

Multiplexed transposon-mediated stable gene transfer in human cells

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Generation of cultured human cells stably expressing one or more recombinant gene sequences is a widely used approach in biomedical research, biotechnology, and drug development. Conventional methods are not efficient and have severe limitations especially when engineering cells to coexpress multiple transgenes or multiprotein complexes. In this report, we harnessed the highly efficient, nonviral, and plasmid-based *piggyBac* transposon system to enable concurrent genomic integration of multiple independent transposons harboring distinct protein-coding DNA sequences. Flow cytometry of cell clones derived from a single multiplexed transfection demonstrated approximately 60% (three transposons) or approximately 30% (four transposons) stable coexpression of all delivered transgenes with selection for a single marker transposon. We validated multiplexed *piggyBac* transposon delivery by coexpressing large transgenes encoding a multisubunit neuronal voltage-gated sodium channel (SCN1A) containing a pore-forming subunit and two accessory subunits while using two additional genes for selection. Previously unobtainable robust sodium current was demonstrated through 38 passages, suitable for use on an automated high-throughput electrophysiology platform. Cotransfection of three large (up to 10.8 kb) *piggyBac* transposons generated a heterozygous SCN1A stable cell line expressing two separate alleles of the pore-forming subunit and two accessory subunits (total of four sodium channel subunits) with robust functional expression. We conclude that the *piggyBac* transposon system can be used to perform multiplexed stable gene transfer in cultured human cells, and this technology may be valuable for applications requiring concurrent expression of multiprotein complexes.

piggyBac | SCN1A | SCN1B | SCN2B | sodium channel

Stable single-gene transfer into cultured cells has revolutionized the study of human disease, provided critical cell models needed for high-throughput screening in drug development, and enabled large-scale production of recombinant proteins and viruses. However, a technology does not currently exist that is capable of simple but efficient engineering of cells to stably coexpress multiple transgenes.

Transposon systems mediate stable integration of exogenous DNA elements into a host cell genome, and have been harnessed as gene transfer vectors for preclinical gene therapy studies and gene discovery applications in mammalian cells (1–4). The two most commonly used transposon systems for gene transfer into human cells include *sleeping beauty* (SB) and *piggyBac* (1, 5, 6). The lepidopteran *piggyBac* transposable element was initially discovered in mutant Baculovirus strains (7). Subsequent work divided the *piggyBac* element into the *piggyBac* transposase that facilitates genomic integration, and 5' and 3' inverted repeat elements (5'IR and 3'IR, respectively) delimiting the transposon cassette for integration (8–10). This arrangement has been harnessed to introduce transgenes between the inverted repeats. *Trans* introduction of the transposase and transposon results in an

efficient “cut and paste” transposition of the transgene into the genome at TTAA nucleotide elements (7, 11).

We and others have previously reported on the high efficiency of the *piggyBac* system and its ability to mediate genomic integration of transposon cassettes in human and mouse cells (3, 12–15). Recent investigations have shown the number of *piggyBac* integrations to be titratable when delivering a single transposon. Transposon elements can allow many (15 or more integrations events) per cell whereas fewer integration events (down to one or two per cell) can be achieved by limiting transposon or transposase availability (13–15). We hypothesized that the ability of *piggyBac* to integrate multiple transposon copies per cell could enable the concurrent integration of multiple independent transposons each carrying distinct transgenes as opposed to the typical paradigm of promoting expression of a single transgene.

In this study, we quantified the ability of *piggyBac* to mediate the stable integration of up to four independent transposons concurrently in human cells following a single transfection. We then harnessed this technology to generate stable cell lines expressing heteromultimeric voltage-gated sodium channels that exhibited robust functional expression of up to four different subunits and were amenable for high-throughput electrophysiological analysis. This technology has potential to enable genetic engineering of cellular models for use in drug discovery applications targeting multiprotein complexes, for simultaneous production of multiple recombinant proteins, and for treatment of genetic disorders that require the stable delivery of multiple genes.

Results

Concurrent Stable Gene Expression from Multiple *piggyBac* Transposons. We tested the capability of the *piggyBac* system to concurrently and stably transfect cells with more than one transposon. HEK-293 cells were transfected with the *piggyBac* transposase (pCMV-*piggyBac*), pT-NeomycinR encoding resistance to the antibiotic G418, and either pT-Luciferase that contains the gene for luciferase but does not confer antibiotic resistance or a non-transposon plasmid encoding luciferase (pCAGGS-Luciferase) as a negative control. Transfected cells were selected for three weeks with G418 to isolate cells stably expressing pT-NeomycinR and were then assayed for luciferase-catalyzed light generation. G418-resistant cells that had been cotransfected with pCAGGS-Luciferase produced only two colonies exhibiting luciferase expression, indicating that *piggyBac* transposition of pT-NeomycinR did not facilitate efficient stable expression from the nontranspo-

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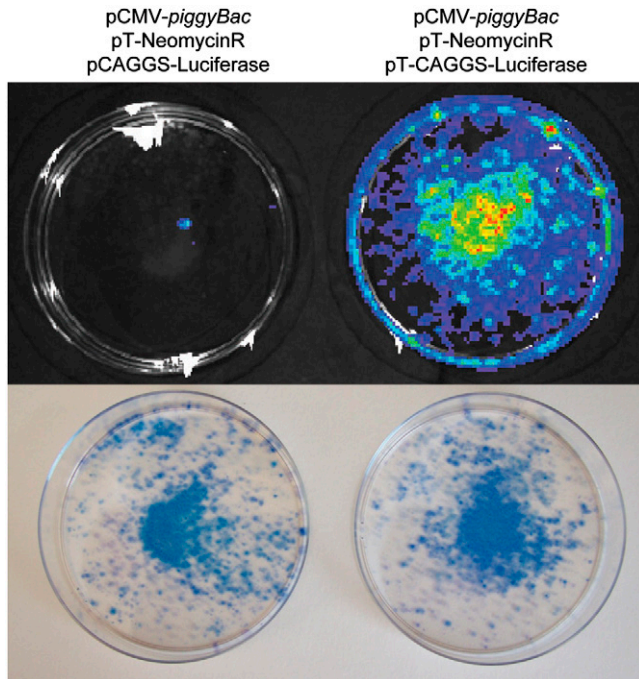


Fig. 1. Simultaneous stable integration of two piggyBac transposons. Luciferase assay comparing the triple transfection of plasmids encoding the *piggyBac* transposase (pCMV-*piggyBac*) and pT-NeomycinR with either a plasmid encoding a luciferase cDNA (pCAGGS-Luciferase, *Left*) or a luciferase transposon (pT-Luciferase) (*Right*). Transfected HEK-293 cells were cultured for 2–3 weeks with G418 to select for stable pT-NeomycinR expression and then assayed for luciferase expression (*Top*). Following imaging, plates were stained with methylene blue to visualize the cells on the plates (*Bottom*). Shown are images representative of three independent experiments.

son pCAGGS-Luciferase (Fig. 1, *Top panel, Left plate*). By contrast, most G418-resistant cell colonies cotransfected with pT-Luciferase readily produced light upon luciferin application presumably due to genomic integration of the luciferase transposon (Fig. 1, *Top panel, Right plate*). We used methylene blue to stain the plates after imaging to demonstrate that similar numbers of G418 resistant cells existed on both plates (Fig. 1, *Bottom panel*). These findings provide a proof-of-principle that *piggyBac* can facilitate the concurrent stable expression of more than one separate transposon in the same cells while only selecting for one transposon.

We next evaluated the efficiency of *piggyBac*-mediated transposition of three to four independent transposons carrying distinct transgenes. For these experiments, we used three different reporter genes including enhanced green fluorescent protein (eGFP), the *Discosoma* red fluorescent protein (DsRed), and the cluster of differentiation 8 (CD8) cell surface receptor. To quantify efficiency of *piggyBac* in stably transfecting cells with three independent transposons, HEK-293 cells were cotransfected with pCMV-*piggyBac*, pT-NeomycinR, pT-eGFP, and pT-DsRed. Importantly, pT-eGFP, pT-DsRed, and pT-CD8 lack neomycin resistance. Transfected cells were selected for 3 weeks with G418 to isolate cells stably expressing pT-NeomycinR, and then flow cytometry was used to quantify eGFP and DsRed expression. As a negative control, nontransfected and nonselected HEK-293 cells were used to establish the thresholds for fluorescent detection. Fig. 2*A Left* illustrates that nontransfected HEK-293 cells are detected within the lower left quadrant of the plot corresponding to the absence of measurable eGFP or DsRed fluorescence. By contrast, in Fig. 2*A Right*, 79% of G418-resistant cells expressed both eGFP and DsRed (upper right quadrant). For three independent experiments, flow cytometry analysis revealed

that $60 \pm 8\%$ (mean \pm SD) of single G418-resistant cells coexpressed eGFP and DsRed, indicating that *piggyBac* mediated efficient concurrent expression from three transposons.

Similarly, we determined the efficiency of *piggyBac* in enabling stable expression from four independent transposons. In these experiments, HEK-293 cells were cotransfected with pCMV-*piggyBac*, pT-NeomycinR, pT-eGFP, pT-DsRed, and pT-CD8 and then selected for 3 weeks with G418 before quantifying eGFP, DsRed, and CD8 expression simultaneously with flow cytometry. Fig. 2*B* illustrates that 43% of the cells expressed both eGFP and DsRed (upper right quadrant). Analysis of eGFP/DsRed positive cells revealed that 89% also expressed CD8. For three independent experiments, $31 \pm 3\%$ (mean \pm SD) of G418-resistant cells coexpressed eGFP, DsRed, and CD8, proving simultaneous expression from four separate transposons in human cells using only a single selectable marker.

We attempted to increase the efficiency of multiple transgene expression by decreasing the amount of the selectable transposon (100 ng), while maintaining 1 μ g each of the reporter gene transposons (pT-eGFP, pT-DsRed, and pT-CD8). However, this only resulted in fewer G418-resistant cells, and the percentage of resistant cells stably expressing the three reporter genes ($30 \pm 6\%$) was not significantly different.

Transposon-Mediated Stable Expression of a Heteromultimeric Protein Complex. To evaluate the utility of *piggyBac* mediated multitransposon delivery, we used this approach to create cell lines stably expressing a human brain voltage-gated sodium channel complex. Sodium channels are heteromultimeric complexes comprised of three component proteins including a pore-forming α -subunit (SCN1A) and two accessory subunits (SCN1B, SCN2B). We constructed two SCN1A transposons in which the sodium channel coding sequence was fused in-frame to either a triple FLAG epitope (pT-SCN1A:3XFLAG) or the fluorescent protein Venus (pT-SCN1A:Venus) and accompanied by a neomycin resistance gene driven by a separate promoter (Fig. S1). We also constructed a separate transposon (pT-SCN1B:cMyc-IRES-SCN2B:HA) containing a bicistronic cassette encoding two epitope tagged accessory subunits and a puromycin resistance gene (Fig. S1).

HEK-293 cells were cotransfected with pCMV-*piggyBac*, pT-SCN1A:3XFLAG, and pT-SCN1B:cMyc-IRES-SCN2B:HA then subjected to dual selection with G418 and puromycin. Only cells transfected with all three plasmids exhibited large numbers of resistant clones (Fig. 3). Omitting the transposase plasmid (middle culture dish in Fig. 3*A*) in what would be the traditional method of constructing stable cell lines was substantially less efficient than *piggyBac* transposition. When plating equivalent numbers of cells (100,000), cotransfection with transposase yielded 650 clones resistant to both G418 and puromycin. In contrast, transfection without transposase yielded a single colony, indicating a >600-fold increase in generating stable cells expressing the sodium channel and accessory subunits when using *piggyBac*. These results demonstrate the substantial increase in efficiency realized in generating multiple-subunit stable cell lines using the *piggyBac* transposon system.

We verified protein expression resulting from *piggyBac* transposition of pT-SCN1A:3XFLAG and pT-SCN1B:cMyc-IRES-SCN2B:HA by immunoblot analysis using antibodies directed against the tag epitopes linked to each distinct sodium channel subunit (Fig. 3*B*). Detection of the endogenous protein transferin served as a gel loading control. In addition to G418 and puromycin resistance, each of 10 selected clones also expressed SCN1A, SCN1B, and SCN2B proteins, demonstrating simultaneous stable expression of five transgenes using *piggyBac*. A saturating exposure was used in Fig. 3*B* to illustrate the variable levels of protein expression for each clone. Varying levels of protein expression may reflect differences in the number of

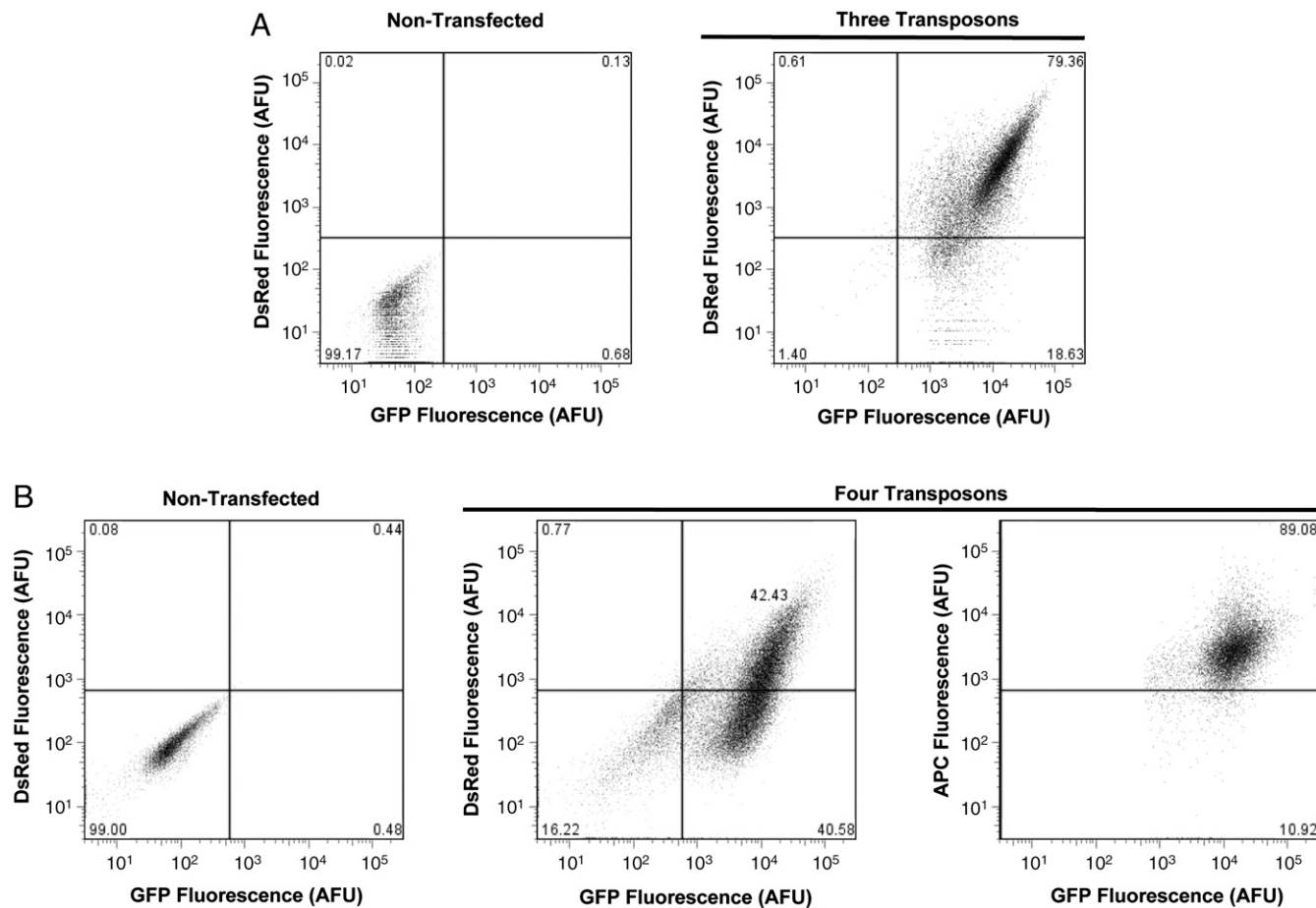


Fig. 2. Concurrent integration of three or four piggyBac transposons. Flow cytometry analysis comparing the delivery and stable coexpression of either three or four *piggyBac* transposons. (A) HEK-293 cells were cotransfected with pCMV-*piggyBac*, pT-NeomycinR, pT-GFP, and pT-DsRed. Transfected cells were selected for pT-NeomycinR expression for 3 weeks with G418. The dual expression of pT-GFP and pT-DsRed was detected in approximately 80% of the G418-resistant cells (Right panel, Upper Right quadrant). Nontransfected and nonselected HEK-293 cells were used to determine the thresholds for fluorescent detection (Left panel, Lower Left quadrant). (B) HEK-293 cells were cotransfected with pCMV-*piggyBac*, pT-NeomycinR, pT-GFP, pT-DsRed, and pT-CD8. Transfected cells were selected for pT-NeomycinR expression for 3 weeks with G418. The dual expression of pT-GFP and pT-DsRed was detected in approximately ~42% of the G418 resistant cells (center panel, upper right quadrant). Dual GFP/DsRed positive cells were analyzed for CD8 expression using an APC conjugated anti-CD8 antibody. Approximately 90% of the GFP/DsRed positive cells also expressed CD8 (right panel, upper quadarant), indicating that 30% of analyzed G418-resistant cells were positive for GFP/DsRed/CD8. Nontransfected and nonselected HEK-293 cells were used to determine the thresholds for fluorescent detection (Left panel, Lower Left quadrant). Panels A and B illustrate representative experiments ($n = 3$), and only singlet cells were subjected to analysis.

transposon integration events or positional effects at the site of integration. Protein expression from *piggyBac* integrated transposons in clone 1 was stable over 38 passages for each of the sodium channel subunits (Fig. S2). Clone 1 was used for subsequent electrophysiology experiments.

Conventional whole-cell patch-clamp recording was used to functionally evaluate the sodium channel stable cell line. Fig. 4A illustrates representative current recordings in response to step depolarizations from a holding potential of -120 mV to between -80 and $+20$ mV. Sodium currents were recorded at passages 1 (Left) and 21 (Right), and Fig. 4B illustrates the average peak current density recorded at -10 mV. This analysis normalizes peak currents by cell capacitance to allow direct comparison of the channel expression level in cells of differing sizes. The current density recorded at passages 1 and 21 were not different (-338 ± 71 pA/pF, $n = 5$ versus -321 ± 58 pA/pF, $n = 5$, respectively), reflecting the high temporal stability of functional protein expression. Moreover, the voltage dependence of sodium channel activation was not changed following 20 passages (passage 1 $V_{1/2} = -23.7 \pm 1.4$ mV, $k = 7.3 \pm 0.4$, $n = 5$; compared to passage 21 $V_{1/2} = -23.8 \pm 0.9$ mV, $k = 6.3 \pm 0.3$, $n = 5$).

High-Throughput Analysis of Sodium Channel Cell Lines. The utility of a sodium channel stable cell line created using transposon technology for high-throughput analysis was investigated using an automated planar patch-clamp system. Fig. 5A illustrates a representative family of sodium current recordings at passages 4 and 38 in response to step depolarizations from a holding potential of -120 mV to between -80 and $+20$ mV. The average current density recorded at passage 4 was -402 ± 64 pA/pF ($n = 27$), and this was not significantly different from that measured by traditional patch clamp. The peak current density-voltage relationship (Fig. 5B) and activation curve (Fig. 5C) for the voltage-gated sodium current expressed by stable cells at passage 4 are indistinguishable from data collected using manual patch clamp. The sodium current density measured at passage 38 was 522 ± 82 pA/pF ($V_{1/2} = -20.3 \pm 2.2$ and $k = 6.1 \pm 0.5$, $n = 21$), further supporting the high temporal stability of *piggyBac*-mediated integration. There were no statistically significant differences between data recorded at passage 4 and passage 38 (current density or activation; Table S1). These data demonstrate that the stable cell line generates voltage-gated sodium currents that are suitable for high-throughput electrophysiology and are potentially useful for drug discovery.

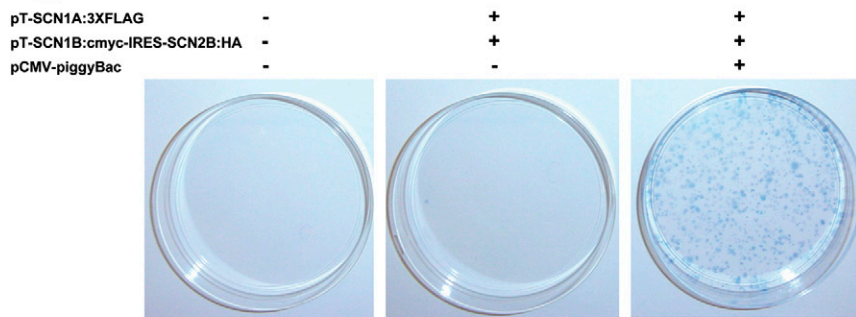
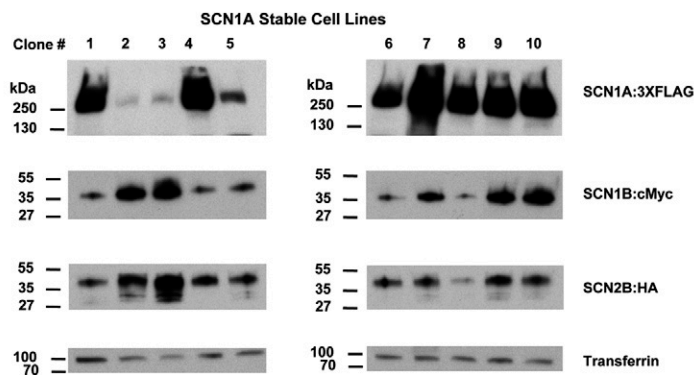
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Fig. 3. Creation of a SCN1A stable cell line by simultaneous genomic integration of two piggyBac transposons. Efficient stable integration of sodium channel transgenes by *piggyBac* transposition. (A) Methylene blue staining of HEK-293 colonies following transfection with the indicated plasmids and selection with G418/Puromycin for 3 weeks. Colonies stably coexpressing pT-SCN1A:3XFLAG and pT-SCN1B:cMyc-IRES-SCN2B:HA were able to grow in the presence of G418/Puromycin selection. (B) Protein expression from each *piggyBac* transposon confirmed for ten selected clones by Western blot analysis. Transgene specific proteins were detected by primary antibodies directed against the appropriate epitope tag (FLAG, cMyc, or HA). Detection of the endogenous protein transferrin confirms protein loading.

Construction of a Heterozygous Sodium Channel Cell Line. We employed *piggyBac* transposons to create cell lines stably expressing two alleles of SCN1A by concurrent integration of three separate

large transposons. HEK-293 cells were cotransfected with pCMV-*piggyBac*, pT-SCN1A:Venus, pT-SCN1A:3XFLAG, and pT-SCN1B:cMyc-IRES-SCN2B:HA. Fig. 6A illustrates immunoblot detection of the four component proteins expressed in G418/puromycin-resistant cells visualized with antibodies directed against the epitopes Venus, FLAG, c-Myc, and HA. Importantly, genomic integration of either pT-SCN1A:Venus or pT-SCN1A:3XFLAG would confer resistance to G418, but due to the efficiency of the *piggyBac* system, every assayed clone expressed both SCN1A alleles. Electrophysiological recording experiments demonstrated robust sodium currents in the cells (Fig. 6). Moreover, these data demonstrate the stable expression of at least six transgenes from 27 kb of DNA encoded in three separate transposons stably transfected into the genome of human cells. These data demonstrate that *piggyBac* is capable of stably transfecting cell lines with multiple transgenes, including those encoded by large cDNAs.

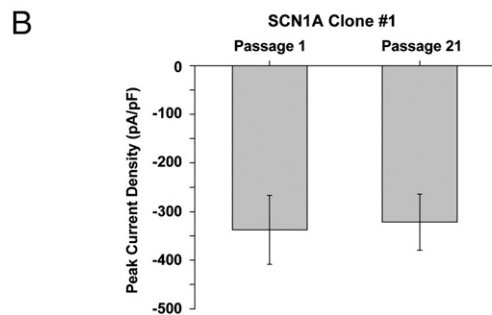
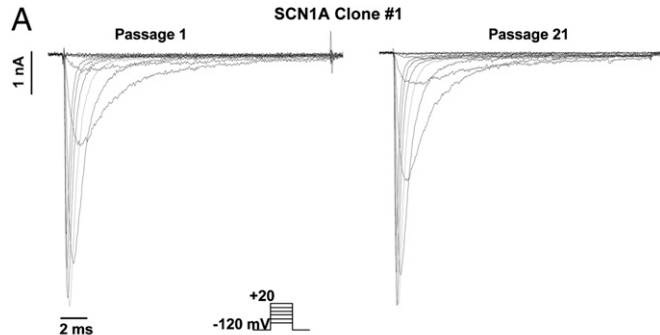


Fig. 4. Functional expression from SCN1A transposons exhibits high temporal stability. Functional analysis of SCN1A clone 1 by conventional whole-cell patch-clamp recording of voltage-gated sodium currents at passage number 1 and 21. (A) Representative voltage-gated sodium currents activated by voltage steps to between -80 and +20 mV from a holding potential of -120 mV (see pulse protocol *Inset*). The current in response to the -10 mV step is shown in red. (B) Average sodium current density recorded at -10 mV plotted at passage number 1 and 21 ($n = 6$).

Discussion

In this study, we demonstrated that the plasmid-based *piggyBac* transposon system can facilitate efficient and concurrent genomic integration of multiple gene segments in human cells. Our results have potential applicability to the creation of cell lines with stable expression of multiprotein complexes useful for drug discovery, developing complex cellular models, and possibly for therapeutic gene transfer when more than one transgene is required.

Specifically, we report on the efficient and stable integration of up to four separate *piggyBac* transposons in human HEK-293 cells. In experiments with reporter genes, having only one of the transposons (pT-NeomycinR) confer resistance to a selectable marker was sufficient to isolate cells stably expressing the other transposons that lacked a means for selection. Our experiments with three independent transposons demonstrated that after selection, approximately 60% of the cells stably express transgenes encoded by the three transposons. As expected, the efficiency decreases when selecting for stable expression of four independent transposons. However, the ability to efficiently stably integrate four independent DNA segments from a single transfection is unprecedented. Moreover, the vectors employed

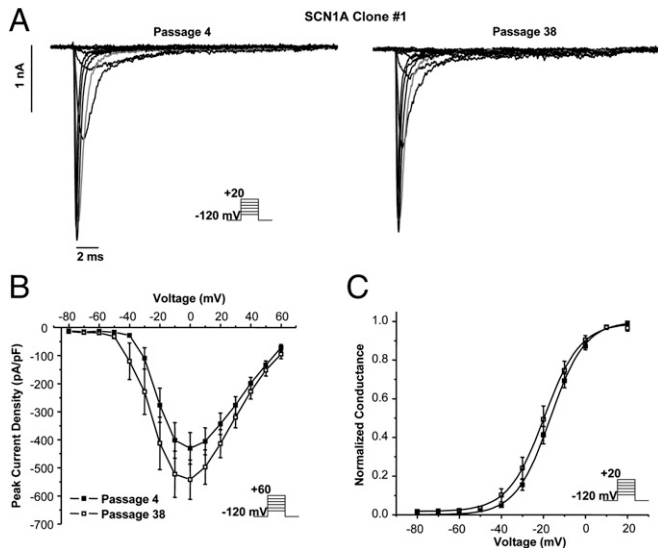


Fig. 5. High-throughput planar patch-clamp analysis of SCN1A transposon expression. Voltage-gated sodium currents from SCN1A clone 1 were measured at passages 4 and 38 using an automated, high-throughput electrophysiology workstation (Nanion Patchliner). (A) Representative voltage-gated sodium currents activated by voltage steps to between -80 and $+20$ mV from a holding potential of -120 mV (see pulse protocol *Inset*). The current in response to the -10 mV step is shown in red. (B) Peak current density elicited by test pulses to various potentials and normalized to cell capacitance. (C) Voltage dependence of channel activation measured during voltage steps to between -80 and $+20$ mV. There were no statistical differences in current density and activation $V_{1/2}$ between data recorded at passage 4 and passage 38 ($n = 27$ and $n = 21$, respectively).

in this study used bicistronic IRES elements. Therefore, our data suggest the possibility of introducing up to eight transgenes on four independent transposon cassettes. The use of polycistronic transposons for generating induced pluripotent stem cells has recently been demonstrated (2, 4). We anticipate the number

of independent transposons might be increased further by implementing innovative cell sorting methodologies to isolate the clonal populations of interest.

Comparisons with Other Gene Transfer Strategies. In comparison to other available systems such as other transposons and viral vectors, *piggyBac* may offer distinct advantages. More than 10 years of research has contributed to engineering SB transposase to produce several hyperactive variants including SB100X that in some cases exhibits 100-fold more activity than the original SB (16). However, the capabilities of SB100X to carry large DNA segments and mediate multiple transposon integration are not known. Research efforts are also underway to enhance *piggyBac* transposase activity and this should further improve efficiency in multigene transfer (17).

Retroviral and lentiviral vectors have also been used for codelivery of multiple genes to cells (18, 19). However, neither of these viral vectors can integrate DNA segments as large as 10 kb, and coinfection with multiple different viruses is required for integration of multiple transgenes. A plasmid-based system has other advantages compared to viral vectors with respect to shorter production time and lower cost.

Applications for Multigene Integration. Multiplexed gene integration has potential for application in the creation of novel cell-based assays for drug discovery when the target is a multiprotein complex such as a G-protein coupled receptor (GPCR). As a single class of proteins, GPCRs represent targets for approximately 30% of all marketed drugs (20). Further, GPCR signaling is important in all cells, but developing cell type specific pathways within a heterologous system can be complex and difficult. Often, commonly used GPCR cell culture models lack critical components necessary to reconstitute the appropriate signaling pathways. These missing components can compromise the drug response and/or prevent the discovery of novel agonists and antagonists. Therefore, the ability to use a plasmid-based system to create stable cell lines expressing multiple transgenes may improve upon current drug discovery methodology (21).

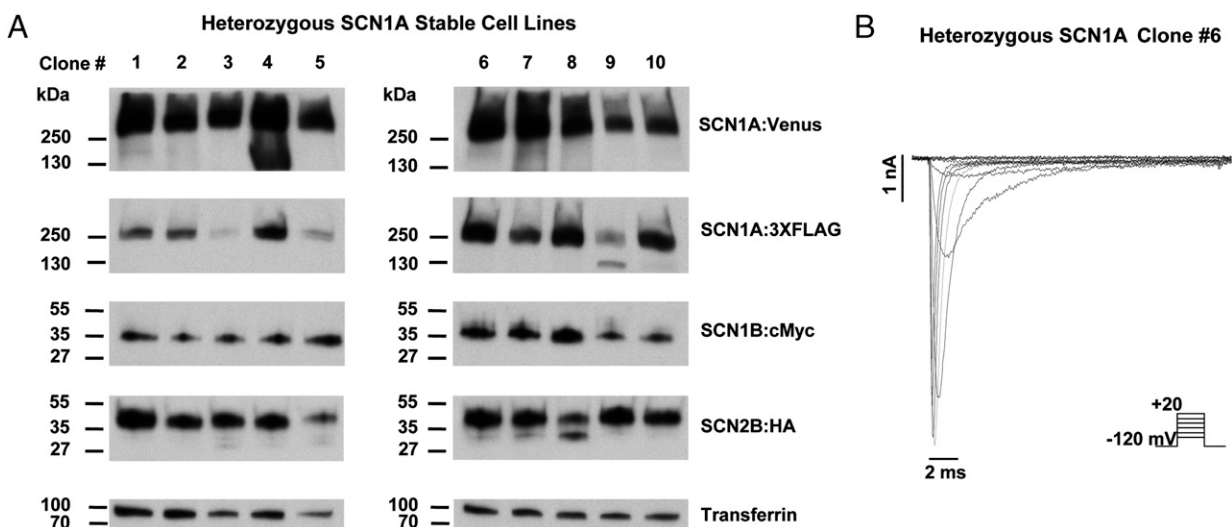


Fig. 6. Creation of a heterozygous SCN1A stable cell line by simultaneous genomic integration of three *piggyBac* transposons. Efficient stable integration of SCN1A transgenes by *piggyBac* transposition. Cells stably coexpressing either pT-SCN1A:Venus or pT-SCN1A:3XFLAG with pT-SCN1B:cMyc-IRES-SCN2B:HA transposons are able to grow in the presence of G418/Puromycin selection. (A) Expression from each *piggyBac* transposon confirmed for ten selected clones by Western blot analysis. Transgene specific proteins were detected by primary antibodies directed against the appropriate epitope tag (Venus, FLAG, cMyc, or HA). Note that every clone expresses both alleles of SCN1A. Detection of the endogenous protein transferrin confirms protein loading. (B) Functional analysis of heterozygous SCN1A clone 6 by conventional whole-cell patch-clamp recording of voltage-gated sodium currents. Representative currents activated by voltage steps to between -80 and $+20$ mV from a holding potential of -120 mV (see pulse protocol *Inset*). The current in response to the -10 mV step is shown in red. The average current density for the -10 mV step recorded at passage 1 was -489 ± 53 pA/pF ($n = 5$). Fitting an activation curve with a Boltzmann equation generated the average fit parameters of $V_{1/2} = -23.6 \pm 1.7$ and $k = 5.7 \pm 0.4$ ($n = 5$).

Multisubunit neurotransmitter receptors (e.g., GABA_A receptors) and ion channels (e.g., voltage-gated sodium, potassium, and calcium channels) are also important therapeutic targets. Discovery of novel compounds that target these proteins has been hampered by low-throughput assay methods and difficulty in reconstituting multiprotein complexes. Recent advances in high-throughput electrophysiology are beginning to address the first of these bottlenecks (22). However, traditional transfection methods are unable to efficiently generate stable cell reagents when the target is comprised of multiple protein subunits. The *piggyBac* transposon system represents a paradigm shift that may overcome this bottleneck in high-throughput drug discovery for ligand and voltage-gated ion channel targets.

In this study, we demonstrated that the *piggyBac* system was able to stably reconstitute a three subunit sodium channel complex. Creation of this cell line required only a single transfection followed by approximately 5 weeks of colony selection and expansion. In addition, sodium channel activity exhibited by this cell line was more robust than typically observed for transient transfection experiments (23). Moreover, the level of the sodium currents were stable for more than 35 passages and were easily

measured using a commercial high-throughput electrophysiology system. Traditional methods of generating this cell line would have required multiple rounds of transfection and selection with a total time commitment of many months. Our strategy employing *piggyBac* transposon technology may also enable creation of biallelic cell models to mimic certain genetic conditions as we have demonstrated by establishing cells with stable expression of two sodium channels combined with their associated proteins. This cell model would have been difficult, if not impossible, to create with traditional methods. From these illustrations, we conclude that *piggyBac* offers distinct advantages over traditional methodologies for the delivery and stable genomic integration of multiprotein complexes.

Materials and Methods

For descriptions of the materials and methods used, see *SI Materials and Methods*.

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