Novel strategies for gene trapping and insertional mutagenesis mediated by *Sleeping Beauty* transposon

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Keywords: insertional mutagenesis, transposon, *Sleeping Beauty*, gene trapping, poly(A) trapping

Submitted: 05/28/2013

Revised: 09/10/2013

Accepted: 09/15/2013

Citation: Song G, Cui Z. Novel strategies for gene trapping and insertional mutagenesis mediated by *Sleeping Beauty* transposon. Mobile Genetic Elements 2013; 3:e26499; <http://dx.doi.org/10.4161/mge.26499>

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Commentary to: Song G, Li Q, Long Y, Gu Q, Hackett PB, Cui Z. Effective gene trapping mediated by *Sleeping Beauty* transposon. PLoS One 2012; 7:e44123; PMID:22952894; <http://dx.doi.org/10.1371/journal.pone.0044123> and Song G, Li Q, Long Y, Hackett PB, Cui Z. Effective expression-independent gene trapping and mutagenesis mediated by *Sleeping Beauty* transposon. J Genet Genomics 2012; 39:503–20; PMID:23021550; <http://dx.doi.org/10.1016/j.jgg.2012.05.010>

Gene and poly(A) trappings are high-throughput approaches to capture and interrupt the expression of endogenous genes within a target genome. Although a number of trapping vectors have been developed for investigation of gene functions in cells and vertebrate models, there is still room for the improvement of their efficiency and sensitivity. Recently, two novel trapping vectors mediated by *Sleeping Beauty* (SB) transposon have been generated by the combination of three functional cassettes that are required for finding endogenous genes, disrupting the expression of trapped genes, and inducing the excision of integrated traps from their original insertion sites and then inserting into another gene. In addition, several other strategies are utilized to improve the activities of two trapping vectors. First, activities of all components were examined *in vitro* before the generation of two vectors. Second, the inducible promoter from the tilapia Hsp70 gene was used to drive the expression of SB gene, which can mediate the excision of integrated transposons upon induction at 37 °C. Third, the Cre/LoxP system was introduced to delete the SB expression cassette for stabilization of gene interruption and bio-safety. Fourth, three stop codons in different reading frames were introduced downstream of a strong splice acceptor (SA) in the gene trapping vector to effectively terminate the translation of trapped endogenous genes. Fifth, the strong splicing donor (SD) and AU-rich RNA-destabilizing element exhibited no obvious insertion

bias and markedly reduced SD read-through events, and the combination of an enhanced SA, a poly(A) signal and a transcript terminator in the poly(A) trapping vector efficiently disrupted the transcription of trapped genes. Thus, these two trapping vectors are alternative and effective tools for large-scale identification and disruption of endogenous genes in vertebrate cells and animals.

The rapid advent of high-throughput DNA sequencing technologies has enabled the completion of genome projects for a large number of species including human and other model species. The availability of these genomes has in turn advanced biological researches into the post-genome era, which integrates classical experimental approaches with those of “omics” technologies to elucidate functions of identified genes in various processes such as embryonic development and human diseases. A number of mutagenic methods have long been developed to decipher functions of genes in model animals. For instance, chemical mutation by N-ethyl-N-nitrosourea (ENU) has produced a large number of zebrafish mutants with distinct phenotypes¹⁻³ but identification of mutated genes responsible for these phenotypes by positional cloning is extremely laborious and time consuming.

To address these limitations, retroviral and transposable DNA elements have been used as insertional mutagens,⁴⁻⁷ which has greatly facilitated the identification of mutated genes since the gene loci are tagged by the inserted DNA

Figure 1. The flowchart of gene trapping with pT2/Gene-Trap. Insertion of the gene-Trap cassette into an intron of transcriptionally active loci can generate a fusion transcript that contains the upstream exon and the reporter marker. The expression of SB11 gene was driven by the heat-inducible promoter TiHsp70. IR/DR(L) and IR/DR(R), left and right inverted repeat/directed repeat of the SB transposon; SA, splice acceptor; IRES, internal ribosome entry site; GFP, GFP reporter gene; poly(A), poly(A) signal; TiHsp70, tilapia Hsp70 promoter; SB11, SB11 transposase gene.

sequence. However, the mutation rates generated by retroviral or transposon vectors are approximately 7–10 fold lower than that of ENU mutation. Insertional mutagenesis mediated by the pseudotyped retrovirus was found to be very effective for the generation of zebrafish mutants with altered phenotypes in 1 out of 80 to 100 such insertions,^{4,8} but for some reason the retroviral vectors have a preference for integrating in the 5'-transcriptional regulatory regions of genes⁹ and tend to create mutations in genes that are expressed during early developmental stages. Apart from the 5' integration bias, retroviral vectors have intrinsic drawbacks such as limited packaging size, gene expression silencing and ectopic reporter gene expression.

Transposon systems have recently been exploited as potential tools for insertional mutagenesis in mouse and zebrafish^{10–12} due to their less demanding of laboratory and technical conditions when compared with those of pseudotyped retrovirus. These systems were also used to develop enhancer-trapping and gene-trapping cassettes and a number of transgenic fish lines with different reporter gene expression patterns were generated.^{13,14} The transgenic efficiency of *Sleeping Beauty* (SB)-based gene delivery vector was improved about 6-fold (from 5% to 31%)¹⁵ and SB-based insertional mutagenesis screens were introduced in mouse¹⁶ and zebrafish¹⁷ to elucidate gene

functions in all kinds of genetic networks and pathways. Indeed, the SB system was successfully used to identify temporal and spatial expression and functions of genes in developmental pathways⁷ and has proved to be a highly instrumental tool to induce tumors in experimental animals for the purpose of uncovering the genetic basis of diverse cancers.^{18,19} In addition, it has been shown that Tol2 transposon-based enhancer trapping renders the comparable mutagenic efficiency with that of retroviral insertions²⁰ and Tol2-based gene trap systems were demonstrated to be useful for the study of developmental processes and gene discovery in zebrafish.^{14,21,22} Therefore, transposon-mediated insertional mutagenesis offers great potential for loss-of-function screening in model animals. However, tremendous efforts are being made to increase the mutagenic potential of transposon-based gene trapping constructs currently available.^{23–25}

The conventional gene trapping vector contains a promoterless marker/reporter gene flanked by an upstream splice acceptor (SA) and a downstream poly(A) signal. Once inserted into an intron or exon of active genes, the trap cassette is transcribed from the endogenous promoter and the SA intercepts endogenous normal splicing through the generation of a fused transcript between upstream exon and the marker/reporter gene. Since the fusion transcript is prematurely terminated

at the foreign poly(A) site, it encodes a truncated and often non-functional version of cellular protein and the marker/reporter.^{26,27} The key cassette in nearly all gene trapping constructs contains a highly efficient SA and a poly(A) signal. Without such a transcriptional termination cassette, the splicing of endogenous gene transcripts around the trapping vector can readily occur and thus result in an insertion that may not effectively interrupt the gene function at the insertion locus.^{6,28}

Recently, we have developed a novel gene trapping vector pT2/Gene-Trap (Fig. 1).²⁹ The activities of all components in this vector were tested in cultured HeLa cells using an exon trapping vector pSPL3.³⁰ When inserted into an intron of the pSPL3, the SA properly spliced with the upstream exon and efficiently terminated the transcription of downstream exons. In the circumstance of integration into an exon of the modified pSPL3, the trapping cassette directly disrupted the exon and blocked the transcription of downstream exons. The activity of this vector in trapping endogenous genes was further examined in HeLa cells and developing zebrafish embryos. A number of transposon inserts were identified in G418-selected cell colonies and the normal splicing of trapped endogenous genes was totally disrupted by the mutation cassette, which contains a SA signal originated from the carp β -actin gene and a short exon sequence. The short exon contains three

Figure 2. The flowchart of gene trapping with pT2/Poly(A)-Trap. Insertion of the poly(A)-trap cassette into an intron before a functional poly(A) sequence for an endogenous gene leads to the generation of a stable pre-mRNA and the proper splicing between the trap SD with a SA downstream of the insertion site and gives rise to a fusion transcript for GFP expression. The proper expression of trapped gene was disrupted by the formation of another fusion transcript that contains exons upstream of the insert site. The expression of SB11 gene was driven by the heat-inducible promoter TiHsp70. IR/DR(L) and IR/DR(R), left and right inverted/directed repeats of the SB transposon; SA, splice acceptor from carp β -actin gene; Exon, the exon 2 from the carp β -actin gene; SV40 poly(A), SV40 polyadenylation sequence; TT, transcript terminator sequence. TiHSP70, tilapia *Hsp70* promoter; SB11, SB11 transposase gene. polyA, a poly(A) signal from carp β -actin gene; SV40, SV40 promoter; GFP, GFP reporter gene; IRES, internal ribosome entry site; SD, splice donor; ARE, AU-rich element.

stop codons in different reading frames (TGA ATT AGT GA) and can efficiently truncate the translation of trapped gene transcripts. It has been shown that the internal ribosomal entry sequence (IRES) inserted in front of a marker/reporter gene can mediate the independent translation of marker/reporter gene and the IRES-based vectors are able to capture a wide range of genes expressed in a variety of tissues and embryos at different developmental stages.^{31,32} In our trapping vector, the IRES containing 12 AUGs and an A6 (AAAAAA) bifurcation loop, which performs better in zebrafish than another IRES element containing 10 AUGs and an A7 (AAAAAAA) bifurcation loop.²⁹ In addition, the SA signal from the carp β -actin gene coupled with a poly(A) signal of ocean pout antifreeze gene can severely disrupt the expression of trapped genes by producing a truncated fusion transcript.²² However, conventional gene trapping is not suitable to capture genes that are not or poorly expressed.

To circumvent the limitation, a number of poly(A)-trap vectors have been generated. A basic poly(A)-trap vector contains a reporter/selectable marker gene flanked by an upstream constitutive promoter and a downstream splice donor (SD). Integration of a

trap cassette upstream of a functional poly(A) signal within a genome leads to the generation of a stable premRNA and the proper splicing of the SD with a SA downstream of the insertion site and thus gives rise to a fusion transcript that encodes the reporter/selectable marker and an N-terminal truncated version of endogenous protein. Theoretically, poly(A)-trapping is able to capture endogenous genes almost equally regardless of their transcriptional status in a target genome.³³⁻³⁶ Nevertheless, later findings indicate that these basic poly(A)-trap vectors have a bias toward the last introns of trapped genes because of the activation of nonsense-mediated mRNA decay (NMD) mechanism.³⁷ Moreover, most of poly(A)-trap vectors currently available still suffer from other problems such as the background SD read-through events. Therefore, we constructed a novel trap vector pT2/poly(A)-Trap (Fig. 2), which can effectively capture endogenous genes independent of their transcriptional status and inevitably disrupted the transcription of trapped genes in HeLa cells and zebrafish.³⁸ Importantly, the poly(A) trap vector contains an AU-rich RNA-destabilizing element that is highly effective for reduction of the background SD read-through events.

The integration of two gene trapping cassettes into target genomes is mediated by the SB transposon. It is known that exons account for about 1% of the human genome and approximately 24% of the genome is spanned by introns.³⁹ There still exist large fractions of non-coding sequences in the human genome. Given that the copy numbers of SB-mediated inserts are usually less than 10 copies per genome,^{40,41} the possibility of mutating a gene across a target genome by limited number of transposon inserts remains relatively low. However, a low copy number of inserts allows easier attribution of a mutation to a phenotypic effect and favors the investigation of gene functions. To balance the requirements, an effective cassette was introduced into our gene- and poly(A)-trapping vectors. This cassette controls the expression of SB11 transposase by a Hsp70 promoter from tilapia. The heat-inducible expression of SB transposase leads to the remobilization of transposons inserted in non-coding regions of a target genome and thus improves the efficiency of trapping endogenous genes. Indeed, the trapping cassette can be excised from the original insertion site and new integration patterns were generated after the inducible expression of SB11 transposase.²⁹ Recently, we have obtained 22 stable

Figure 3. The schematic of gene trapping in zebrafish. The transposon-based gene trapping constructs pT2/Gene-Trap or pT2/Poly(A)-Trap and in vitro synthesized SB11 mRNA were co-microinjected into one-cell stage zebrafish embryos to generate transgenic zebrafish lines carrying the gene trapping cassette. Transgenic individuals expressing GFP can be directly used for the analysis of interrupted genes, while other adults showing no GFP expression can be treated with heat-shock during spermatogenesis or oogenesis to obtain offspring with novel insertions and GFP expression. A few of zebrafish individuals expressing GFP in distinct tissues are shown in the right panel.

zebrafish lines from 300 individuals with different patterns of GFP expression by using these two novel trapping vectors (Fig. 3), their trapping rates were about the same as that of the gene trap vector pT2/PAT5.⁷ To avoid the leaky expression of SB transposase gene, the TiHsp7-SB11 cassette was flanked by two Cre/loxP sites for inducible deletion of this cassette by microinjection of capped Cre recombinase mRNA into zebrafish embryos to stabilize the mutants.

DNA transposons have a unique ability to move about in the genome. This inherent feature allows these transposable elements to be harnessed as gene vectors for genetic manipulation. In invertebrate models such as *C. elegans* and *Drosophila*, transposons

have been successfully employed for transgenesis and insertional mutagenesis, but until the reactivation of the Sleeping Beauty transposon in 1997,⁴² there was no indication of such transposons sufficiently active in vertebrates. The reconstructed SB system is composed of a DNA transposon and a transposase that are both indispensable for transposition. The transposon contains two inverted repeat/direct repeat (IR/DR) elements, in which any DNA sequence such as gene of interest or trapping cassette can be introduced. The transposase was resurrected through the correction of accumulated mutations in the genomes of salmonid fish. Typically, binding of two transposase molecules to

each IR/DR is required for SB transposition via a “cut-and-paste” mechanism. This system was later improved by further corrections of mutation in IR/DR and SB gene to generate the T2 transposon,⁴³ SB11 transposase⁴⁴ and hyperactive SB 100X transposase.⁴⁵ These new versions of the SB system appear to be more active than the original one and are highly effective in vertebrates.^{45–48}

Over the past two decades, the SB transposon system was found to be highly active in human cells, fish, mouse, frog and rat.⁴⁹ Accordingly, the SB transposon system is successfully used for long-term expression in transgenesis,^{47,48,50} insertional mutagenesis in animals¹¹ and therapeutic

somatic gene vehicle.⁵¹ Moreover, SB-mediated integration exhibits less regional preference than retroviruses and is not significantly influenced by transcriptional activity.^{52,53} Therefore, the SB transposon is widely accepted as

one of ideal tools for gene transfer and insertional mutagenesis. Obviously, our SB-based trapping vectors are alternative tools for large-scale mutagenesis in cells and vertebrates and will contribute to the saturation gene mutation across the

genomes of model animals including mouse and zebrafish.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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