or random integration may occur if drug selection (i.e., BSD in this case) starts too early. Thus, we started drug selection at different time points, and found that co-transfection of pCMV-PBase yielded significantly lower numbers of stable clones than that by Ad-PBase-mediated transduction (Fig. 2A, a, c, e, *vs*. b, d, f). The drug selection commenced at 2, 4 and 6 days after transfection/infection seemed to yield slightly increased numbers of stable clones than that at 1 day after transfection (data not shown), although no apparent increase in colony numbers when drug selection was started at 6 days after transfection/infection (Fig. 2A). This suggested that the PBase-mediated integration reaches a peak between 2 and 4 days after transfection. Similar results were obtained when MPB-mRFP was tested in iMEF cells (Fig. 2B). Thus, these results strongly suggest that adenovirus-mediated delivery of PBase may significantly facilitate *piggyBac* transposon-mediated transgene integrations.

### *piggyBac*-mediated stable integration is significantly more efficient than that mediated by a retroviral vector

By generating stable lines expressing firefly luciferase in iMEFs, we quantitatively analyzed the efficiency and level of stable gene expression affected by the different approaches of PBase delivery, as well as the timing of drug selection. Using the same amount of *piggyBac* transfer vector for transfection of the iMEFs, we found that adenovirus-mediated expression of PBase constantly yielded significantly higher levels of luciferase activity than that by co-transfection with pCMV-PBase (*p < 0.001*) (Fig. 3A). Furthermore, adding selection drug at day 4 or 6 led to slightly higher gene expression, compared with that at day 2, especially in the Ad-PBase infection groups (*p < 0.05*). These results suggest that, while adenovirus-mediated PBase expression is superior to the co-transfection of CMV-PBase plasmid, a slightly delayed drug selection may increase the efficiency of transposon-mediated stable transgene integration.

We next compared the efficiency of transgene integration mediated by *piggyBac* transposon *vs*. retroviral vector. To determine the relative integration efficiency, we isolated the genomic DNA from blasticidin-resistant stable iMEF cells lines generated by co-transfection, transfection/infection, and retroviral infection. Semi-quantitative PCR was conducted to determine the relative levels of the

**Fig. 2** Adenovirus-mediated expression of PBase significantly increases the efficiency of stable gene expression. (A) Comparison of stable clone formation between CMV-PBase co-transfection and Ad-PBase infection. Subconfluent iMEFs were plated in 12-well plates, and either transfected with MPB vector and CMV-PBase (co-transfection) or infected with Ad-PBase (transfection/infection). Blasticidin selection was initiated at 2, 4, and 6 days after transfection. The colonies were stained with Crystal violet. (B). Subconfluent iMEFs were plated in 12-well plates and transfected with MPB-RFP vector and CMV-PBase or infected with Ad-PBase. Blasticidin selection was initiated at 2, 4, and 6 days after transfection. Representative results are shown.

blasticidin drug marker in the analyzed genomic DNA samples. We found that Ad-PBase transduction led to the highest level of blasticidin marker integration, followed by the CMV-PBase co-transfection, while retroviral infection yielded the lowest (Fig. 3B, panel *a*). Quantitative analysis indicated that the relative integration copy number for Ad-PBase mediated transposition is 2.8 times of that by CMV-PBase co-transfection, and 10.4 times of that mediated by retroviral infection ($p < 0.001$) (Fig. 3B, panel *b*). These results provide further evidence supporting that *piggyBac* transposon-mediated stable transgene expression is superior to that of retroviral vectors.

**Adenovirus-mediated expression of *piggyBac* transposase significantly enhances transposon-mediated stable transgene expression *in vivo***

The above *in vitro* assays indicate that the delivery efficiency of PBase into target cells may determine the efficacy and stable expression level of the transgene of interest. We further tested if this phenomenon held true *in vivo*. We established stable human osteosarcoma 143B cells by transfecting with MPB-FLuc, along with CMV-PBase co-transfection or Ad-PBase infection. The resultant stable 143B-FLuc lines were injected intramuscularly into the quadriceps of athymic nude mice. When the injected mice were subjected to whole body Xenogen bioluminescence imaging, we found that the Ad-PBase mediated transposition yielded a significantly higher level of luciferase activity than that mediated by CMV-PBase co-transfection *in vivo* (Fig. 4A). Quantitative analysis showed that the

relative luciferase activity in the Ad-PBase-mediated transposition group is 15.9 times of that in the CMV-PBase co-transfection group ($p < 0.001$) (Fig. 4B). It is noteworthy that similar results were obtained from the experiments in several other cell lines, including iMEFs and human osteosarcoma line MG63 (data not shown). Taken togther, these *in vivo* results further confirm that adenovirus-mediated expression of transposase significantly enhances the efficacy of stable transgene expression.

**Multiple transgenes can be efficiently and stably expressed by using a single *piggyBac* transposon vector**

One of the major advantages of the *piggyBac* transposon system over retroviral or lentiviral vectors is the *piggyBac* transposon's capability of accommodating large cargo sizes. It is conceivable that multiple transgene expression cassettes can be integrated into target cells using a single transposon vector. Using our modified *piggyBac* vector MPB4, we sequentially subcloned three genes, including human KRAS-G12D, human MDM2, and a dominant-negative mutant of mouse Runx2, into the linker sites of this vector, resulting in MPB-KMR (Fig. 5A).

It is conceivable that the amount of *piggyBac* transfer vector used in transfection may affect the integration copy numbers and hence the transgene expression levels. To test this possibility, we transfected varied amounts of MPB-KMR vector into the iMEF cells, which were infected with the same titer of Ad-PBase, and subsequently selected with blasticidin S. The empty vector was used as a negative control
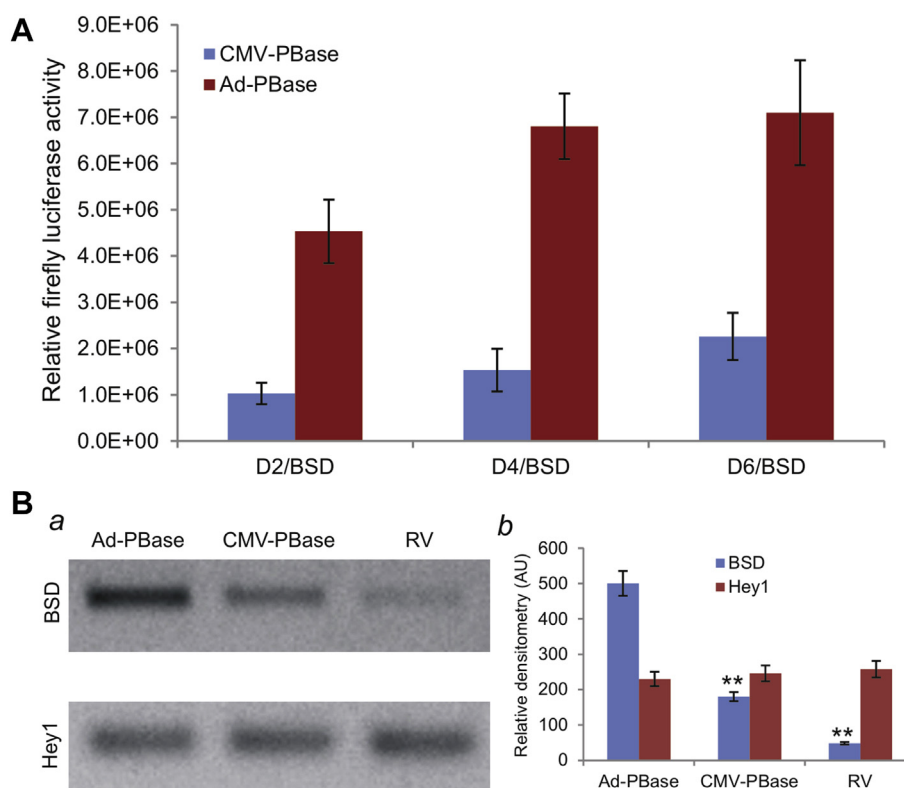
**Fig. 3**  *piggyBac*-mediated stable integration is significantly more efficient than that mediated by retroviral vector. (A) Quantitative analysis of luciferase activity mediated by *piggyBac* vectors. Subconfluent iMEFs were plated in 12-well plates, and either transfected with MPB-FLuc vector and CMV-PBase or infected with Ad-PBase. Blasticidin selection was initiated at 2, 4, and 6 days after transfection. The stable cells were replated in 24-well plates for 24 h and lysed for firefly luciferase assay using the firefly Luciferase Assay kit (Promega). Each assay condition was done in triplicate. (B) Comparison of *piggyBac* and retroviral vector-mediated gene integration. Genomic DNA was isolated from Blasticidin-resistant stable iMEF lines generated by co-transfection, transfection/infection, and retroviral infection (multiple rounds). The isolated genomic DNA was subjected to semi-quantitative touchdown PCR using the blasticidin-specific primers. All samples were normalized with mouse Hey1 primers specific for genomic DNA (*a*). The gel band intensity was quantitatively determined (*b*). "**", $p < 0.001$ for the relative BSD genomic copy number in the Ad-PBase group vs. the CMV-PBase or RV group.

(i.e., iMEF-MPB). Total RNA was isolated from exponentially growing iMEF-KMR or iMEF-MPB cells, and subjected to semi-quantitative RT-PCR analysis using gene-specific primers. We found that there was a trend of elevated transgene expression with the increasing amounts of MPB-KMR vector used, peaking at 1.0 μg per well of the 6-well plates (Fig. 5B). Furthermore, we found that all three transgenes were expressed at a high level, compared with that in the control cell line (Fig. 5B). Thus, our results strongly suggest that the modified *piggyBac* vectors may provide a useful platform for efficient multiple transgene expression, which has been significantly enhanced by the adenovirus-mediated transduction of the *piggyBac* transposase.

## The *piggyBac* transposon system offers significant advantages over the retroviral and lentiviral systems in establishing a sustained high level of transgene expression

We have plenty of experiences using retroviruses and have previously used the retroviral vector-mediated expression of SV40 T antigen to immortalize several sources of progenitor cells, including MEFs.[19,42,44,46,47,52] However, the immortalization efficiency was relatively low because of the

low retrovirus titers associated with the large cargo size for packaging.

The *piggyBac* transposon system should have several distinct advantages over the retroviral system.[10,11] First, *piggyBac* vector can deliver large cargo sizes, up to 100 kb of DNA fragments, into mammalian cells.[10] Second, unlike retroviral infection, liposome-based transfection is more efficient than retroviral vector-mediated infection *in vitro* and *piggyBac* vectors can be delivered into cells with multiple copies so it is easy to achieve high levels of transgene expression. Third, *piggyBac* exhibits non-random AT-rich integration site selectivity and has a higher preference for integrations in regions surrounding transcription start sites.[18] Lastly, it is conceivable that *piggyBac* transposon can be removed from the host genome by its transposase and thus leaves no footprint. The excision-only/dominant forms of mutant *piggyBac* transposase have recently been reported.[61,62].

## Technical considerations for using the optimized *piggyBac* transposon system

In this study, we demonstrate that adenovirus-mediated expression of *piggyBac* transposase significantly enhances the efficiency of the transposon-mediated transgene
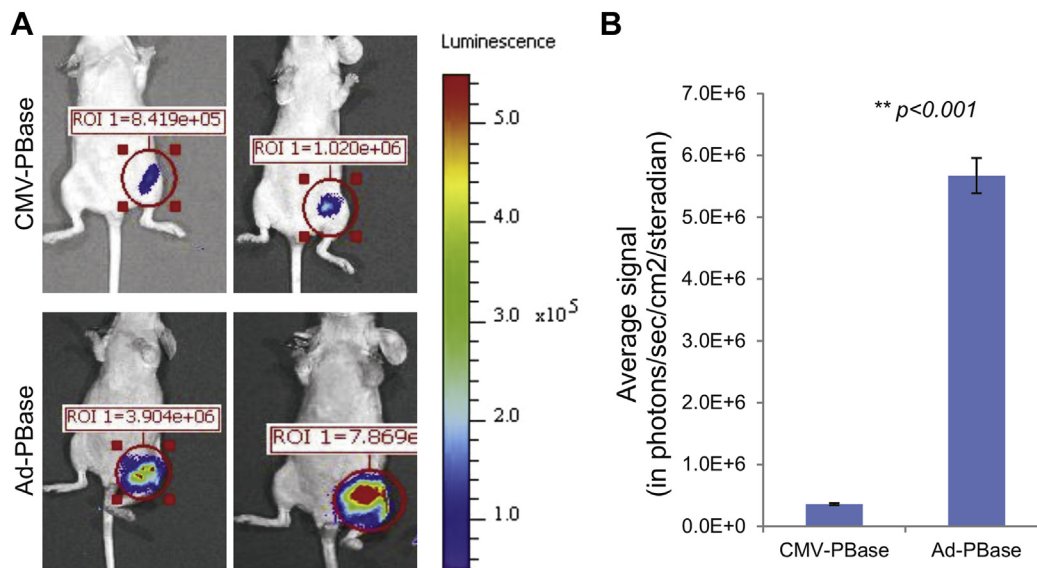
**Fig. 4** Adenovirus-mediated expression of transposase significantly enhances stable transgene expression *in vivo*. Human osteosarcoma 143B cells stably expressing firefly luciferase were established by either co-transfecting pCMV-PBase or infecting with Ad-PBase. Approximately 10e6 exponentially growing cells were injected intramuscularly into the quadriceps of athymic mice nude mice (male, 4–6 week old, 5 mice/group). Whole body bioluminescence imaging was conducted by using Xenogen IVIS 200 Imaging system at 2 weeks after injection (A). The acquired signal data were quantitatively determined by using Xenogen's Living Image software (B). Representative images are shown.

integration, as well as the sustained high expression levels of transgenes. In order to achieve optimal outcomes, several technical considerations are strongly suggested. First, the Ad-PBase viral vector should be titrated in the target cells or the cells to be used for making stable lines. The ideal titers (or MOI, multiplicity of infection) may vary dramatically among cell lines; and the optimal infection efficiency is that about 50%–70% of the cells are positive for
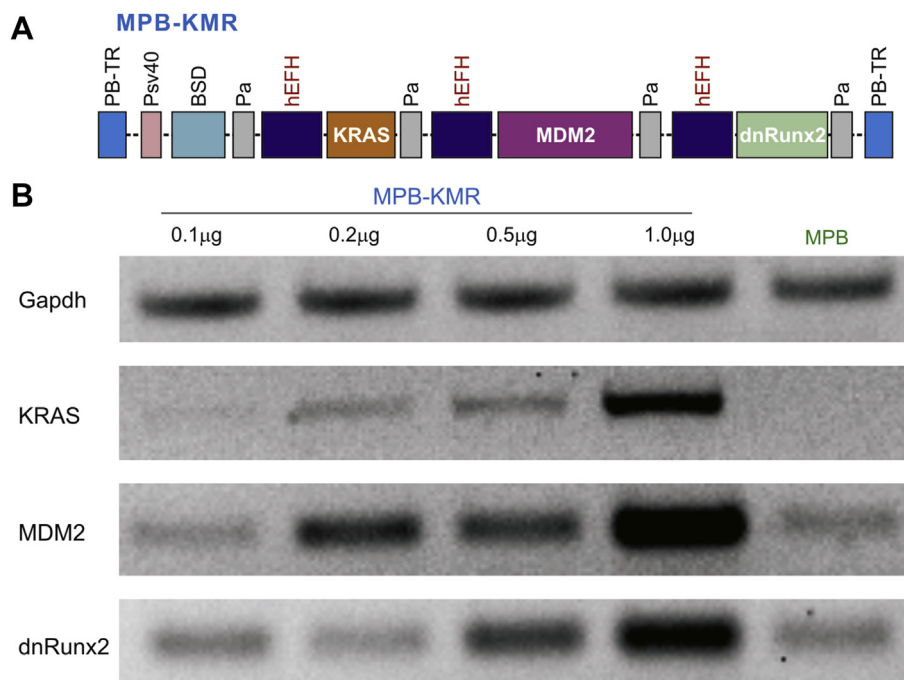


**Fig. 5** The optimized *piggyBac* transposon system effectively expresses multiple transgenes in a single vector. (A) The coding regions of human oncogenic KRAS-G12D, human MDM2, and dominant-negative mouse Runx2 were PCR amplified and subcloned into the linker sites of the MPB4 vector, resulting in MPB-KMR. (B) Stable cell line iMEF-KMR was established by transfecting with varied doses of MPB-KMR into iMEFs in 6-well culture plates and infecting with Ad-PBase, followed by blasticidin S selection. The empty vector was used as a negative control (i.e., iMEF-MPB). Total RNA was isolated from exponentially growing iMEF-KMR or iMEF-MPB cells, and subjected to semi-quantitative RT-PCR analysis using gene-specific primers. Representative results are shown.

GFP signal at 24 h post infection. Second, the amount of *piggyBac* transfer vectors used for transfection should be sufficiently high so maximal numbers of cells can be transfected. As adenoviral infection should be more efficient than transfection, it is generally assumed that most, if not all, of the transfected cells are infected by Ad-PBase. Lastly, the start points of drug selection may also affect the quality of the stable lines. As it will take time for PBase to express and carry out the transposition function, adding selection antibiotics too early (e.g., within 24 h after transfection/infection) may generate drug-resistant cells that survive the selection by forced random integration, rather than true transposition, of the *piggyBac* transfer vectors. Our results indicate that drug selection should be initiated between 2 and 4 days after transfection/infection. It's noteworthy that the *piggyBac* vectors can be further engineered in such to stably expression sgRNAs for the CRISPR genome-editing system. Taken together, the above findings demonstrate that the optimized *piggyBac* transposon system is a valuable tool for making stable cell lines with sustained and high transgene expression.

## Conflicts of interest

The authors declare no conflict of interest.

## Acknowledgments

## Appendix Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.gendis.2014.12.001.

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