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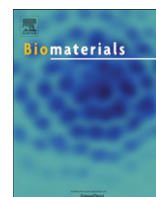
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A gene delivery system for human cells mediated by both a cell-penetrating peptide and a *piggyBac* transposase

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ABSTRACT

The *piggyBac* (PB) transposable element has recently accumulated enormous attention as a tool for the transgenesis in various eukaryotic organisms. Arginine-rich cell-penetrating peptides (CPPs) are protein transduction domains containing a large amount of basic amino acids that were found to be capable of delivering biologically active macromolecules into living cells. In this study, we demonstrate a strategy, which we called “transposoduction”, which is a one-plasmid gene delivery system mediated by the nontoxic CPP-*piggyBac* transposase (CPP-PBase) fusion protein to accomplish both protein transduction and transposition. CPPs were proven to be able to synchronously deliver covalently linked PBase and noncovalently linked a *cis* plasmid into human cells. The expression of promoterless reporter genes coding for red (dTomato) and yellow (mOrange) fluorescent proteins (RFP and YFP) with PB elements could be detected in cells treated with the PBase-expressing plasmid after 3 days indicating transposition of coding regions to downstream of endogenous promoter sequences. An enhanced green fluorescent protein (EGFP) plasmid-based excision assay further confirmed the efficiency of the bifunctional CPP-PBase fusion protein. In conclusion, this strategy representing a combinational concept of both protein transduction and mobile transposition may provide tremendous potential for safe and efficient cell line transformation, gene therapy and functional genomics.

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1. Introduction

DNA transposable elements have been extensively studied and applied for genetics and functional genomics in numerous organisms, since the controlling elements of *Ac-Ds* family in maize were first discovered by McClintock in 1950 [1]. These genetic elements were later called jumping genes or transposons, which in general consist of inverted terminal repeats (ITRs) flanking a *transposase* coding sequence [2]. This transposase follows a cut-and-paste mechanism, also called nonreplicative transposition, to excise the DNA transposon

Abbreviations: BGH, bovine growth hormone; CMV, cytomegalovirus; CNPT, covalent and noncovalent protein transductions; CPP, cell-penetrating peptide; EGFP, enhanced green fluorescent protein; ITR, inverted terminal repeat; *itrPB*, the most minimal 5' and 3' ITRs of PB; mPB, mouse codon-optimized *piggyBac* transposase; PB, *piggyBac*; PBase, *piggyBac* transposase; PBS, phosphate buffered saline; R9, nona-arginine; RFP, red fluorescent protein; SB, *Sleeping Beauty*; SR9, synthetic nona-arginine; SRB, sulforhodamine B; Tat, transactivator of transcription; YFP, yellow fluorescent protein.

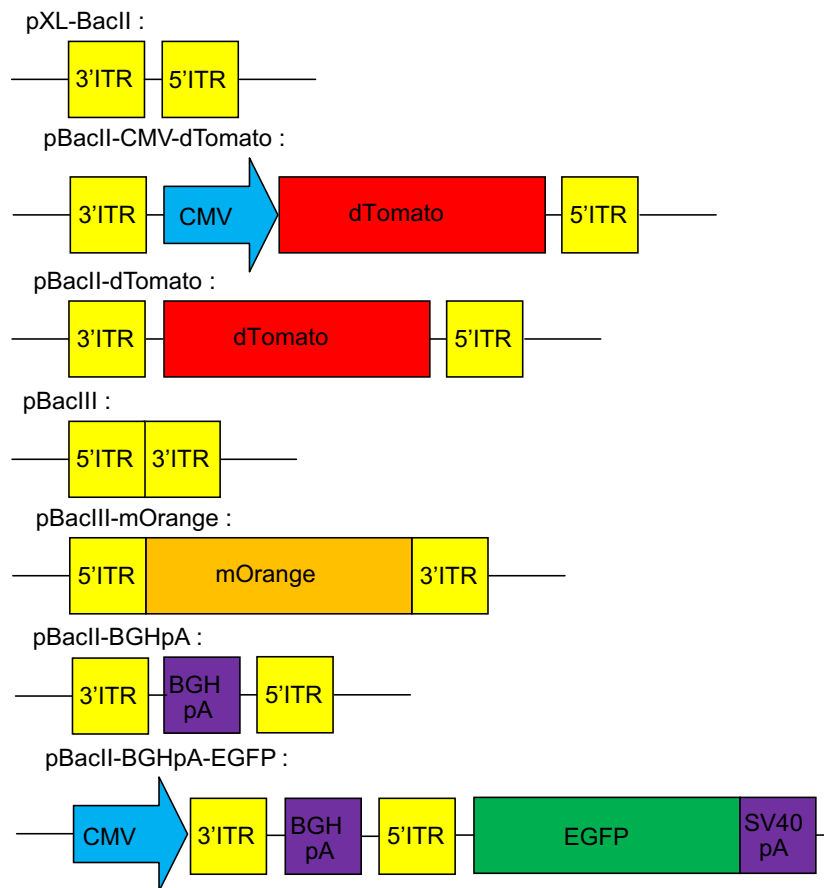
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from its original genomic location and then insert it into a new target site. A synthetic transposon *Sleeping Beauty* (SB) derived from the salmonid subfamily of fish elements was the first DNA transposon proven to be functional in mammalian cells [3]. Though applications of SB in mouse genome-wide screening were restricted due to its low transposition efficiency, limited insert-size capacity and strong local hopping tendency [4–6], SB has been successfully used to identify new cancer genes in mouse tumors [7,8].

The *piggyBac* (PB) transposable element was originally isolated from genomes of baculoviruses that infect cabbage looper moth *Trichoplusia ni* [9]. The mobile element of PB is 2,427 bp in length with specific 13 bp ITRs and a *piggyBac* transposase (PBase) of 594 amino acids with a predicted molecular mass of 68 kDa. Cut-and-paste reaction of PB that inserts the transposable DNA element into TTAA target sequences which are duplicated upon insertion, is thought to be the mechanism catalyzed by PBase. PB has recently accumulated much attention as a genetic tool due to its high functionality in various organisms [4–6,10,11], capacity to transpos sequences up to 18 kb [12], stable gene expression through generations of breeding in mammals [6], applications in whole-genome functional analysis [13] and cancer gene discovery [14].

A *cis* (donor) plasmids



B *trans* (helper) plasmids

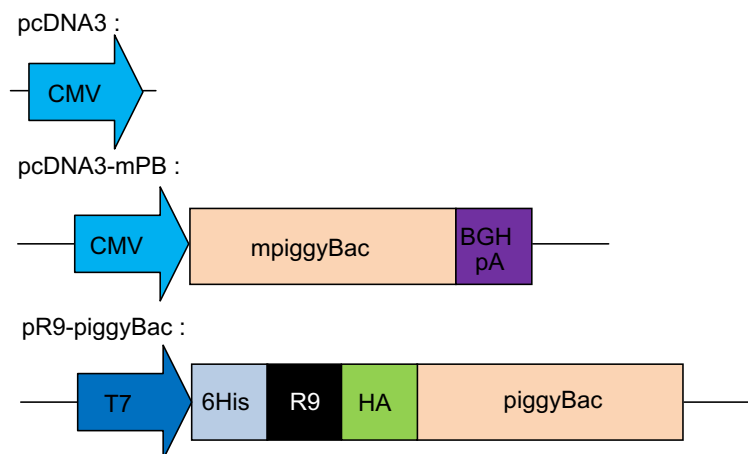


Fig. 1. Schematic structure of constructed plasmids. **A:** *cis* (donor) plasmids. The pBacII-CMV-dTomato, pBacII-dTomato and pBacII-BGHpA plasmids contain coding regions of the RFP expression cassette, the promoterless RFP coding sequence and the polyadenylation (pA) signal of bovine growth hormone (BGH) flanked by both 5' and 3' ITRs of PB from the pXL-BacII vector [9], respectively. The pBacIII-mOrange plasmid consists of the promoterless YFP coding sequence flanked by the most minimal 5' and 3' ITRs of PB (*itrPB*). The pBacII-BGHpA-EGFP plasmid has the BGH polyadenylation signal flanked by both 5' and 3' ITRs between the cytomegalovirus (CMV) promoter and the enhanced green fluorescent protein (EGFP) coding sequence of the pEGFP-N1 plasmid. **B:** *trans* (helper) plasmids. The pcDNA3-mPB plasmid consists of a coding sequence of the mouse codon-optimized PBase (mPB) under the control of the CMV promoter [42]. The pR9-piggyBac plasmid contains coding regions of the hexa-histidine (6His), nona-arginine (R9), haemagglutinin (HA) tags and the full-length of PB transposase (PBase). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Cell-penetrating peptides (CPPs), also called protein transduction domains (PTDs), are small peptides containing a large number of basic amino acids [for recent reviews 15–17]. CPPs were originally discovered in the studies of the transactivator of transcription (Tat) protein of the human immunodeficiency virus type 1

[18,19]. Subsequently, many CPPs were found to be able to deliver biologically active macromolecules, such as proteins, nucleic acids and nanoparticles, into cells [17,20,21]. Among short basic peptide sequences, polyarginines are more efficient than polyhistidine and polyornithine in facilitating cellular uptake [22]. Among

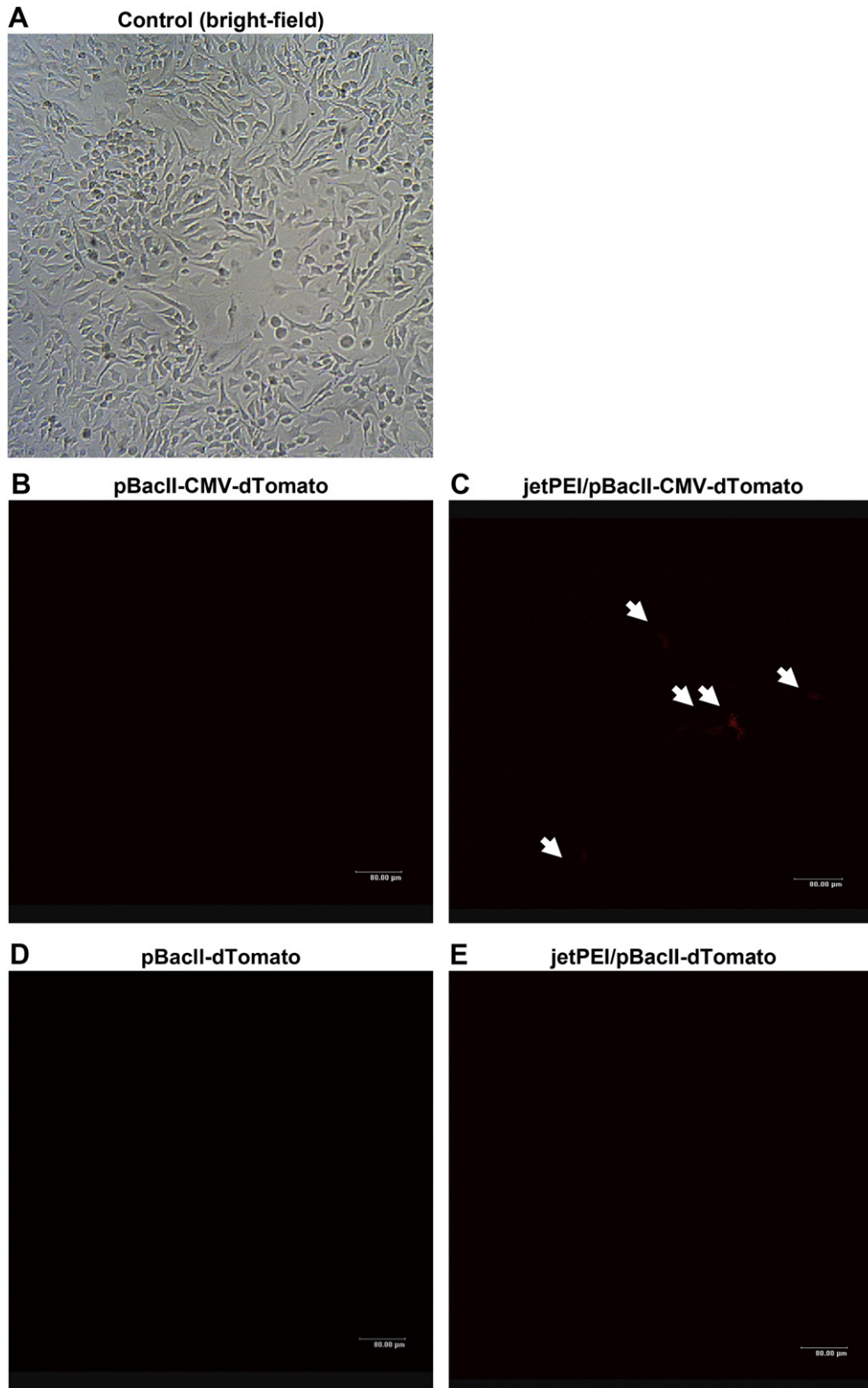


Fig. 2. Confocal microscopy of RFP and YFP gene expression after jetPEI transfection. Human A549 cells (A) were treated with the pBacII-CMV-dTomato plasmid alone (B), jetPEI/pBacII-CMV-dTomato complex (C), pBacII-dTomato plasmid alone (D), jetPEI/pBacII-dTomato complex (E), pBacIII-mOrange plasmid alone (F) or jetPEI/pBacIII-mOrange complex (G). The scale bars are 80 and 10 μm for A–E and F–G, respectively.

polyarginines with various chain lengths, nona-arginine (R9) peptides showed the highest internalization efficiency. However, the exact mechanism(s) by which CPPs enter cells is still incompletely understood [23].

In our previous studies, arginine-rich CPPs have been shown to act in a covalent protein transduction (CPT) manner to efficiently deliver covalently fused proteins into several types of cells [24–28]. We further demonstrated that nontoxic arginine-rich CPPs could

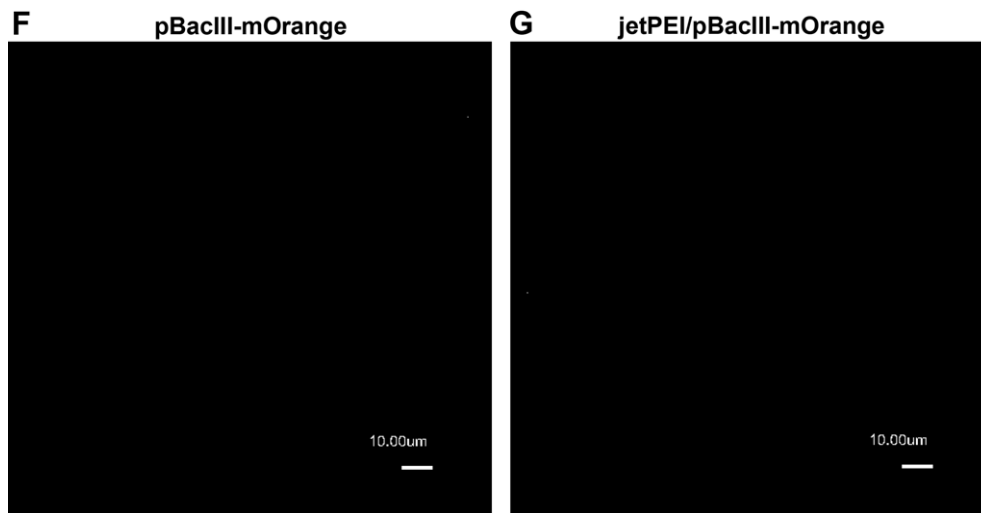


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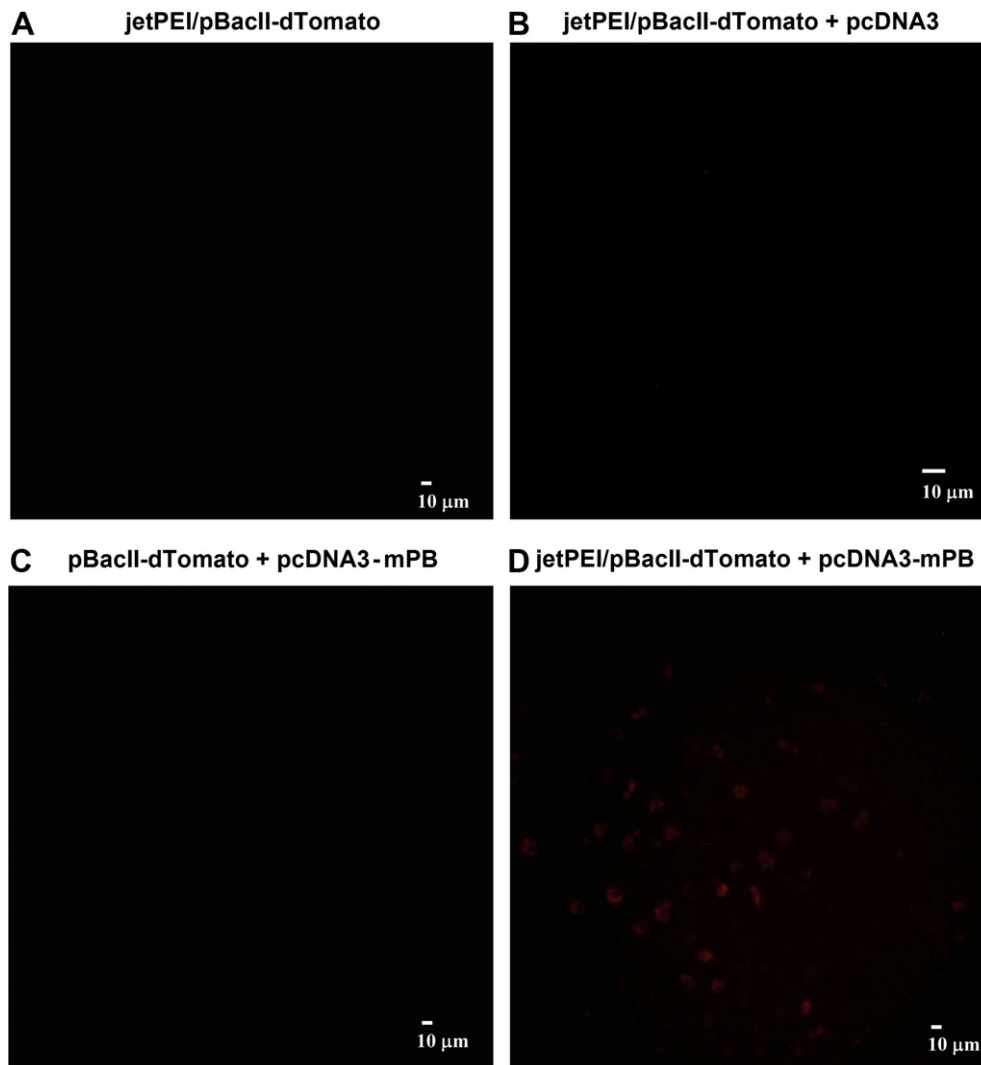


Fig. 3. Fluorescent microscopy of transposition by a two-plasmid system. Cells were treated with jetPEI/pBacII-dTomato complex (A), jetPEI/pBacII-dTomato plus pcDNA3 complex (B), pBacII-dTomato plus pcDNA3-mPB plasmids (C), jetPEI/pBacII-dTomato plus pcDNA3-mPB complex (D), jetPEI/pBacIII-mOrange complex (E), jetPEI/pBacIII-mOrange plus pcDNA3 complex (F), pBacIII-mOrange plus pcDNA3-mPB plasmids (G) or jetPEI/pBacIII-mOrange plus pcDNA3-mPB complex (H). The scale bar is 10 μm.

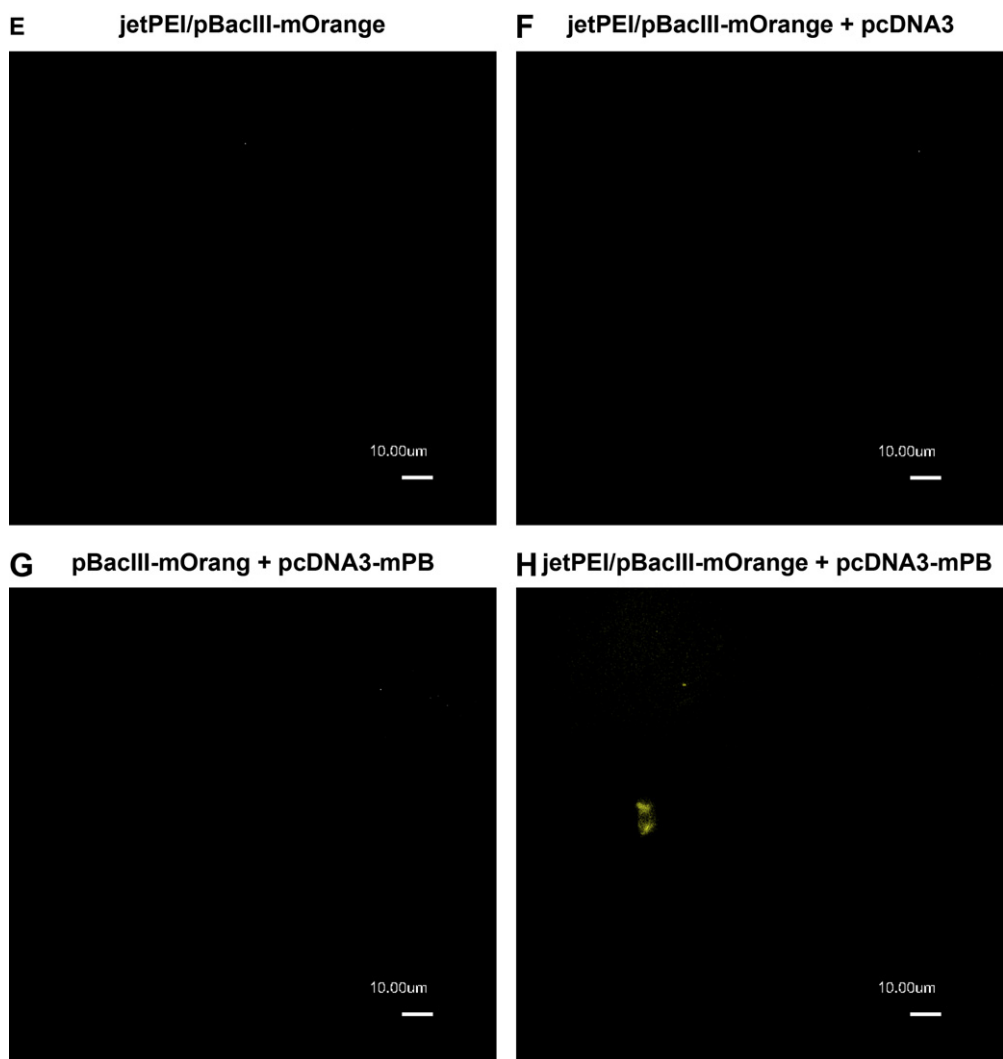


Fig. 3. (continued).

function in a noncovalent protein transduction (NPT) fashion to effectively transport noncovalently conjugated proteins [27–32], RNA [33] or quantum dots [34–37] into living cells or tissues. Moreover, arginine-rich CPPs were able to directly deliver plasmid DNA through the cell wall, membrane and into nuclei of plant cells noncovalently [38]. In addition, we recently demonstrated that arginine-rich CPPs can deliver cargo proteins into living cells in both covalent and noncovalent protein transductions (CNPT), synchronously [27,28]. These data agree with other studies that CPPs are capable of delivering DNA *in vivo* [39] and *in vitro* [20,40,41].

In this study, we test the hypothesis that arginine-rich CPPs can simultaneously deliver covalently linked PBase and noncovalently linked DNA into human cells. We name this CPP-PBase system of transposon and protein transduction mediated gene delivery as “transposoduction”. According to a two-plasmid donor-helper system [42], also called two-component system [43], we constructed the *cis* (or called the donor) vector containing a red (RFP; dTomato) or yellow fluorescent protein (YFP; mOrange) reporter gene in the presence or absence of the cytomegalovirus (CMV) promoter flanked by ITRs of PB. An enhanced green fluorescent protein (EGFP) expression plasmid was constructed to easily monitor the function of PBase. The *trans* (or called the helper) vector was generated to fuse in-frame with coding sequences of

both R9 and PBase. The function of nontoxic CPP-PBase fusion protein in DNA delivery and subsequent transposition was investigated in human cells.

2. Materials and methods

2.1. Plasmid construction

The pDs-CMV-EGFP-Ub-Bsd (kindly provided by Dr. Serguei Parinov, National University of Singapore, Singapore) [43], dTomato and mOrange (kindly provided by Dr. Roger Y. Tsien, University of California, San Diego, CA, USA) [44], pXL-BacII and pBSII-IFP2-orf (kindly provided by Dr. Malcolm J. Fraser Jr., University of Notre Dame, Notre Dame, IN, USA) [9], pcDNA3-mPB (kindly provided by Dr. Allan Bradley, Wellcome Trust Sanger Institute, Cambridgeshire, UK) [45] and pR9-RFP [29] plasmids were described previously. To construct *cis* (donor) plasmids, the insert of the pDs-CMV-EGFP-Ub-Bsd plasmid containing the minimal ITRs recognized by maize transposase was removed by the digestion of *Sma*I restriction enzyme followed by the self-ligation to generate the pDs-CMV plasmid (Fig. 1S). The pDs-CMV-dTomato plasmid was constructed by the insertion of a coding region of RFP released from the dTomato plasmid by the digestion of *Bam*HI and *Hind*III restriction enzymes into the pDs-CMV plasmid at *Bgl*III and *Hind*III sites. Finally, the pBacII-CMV-dTomato plasmid containing the RFP cassette flanked by both 5' and 3' ITRs of PB was generated by the transfer of a coding region of CMV-dTomato from the pDs-CMV-dTomato plasmid into the pXL-BacII plasmid at *Xho*I and *Hind*III sites.

In order to assay PBase activity, the CMV promoter of the pBacII-CMV-dTomato plasmid was removed by the digestion of *Xho*I and *Nhe*I restriction enzymes, blunting of ends and self-ligation to generate the promoterless pBacII-dTomato plasmid. The pBacIII plasmid containing the most minimal 5' and 3' ITRs of PB, which we called *itrPB*,

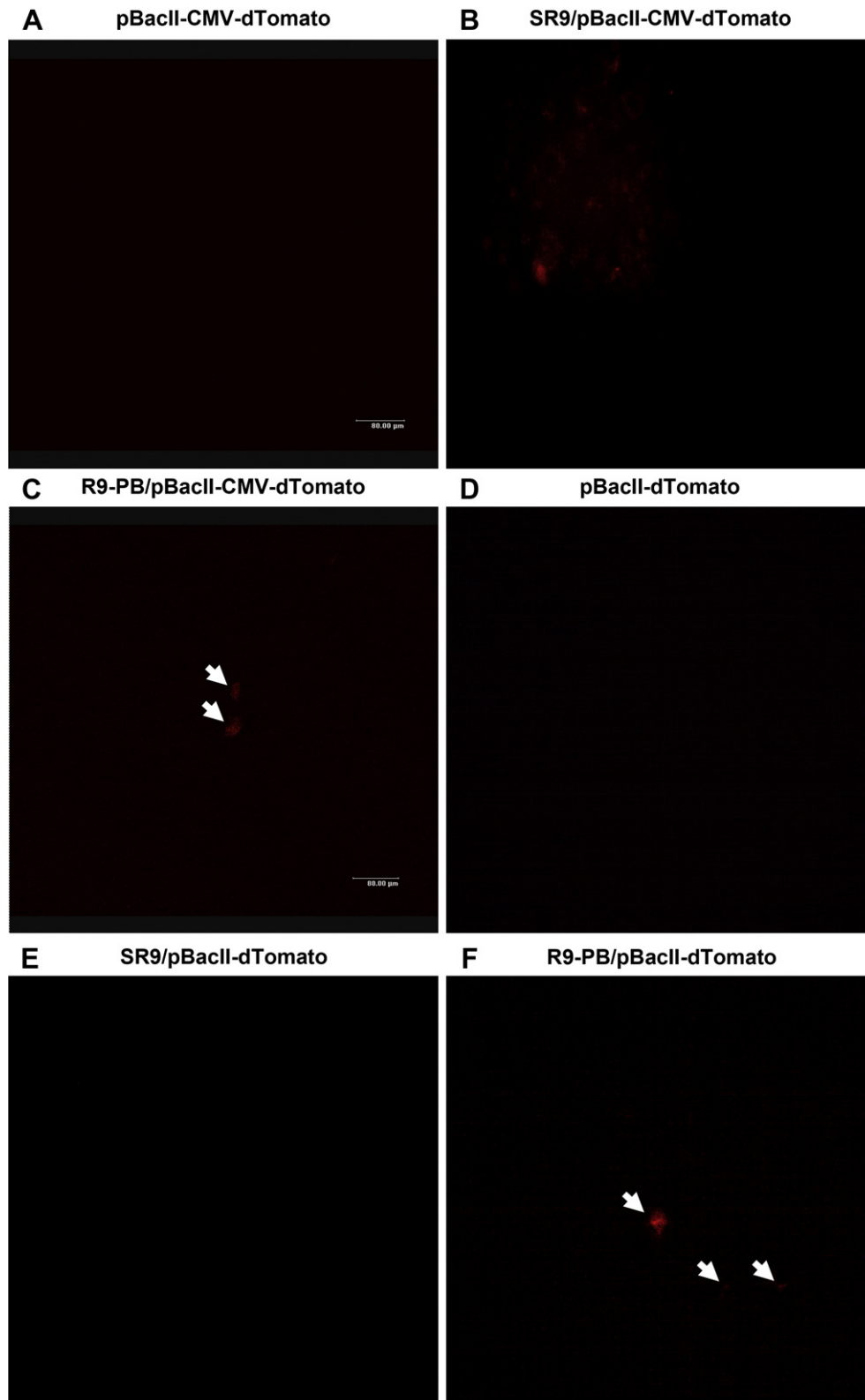


Fig. 4. Microscopy of transposition by a one-plasmid system with RFP and YFP gene expression mediated by CPP or CPP-PBase protein. Cells were treated with the pBacII-CMV-dTomato plasmid (A), SR9/pBacII-CMV-dTomato complex (B), R9-PBase/pBacII-CMV-dTomato complex at an N/P ratio of 8 (C), pBacII-dTomato plasmid (D), SR9/pBacII-dTomato complex (E), R9-PBase/pBacII-dTomato complex (F), pBacIII-mOrange plasmid (G), SR9/pBacII-dTomato complex (H) or R9-PBase/pBacIII-mOrange complex (I).

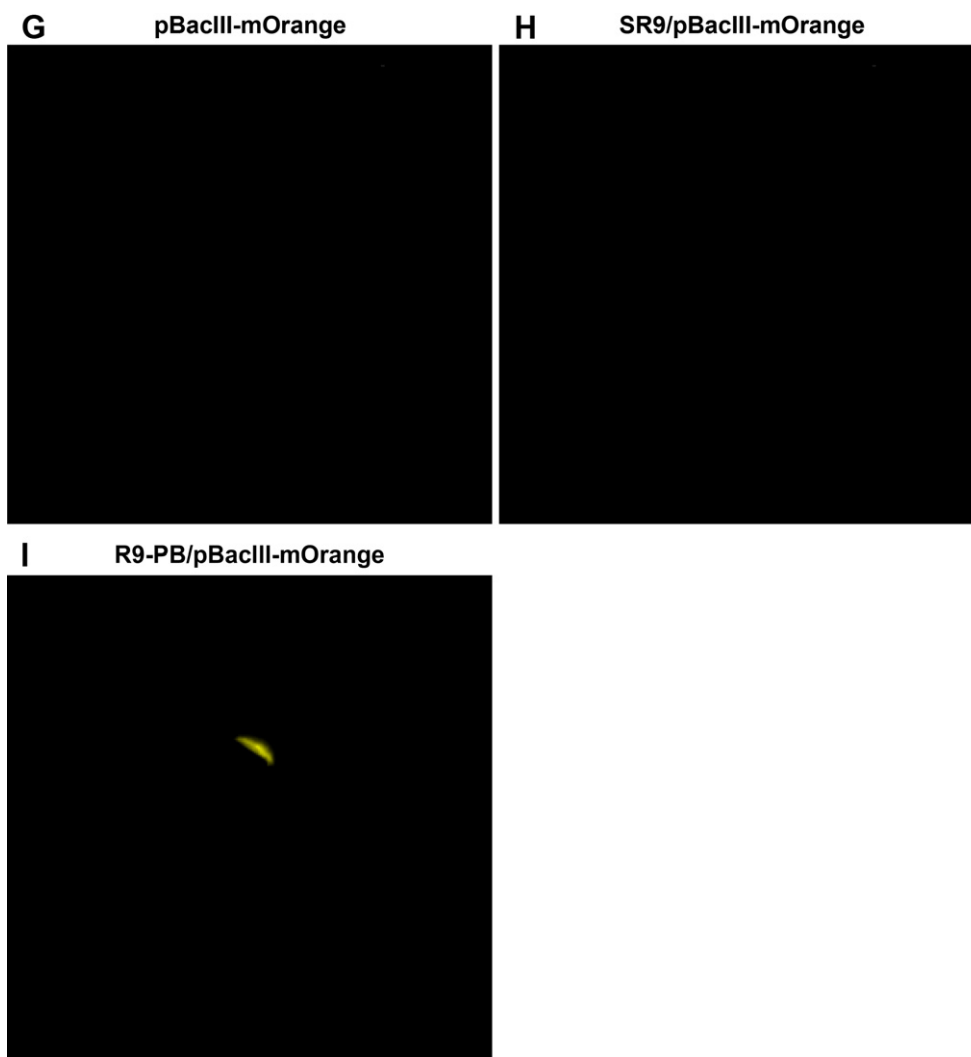


Fig. 4. (continued).

was constructed by the insertion of the PCR product with two partially overlapped 5'PiggyBac (5'-CCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGGATCCITTAAGCTTATGCGTCAATTTTACAC-3' with *Bam*HI and *Hind*III sites underlined and the overlapped sequence shadowed) and 3'PiggyBac (5'-CCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGTGTTAAAATTGACGCATAAG-3') primers into the pGEM-T easy vector (Promega, Madison, WI, USA). Then, another promoterless pBacIII-mOrange plasmid was generated by the transfer of the *YFP* coding region into the pBacIII plasmid at *Bam*HI and *Hind*III sites.

To apply the cut-and-paste mechanism of *PB*, the pBacII-BGHpA plasmid containing the polyadenylation signal of bovine growth hormone (BGH) flanked by *PB*-ITRs was constructed by the insertion of the *Hind*III- and *Xba*I-digested annealing of BGH-U (5'-TTAAGCTTATCAGCCTCGACTGTGCCCTTCTAGTTGCCA-3' with the *Hind*III site underlined) and BGH-D (5'-AATCTAGACAGCATGCCCTGCTATTGTCTTCCCAATCT-3' with the *Xba*I site underlined) primers into the pXL-BacII plasmid at *Hind*III and *Xba*I sites. The pBacII-BGHpA-EGFP plasmid was then generated by the insertion of the *Bgl*III- and *Kpn*I-digested PCR product from the template pBacII-BGHpA plasmid plus Bac-U (5'-TTAGATCTTTAACCTAGAAA-GATAGTCTGCGTAAAATT-3' with the *Bgl*III site underlined and the TTA target sequence shadowed) and Bac-D (5'-AAGGTACCTTAACCCTAGAAAAGATAATCATATTGTGACG-3' with the *Kpn*I site underlined and the TTA target sequence shadowed) primers covering both 5' and 3' *PB*-ITRs into the pEGFP-N1 vector (Invitrogen, Carlsbad, CA, USA) at *Bgl*III and *Kpn*I sites.

The pcDNA3-mPB plasmid is a *trans* (helper) vector containing a coding sequence of the mouse codon-optimized PBase (mPB) under the control of the CMV promoter in a pcDNA3 backbone [45]. To construct another *trans* plasmid, the pR9-piggyBac plasmid was generated by the replacement of the *RFP* coding region of the pR9-RFP plasmid with the PBase coding region from the pBSII-IFP2-orf plasmid at *Sma*I and *Not*I sites. Thus, the expression of the pR9-piggyBac plasmid in prokaryotic

cells produced an open reading frame encoding the hexa-histidine (6His), R9 (a CPP) tags and the full-length of PBase [9]. All constructs were confirmed by DNA sequencing.

2.2. Protein expression and purification

The pR9-piggyBac plasmid was transformed into the *Escherichia coli* KRX strain (Promega). R9-PBase protein expression and purification were described previously with modifications [27,46]. Bacteria were grown to the $OD_{600} = 0.6$ and induced with 0.05% (w/v) of rhamnose overnight at 16 °C. The binding, wash and elute buffers were replaced by the TSG (10% glycerol, 0.5 M NaCl and 20 mM Tris-HCl), TSG-50 (50 mM imidazole, 10% glycerol, 0.5 M NaCl and 20 mM Tris-HCl) and TSG-200 (200 mM imidazole, 10% glycerol, 0.5 M NaCl and 20 mM Tris-HCl) buffers, respectively. Finally, purified proteins were concentrated and dialyzed using the Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA, USA).

2.3. Cell culture

Human lung carcinoma A549 cells (American Type Culture Collection, Manassas, VA, USA; CCL-185) were cultured as previously described [29]. Cells were seeded at a density of 3×10^5 per 30-mm petri dish.

2.4. Transient DNA transfection

Plasmid DNA was transfected into cells with either the jetPEI transfection reagent (Polyplus-transfection, San Diego, CA, USA) according to the manufacturer's instructions [27,33] or the CPP-mediated DNA delivery method [38]. For the former one, the highly purified pBacII-CMV-dTomato plasmid or pBacII-dTomato and

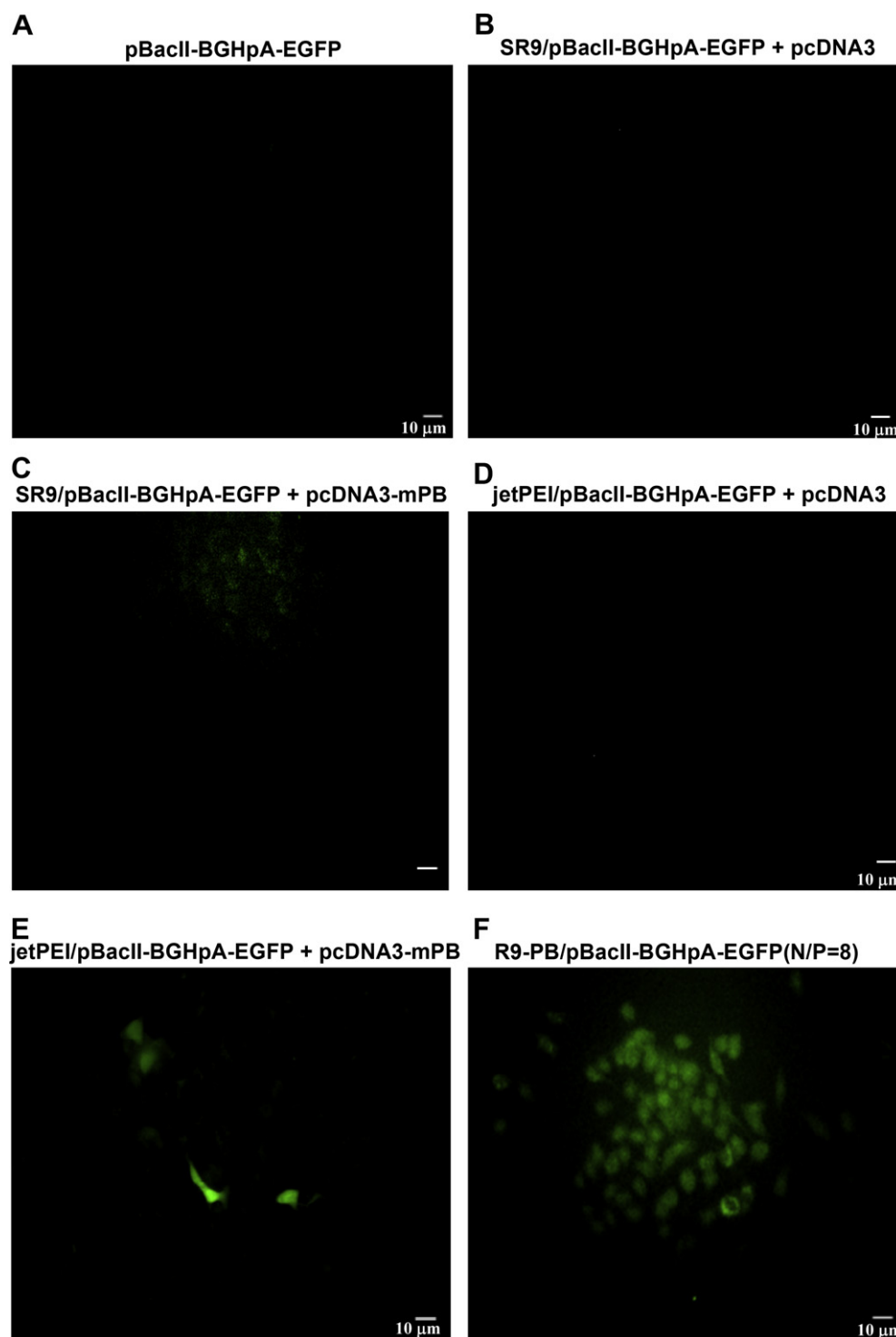


Fig. 5. Microscopy of transposition by a one-plasmid system with EGFP gene expression mediated by jetPEI, CPP or CPP-PBase protein. Cells were treated with the pBacII-BGHpA-EGFP plasmid (A), SR9/pBacII-BGHpA-EGFP plus pcDNA3 complex (B), SR9/pBacII-BGHpA-EGFP plus pcDNA3-mPB complex (C), jetPEI/pBacII-BGHpA-EGFP plus pcDNA3 complex (D), jetPEI/pBacII-BGHpA-EGFP plus pcDNA3-mPB complex (E), R9-PBase/pBacII-BGHpA-EGFP complex at an N/P ratio of 8 (F), R9-PBase/pBacII-BGHpA-EGFP complex at an N/P ratio of 12 (G) or R9-PBase/pBacII-BGHpA-EGFP complex at an N/P ratio of 20 (H) in the presence of the modified Buffer A. The scale bar is 10 μm .

pcDNA3-mPB plasmids were transfected into cells with the jetPEI reagent according to a two-plasmid system [42,43]. For the later one, we want to apply the CPP-mediated DNA transfection method, which is a combination of both covalent protein transduction and noncovalent plasmid DNA delivery. R9-PBase fusion protein (0, 59, 119, 178 and 295 nM) was incubated with highly purified plasmid DNA (1 μg) in a final volume of 500 μl of the modified Buffer A (10 mM of MgCl_2 , 133 mM of NaCl, 2 mM of EDTA, 50 mM of Tris-HCl and 50% glycerol, pH 7.5) [47] at 37 $^\circ\text{C}$ for 3 h prior to treatment. Cells were treated with R9-PBase protein and plasmid DNA at

N/P (NH_3^+ per PO_4^-) ratios of 0, 4, 8, 12 or 20, respectively [38]. Cells were incubated for 48 h after transfection in a 37 $^\circ\text{C}$ incubator.

2.5. Confocal and fluorescent microscopes

Cells were observed using the TCS SL confocal microscope system (Leica, Wetzlar, Germany) 48 h after transfection as previously described [27]. For the RFP channel, we set excitation at 543 nm and emission at 580–650 nm bandpass barrier

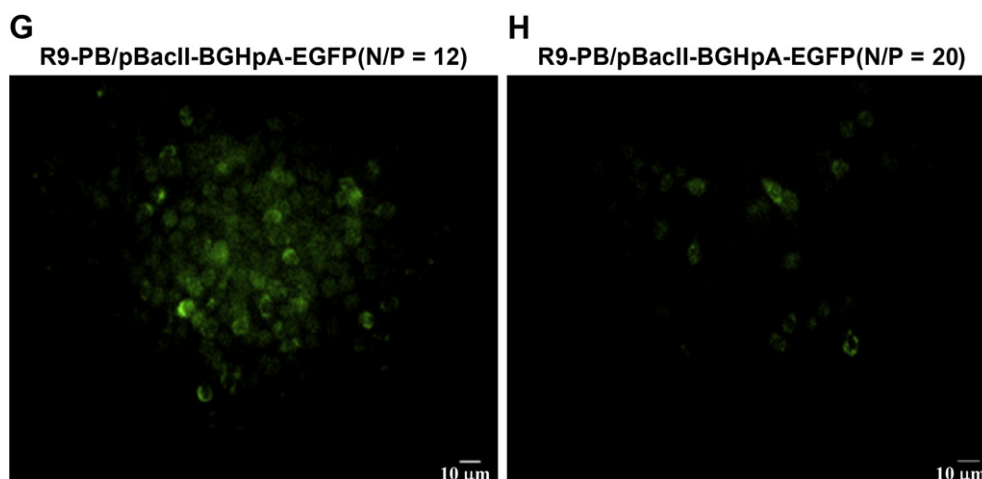


Fig. 5. (continued).

filters. Cells were also observed using the Eclipse E600 fluorescent microscope (Nikon, Melville, NY, USA) which equipped with a filter set providing excitation at 510–560 nm and emission above 590 nm. Fluorescent images were recorded using the Penguin 150CL cooled CCD camera (Pixera, Los Gatos, CA, USA) as previously described [32].

2.6. Flow cytometric analysis

To monitor the functional activity of PBase, flow cytometry was conducted using the Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) as previously described [34]. For EGFP detection, we set excitation at 488 nm and emission at 513 nm with FL1 filters. Results are reported as the percentage of total cell population.

2.7. Cytotoxicity assay

To determine cell viability, the sulforhodamine B (SRB) assay was performed as previously described [27]. Cells were treated with phosphate buffered saline (PBS) as a negative control and 70% alcohol as a positive control. For experimental groups, cells were treated with various concentrations of R9-PBase and then incubated at 37 °C for 48 h. The absorbance of the SRB dye solution was measured at 550 nm using the DU800 spectrophotometer (Beckman Coulter).

2.8. Statistical analysis

Data were expressed as means \pm standard deviations. Statistical comparisons between the control and treated groups were performed using the Student's *t*-test. Mean values and standard deviations were calculated for each sample examined from at least three independent experiments. The levels of statistical significance were set at $P < 0.05$ (*) or 0.01 (**).

3. Results

3.1. Plasmid construction

To test the gene delivery mediated by both CPP and PBase, several *cis* vectors were constructed (Fig. 1A). The pBacII-CMV-dTomato plasmid and the promoterless pBacII-dTomato plasmid, both containing the RFP coding sequence flanked by PB-ITRs, were constructed. Another promoterless pBacIII-mOrange plasmid was generated from the newly constructed pBacIII vector containing the minimal PB-ITRs (*itrPB*) as the third generation of pBac system. Moreover, the pBacII-BGHpA plasmid was first generated to contain the BGH polyadenylation signal flanked by both 5' and 3' ITRs, and then the pBacII-BGHpA-EGFP plasmid was constructed by the insertion of this polyadenylation signal flanked by both 5' and 3' ITRs between the CMV promoter and the EGFP coding sequence of the pEGFP-N1 plasmid. As a result, the pBacII-BGHpA-EGFP plasmid is an excision EGFP marker plasmid which can be used to monitor the function of PBase. The EGFP coding sequence of the pBacII-

BGHpA-EGFP plasmid can not be expressed due to the blockage of the polyadenylation signal downstream of the CMV promoter. However, the EGFP coding sequence can be expressed under the control of the CMV promoter, when PBase causes transposition of the polyadenylation signal out of the pBacII-BGHpA-EGFP plasmid followed by self-ligation of the post-transposed plasmid according to the cut-and-paste mechanism of PB [2].

A bacterial expression vector, the pR9-piggyBac, was generated to produce R9-PBase bifunctional fusion protein (cellular uptake of plasmids by R9 as well as transposition by PBase) (Fig. 1B).

3.2. Analysis of RFP and YFP gene expression after transfection

To reveal transient gene expression profile of *cis* vectors, human A549 cells (Fig. 2A) were transfected with the pBacII-CMV-dTomato, pBacII-dTomato or pBacIII-mOrange plasmid by the jetPEI transfection reagent. No fluorescence could be detected in cells treated with the pBacII-CMV-dTomato (Fig. 2B), pBacII-dTomato (Fig. 2D), pBacIII-mOrange (Fig. 2F) plasmid alone, jetPEI/pBacII-dTomato (Fig. 2E) or jetPEI/pBacIII-mOrange (Fig. 2G) complexes. In contrast, cells treated with jetPEI/pBacII-CMV-dTomato complex exhibited red fluorescence (Fig. 2C). These results indicate that the pBacII-CMV-dTomato plasmid can be expressed in cells after jetPEI transfection, but both promoterless pBacII-dTomato and pBacIII-mOrange plasmids can not.

3.3. Transposition by a two-plasmid system

To determine whether the ITR elements of PB were capable of helper-dependent transposition or not, cells were transfected with the pBacII-dTomato or pBacIII-mOrange and pcDNA3-mPB or pcDNA3 plasmids by jetPEI reagent according to a two-plasmid system. No fluorescence was detected in the cells treated with jetPEI/pBacII-dTomato (Fig. 3A), jetPEI/pBacII-dTomato plus pcDNA3 (Fig. 3B), jetPEI/pBacIII-mOrange (Fig. 3E), jetPEI/pBacIII-mOrange plus pcDNA3 (Fig. 3F) complex, pBacII-dTomato plus pcDNA3-mPB (Fig. 3C) or pBacIII-mOrange plus pcDNA3-mPB (Fig. 3G) plasmids. On the other hand, the cells treated with jetPEI/pBacII-dTomato plus pcDNA3-mPB (Fig. 3D) or jetPEI/pBacIII-mOrange plus pcDNA3-mPB (Fig. 3H) complex displayed red and yellow fluorescence, respectively. These data confirm that PBase expressed from the pcDNA3-mPB plasmid in the cells has functional transposition activity toward PB mobile elements. With the help of PBase, both *dTomato* and *mOrange* coding regions might

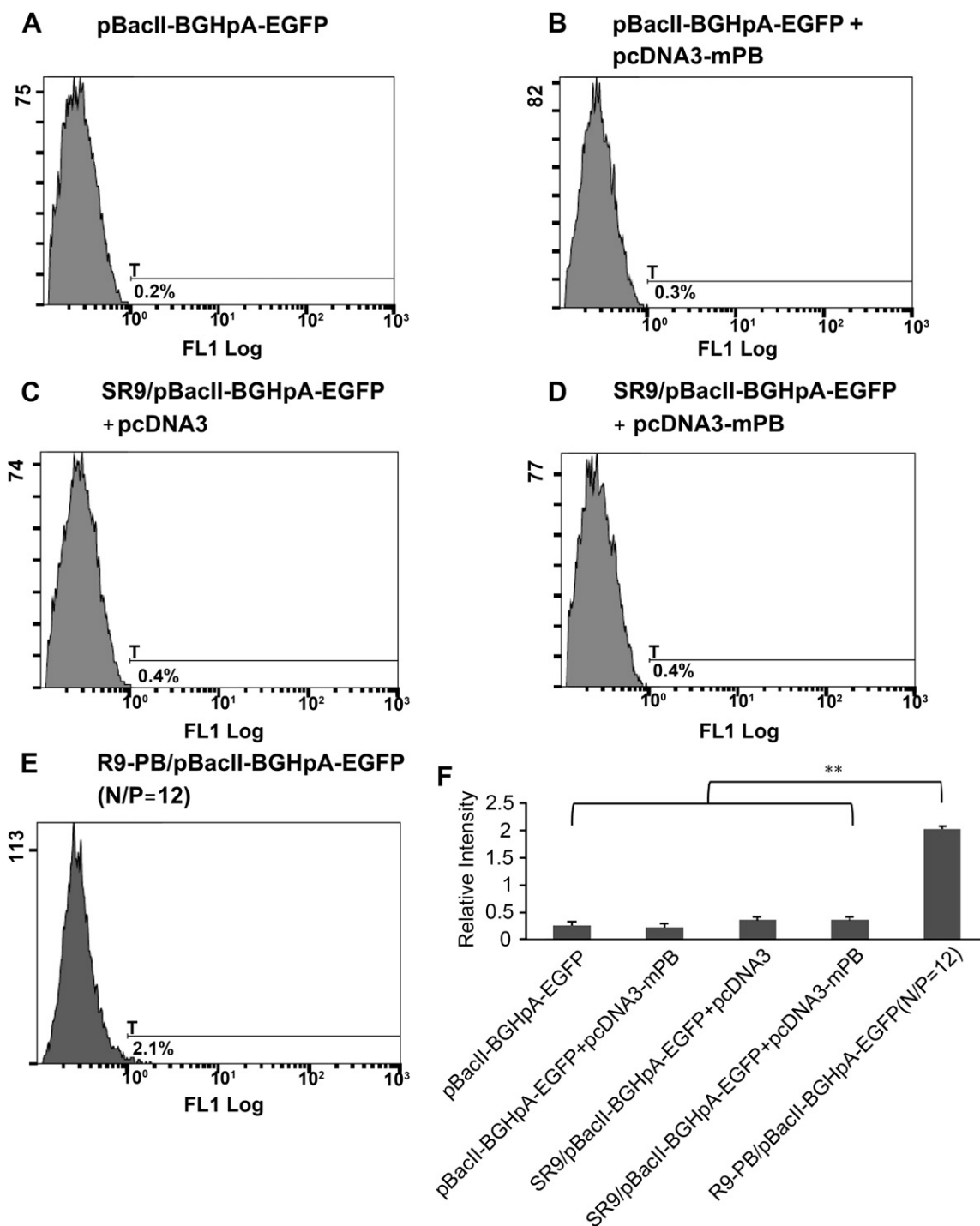


Fig. 6. Flow cytometric analysis of transposition by a one-plasmid system with EGFP gene expression mediated by CPP or CPP-PBase protein. Profiles of cells treated with the pBacII-BGHpA-EGFP plasmid (A), pBacII-BGHpA-EGFP plus pcDNA3-mPB plasmids (B), SR9/pBacII-BGHpA-EGFP plus pcDNA3 complex (C), SR9/pBacII-BGHpA-EGFP plus pcDNA3-mPB complex (D) or R9-PBase/pBacII-BGHpA-EGFP complex at an N/P ratio of 12 (E) in the absence of the modified Buffer A are shown. T represents the mean fluorescent intensity of a population over the fluorescence gate region FL1 > 10⁰ at the x-axis. (F) Comparative fluorescent intensity derived from combined profiles. Significant differences were determined at $P < 0.01$ (**). Data are presented as mean \pm standard deviations from three independent experiments.

be integrated into the host genome after transposition and then expressed under the control of endogenous promoters.

3.4. Transposition by a one-plasmid system mediated via CPP-PBase protein

To investigate the CPP-mediated DNA delivery system, cells were treated with different gene constructs in the presence or absence of

the synthetic nona-arginine (SR9) peptide [30] or the purified R9-PBase protein at various ratios. No fluorescent signal was detected in the cells treated with the pBacII-CMV-dTomato (Fig. 4A), pBacII-dTomato (Fig. 4D), pBacII-mOrange (Fig. 4G) plasmids alone, SR9/pBacII-dTomato (Fig. 4E) or SR9/pBacII-mOrange (Fig. 4H) complexes for 3 days. On the other hand, red fluorescence was observed in the cells treated with SR9/pBacII-CMV-dTomato (Fig. 4B), R9-PBase/pBacII-CMV-dTomato (Fig. 4C) or R9-PBase/pBacII-d

Tomato (Fig. 4F) complex, and yellow fluorescence was observed in the cells treated with R9-PBase/pBacIII-mOrange complex (Fig. 4I). These results suggest that the bifunctional R9-PBase fusion protein not only can deliver DNA (donor vector) into cells via R9 functionality, but also induce transposition of *PB* elements.

To further confirm that mobile *PB* elements are capable of transposition with the help of R9-PBase protein, cells were treated with an excision marker plasmid alone, SR9/plasmid, jetPEI/plasmid or R9-PBase/plasmid complex at N/P ratios of 0, 4, 8, 12 and 20 for 3 days. No fluorescent image was noticed in the cells treated with the pBacII-BGHpA-EGFP plasmid alone (Fig. 5A), SR9/pBacII-BGHpA-EGFP plus pcDNA3 (Fig. 5B) or jetPEI/pBacII-BGHpA-EGFP plus pcDNA3 (Fig. 5D) complexes. On the other hand, green fluorescent image was observed in the cells treated with SR9/pBacII-BGHpA-EGFP plus pcDNA3-mPB (Fig. 5C), jetPEI/pBacII-BGHpA-EGFP plus pcDNA3-mPB (Fig. 5E) or R9-PBase/pBacII-BGHpA-EGFP complex at N/P ratios of 8 (Fig. 5F), 12 (Fig. 5G) and 20 (Fig. 5H). Together, these results demonstrate again that not only can R9-PBase protein deliver a donor plasmid into cells but it also expresses active PBase activity after cellular internalization. Noticeably, the one-plasmid system mediated by CPP-PBase protein (Fig. 5G) is more effective than the two-plasmid system mediated by either SR9 (Fig. 5C) or jetPEI (Fig. 5E).

The hypothesis was further characterized using a new reporter pBacII-BGHpA-EGFP plasmid, which monitors the functional activity of PBase. Flow cytometric analysis demonstrated little fluorescence in the cells treated with pBacII-BGHpA-EGFP (Fig. 6A; 0.27 ± 0.06), pBacII-BGHpA-EGFP plus pcDNA3-mPB (Fig. 6B; 0.23 ± 0.06) plasmids alone, SR9/pBacII-BGHpA-EGFP plus pcDNA3 (Fig. 6C; 0.37 ± 0.06) or SR9/pBacII-BGHpA-EGFP plus pcDNA3-mPB (Fig. 6D; 0.37 ± 0.06) complexes. In contrast, the cells treated with the R9-PBase/pBacII-BGHpA-EGFP complex at an N/P ratio of 12 (Fig. 6E; 2.03 ± 0.06) exhibited about 6.5-fold higher fluorescent intensity than others (Fig. 6F). These data indicate that the bifunctional CPP-PBase fusion protein is an efficient tool in both gene delivery and transposase activity.

To reveal any cytotoxicity caused by CCP-PBase, cells were treated with PBS as a negative control, 70% alcohol as a positive control or R9-PBase fusion protein, and analyzed by the SRB assay (Fig. 7). Cells treated with various amounts of R9-PBase protein (0, 0.2, 1, 10 or 20 μ M) did not result in any cytotoxicity. These results indicate that R9-PBase protein does not cause cytotoxicity.

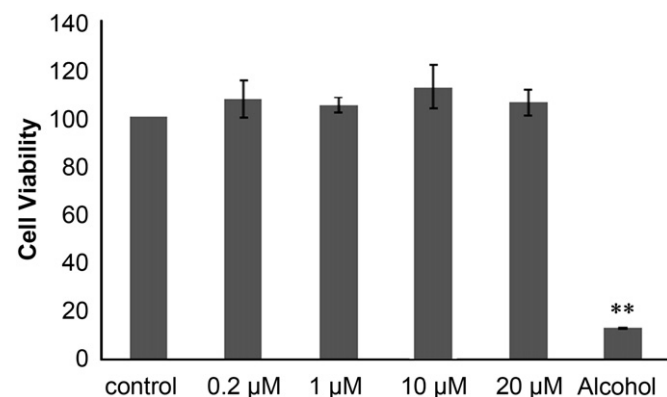


Fig. 7. Cytotoxicity of CPP-PBase protein. Cells were treated with phosphate buffered saline (PBS) as a negative control and 70% alcohol as a positive control. For experimental groups, cells were treated with 0, 0.2 (corresponding to the working concentration used at an N/P ratio of 20), 1 (corresponding to 5 times the working concentration), 10 (corresponding to 50 times the working concentration) or 20 μ M (corresponding to 100 times the working concentration) of R9-PBase in the absence of the modified Buffer A and analyzed by the SRB assay.

4. Discussion

In this report, we demonstrated a strategy for gene delivery mediated by CPP-PBase into human cells. The *in vitro* expressed CPP-PBase fusion protein is a bifunctional device. R9 peptide, an effective CPP, can transport PBase protein covalently and DNA noncovalently across the cell membrane in a CNPT manner. Subsequently, the internalized PBase is capable of transposing specific DNA fragments flanked by mobile *PB* elements into genome. This one-plasmid system mediated by CPP-PBase protein consists of two functional applications, protein transduction and mobile transposition may have important potential for efficient gene therapy in the future.

To date, gene therapy applications rely heavily on highly efficient tools for safe and stable delivery of genetic information into eukaryotic genome [48]. Most current gene therapies are based on efficient viral vectors for permanent or transient transfer of nucleic acids. However, viral vectors have exhibited major drawbacks which have limited their clinical applications. For instance, viral vectors can cause problems like high immunogenicity, risk of insertional oncogenicity and limited cargo capacity in the range of 8–10 kb. These restrictions have prompted a search for nonviral vectors in gene therapy applications. In general, nonviral vectors have relatively low efficiency and high toxicity. CPPs are nonviral vectors with no cytotoxicity and very attractive tools for delivering therapeutic macromolecules such as proteins and nucleic acids [15–17,49]. Recently, we explored the possibility of using the nontoxic CPP-based system to deliver nucleic acids into cells [33,38]. In this study, a viability assay showed that the R9-PBase fusion protein does not cause cytotoxicity (Fig. 7). Those results and our data presented in the present study are in concert with other accomplished studies indicating the CPP-based system is able to safely delivery DNA *in vivo* [39] and *in vitro* [20,40,41].

It has long been a difficult task to determine transposase activity [50,51]. Both promoterless pBacII-dTomato and pBacIII-mOrange plasmids were generated for the promoter-trap assay [52] in this study. Both *RFP* and *YFP* coding regions will be transcribed only when they are under the control of endogenous promoters after transposition into the host genome mediated by PBase action (Figs. 2–4). Another possibility mediated by PBase is that both *RFP* and *YFP* coding regions may possibly jump into the co-inoculated pcDNA3-mPB plasmid downstream of the CMV promoter to produce gene expression. The pXL-BacII plasmid was a popular *PB* minimal construct [9]. However, this second generation of pBac plasmid contains more than 200 bp ITRs of *PB* flanking the multiple cloning sites. The long ITR may be a hindrance between the promoter and the coding sequence after proper insertion. Therefore, the pBacIII plasmid containing the *itrPB*, the most minimal 5' and 3' ITRs of *PB*, was constructed in the present report as the third generation of pBac system in order to shorten ITRs for the promoter-trap assay. In addition, the pBacII-BGHpA-EGFP plasmid generated in the present study provides a good candidate reporter gene for molecular confirmation of excision by the plasmid excision assay [51].

More recently, transposons have been developed as gene delivery vehicles and offer the prospect of repairing mutations in gene therapy applications [48]. Further, the transposon-based mutagenesis has emerged as a powerful molecular genetic tool for eukaryotic transgenesis [13,53,54]. This transposon-based system has been extensively used to create gene disruption, promoter-and-enhancer traps and generate gene functions in model organisms, such as *Drosophila*, yeast, mouse and human cells [10,55–57]. The strategy presented in this report may provide an alternative and highly efficient approach in transposon-based mutagenesis to identify critical biological processes. In particular,

PB was recently engineered to be highly active in mammalian cells [6,45,58] and has become widely used to manipulate large-scale mutagenesis screens in different genomes [4,5,10,13,51,59,60]. Insertional mutagenesis with PB has recently been used for cancer gene discovery in PBase knockin mice [14]. Thus, the application of our strategy shall reveal valuable information in functional genomics studies.

5. Conclusions

We have demonstrated that CPPs are able to deliver covalently linked PBase and noncovalently linked a *cis* plasmid containing PB mobile elements into human cells synchronously. The CPP-PBase fusion protein is nontoxic and a bifunctional device to accomplish both protein transduction and transposition. Thus, this strategy representing a combinational concept of intracellular delivery and mobile transposition may provide tremendous potential for safe and efficient gene therapy and functional genomics in the future.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.05.012.

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