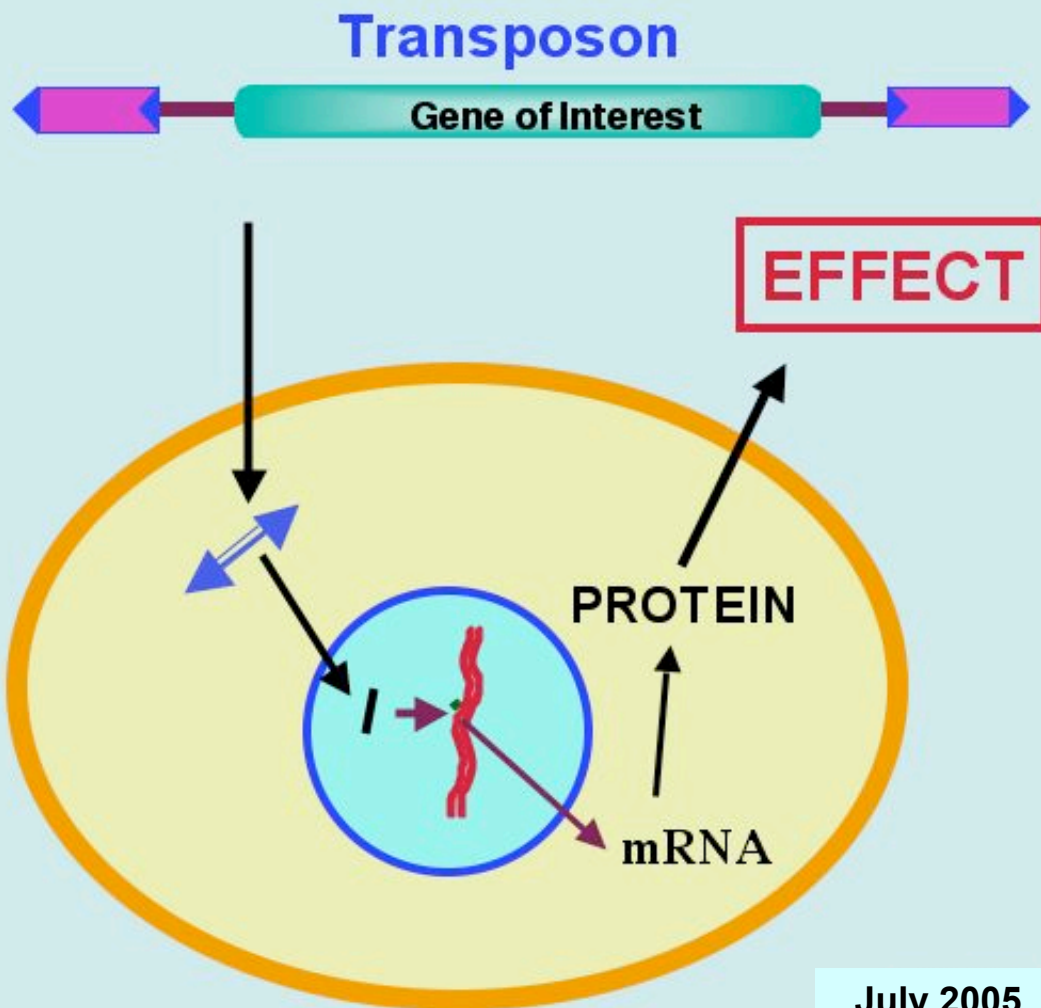


Discovery Genomics, Inc.

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For the

# The *Sleeping Beauty* Transposon System



**A. Mechanisms of Action of the Sleeping Beauty Transposon System**

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#### **E. Other Sleeping Beauty Transposon References**

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**DISCOVERY GENOMICS, INC.****References on the *Sleeping Beauty* Transposon System****Mechanisms of Action of SBTS:**

- 1. Ivics, Z., P. B. Hackett, R. H. Plasterk and Z. Izsvak (1997). "Molecular reconstruction of *Sleeping Beauty*, a Tc1-like transposon from fish, and its transposition in human cells." *Cell* 91: 501-510. [SEMINAL REPORT]**

Members of the Tc1/mariner superfamily of transposons isolated from fish appear to be transpositionally inactive due to the accumulation of mutations. Molecular phylogenetic data were used to construct a synthetic transposon, *Sleeping Beauty*, which could be identical or equivalent to an ancient element that dispersed in fish genomes in part by horizontal transmission between species. A consensus sequence of a transposase gene of the salmonid subfamily of elements was engineered by eliminating the inactivating mutations. *Sleeping Beauty* transposase binds to the inverted repeats of salmonid transposons in a substrate-specific manner, and it mediates precise cut-and-paste transposition in fish as well as in mouse and human cells. *Sleeping Beauty* is an active DNA-transposon system from vertebrates for genetic transformation and insertional mutagenesis.

- 2. Izsvak, Z., Z. Ivics and R. H. Plasterk (2000). "*Sleeping Beauty*, a wide host-range transposon vector for genetic transformation in vertebrates." *J. Mol. Biol.* 302: 93-102.**

In contrast to mariner transposons, which are regulated by overexpression inhibition, the frequency of *Sleeping Beauty* (SB) transposition was found to be roughly proportional to the amount of transposase present in cells. Unlike Tc1 and mariner elements, SB contains two binding sites within each of its terminal inverted repeats, and we found that the presence of both of these sites is a strict requirement for mobilization. In addition to the size of the transposon itself, the length as well as sequence of the DNA outside the transposon have significant effects on transposition. As a general rule, the closer the transposon ends are, the more efficient transposition is from a donor molecule. We have found that SB can transform a wide range of vertebrate cells from fish to human. However, the efficiency and precision of transposition varied significantly among cell lines, suggesting potential involvement of host factors in SB transposition. A positive-negative selection assay was devised to enrich populations of cells harboring inserted transposons in their chromosomes. Using this assay, of the order of 10,000 independent transposon insertions can be generated in human cells in a single transfection experiment. *Sleeping Beauty* can be a powerful alternative to other vectors that are currently used for the production of transgenic animals and for human gene therapy.

- 3. Karsi A., B. Moav, P.B. Hackett and Z. Liu (2001) "Effects of insert size on transposition efficiency of the *Sleeping Beauty* transposon in mouse cells." *Mar. Biotechnol.* 3: 241-245.**

Transposon vectors are widely used in prokaryotic and lower eukaryotic systems. However, they were not available for use in vertebrate animals until the recent reconstitution of a synthetic fish transposon, *Sleeping Beauty* (SB). The reacquisition of transposability of the SB transposase fostered great enthusiasm for using transposon vectors as tools in

vertebrate animals, particularly for gene transfer to facilitate accelerated integration of transgenes into chromosomes. Here, we report the effects of insert sizes on transposition efficiency of *SB*. A significant effect of insert size on efficiency of transposition by *SB* was found. The *SB* transposase enhanced the integration efficiency effectively for *SB* transposon up to approximately 5.6 kb, but lost its ability to enhance the integration efficiency when the transposon size was increased to 9.1 kb. This result indicates that the *SB* transposon system is highly applicable for transferring small genes, but may not be applicable for transferring very large genes.

**4. Cui, Z., A. M. Geurts, G. Liu, C. D. Kaufman and P. B. Hackett (2002). "Structure-function analysis of the inverted terminal repeats of the sleeping beauty transposon." J. Mol. Biol. 318: 1221-1235.**

Translocation of *Sleeping Beauty* (*SB*) transposon requires specific binding of *SB* transposase to inverted terminal repeats (ITRs) of about 230 bp at each end of the transposon, which is followed by a cut-and-paste transfer of the transposon into a target DNA sequence. The ITRs contain two imperfect direct repeats (DRs) of about 32 bp. The outer DRs are at the extreme ends of the transposon whereas the inner DRs are located inside the transposon, 165-166 bp from the outer DRs. Here we investigated the roles of the DR elements in transposition. Although there is a core transposase-binding sequence common to all of the DRs, additional adjacent sequences are required for transposition and these sequences vary in the different DRs. As a result, *SB* transposase binds less tightly to the outer DRs than to the inner DRs. Two DRs are required in each ITR for transposition but they are not interchangeable for efficient transposition. Each DR appears to have a distinctive role in transposition. The spacing and sequence between the DR elements in an ITR affect transposition rates, suggesting a constrained geometry is involved in the interactions of *SB* transposase molecules in order to achieve precise mobilization. Transposons are flanked by TA dinucleotide base-pairs that are important for excision; elimination of the TA motif on one side of the transposon significantly reduces transposition while loss of TAs on both flanks of the transposon abolishes transposition. These findings have led to the construction of a more advanced transposon that should be useful in gene transfer and insertional mutagenesis in vertebrates.

**5. Harris, J. W., D. D. Strong, M. Amoui, D. J. Baylink and K. H. Lau (2002). "Construction of a Tc1-like transposon *Sleeping Beauty*-based gene transfer plasmid vector for generation of stable transgenic mammalian cell clones." Anal. Biochem. 310: 15-26.**

We have constructed a single plasmid-, Tc1-like transposon-based gene transfer vector, termed the Prince Charming vector (pPC). The pPC vector was constructed by ligating the CMV-driven "*Sleeping Beauty*" transposase gene downstream to the Tc1-like transposon inverted repeat (IR) elements and by inserting the RSV promoter (to drive expression of the gene-of-interest) along with a multiple cloning site (MCS), a polyadenylation signal, and the SV40 promoter-driven neomycin gene, at a site flanked by the transposon IR elements. To assess the utility of the pPC vector, we cloned a red fluorescent protein (RFP) gene into the pPC vector at the MCS and transfected human TE85 osteosarcoma cells with the pPC-RFP expression vector using Effectene. Stable transgenic cell clones expressing RFP were selected with G418 sulfate and individual clones were isolated. After 4 weeks of clonal isolation and expansion, 99% of cells in each randomly selected clone expressed RFP

strongly. Aliquots of each clone were then maintained in either the presence or the absence of G418 sulfate and were passaged weekly. Even after 6 months in culture in the absence of G418 sulfate, approximately 90% of the cells in each clone still maintained a strong expression level of RFP, indicating that these transgenic cell clones were stable and that the clonal stability of these clones did not require a constant selection pressure. In conclusion, we have developed a single plasmid-, Tc1-like transposon-based gene transfer vector that can be used to generate stable transgenic mammalian cell clones.

**6. Izsvak, Z., D. Khare, J. Behlke, U. Heinemann, R. H. Plasterk and Z. Ivics (2002). "Involvement of a bifunctional, paired-like DNA-binding domain and a transpositional enhancer in *Sleeping Beauty* transposition." *J. Biol. Chem.* **277**: 34581-34588.**

*Sleeping Beauty* (SB) is the most active Tc1/mariner-like transposon in vertebrate species. Each of the terminal inverted repeats (IRs) of SB contain two transposase binding sites (DRs). This feature, termed the IR/DR structure, is conserved in a group of Tc1-like transposons. The DNA-binding region of SB transposase, similar to the paired domain of Pax proteins, consists of two helix-turn-helix subdomains (PAI+RED=PAIRED). The N-terminal PAI subdomain was found to play a dominant role in contacting the DRs. Transposase was able to bind to mutant sites retaining the 3-part of the DRs; thus, primary DNA-binding is not sufficient to determine the specificity of the transposition reaction. The PAI subdomain was also found to bind to a transpositional enhancer-like sequence within the left IR of SB, and to mediate protein-protein interactions between transposase subunits. A tetrameric form of the transposase was detected in solution, consistent with an interaction between the IR/DR structure and a transposase tetramer. We propose a model in which the transpositional enhancer and the PAI subdomain stabilize complexes formed by a transposase tetramer bound at the IR/DR. These interactions may result in enhanced stability of synaptic complexes, which might explain *Sleeping Beauty*'s efficient transposition in vertebrate cells.

**7. Vigdal, T. J., C. D. Kaufman, Z. Izsvak, D. F. Voytas and Z. Ivics (2002). "Common physical properties of DNA affecting target site selection of *Sleeping Beauty* and other Tc1/mariner transposable elements." *J. Mol. Biol.* **323**: 441-452.**

*Sleeping Beauty* (SB) is the most active Tc1/mariner-type transposable element in vertebrates, and is therefore a valuable vector for transposon mutagenesis in vertebrate models and for human gene therapy. We have analyzed factors affecting target site selection of SB in mammalian cells, by generating transposition events from extrachromosomal plasmids to chromosomes. In contrast to the local hopping observed when transposition is induced from a chromosomal context, mapping of 138 unique SB insertions on human chromosomes showed a fairly random genomic distribution, and a 35% occurrence of transposition into genes. Inspection of the DNA flanking the sites of element integration revealed significant differences from random DNA in both primary sequence and physical properties. The consensus sequence of SB target sites was found to be a palindromic AT-repeat, ATATATAT, in which the central TA is the canonical target site. We found however, that target site selection is determined primarily on the level of DNA structure, and not by specific base-pair interactions. Computational analyses revealed that insertion sites tend to have a bendable structure and a palindromic pattern of potential hydrogen-bonding sites in the major groove of the DNA. These features appear conserved in

the Tc1/mariner family of transposons and in other, distantly related elements that share a common catalytic domain of the transposase, and integrate fairly randomly. No similar target site preference was found for non-randomly integrating elements. Our results suggest common factors influencing target site selection of a wide range of transposable elements.

**8. Zayed, H., Z. Izsvak, D. Khare, U. Heinemann and Z. Ivics (2003). "The DNA-bending protein HMGB1 is a cellular cofactor of *Sleeping Beauty* transposition." *Nucl. Acids Res.* 31: 2313-2322.**

*Sleeping Beauty* (SB) is the most active Tc1/ mariner-type transposon in vertebrates. SB contains two transposase-binding sites (DRs) at the end of each terminal inverted repeat (IR), a feature termed the IR/DR structure. We investigated the involvement of cellular proteins in the regulation of SB transposition. Here, we establish that the DNA-bending, high-mobility group protein, HMGB1 is a host-encoded cofactor of SB transposition. Transposition was severely reduced in mouse cells deficient in HMGB1. This effect was rescued by transient over-expression of HMGB1, and was partially complemented by HMGB2, but not with the HMGA1 protein. Over-expression of HMGB1 in wild-type mouse cells enhanced transposition, indicating that HMGB1 can be a limiting factor of transposition. SB transposase was found to interact with HMGB1 in vivo, suggesting that the transposase may recruit HMGB1 to transposon DNA. HMGB1 stimulated preferential binding of the transposase to the DR further from the cleavage site, and promoted bending of DNA fragments containing the transposon IR. We propose that the role of HMGB1 is to ensure that transposase-transposon complexes are first formed at the internal DRs, and subsequently to promote juxtaposition of functional sites in transposon DNA, thereby assisting the formation of synaptic complexes.

**9. Geurts, A.M., Y. Yang, K.J. Clark, Z. Cui, A.J. Dupuy, D.A. Largaespada and P.B. Hackett (2003). "Gene transfer into genomes of human cells by the *Sleeping Beauty* transposon system." *Mol. Therapy* 8: 108-117.**

The *Sleeping Beauty* (SB) transposon system, derived from teleost fish sequences, is extremely effective at delivering DNA to vertebrate genomes including those of humans. We have examined several parameters of the SB system to improve it as a potential, non-viral vector for gene therapy. Our investigation centered on three features, the carrying capacity of the transposon for efficient integration into chromosomes of HeLa cells, the effects of overexpression of the SB transposase gene on transposition rates, and improvements in the activity of SB transposase to increase insertion rates of transgenes into cellular chromosomes. We found that SB transposons of about 6 kbp retained 50% of the maximal efficiency of transposition, which is sufficient to deliver 70% - 80% of identified human cDNAs with appropriate transcriptional regulatory sequences. Overexpression inhibition studies revealed that there are optimal ratios of SB transposase to transposon for maximal rates of transposition, suggesting that conditions of delivery of the two-part transposon system are important for the best gene-transfer efficiencies. We further refined the SB transposase to incorporate several amino acid substitutions, the result of which led to an improved transposase called SB11. With SB11 we are able to achieve transposition rates that are about 100-fold above those achieved with plasmids that insert into chromosomes by random recombination. With the recently described improvements to the transposon itself, the SB system appears to be a potential gene-transfer tool for human gene therapy.

**10. Ivics Z., C.D. Kaufman, H. Zayed, C. Miskey, O. Walisko, Z. Izsvak Z. (2004). "The *Sleeping Beauty* transposable element: evolution, regulation and genetic applications." *Curr. Issues Mol. Biol.* 6: 43-55.**

Members of the Tc1/mariner superfamily of transposable elements isolated from vertebrate species are inactive due to the accumulation of mutations. A representative of a subfamily of fish elements estimated to be last active > 10 million years ago has been reconstructed, and named *Sleeping Beauty* (SB). This element opened up new avenues for studies on DNA transposition in vertebrates, and for the development of transposon tools for genetic manipulation in important model species and in humans. Multiple transposase binding sites within the terminal inverted repeats, a transpositional enhancer sequence, unequal affinity of the transposase to the binding sites and the activity of the cellular HMGB1 protein all contribute to a highly regulated assembly of SB synaptic complexes, which is likely a requirement for the subsequent catalytic steps. Host proteins involved in double-strand DNA break repair are limiting factors of SB transposition in mammalian cells, underscoring evolutionary, structural and functional links between DNA transposition, retroviral integration and V(D)J recombination. SB catalyzes efficient cut-and-paste transposition in a wide range of vertebrate cells in tissue culture, and in somatic tissues as well as the germline of the mouse and zebrafish in vivo, indicating its usefulness as a vector for transgenesis and insertional mutagenesis.

**11. Yant, S.R., and M.A. Kay (2003). "Nonhomologous-end-joining factors regulate DNA repair fidelity during *Sleeping Beauty* element transposition in mammalian cells." *Mol. Cell Biol.* 23: 8505-8518.**

Herein, we report that the DNA-dependent protein kinase (DNA-PK) regulates the DNA damage introduced during *Sleeping Beauty* (SB) element excision and reinsertion in mammalian cells. Using both plasmid- and chromosome-based mobility assays, we analyzed the repair of transposase-induced double-stranded DNA breaks in cells deficient in either the DNA-binding subunit of DNA-PK (Ku) or its catalytic subunit (DNA-PKcs). We found that the free 3' overhangs left after SB element excision were efficiently and accurately processed by the major Ku-dependent nonhomologous-end-joining pathway. Rejoining of broken DNA molecules in the absence of Ku resulted in extensive end degradation at the donor site and greatly increased the frequency of recombination with ectopic templates. Therefore, the major DNA-PK-dependent DNA damage response predominates over more-error-prone repair pathways and thereby facilitates high-fidelity DNA repair during transposon mobilization in mammalian cells. Although transposable elements were not found to be efficiently circularized after transposase-mediated excision, DNA-PK deficiency supported more-frequent transposase-mediated element insertion than was found in wild-type controls. We conclude that, based on its ability to regulate excision site junctional diversity and transposon insertion frequency, DNA-PK serves an important protective role during transpositional recombination in mammals.

**12. Izsvak Z., E.E. Stuwe, D. Fiedler., A. Katzer, P.A. Jeggo and Z. Ivics (2004). "Healing the wounds inflicted by *Sleeping Beauty* transposition by double-strand break repair in mammalian somatic cells." *Mol. Cell* 13: 279-290.**

Healing the wounds inflicted by sleeping beauty transposition by double-strand break repair in Mammalian somatic cells. The *Sleeping Beauty* (SB) element is a useful tool to probe transposon-host interactions in vertebrates. We investigated requirements of DNA repair factors for SB transposition in mammalian cells. Factors of nonhomologous end joining (NHEJ), including Ku, DNA-

PKcs, and Xrcc4 as well as Xrcc3/Rad51C, a complex that functions during homologous recombination, are required for efficient transposition. NHEJ plays a dominant role in repair of transposon excision sites in somatic cells. Artemis is dispensable for transposition, consistent with the lack of a hairpin structure at excision sites. Ku physically interacts with the SB transposase. DNA-PKcs is a limiting factor for transposition and, in addition to repair, has a function in transposition that is independent from its kinase activity. ATM is involved in excision site repair and affects transposition rates. The overlapping but distinct roles of repair factors in transposition and in V(D)J recombination might influence the outcomes of these mechanistically similar processes.

**13. Zayed H., Z. Izsvak, O. Walisko and Z. Ivics (2004). "Development of hyperactive *Sleeping Beauty* transposon vectors by mutational analysis." *Mol. Ther.* 9: 292-304.**

The *Sleeping Beauty* (SB) transposable element is a promising vector for transgenesis in vertebrates and is being developed as a novel, nonviral system for gene therapeutic purposes. A mutagenesis approach was undertaken to improve various aspects of the transposon, including safety and overall efficiency of gene transfer in human cells. Deletional analysis of transposon sequences within first-generation SB vectors showed that the inverted repeats of the element are necessary and sufficient to mediate high-efficiency transposition. We constructed a "sandwich" transposon, in which the DNA to be mobilized is flanked by two complete SB elements arranged in an inverted orientation. The sandwich element has superior ability to transpose >10-kb transgenes, thereby extending the cloning capacity of SB-based vectors. We derived hyperactive versions of the SB transposase by single-amino-acid substitutions. These mutations act synergistically and result in an almost fourfold enhancement of activity compared to the wild-type transposase. When combined with hyperactive transposons and transiently overexpressed HMGB1, a cellular cofactor of SB transposition, hyperactive transposases elevate transposition by almost an order of magnitude compared to the first-generation transposon system. The improved vector system should prove useful for efficient gene transfer in vertebrates.

**14. Heggestad A.D., L. Notterpek and B.S. Fletcher (2004). "Transposon-based RNAi delivery system for generating knockdown cell lines." *Biochem. Biophys. Res. Comm.* 316: 643-650.**

RNA interference is rapidly becoming a powerful tool for genetic analyses in mammalian systems. A potential drawback to transient small inhibitory RNA silencing is the short duration of downregulation it confers, usually only 24-72h. Viral-based vector systems for the long-term delivery of RNA hairpins have been developed, yet they require expertise in viral production and transduction. Here we describe a simple plasmid-based system for the generation of long-term gene knockdown utilizing RNA interference combined with the gene delivery capabilities of the mammalian Tc1-like transposon *Sleeping Beauty*. Designated Maleficent, this system is shown to downregulate exogenous expression of GFP in a constitutively positive cell line. In addition, targeting of the endogenously expressed lamin A gene results in long-term silencing with significant reduction in protein levels (> 95%). Maleficent therefore provides a relatively easy, efficient, and stable means of delivering RNAi hairpins to generate long-term gene-specific knockdown cell lines.

**15. Masuda, K., S. Yamamoto, M. Endoh and Y. Kaneda (2004). "Transposon-independent increase of transcription by the *Sleeping Beauty* transposase." *Biochem. Biophys. Res. Comm.* 317:796-800.**

When a plasmid encoding the *Sleeping Beauty* (SB) transposase (pCMV-SB) was coinroduced with luciferase expression plasmid DNA into mouse skeletal muscle at a molar ratio of 4:1, luciferase gene expression was 5 times higher than the expression without

pCMV-SB on day 28. This enhancement was not dependent on the presence of transposon (Tn) sequence in luciferase expression plasmid. Southern blot analysis failed to detect luciferase gene insertion into the host genome. Then, expression, a luciferase expression plasmid without Tn, was cointroduced into HeLa cells with or without pCMV-SB. With pCMV-SB, the mRNA amount and the luciferase activity were 1.5 times and 2 times higher, respectively, than without pCMV-SB, even though the cells with pCMV-SB had a smaller copy number of luciferase plasmids than the cells without pCMV-SB. These results suggest that SB transposase enhances the transcription of an exogenous gene regardless of the presence of the Tn sequence.

**16. Ivics, Z. and Z. Izsvak (2004). "Transposable elements for transgenesis and insertional mutagenesis in vertebrates: a contemporary review of experimental strategies." *Methods Mol. Biol.* 260: 255-276.**

Functional genomic analyses in vertebrate model systems, including fish, frogs, and mice, have greatly contributed to our understanding of embryonic development and human disease. However, new molecular tools and strategies are needed to meet the increasing demands of linking sequence information to gene function. Transposable elements (TEs) are very efficient at integrating into DNA, and are therefore useful vectors for transferring new genetic material into genomes. In particular, members of the Tc1/mariner superfamily of elements are able to transpose in species other than their hosts, and are therefore emerging tools for functional genomics in several organisms. This chapter describes strategies of using retrovirus vectors and DNA-based TEs for transgenesis and insertional mutagenesis in vertebrates, with special emphasis on the *Sleeping Beauty* (SB) element, a reconstructed Tc1/mariner-like transposon from fish. SB jumps efficiently in cells of diverse vertebrate species in culture, as well as in somatic and germline tissues of the mouse in vivo. Simple structure and easy laboratory handling of transposon vectors are coupled with efficient and stable transgene integration and persistent, long-term transgene expression by transposon-mediated gene transfer. These features all contribute to the usefulness of TEs as tools for vertebrate functional genomics, as well as for animal biotechnology and human gene therapy.

**17. Yusa, K., J. Takeda and K. Horie (2004). "Enhancement of *Sleeping Beauty* transposition by CpG methylation: possible role of heterochromatin formation." *Mol. Cell Biol.* 24: 4004-4018.**

The *Sleeping Beauty* (SB) transposase is the most active transposase in vertebrate cells, and the SB transposon system has been used as a tool for insertional mutagenesis and gene delivery. Previous studies have indicated that the frequency of chromosomal transposition is considerably higher in mouse germ cells than in mouse embryonic stem cells, suggesting the existence of unknown mechanisms that regulate SB transposition. Here, we demonstrated that CpG methylation of the transposon region enhances SB transposition. The transposition efficiencies of a methylated transposon and an unmethylated transposon which had been targeted in the same genomic loci by recombination-mediated cassette exchange in mouse erythroleukemia cells were compared, and at least a 100-fold increase was observed in the methylated transposon. CpG methylation also enhanced transposition from plasmids into the genome. Chromatin immunoprecipitation assays revealed that histone H3 methylated at lysine-9, a hallmark of condensed heterochromatin, was enriched at the methylated transposon, whereas the unmethylated transposon formed a relaxed euchromatin structure,

as evidenced by enrichment of acetylated histone H3 and reporter gene expression. Possible roles of heterochromatin formation in the transposition reaction are discussed. Our findings indicate a novel relationship between CpG methylation and transposon mobilization.

**18. Yant, S.R., J. Park, J.G. Mikkelsen and M.A. Kay, M.A. (2004). "Mutational analysis of the N-terminal DNA-binding domain of *Sleeping Beauty* transposase: critical residues for DNA binding and hyperactivity in mammalian cells." *Mol. Cell Biol.* 24: 9239-9247.**

The N-terminal domain of the *Sleeping Beauty* (SB) transposase mediates transposon DNA binding, subunit multimerization, and nuclear translocation in vertebrate cells. For this report, we studied the relative contributions of 95 different residues within this multifunctional domain by large-scale mutational analysis. We found that each of four amino acids (leucine 25, arginine 36, isoleucine 42, and glycine 59) contributes to DNA binding in the context of the N-terminal 123 amino acids of SB transposase, as indicated by electrophoretic mobility shift analysis, and to functional activity of the full-length transposase, as determined by a quantitative HeLa cell-based transposition assay. Moreover, we show that amino acid substitutions within either the putative oligomerization domain (L11A, L18A, L25A, and L32A) or the nuclear localization signal (K104A and R105A) severely impair its ability to mediate DNA transposition in mammalian cells. In contrast, each of 10 single amino acid changes within the bipartite DNA-binding domain is shown to greatly enhance SB's transpositional activity in mammalian cells. These hyperactive mutations functioned synergistically when combined and are shown to significantly improve transposase affinity for transposon end sequences. Finally, we show that enhanced DNA-binding activity results in improved cleavage kinetics, increased SB element mobilization from host cell chromosomes, and dramatically improved gene transfer capabilities of SB in vivo in mice. These studies provide important insights into vertebrate transposon biology and indicate that *Sleeping Beauty* can be readily improved for enhanced genetic research applications in mammals.

**19. Liu, G, A. Geurts, K. Yae, A.R. Srinivasan, S.C. Fahrenkrug, D.A. Largaespada, J. Takeda, K. Horie, W.K. Olson, and P.B. Hackett (2005). "Target-Site preferences of *Sleeping Beauty* transposons." *J. Mol. Biol.* 346: 161-173.**

The *Sleeping Beauty* (SB) transposon is a *Tc1/mariner* family transposon that has applications in vertebrate animals for gene-transfer, gene-tagging, and human gene therapy. In this study, we analyzed the target-site preference of the SB transposon. At the genomic level, integration of SB transposons with respect to genes (exons and introns) and intergenic regions appears fairly random but not on a micro-scale. Although there appears to be a consensus sequence around the vicinity of the target sites, the primary sequence is not the determining factor for target selection. When integrations are examined over a limited topography, the sites most often used for integration did not match the consensus sequence. Rather, a unique deformation inherent in the sequence may be a recognition signal for target selection. The deformation is characterized by an angling of the target site such that the axis around the insertion site is off center, the rotation of the helix (twisting) is non-uniform and there is an increase in the distance between the central base-pairs. Our observations offer several hypothetical insights into the transposition process. Our observations suggest that particular deformations of the double helix predicted by the

V(step) algorithm can distinguish TA sites that vary by about 16-fold in their preferences for accommodating insertions of SB transposons.

**20. Yant, S.R., X. Wu, Y. Huang, B.A. Garrison, S.M. Burgess and M.A. Kay (2005). “High-resolution genome-wide mapping of transposon integration in mammals. *Mol. Cell Biol.* 25: 2085-2094.**

The *Sleeping Beauty* (SB) transposon is an emerging tool for transgenesis, gene discovery, and therapeutic gene delivery in mammals. Here we studied 1,336 SB insertions in primary and cultured mammalian cells in order to better understand its target site preferences. We report that, although widely distributed, SB integration recurrently targets certain genomic regions and shows a small but significant bias toward genes and their upstream regulatory sequences. Compared to those of most integrating viruses, however, the regional preferences associated with SB-mediated integration were much less pronounced and were not significantly influenced by transcriptional activity. Insertions were also distinctly nonrandom with respect to intergenic sequences, including a strong bias toward microsatellite repeats, which are predominantly enriched in noncoding DNA. Although we detected a consensus sequence consistent with a twofold dyad symmetry at the target site, the most widely used sites did not match this consensus. In conjunction with an observed SB integration preference for bent DNA, these results suggest that physical properties may be the major determining factor in SB target site selection. These findings provide basic insights into the transposition process and reveal important distinctions between transposon- and virus-based integrating vectors.

**21. Park, C.W., B.T. Kren, D.A. Largaespada and C.J. Steer (.....). “Sleeping Beauty transposition and differential expression of the Agouti transgene in mice occur without changes in DNA methylation.” (submitted).**

The *Sleeping Beauty* transposon is a recently developed non-viral vector that can mediate insertion of therapeutic transgenes into mammalian genomic DNA. Foreign DNA elements introduced into the mammalian genome tend to invoke a host-defense mechanism resulting in epigenetic changes, such as DNA methylation. Methylation of DNA is closely associated with the transcriptional inactivation of many mammalian genes. To assess potential epigenetic modifications associated with *Sleeping Beauty* transposition, we investigated the DNA methylation pattern of the transgene inserted into the mouse genome as well as genomic regions flanking the insertion sites. *Sleeping Beauty* carrying an *Agouti* transgene was used to create transgenic mouse lines via transposition of the transposons. DNA isolated from transgenic or wild type control mice was subjected to bisulfite-mediated genomic sequencing. Only a low level of DNA methylation was observed in the *keratin-14* promoter driving expression of the *Agouti* transgene; and no DNA methylation was detected in the *Agouti* transgene regardless of its level of expression. Two different mouse genomic loci flanking the insertion sites demonstrated patterns of DNA methylation similar to those observed from wild type mice. These results indicate that transposition via *Sleeping Beauty* into the mouse genome does not result in either a significant level of *de novo* DNA methylation of the transgene, nor changes in CpG methylation within the flanking genomic regions. In addition, varied expression of the *Agouti* transgene does not appear to be regulated by DNA methylation. Thus, *Sleeping Beauty* may be a more advantageous method of expressing a transgene than other viral gene delivery vectors that may induce potentially harmful changes in DNA methylation.

**22. Wilber, A.C. J.L. Frandsen, J.L. Geurts, D.A. Largaespada, P.B. Hackett and R.S. Mclvor (.....). "RNA as a source of transposase for *Sleeping Beauty*-mediated gene insertion and expression in somatic cells and tissues." (submitted)**

In all reports of *Sleeping Beauty* (SB)-mediated gene insertion in somatic cells, the transposase component has been provided through expression of a co-delivered DNA molecule. Although this approach has been quite successful, there is limited control over the transient levels of transposase expressed in target cells, and the potential for genomic integration of the transposase encoding sequence resulting in sustained transposase expression and potential destabilizing effects on the integrated transgene or host genome integrity. Here we address these potential problems by supplying the transposase-encoding molecule in the form of RNA. Using rapid, high-volume injection into the tail vein of mice as a model for efficient gene transfer in the liver, we have optimized delivery and expression of RNA using constructs that promote stability and translation. Real-time, bioluminescence imaging of luciferase activity determined that incorporation of 5' and 3' UTR elements from the b-globin gene and addition of RNase inhibitor or decoy RNA provided luciferase protein expression mimicking levels achieved using promoters known to provide optimum transposase expression for in vivo transposition. Using this genetic arrangement, transposase-encoding mRNA was co-transfected with a transposon plasmid encoding a puromycin resistance gene into cultured human HT1080 cells, resulting in a 9-fold increase in drug-resistant clones relative to controls. To confirm that puromycin resistance was conferred by transposition, several transposon integrants were recovered using a plasmid rescue strategy that facilitates the cloning and sequencing of cellular-transposon junction fragments. Transposase-encoding mRNA was also co-delivered with a luciferase transposon to the liver of Mx1Cre transgenic mice in a system that allows silencing of untransposed luciferase sequences by induction of Cre recombinase. Induction of Cre recombinase resulted in a 1400-fold reduction of luciferase expression in animals infused with the transposon alone, while in animals co-injected with transposase-encoding mRNA there was only a 45-fold reduction of luciferase expression. This remaining luciferase expression is likely attributable to transposition mediated by SB translated from co-delivered SB-encoding mRNA. We conclude that in vitro transcribed mRNA can be used as an effective and less risky source of transposase for SB mediated transposition in vitro and in vivo.

**23. Converse, A., L. Belur, J. Gori, G. Liu, F. Amaya, E. Aguilar-Cordova, P.B. Hackett, and R.S. Mclvor (2005). "Counterselection and co-delivery of transposon and transposase functions for the study of *Sleeping Beauty*-mediated transposition in cultured mammalian cells." *Som. Cell Mol. Genet.* (in press).**

*Sleeping Beauty* (SB) is a gene-insertion system reconstructed from transposon sequences found in teleost fish and is capable of mediating the transposition of DNA sequences from transfected plasmids into the chromosomes of vertebrate cell populations (Ivics et al, Cell 91: 501, 1997). We carried out a series of studies to further characterize SB-mediated transposition as a tool for gene transfer to chromosomes and ultimately for human gene therapy. Transfection of mouse 3T3 cells, HeLa cells, and human A549 lung carcinoma cells with a transposon containing the neomycin phosphotransferase (NEO) gene resulted in a several-fold increase in drug-resistant colony formation when co-transfected with a plasmid expressing the SB transposase. A transposon containing a methotrexate-resistant dihydrofolate reductase gene was also found to confer an increased frequency of

methotrexate-resistant colony formation when co-transfected with SB transposase-encoding plasmid. A plasmid containing a herpes simplex virus thymidine kinase gene as well as a transposon containing a NEO gene was used for counterselection against random recombinants (NEO+TK+) in medium containing G418 plus ganciclovir. Effective counterselection required a recovery period of 5 days after transfection before shifting into medium containing ganciclovir to allow time for transiently expressed thymidine kinase activity to subside in cells not stably transfected. Southern analysis of clonal isolates indicated a shift from random recombination events toward transposition events when clones were isolated in medium containing ganciclovir as well as G418. We found that including both transposon and transposase functions on the same plasmid substantially increased the stable gene transfer frequency in Huh7 human hepatoma cells. The results from these experiments contribute technical and conceptual insight into the process of transposition in mammalian cells, and into the optimal provision of transposon and transposase functions that may be applicable to gene therapy studies.

**24. Wilber, A.M. J.L. Frandsen, K. Wangensteen, S.C. Ekker, X. Wang and R.S. McIvor (2005). "Dynamic gene expression following systemic delivery of plasmid DNA as determined by *in vivo* bioluminescence imaging." *Mol. Therap.* (submitted).**

Studies focusing on gene expression have shown that *in vivo* Luc activity could be used as a marker to monitor stable transgene expression from lentiviral vectors encoding the reporter alone or when co-expressed with the gene encoding human clotting factor IX, and non-virally by direct injection of a polyethylenimine complexed *Sleeping Beauty* transposon vector into tumors grown in nude mice. These applications suggested that *in vivo* imaging of luciferase enzyme activity could be used to monitor transcriptional activities of different elements designed for the purpose of non-viral gene expression. Here we describe experiments combining the efficiency of liver-directed gene transfer achieved by rapid, high-volume systemic injection with *in vivo* imaging to quantitatively study the dynamics of transgene expression in mouse liver.

## B. Activity of SBTS in Mice:

1. Luo, G., Z. Ivics, Z. Izsvak and A. Bradley (1998). "Chromosomal transposition of a Tc1/mariner-like element in mouse embryonic stem cells." *Proc. Natl. Acad. Sci. USA* **95**: 10769-10773.

Mouse has become an increasingly important organism for modeling human diseases and for determining gene function in a mammalian context. Unfortunately, transposon-tagged mutagenesis, one of the most valuable tools for functional genomics, still is not available in this organism. On the other hand, it has long been speculated that members of the Tc1/mariner-like elements may be less dependent on host factors and, hence, can be introduced into heterologous organisms. However, this prediction has not been realized in mice. We report here the chromosomal transposition of the *Sleeping Beauty* (SB) element in mouse embryonic stem cells, providing evidence that it can be used as an in vivo mutagen in mice.

2. Dupuy, A. J., S. Fritz and D. A. Largaespada (2001). "Transposition and gene disruption in the male germline of the mouse." *Genesis* **30**: 82-88.

We have tested a synthetic, functional, transposon called *Sleeping Beauty* for use in mice as a germline insertional mutagen. We describe experiments in which mutagenic, polyadenylation-site trapping, transposon vectors were introduced into the germline of mice. When doubly transgenic males, expressing the *Sleeping Beauty* transposase gene (SB10) and harboring poly(A)-trap transposon vectors, were outcrossed to wild-type females, offspring were generated with new transposon insertions. The frequency of new transposon insertion is roughly two per male gamete. These new insertions can be passed through the germline to the next generation and can insert into or near genes. We have generated a preliminary library of 24 mice harboring 56 novel insertion sites, including one insertion into a gene represented in the EST database and one in the promoter of the galactokinase (Gck) gene. This technique has promise as a new strategy for forward genetic screens in the mouse or functional genomics.

3. Fischer, S. E., E. Wienholds and R. H. Plasterk (2001). "Regulated transposition of a fish transposon in the mouse germ line." *Proc. Natl. Acad. Sci. USA* **98**: 6759-6764.

*Tc1/mariner* elements are able to transpose in species other than the host from which they were isolated. As potential vectors for insertional mutagenesis and transgenesis of the mouse, these cut-and-paste transposons were tested for their ability to transpose in the mouse germ line. First, the levels of activity of several Tc1/mariner elements in mammalian cells were compared; the reconstructed fish transposon *Sleeping Beauty* (SB) was found to be an order of magnitude more efficient than the other tested transposons. SB then was introduced into the mouse germ line as a two-component system: one transgene for the expression of the transposase in the male germ line and a second transgene carrying a modified transposon. In 20% of the progeny of double transgenic male mice the transposon had jumped from the original chromosomal position into another locus. Analysis of the integration sites shows that these jumps indeed occurred through the action of SB transposase, and that SB has a strong preference for intrachromosomal transposition. Analysis of the excision sites suggests that double-strand breaks in haploid spermatids are

repaired via nonhomologous end joining. The SB system may be a powerful tool for transposon mutagenesis of the mouse germ line.

- 4. Horie, K., A. Kuroiwa, M. Ikawa, M. Okabe, G. Kondoh, Y. Matsuda and J. Takeda (2001). "Efficient chromosomal transposition of a Tc1/mariner-like transposon *Sleeping Beauty* in mice." *Proc. Natl. Acad. Sci. USA* 98: 9191-9196.**

The presence of mouse embryonic stem (ES) cells makes the mouse a powerful model organism for reverse genetics, gene function study through mutagenesis of specific genes. In contrast, forward genetics, identification of mutated genes responsible for specific phenotypes, has an advantage to uncover novel pathways and unknown genes because no a priori assumptions are made about the mutated genes. However, it has been hampered in mice because of the lack of a system in which a large-scale mutagenesis and subsequent isolation of mutated genes can be performed efficiently. Here, we demonstrate the efficient chromosomal transposition of a *Tc1/mariner*-like transposon, *Sleeping Beauty*, in mice. This system allows germ-line mutagenesis in vivo and will facilitate certain aspects of phenotype-driven genetic screening in mice.

- 5. Dupuy, A. J., K. Clark, C. M. Carlson, S. Fritz, A. E. Davidson, K. M. Markley, K. Finley, C. F. Fletcher, S. C. Ekker, P. B. Hackett, S. Horn and D. A. Largaespada (2002). "Mammalian germ-line transgenesis by transposition." *Proc. Natl. Acad. Sci. USA* 99: 4495-4499.**

Transposons have been used in invertebrates for transgenesis and insertional mutagens in genetic screens. We tested a functional transposon called *Sleeping Beauty* in the one-cell mouse embryo. In this report, we describe experiments in which transposon vectors were injected into one-cell mouse embryos with mRNA expressing the SB10 transposase enzyme. Molecular evidence of transposition was obtained by cloning of insertion sites from multiple transgenic mice produced by SB10 mRNA/transposon coinjection. We also demonstrate germ-line transmission and expression from transposed elements. This technique has promise as a germ-line transgenesis method in other vertebrate species and for insertional mutagenesis in the mouse.

- 6. Roberg-Perez, K., C.M. Carlson and D.A. Largaespada (2003). "MTID: a database of *Sleeping Beauty* transposon insertions in mice." *Nucl. Acids Res.* 31: 78-81.**

The *Sleeping Beauty* (SB) transposon system provides the first random insertional mutagen available for germline genetic screens in mice. In preparation for a large scale project to create, map and manage up to 5000 SB insertions, we have developed the Mouse Transposon Insertion Database (MTID; <http://mouse.ccgb.umn.edu/transposon/>). Each insertion's genomic position, as well as the distance between the insertion and the nearest annotated gene, are determined by a sequence analysis pipeline. Users can search the database using a specified nucleotide or genetic map position to identify the nearest insertion. Mouse reports describe insertions carried, strain, genotype and dates of birth and death. Insertion reports describes chromosome, nucleotide and genetic map positions, as well as nearest gene data from Ensembl, NCBI and Celera. The flanking sequence used to map the insertion is also provided. Researchers will be able to identify insertions of interest and request mice or frozen sperm that carry the insertion.

**7. Carlson, C, A., Dupuy, S. Fritz, K. Roberg-Perez, C.F. Fletcher and D.A. Largaespada (2003). "Transposon mutagenesis of the mouse germline." *Genetics* 165: 243-256.**

*Sleeping Beauty* is a synthetic "cut and paste" transposon of the Tc1/mariner class. The *Sleeping Beauty* transposase (SB) was constructed based on a consensus sequence obtained from an alignment of 12 remnant elements cloned from the genomes of eight different fish species. Transposition of *Sleeping Beauty* elements has been observed in cultured cells, hepatocytes of adult mice, one-cell mouse embryos, and the germline of mice. SB has potential as a random germline insertional mutagen useful for *in vivo* gene trapping in mice. Previous work in our lab has demonstrated transposition in the male germline of mice and transmission of novel transposon insertion sites in offspring. In order to determine sequence preferences and mutagenicity of SB-mediated transposition, we cloned and analyzed 44 gene-trap transposon insertion sites from a panel of 30 mice. The distribution and sequence content flanking these cloned insertion sites was compared to 44 mock insertion sites randomly selected from the genome. We find that germline SB transposon insertion sites are AT-rich and the sequence ANNTANNT is favored compared to other TA dinucleotides. Local transposition occurs with insertions closely linked to the donor site roughly one third of the time. We find roughly 27% of the transposon insertions are in transcription units. Finally, we characterize an embryonic lethal mutation caused by endogenous splicing disruption in mice carrying a particular intron-inserted gene-trap transposon.

**8. Horie, K., K. Yusa, K. Yae, j. Odajima, S.E.J. Fischer, V.W. Keng, T. Hayakawa, S. Mizuno, G. Kondoh, T. Ijiri, Y. Matsuda, R.H.A. Plasterk and J. Takeda (2003). "Characterization of *Sleeping Beauty* transposition and its application to genetic screening in mice." *Mol. Cell. Biol.* 23: 9189-9207.**

The use of mutant mice plays a pivotal role in determining the function of genes, and the recently reported germ line transposition of the *Sleeping Beauty* (SB) transposon would provide a novel system to facilitate this approach. In this study, we characterized SB transposition in the mouse germ line and assessed its potential for generating mutant mice. Transposition sites not only were clustered within 3 Mb near the donor site but also were widely distributed outside this cluster, indicating that the SB transposon can be utilized for both region-specific and genome-wide mutagenesis. The complexity of transposition sites in the germ line was high enough for large-scale generation of mutant mice. Based on these initial results, we conducted germ line mutagenesis by using a gene trap scheme, and the use of a green fluorescent protein reporter made it possible to select for mutant mice rapidly and non-invasively. Interestingly, mice with mutations in the same gene, each with a different insertion site, were obtained by local transposition events, demonstrating the feasibility of the SB transposon system for region-specific mutagenesis. Our results indicate that the SB transposon system has unique features that complement other mutagenesis approaches.

**9. Dupuy, A.J., K. Akagi, D.A. Largaespada, N.G. Copeland and N.A. Jenkins (2005). "Mammalian mutagenesis using a highly mobile somatic *Sleeping Beauty* transposon system." *Nature* 436: 221-226.**

Transposons have provided important genetic tools for functional genomic screens in lower eukaryotes but have proven less useful in higher eukaryotes because of their low transposition frequency. Here we show that Sleeping Beauty (SB), a member of the Tc1/mariner class of transposons, can be mobilized in mouse somatic cells at frequencies high enough to induce embryonic death and cancer in wild-type mice. Tumours are aggressive, with some animals developing two or even three different types of cancer within a few months of birth. The tumours result from SB insertional mutagenesis of cancer genes, thus facilitating the identification of genes and pathways that induce disease. SB transposition can easily be controlled to mutagenize any target tissue and can therefore, in principle, be used to induce many of the cancers affecting humans, including those for which little is known about the aetiology. The uses of SB are also not restricted to the mouse and could potentially be used for forward genetic screens in any higher eukaryote in which transgenesis is possible.

**10. Collier\*, L.S., C.M. Carlson\*, S. Ravimohan, A.J. Dupuy and D.A. Largaespada (2005). "Cancer gene discovery in solid tumors using transposon-based somatic mutagenesis in the mouse." *Nature* 436:272-276.**

Retroviruses, acting as somatic cell insertional mutagens, have been widely used to identify cancer genes in the haematopoietic system and mammary gland. An insertional mutagen for use in other mouse somatic cells would facilitate the identification of genes involved in tumour formation in a wider variety of tissues. Here we report the ability of the Sleeping Beauty transposon to act as a somatic insertional mutagen to identify genes involved in solid tumour formation. A Sleeping Beauty transposon, engineered to elicit loss-of-function or gain-of-function mutations, transposed in all somatic tissues tested and accelerated tumour formation in mice predisposed to cancer. Cloning transposon insertion sites from these tumours revealed the presence of common integration sites, at known and candidate cancer genes, similar to those observed in retroviral mutagenesis screens. Sleeping Beauty is a new tool for unbiased, forward genetic screens for cancer genes in vivo.

**11. Weiser, K.C. and M.J. Justice (2005). "Sleeping Beauty awakens." *Nature* 436: 184-186.**

Ancient jumping DNA found napping in fish has been revived and is being used to identify cancer genes in mice. But the benefits of this aptly named 'Sleeping Beauty' system could reach far beyond cancer.

**12. Wacnik, P.W., C.M. Baker, M.J. Herron, B.T. Kren, B.R. Blazar, G.L. Wilcox, M.K. Hordinsky, A.J. Beitz, A.J. and M.E. (2005). "Tumor-induced mechanical hyperalgesia involves CGRP receptors and altered innervation and vascularization of DsRed2 fluorescent hindpaw tumors." *Pain* 115: 95-106.**

Functional and anatomical relationships among primary afferent fibers, blood vessels, and cancers are poorly understood. However, recent evidence suggests that physical and biochemical interactions between these peripheral components are important to both tumor biology and cancer-associated pain. To determine the role of these peripheral components in a mouse model of cancer pain, we quantified the change in nerve and blood vessel density

within a fibrosarcoma tumor mass using stereological analysis of serial confocal optical sections of immunostained hind paw. To this end we introduced the Discoma coral-derived red fluorescent protein (DsRed2) into the NCTC 2472 fibrosarcoma line using the *Sleeping Beauty* transposon methodology, thus providing a unique opportunity to visualize tumor-nerve-vessel associations in context with behavioral assessment of tumor-associated hyperalgesia. Tumors from hyperalgesic mice are more densely innervated with calcitonin gene related peptide (CGRP)-immunoreactive nerve fibers and less densely vascularized than tumors from non-hyperalgesic mice. As hyperalgesia increased from Day 5 to 12 post-implantation, the density of protein gene product 9.5 (PGP9.5)-immunoreactive nerves and CD31-immunoreactive blood vessels in tumors decreased, whereas CGRP-immunoreactive nerve density remained unchanged. Importantly, intra-tumor injection of a CGRP1 receptor antagonist (CGRP 8-37) partially blocked the tumor-associated mechanical hyperalgesia, indicating that local production of CGRP may contribute to tumor-induced nociception through a receptor-mediated process. The results describe for the first time the interaction among sensory nerves, blood vessels and tumor cells in otherwise healthy tissue, and our assessment supports the hypothesis that direct tumor cell-axon communication may underlie, at least in part, the occurrence of cancer pain.

**13. Carlson, C.S. and D.A. Largaespada (2005). "Insertional mutagenesis in mice: new perspectives and tools." *Nature Rev. Genet.* 6: 568-580.**

Retroviruses, acting as somatic cell insertional mutagens, have been widely used to identify cancer genes in the haematopoietic system and mammary gland. An insertional mutagen for use in other mouse somatic cells would facilitate the identification of genes involved in tumour formation in a wider variety of tissues. Here we report the ability of the *Sleeping Beauty* transposon to act as a somatic insertional mutagen to identify genes involved in solid tumour formation. A *Sleeping Beauty* transposon, engineered to elicit loss-of-function or gain-of-function mutations, transposed in all somatic tissues tested and accelerated tumour formation in mice predisposed to cancer. Cloning transposon insertion sites from these tumours revealed the presence of common integration sites, at known and candidate cancer genes, similar to those observed in retroviral mutagenesis screens. *Sleeping Beauty* is a new tool for unbiased, forward genetic screens for cancer genes in vivo.

**14. Carlson, C.M. J.L. Frandson, R.S. McIvor and D.A. Largaespada (2005). "Somatic integration of an oncogene-harboring *Sleeping Beauty* transposon to model tumor development in the mouse." (submitted).**

Insertional mutagenesis has been at the core of functional genomics in many species. In the mouse, improved vectors and methodologies allow easier genome-wide and phenotype-driven insertional mutagenesis screens. The ability to generate homozygous diploid mutations in mouse embryonic stem cells allows prescreening for specific null phenotypes prior to in vivo analysis. In addition, the discovery of active *Sleeping Beauty* transposable elements in vertebrates, and their development as genetic tools, has led to in vivo forward insertional mutagenesis screens in the mouse. These new technologies will greatly contribute to the speed and ease with which we achieve complete functional annotation of the mouse genome.

**15. Geurts\*, A.M., C.S. Hackett\*, J.B. Bell, C.M. Carlson, L.S. Collier, D.A. Largaespada and P.B. Hackett. (2005). "Integration-site preferences of *Sleeping Beauty* transposons in mouse chromosomes." (submitted).**

The *Sleeping Beauty* (SB) transposon is a *Tc1/mariner* family transposon that has utility as a non-viral, integrating vector for human gene therapy and as a vector for gene tagging in functional genomics studies. At a genomic level, integration of SB transposons appears fairly random at TA dinucleotide basepairs. However, on a micro-scale, there appears to be a consensus sequence around the TA-integration sites. In an earlier study {Liu et al. (2005) J. Mol. Biol. 346: 161-173}, we found that preferential sites had a unique deformation pattern that could be predicted by the  $V_{\text{step}}$  algorithm. By creating a Perl script program, we have been able to extend our analysis of the previous dataset to refine our ability to predict SB transposon insertion sites for any target sequence. Here we report our results of preferential integrations from two additional datasets. The first integration set was identified in certain TA sites in the ninth intron of the mouse *Braf* gene. Transpositions occurred as a result of remobilization, by SB10 transposase, of "insertionally oncogenic" transposons from two distinct donor chromosome loci on mouse chromosomes 1 and 15, landing in the mouse *Braf* gene on chromosome 6 {Collier and Carlson et al. (2005) Nature (in press)}. Twenty-five oncogenic, gain-of-function insertions into *Braf* in *p19arf<sup>-/-</sup>* mice were mapped. Similar target preferences were found in second dataset of 34 transposon insertions into a region of 3.2 Mbp of mouse chromosome-1 that has 208,299 TA sites. These data suggest about a 10-fold preference for integration at certain sites in chromatin that can be predicted by  $V_{\text{step}}$  patterns and that hotspots for integration of SB transposons can be predicted.

## C. Activity of SBTS for Gene Therapy (in mice):

1. **Yant, S. R., L. Meuse, W. Chiu, Z. Ivics, Z. Izsvak and M. A. Kay (2000). "Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system." *Nature Genetics* 25: 35-41.**

The development of non-viral gene-transfer technologies that can support stable chromosomal integration and persistent gene expression in vivo is desirable. Here we describe the successful use of transposon technology for the nonhomologous insertion of foreign genes into the genomes of adult mammals using naked DNA. We show that the *Sleeping Beauty* transposase can efficiently insert transposon DNA into the mouse genome in approximately 5-6% of transfected mouse liver cells. Chromosomal transposition resulted in long-term expression (>5 months) of human blood coagulation factor IX at levels that were therapeutic in a mouse model of hemophilia B. Our results establish DNA-mediated transposition as a new genetic tool for mammals, and provide new strategies to improve existing non-viral and viral vectors for human gene therapy applications.

2. **Montini, E., P. K. Held, M. Noll, N. Morcinek, M. Al-Dhalimy, M. Finegold, S. R. Yant, M. A. Kay and M. Grompe (2002). "In Vivo Correction of Murine Tyrosinemia Type I by DNA-Mediated Transposition." *Mol. Therapy* 6: 759-769.**

Gene therapy applications of naked DNA constructs for genetic disorders have been limited because of lack of permanent transgene expression. This limitation, however, can be overcome by the *Sleeping Beauty* (SB) transposable element, which can achieve permanent transgene expression through genomic integration from plasmid DNA. To date, only one example of an in vivo gene therapy application of this system has been reported. In this report, we have further defined the activity of the SB transposon in vivo by analyzing the expression and integration of a fumarylacetoacetate hydrolase (FAH) transposon in FAH-deficient mice. In this model, stably corrected FAH(+) hepatocytes are clonally selected and stable integration events can therefore be quantified and characterized at the molecular level. Herein, we demonstrate that SB-transposon-transfected hepatocytes can support significant repopulation of the liver, resulting in long-lasting correction of the FAH-deficiency phenotype. A single, combined injection of an FAH-expressing transposon plasmid and a transposase expression construct resulted in stable FAH expression in approximately 1% of transfected hepatocytes. The average transposon copy number was determined to be approximately 1/diploid genome and expression was not silenced during serial transplantation. Molecular analysis indicated that high-efficiency DNA-mediated transposition into the mouse genome was strictly dependent on the expression of wild-type transposase.

3. **Yant, S. R., A. Ehrhardt, J. G. Mikkelsen, L. Meuse, T. Pham and M. A. Kay (2002). "Transposition from a gutless adeno-transposon vector stabilizes transgene expression in vivo." *Nature Biotech.* 20: 999-1005.**

A major limitation of adenovirus-mediated gene therapy for inherited diseases is the instability of transgene expression in vivo, which originates at least in part from the loss of the linear, extrachromosomal vector genomes. Herein we describe the production of a gene-deleted adenovirus-transposon vector that stably maintains virus-encoded transgenes in vivo through integration into host cell chromosomes. This system utilizes a donor transposon vector that undergoes Flp-mediated recombination and excision of its therapeutic payload in the presence of the Flp and *Sleeping Beauty* recombinases. Systemic

in vivo delivery of this system resulted in efficient generation of transposon circles and stable transposase-mediated integration in mouse liver. Somatic integration was sufficient to maintain therapeutic levels of human coagulation Factor IX for more than six months in mice undergoing extensive liver proliferation. These vectors combine the versatility of adenoviral vectors with the integration capabilities of a eukaryotic DNA transposon and should prove useful in the treatment of genetic diseases.

**4. Ortiz, S., Q. Lin, S.R. Yant, D. Keene, M.A. Kay and P.A. Khavari (2003).**

**“Sustainable correction of junctional epidermolysis bullosa via transposon-mediated nonviral gene transfer.” *Gene Therapy* 10: 1099-1104.**

Sustainable correction of severe human genetic disorders of self-renewing tissues, such as the blistering skin disease *junctional epidermolysis bullosa* (JEB), is facilitated by stable genomic integration of therapeutic genes into somatic tissue stem cells. While integrating viral vectors can achieve this, they suffer from logistical and biosafety concerns. To circumvent these limitations, we used the *Sleeping Beauty* transposable element to integrate the LAMB3 cDNA into genomes of epidermal holoclones from six unrelated JEB patients. These cells regenerate human JEB skin that is normalized at the level of laminin 5 protein expression, hemidesmosome formation and blistering. Transposon-mediated gene delivery therefore affords an opportunity for stable gene delivery in JEB and other human diseases.

**5. Belur, L., J.L. Frandsen, A. Dupuy, D.H. Ingbar, D.L. Largaespada, P.B. Hackett and R.S. Mclvor (2003). “Integration and long-term expression in lung mediated by the *Sleeping Beauty* transposon system.” *Mol. Therapy* 8: 501-507.**

Gene transfer to the lung could provide important new treatments for chronic and acquired lung diseases such as cystic fibrosis, alpha-1-antitrypsin deficiency, emphysema and cancer. DNA-mediated gene transfer to the lung has been previously demonstrated, but anticipated effectiveness has been limited by low gene transfer efficiencies and by transient expression of the transgene. Here, we combine plasmid based gene transfer with the integrating capacity of the non-viral *Sleeping Beauty* (SB) transposon vector system to mediate gene insertion and long-term gene expression in mouse lung. Transgene expression was observed after 24 hrs in lungs of all animals injected with the luciferase transposon (pTL), but expression for up to 3 months required co-delivery of a plasmid encoding the *Sleeping Beauty* transposase. Long-term expression was also observed in pTL-injected animals transgenic for SB transposase. Transgene expression was localized to the alveolar region of the lung, with transfection mainly including type II pneumocytes. A linker-mediated PCR technique was used to recover transposon-flanking sequences, demonstrating transposition of TL into mouse chromosomal DNA of the lung.

**6. Mikkelsen, J.G., S.R. Yant, L. Meuse, Z. Huang, H. Xu and M.A. Kay (2003) “Helper-independent *Sleeping Beauty* transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression in vivo.” *Mol. Therapy* 8: 654-665.**

Transposon-based vectors represent promising new tools for chromosomal transgene insertion and establishment of persistent gene expression in vivo. Here, we report the development of helper-independent transposon-transposase (HITT) vectors, which contain

on single plasmids (i) a *Sleeping Beauty* (SB) transposon containing the transgene and (ii) a SB transposase expression cassette. To obtain an optimal level of transposase expression from HITT vectors, we determined the relative strength of a panel of different promoters in mouse liver and used these promoters to drive transposase expression from injected HITT vectors carrying a human alpha(1)-antitrypsin (hAAT) expression cassette flanked by transposon inverted repeats. By correlating promoter strength with stabilized serum hAAT levels, a narrow expression window supporting high-level transposition in the liver was defined. Peak levels of long-term gene expression were obtained with promoters 30- to 40-fold less active than CMV in mouse liver, whereas reduced stable levels of hAAT were detected with both weaker and stronger promoters. Injected HITT vectors induced transposase-dependent insertion of transposon DNA into the genome of at least 5-6% of transfected hepatocytes, generating levels of persistent hAAT expression that were 2- to 4-fold higher than with an optimized two-plasmid approach. In addition, we show that HITT vectors carrying a human factor IX (hFIX)-containing transposon support (i) long-term hFIX expression in normal mice and (ii) partial phenotypic correction in a mouse model of hemophilia B. SB-based HITT vectors represent a major advance in the establishment of persistent transgene expression from nonviral gene delivery systems and should prove useful for gene transfer to tissues or cell types in which transfection efficiencies are low.

7. **Kren, B.T., S.S. Ghosh, C.L. Linehan, N. Roy-Chowdhury, P.B. Hackett, J. Roy-Chowdhury and C.J. Steer (2003). "Hepatocyte-targeted delivery of *Sleeping Beauty* mediates efficient gene transfer *in vivo*." *Gene Ther. Mol. Biol.* 7: 229-238.**

Currently, most gene therapy studies utilize viral vectors that can potentially produce immunological and toxic side effects. To circumvent these limitations, we evaluated the efficiency of nonviral hepatocyte-targeted *in vivo* delivery of plasmids that mediate stable genomic integration of transgenes via the *Sleeping Beauty* (SB) transposon system. We constructed plasmids that express a reporter green fluorescent protein (GFP) transposon and the SB transposase, required for transgene insertion into genomic DNA, from either a single plasmid (*cis*) or two different plasmids (*trans*). The constructs were compacted to an average diameter of <50 nm with lactosylated polyethyleneimine, a polycation, for targeting to the hepatocyte asialoglycoprotein receptor. Intravenous administration of the *cis* plasmid resulted in greater efficiency of transgene integration in mouse liver compared to transposase expression from a separate plasmid. Furthermore, by western blot analysis and fluorescence microscopy, delivery of the *cis* plasmid to rat livers resulted in transgene expression that persisted for months even after regeneration from partial hepatectomy. Southern blot analysis of the regenerated livers indicated that SB mediated genomic integration of the GFP transgene at random sites, and this correlated with disappearance of SB transposase. In conclusion, receptor-mediated targeted delivery of a transposon system capable of transgene integration and stable expression provides an attractive alternative to viral vectors for gene therapy to the liver.

8. **Izsvak, Z. and Z. Ivics (2004). "*Sleeping Beauty* transposition: biology and applications for molecular therapy." *Mol. Therap.* 9: 147-156.**

Transposable elements can be considered as natural, nonviral gene-delivery vehicles and are valuable and widely used tools for germ-line transgenesis and insertional mutagenesis in invertebrate systems such as flies and worms. Such tools were not available for genome

manipulations in vertebrates until recently, when an active element was resurrected from transposon fossils found in fish genomes. This element, the Sleeping Beauty transposon, shows efficient transposition in cells of a wide range of vertebrates, including humans. *Sleeping Beauty* transposition is a cut-and-paste process, during which the element "jumps" from one DNA molecule to another. Transposon integration into chromosomes provides the basis for long-term, or possibly permanent, transgene expression in transgenic cells and organisms. Thus, the reconstruction of the Sleeping Beauty element generated considerable interest in developing efficient and safe vectors for vertebrate transgenesis as well as for human gene therapy. In this review we summarize our current knowledge of Sleeping Beauty biology and describe the strengths and current limitations of transposon technology for gene therapeutic applications.

**9. He, C.X., D. Shi, W.J. Wu, Y.F. Ding, D.M. Feng, B. Lu, H.M. Chen, J.H. Yao, Q. Shen, D.R. Lu and J.L. Xue (2004). "Insulin expression in livers of diabetic mice mediated by hydrodynamics-based administration." *World J. Gastroenterol.* 10: 567-572.**

**AIM:** Transfer and expression of insulin gene in vivo are an alternative strategy to improve glycemic control in type 1 diabetes. Hydrodynamics-based procedure has been proved to be very efficient to transfer naked DNA to mouse livers. The basal hepatic insulin production mediated by this rapid tail vein injection was studied to determine its effect on the resumption of glycemic control in type 1 diabetic mice. **METHODS:** Engineered insulin cDNA was inserted into plasmid vectors under a CMV promoter, and transferred into STZ induced diabetic mice by hydrodynamic procedure. Glucose levels, body weight of treated mice, insulin levels, immunohistology of the liver, and quantity of insulin mRNA in the liver were assayed to identify the improvement of hyperglycemic complication after plasmid administration. *Sleeping Beauty*, a transposon system, was also used to prolong the insulin expression in the liver. **RESULTS:** After plasmid administration, Plasma insulin was significantly increased in the diabetic mice and the livers were insulin-positive by immunostaining. At the same time the hyperglycemic complication was improved. The blood glucose levels of mice were reduced to normal. Glucose tolerance of the treated diabetic mice was improved. Body weight loss was also ameliorated. The rapid tail vein injection did not cause any fatal result. **CONCLUSION:** Our results suggested that insulin gene could be efficiently transferred into the livers of diabetic mice via rapid tail vein injection and it resulted in high level of insulin expression. The basal hepatic insulin production mediated by hydrodynamics-based administration improved the glycemic control in type 1 diabetes dramatically and ameliorated diabetic syndromes. Hydrodynamics-based administration offers a simple and efficient way in the study of gene therapy for type 1 diabetes.

**10. Liu, G, Z. Cui, E.L. Aronovich, C.B. Whitley and P.B. Hackett (2004). "Excision of *Sleeping Beauty* transposons: parameters and applications to gene therapy." *J. Gene Med.* 6:574-583.**

A major problem in gene therapy is the determination of the rates at which gene transfer has occurred. Our work has focused on applications of the *Sleeping Beauty* (SB) transposon system as a non-viral vector for gene therapy. Excision of a transposon from a donor molecule and its integration into a cellular chromosome are catalyzed by SB transposase. In this study, we used a plasmid-based excision assay to study the excision step of transposition. We used the excision assay to evaluate the importance of various sequences

that border the sites of excision inside and outside the transposon in order to determine the most active sequences for transposition from a donor plasmid. These findings together with our previous results in transposase-binding to the terminal repeats suggest that the sequences in the transposon-junction of SB are involved in steps subsequent to DNA binding but before excision, and that they may have a role in transposase-transposon interaction. We found that SB transposons leave characteristically different footprints at excision sites in different cell types, suggesting that alternative repair machineries operate in concert with transposition. Most importantly, we found that the rates of excision correlate with the rates of transposition. We used this finding to assess transposition in livers of mice that were injected with the SB transposon and transposase. The excision assay appears to be a relatively quick and easy method to optimize protocols for delivery of genes in SB transposons to mammalian chromosomes in living animals.

**11. Liu L., Sanz S., Heggestad A.D., Antharam V., Notterpek L., Fletcher B.S. (2004). "Endothelial targeting of the *Sleeping Beauty* transposon within lung." *Mol. Ther.* 10:97-105.**

Endothelial cells have complex roles in the pathophysiology of vascular and heart disease and are increasingly being recognized as targets for gene therapy. The intravenous administration of plasmid DNA complexed to lipid tends to target transfection of endothelial cells within the lung; however, expression from the transgene remains transient. Here we utilize the integrating capability of the *Sleeping Beauty* (SB) transposon for durable gene transfer within lung endothelia. To restrict expression of the transgene, an endothelial cell-specific promoter, endothelin-1, was placed within the transposon. Further refinements to the transposon increased in vitro transposition efficiency by 3.6-fold. Utilizing this optimized transposon we evaluated the expression of two reporter molecules, secreted alkaline phosphatase (SEAP) and intracellular GFP, following administration of DNA-polyethylenimine complexes to mice. Long-term expression (>2 months) of SEAP occurred only with cotransfection of adequate amounts of transposase. Localization studies using the GFP reporter, at 3 days and 6 weeks postinjection, demonstrated that the majority of transgene-expressing cells were of endothelial origin, while the second most abundant cell type was type II pneumocyte. These results suggest that the SB transposon can be adapted to target particular cell types, in this case, endothelial cells. Such an approach may be useful for gene therapy paradigms involving the long-term modulation of vascular and endothelial cell biology.

**12. Ohlfest, J.R., P. Lobitz, S. Perkinson and D.A. Largaespada (2004). "Integration and long-term expression in xenografted human glioblastoma cells using a plasmid-based transposon system." *Mol. Therapy* 10: 260-268.**

Gene therapy has the potential to become an effective component of cancer treatment by transferring genes that cause immunomodulation, tumor cell death, or inhibit angiogenesis into tumor cells or tumor-associated stroma. Viral vectors have been the primary gene transfer vehicles used for intratumoral gene transfer to date. Plasmid-based vectors may be safer and more scalable than viral vectors. However, attempts at plasmid-based intratumoral gene transfer have been met with transient expression and poor gene transfer efficiency. Here we report integration and long-term expression of reporter genes into human glial tumors, growing in nude mice, using the *Sleeping Beauty* (SB) transposon system. A two-plasmid system was used, in which linear polyethylenimine (L-PEI) was complexed with a GFP, NEO, or luciferase transposon plasmid and a SB transposase-expressing plasmid. SB-

mediated transposition led to chromosomal integration of the NEO transgene in roughly 8% of tumor cells. SB-mediated insertions were cloned from the genomes of glial tumor cells to provide molecular proof of transposase-mediated integration. Luciferase studies showed that SB facilitated long-term expression of the transgene in glial tumors. SB-mediated intratumoral gene transfer is a novel, non-viral technique that could be used to augment conventional therapy for glioblastoma or other cancers.

**13. Ohlfest, J.R., J.L. Frandsen, S. Fritz, P.D. Lobitz, S.G. Perkinson, K.J. Clark, N.S. Key, R.S. Mclvor, P.B. Hackett and D.A. Largaespada (2004). "Phenotypic correction and long-term Factor VIII expression in hemophilic mice by immunotolerization and nonviral gene transfer using the *Sleeping Beauty* transposon system." *Blood* 105: 2691-2698**

Hemophilia A is a lead candidate for treatment by gene therapy as the missing protein product, coagulation factor VIII (FVIII), is secreted. Clinically relevant therapy might be achieved by stably delivering a FVIII cDNA to correct the bleeding disorder. We used the *Sleeping Beauty* (SB) transposon, delivered as naked plasmid DNA by high-pressure tail vein injection, to integrate B-domain deleted FVIII genes into the chromosomes of hemophilia A mice and correct the phenotype. Since FVIII protein is a neoantigen to these mice, sustaining therapeutic plasma FVIII levels was problematic due to inhibitory antibody production. We circumvented this problem by tolerizing neonates by a facial vein injection of recombinant FVIII within 24 hours of birth. Achievement of high-level (42-100% normal by clotting) FVIII expression and phenotypic correction required co-injection of a SB transposase-expressing plasmid to facilitate transgene integration in immunotolerized animals. Linker-mediated PCR was used to clone FVIII transposon insertion sites from liver genomic DNA, providing molecular evidence of transposition. Thus SB provides a nonviral means for FVIII gene delivery in a mouse model of hemophilia A if the immune response is prevented. We also conclude that tolerization of neonatal mice prior to vector administration allows the efficacy of gene delivery to be determined and should prove useful in gene therapy studies when the vector delivers a neoantigen-encoding gene.

**14. Hackett, P.B., S.C. Ekker, D.A. Largaespada and R.S. Mclvor (2005) "*Sleeping Beauty* transposon-mediated gene therapy for prolonged expression." *Adv. Genet.* 54: 187-229.**

In the 21st century, we can expect a revolution in delivery of therapeutics. We can expect genetic medicines that will confer permanent solutions to chronic and acute ailments. How these genetic medicines will be delivered and controlled, without adverse side effects, are the pressing issues facing modern medicine. Gene therapy theoretically represents the best form of treatment for some medical disorders because natural biological products rather than chemicals are employed to do their natural jobs. The delivery of the therapeutic is relatively constant at physiologically effective levels, rather than cycles of high and low concentrations that result from introduction of drugs or other therapeutics at periodic intervals. Conceptually, gene therapy has the potential to provide a marked improvement over infused recombinant enzymes used in enzyme-replacement therapies. The essential goal of gene therapy is to provide what all patients want, an improved quality of life. For these reasons gene therapy will become the treatment of choice for disorders such as hemophilia.[Mannucci, 2001 #653] For some patients, gene therapy can mean the difference between life and death. Gene therapy, applicable to both genetic and acquired diseases, is a form of molecular medicine, which will have a major impact on human health in

the coming decades. In this chapter we review a new vector for non-viral gene therapy, the *Sleeping Beauty* transposon system. This vector combines the advantages of viral vectors, directed integration of single copies of a therapeutic gene, with the advantages of non-viral vectors, the absence of protein factors that elicit immune responses.

**15. Chen, Z.J., B.T. Kren, P.Y-P. Wong, W.C. Low and C.J. Steer (2005). “*Sleeping Beauty*-mediated down-regulation of huntingtin expression by RNA interference.” *Biochem. Biophys. Res. Comm.* **329**: 646-652.**

Huntington disease (HD) is a devastating neurologic disorder that is characterized by abnormal expansion of a CAG nt repeat in the first exon of the huntingtin (*htt*) gene, producing a mutant protein with an elongated polyglutamine stretch. The presence of this mutant protein is correlated with the characteristic loss of striatal neurons and the clinical manifestation of D. Currently there is no effective treatment for the associated cell death. The aim of this study was to evaluate an innovative strategy combining RNA interference (RNAi) and gene transfer via the nonviral *Sleeping Beauty* (SB) transposon system to down-regulate Htt expression. siRNA expression vectors were designed to target exons 1, 4, 6, and 62 of the *htt* gene. Real-time RT-PCR and Western blot analysis were used to quantify Htt mRNA and protein levels, respectively, in human cell lines. The results indicated that selected siRNA constructs significantly decreased Htt mRNA and protein levels relative to controls. In addition, SB transposition of the siRNA constructs into the genome reduced long-term protein expression of Htt by about 90%. The combination of siRNA, the SB transposon, and an accurate transgenic mouse model may permit evaluation of this approach in preventing the pathogenesis associated with expression of mutant Htt.

**16. Ehrhardt, A., H. Xu, Z. Huang, J.A. Engler and M.A. Kay (2005). “A direct comparison of two nonviral gene therapy vectors for somatic integration: in vivo evaluation of the bacteriophage integrase varphiC31 and the *Sleeping Beauty* transposase” *Mol. Therap.* **11**: 695-706.**

In this study we performed a head-to-head comparison of the integrase varphiC31 derived from a *Streptomyces* phage and the *Sleeping Beauty* (SB) transposase, a member of the TC1/mariner superfamily of transposable elements. Mouse liver was cotransfused with a vector containing our most robust human coagulation factor IX expression cassette and the appropriate recombinase recognition site and either a varphiC31- or a SB transposase-expressing vector. To analyze transgene persistence and to prove somatic integration in vivo we induced cell cycling of mouse hepatocytes and found that the transgene expression levels dropped by only 16 to 21% and 56 to 66% in mice that received varphiC31 and SB, respectively. Notably, no difference in the toxicity profile was detected in mice treated with either recombinase. Moreover we observed that with the integrase-mediated gene transfer, transgene expression levels were dependent on the remaining noncoding vector sequences, which also integrate into the host genome. Further analyses of a hot spot of integration after varphiC31-mediated integration revealed small chromosomal deletions at the target site and that the recombination process was not dependent on the orientation in which the varphiC31 recognition site attached to the pseudo-recognition sites in the host genome. Coupled together with ongoing improvements in both systems this study suggests that both nonviral vector systems will have important roles in achieving stable gene transfer in vivo.

- 17. Tolar, J., M. Osborn, S. Bell, R. McElmurry, L. Xia, M. Riddle, A. Panoskaltisis-Motari, Y. Jiang, R.S. Mclvor, C.H. Contag, S.R. Yant, M.A. Kay, C.M. Verfaillie and B.R. Blazar (2005). "Real time in vivo imaging of stem cells following transgenesis by transposition" *Mol. Therap.* 11: 42-48.**

Previous studies have identified *Sleeping Beauty* transposons as efficient vectors for non-viral gene delivery in mammalian cells. However, studies demonstrating the usefulness of transposons as gene delivery vehicles into adult stem cells are lacking. Multipotent Adult Progenitor Cells (MAPC) are non-hematopoietic stem cells with the capacity to form most, if not all, cell types of the body and as such hold great therapeutic potential. The biodistributive properties of MAPC are unknown and prior to any clinical application, it is essential to map their biodistribution in live organisms. We have nucleofected murine MAPC with two plasmid-based *Sleeping Beauty* transposons encoding the red fluorescent protein (DsRed2) and luciferase. Transgenic MAPC clones were euploid, and maintained their characteristic multilineage differentiation potential in vitro. DsRed2 and luciferase expression allowed for MAPC detection in vivo and in tissue sections of the lung. To confirm that transgenesis occurred by transposition into the genome of MAPC, we mapped *Sleeping Beauty* transposon integration sites in two MAPC clones using splinkerette PCR. This novel dual-reporter imaging system based on transgenesis of MAPC with *Sleeping Beauty* transposons sheds light on the homing patterns of MAPC and paves the way for quantification of MAPC engraftment in real-time in vivo.

- 18. Rumpold, H., A.M. Wolf, K. Gruenewald, G. Gastl, E. Gunsilius and D. Wolf (2005). "RNAi-mediated knockdown of P-glycoprotein using a transposon-based vector system durably restores imatinib sensitivity in imatinib-resistant CML cell lines." *Exp. Hematol.* 33: 767-775.**

Resistance to therapeutic drugs is a frequent phenomenon in hematologic malignancies, causing treatment failure in patients with leukemias and lymphomas. Overexpression of the multidrug-resistance gene (MDR-1) and its translational product P-glycoprotein (PgP) represents one mechanism of fatal drug resistance. We constructed a nonviral, *Sleeping Beauty* transposon-based vector system for the stable knockdown of PgP in chronic myeloid leukemia cell lines resistant to imatinib and doxorubicin. Using this strategy, PgP expression was completely knocked down 72 hours after vector inoculation and lasted for several months. Cellular efflux of the PgP substrates rhodamine and doxorubicin was abolished. Vector-treated cells were resensitized to imatinib- and doxorubicin-induced cell death. CONCLUSION: Using chronic myeloid leukemia as a model, we show that PgP-mediated resistance to imatinib and anthracyclines can be durably reversed by nonviral, transposon-based knockdown of PgP in malignant cells.

- 19. Essner, J.J., R.S. Mclvor and P.B. Hackett (2005). "Awakening gene therapy with *Sleeping Beauty* transposons" *Curr. Opin. Pharmacol.* 5 (5): (in press)**

The *Sleeping Beauty* transposons have the potential for use as chromosome-integrating vectors for non-viral gene therapy. Recent preclinical data from mouse models for human genetic disorders has shown efficacy for the *Sleeping Beauty* transposon system in the treatment of hemophilia, tyrosinemia type I, junctional epidermolysis bullosa, and type 1 diabetes. Methods have also been developed to deliver *Sleeping Beauty* transposons to the lung, liver, and tumors for treatments for cystic fibrosis, cardiovascular and metabolic diseases, and cancer. Recent studies characterizing site selection for integration and

insertional mutagenesis indicate that the *Sleeping Beauty* transposon system may be a safer alternative to viral approaches for gene therapy.

- 20. Ohlfest, J.R., Z.L. Demorest, Y. Motooka, I. Vengco, S. Oh, E. Chen, F.A. Scappaticci, R.J. Saplis, S.C. Ekker, W.C. Low, A.B. Freese, and D.A. Largaespada (2005). "Combinatorial anti-angiogenic gene therapy by nonviral gene transfer using the *Sleeping Beauty* transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma." *Mol. Therapy* 12: (in press).**

Glioblastoma is a fatal brain tumor that becomes highly vascularized by secreting proangiogenic factors and depends on continued angiogenesis to increase in size. Consequently, a successful anti-angiogenic therapy should provide long-term inhibition of tumor-induced angiogenesis, suggesting long-term gene transfer as a therapeutic strategy. In this study a soluble vascular endothelial growth factor receptor (sFlt-1) and an angiostatin/endostatin fusion gene (Statin-AE) were co-delivered to human glioblastoma xenografts by nonviral gene transfer using the *Sleeping Beauty* (SB) transposon. In subcutaneously implanted xenografts, co-injection of both transgenes showed marked anti-tumor activity as demonstrated by reduction of tumor vessel density, inhibition or abolition of glioma growth, and an increase in animal survival ( $p=0.003$ ). Using luciferase-stable engrafted intracranial gliomas, the anti-tumor effect of convection enhanced delivery of plasmid DNA into the tumor was assessed by luciferase *in vivo* imaging. Sustained tumor regression of intracranial gliomas was achieved only when Statin-AE and sFlt-1 transposons were co-administered with SB-transposase-encoding DNA to facilitate long-term expression. We show that SB can be used to significantly increase animal survival ( $p=0.008$ ) by combinatorial anti-angiogenic gene transfer in an intracranial glioma model.

## **D. Activity of the SBTS in Fish:**

- 1. Davidson, A.E., D. Balciunas, D. Mohn, J. Shaffer, S. Hermanson, S. Sivasubbu, M.P. Cliff, P.B. Hackett, and S.C. Ekker (2003). "Efficient gene delivery and gene expression in zebrafish using the *Sleeping Beauty* Transposon." *Dev. Biol.* **263:191-202.****

We used the *Tc1/mariner* family transposable element *Sleeping Beauty* (SB) for transgenesis and long-term expression studies in the zebrafish (*Danio rerio*), a popular organism for clinical disease, vertebrate patterning, and cell biology applications. SB transposase enhanced the transgenesis and expression rate six-fold (from 5% to 31%) and more than doubled the total number of tagged chromosomes over standard, plasmid injection-based transgenesis methods. Molecular analysis of these loci demonstrated a precise integration of these elements into recipient chromosomes with genetic footprints diagnostic of transposition. GFP expression from transposase-mediated integrants was Mendelian through the eighth generation. A blue-shifted GFP variant (BFP) and a red fluorescent protein (DsRed) were also useful transgenesis markers, indicating multiple reporters are practical for use with SB in zebrafish. We showed SB is suitable for tissue-specific transgene applications using an abbreviated gamma-crystallin GFP cassette. Finally, we describe a general utility transposon vector for chromosomal engineering and molecular genetics experiments in zebrafish. Together, these data indicate that SB is an efficient tool for transgenesis and expression in zebrafish, and that the transposon will be useful for gene expression in cell biology applications as well as an insertional mutagen for gene discovery during development.

- 2. Grabher C, T. Henrich, T. Sasado, A. Arenz, J. Wittbrodt and M. Furutani-Seiki (2003). "Transposon-mediated enhancer trapping in medaka." *Gene.* **322: 57-66.****

We tested the *Sleeping Beauty* transposable element for its ability to efficiently insert transgenes into the genome of medaka (*Oryzias latipes*), an important model system for vertebrate development. We show that the SB transposon efficiently mediates integration of a reporter gene into the fish germ line. In pilot experiments, we established 174 transgenic lines with a transgenesis efficiency of 32%. Transgenes are stably transmitted to, and expressed in, subsequent generations. Interestingly, the transgenic lines show novel expression patterns with temporal and spatial specificity at a rate of 12% (21/174), likely due to both, enhancing and silencing position effects. Furthermore, promoter-dependent GFP expression in injected fish embryos is tightly correlated with germ line transmission, facilitating easy selection of founder fish. Thus, the SB transposon/transposase system provides a highly efficient tool for transgenesis in general and for the generation of novel reporter gene expression patterns in particular.

- 3. Clark, K.J. A.M. Geurts, J. Bell, and P.B. Hackett (2004). "Transposon vectors for gene-trap insertional mutagenesis in vertebrates." *Genesis* **29: 225-233.****

The function of most vertebrate genes remains unknown or uncertain. Insertional mutagenesis offers one approach to identify and understand the function of these genes. Transposons have been used successfully in lower organisms and plants for insertional mutagenesis, but until activation of the *Sleeping Beauty* (SB) transposon system, there was no indication of active DNA-based transposons in vertebrates. Investigator-driven insertional mutagenesis in vertebrates has relied on retroviral insertions or selection of low

frequency integration of naked DNA in ES cell lines. We have combined the highly active SB transposon with gene-trapping technology to demonstrate that transposon traps can be used for insertional mutagenesis screens in vertebrates. In our studies about one fourth of the trap insertions appear to be in transcriptional units, a rate that is commensurate with random integration. We show that gene-traps coupled to a fluorescent protein reporter gene can be used to detect insertions into genes active in specific cells of living zebrafish embryos, supporting use of our transposon traps for high throughput functional genomic screens in vertebrates.

- 4. Hackett, P.B., K.J. Clark, S.C. Ekker and J.J. Essner (2004). "Applications of transposable elements in fish for transgenesis and functional genomics." In *Fish Developmental Biology and Genetics*. (Zhiyuan Gong and Vladimir Korzh, eds.) Chapter 16, pp 532-580.**

Transgenic fish were first made more than 30 years ago. Since then a variety of methods and constructs have been tested for introducing genetic sequences into fish for scientific investigations as well as commercial purposes. Here we review transposable elements and their applications in fish. Transposons can be used to deliver genes to chromosomes to confer new traits or as insertional agents and traps to uncover the functions and expression patterns of natural genes in chromosomes. Two DNA transposons have been characterized for transposon-based gene transfer and insertional mutagenesis. The first is the *Sleeping Beauty* transposon system that was reconstituted from a *Tc1/mariner*-like relic in salmonid genomes after more than a 10 million year evolutionary sleep. The second is a naturally occurring transposon from medaka, the *Tol2* transposon that belongs to the *hAT* family of mobile elements. In comparison with random integration of plasmid sequences and pseudotyped retroviral genomes, transposons have several advantages for genetic studies in fish. These include introduction of a single, defined DNA sequence into a cellular chromosome, stable expression from the integrant for multiple generations, no absolute size restrictions on the transferred gene, ease in construction and use, and safety. Early experiments have validated the versatility of the *Sleeping Beauty* transposon for all of these purposes. The applications of transposon systems surpass use just in fish; the *Sleeping Beauty* transposon system is being used in mice to discover functions of genes and is being developed for gene therapy in humans.

- 5. Balciunas, D., A.E. Davidson, S. Sivasubbu, S.B. Hermanson, Z. Welle and S.C. Ekker (2004). "Enhancer trapping in zebrafish using the *Sleeping Beauty* transposon." *BMC Genomics* 5: 62.**

Among functional elements of a metazoan gene, enhancers are particularly difficult to find and annotate. Pioneering experiments in *Drosophila* have demonstrated the value of enhancer "trapping" using an invertebrate to address this functional genomics problem. RESULTS: We modulated a *Sleeping Beauty* transposon-based transgenesis cassette to establish an enhancer trapping technique for use in a vertebrate model system, zebrafish *Danio rerio*. We established 9 lines of zebrafish with distinct tissue- or organ- specific GFP expression patterns from 90 founders that produced GFP-expressing progeny. We have molecularly characterized these lines and show that in each line, a specific GFP expression pattern is due to a single transposition event. Many of the insertions are into introns of zebrafish genes predicted in the current genome assembly. We have identified both previously characterized as well as novel expression patterns from this pilot screen. For

example, the ET7 line harbors a transposon insertion near the *mkp3* locus and expresses GFP in the midbrain-hindbrain boundary, forebrain and the ventricle, matching a subset of the known FGF8-dependent *mkp3* expression domain. The ET2 line, in contrast, expresses GFP specifically in caudal primary motoneurons due to an insertion into the poly(ADP-ribose) glycohydrolase (PARG) locus. This surprising expression pattern was confirmed using in situ hybridization techniques for the endogenous PARG mRNA, indicating the enhancer trap has replicated this unexpected and highly localized PARG expression with good fidelity. Finally, we show that it is possible to excise a *Sleeping Beauty* transposon from a genomic location in the zebrafish germline. CONCLUSIONS: This genomics tool offers the opportunity for large-scale biological approaches combining both expression and genomic-level sequence analysis using as a template an entire vertebrate genome.

**6. Grabher, C. and J. Wittbrodt (2004). "Efficient activation of gene expression using a heat-shock inducible Gal4/Vp16-UAS system in medaka." *BMC Biotechnol.* 4: 26**

BACKGROUND: Genetic interference by DNA, mRNA or morpholino injection is a widely used approach to study gene function in developmental biology. However, the lack of temporal control over the activity of interfering molecules often hampers investigation of gene function required during later stages of embryogenesis. To elucidate the roles of genes during embryogenesis a precise temporal control of transgene expression levels in the developing organism is on demand. RESULTS: We have generated a transgenic Gal4/Vp16 activator line that is heat-shock inducible, thereby providing a tool to drive the expression of specific effector genes via Gal4/Vp16. Merging the Gal4/Vp16-UAS system with the I-SceI meganuclease and the *Sleeping Beauty* transposon system allows inducible gene expression in an entirely uniform manner without the need to generate transgenic effector lines. Combination of this system with fluorescent protein reporters furthermore facilitates the direct visualization of transgene expressing cells in live embryos. CONCLUSION: The combinatorial properties of this expression system provide a powerful tool for the analysis of gene function during embryonic and larval development in fish by ectopic expression of gene products.

**7. Hermanson, S., Davidson, A.E., Sivasubbu, S., Balciunas, D., Ekker, S.C. (2004). "*Sleeping Beauty* transposon for efficient gene delivery." *Methods Cell Biol.* 2004;77: 349-362.**

Review article on SBT in fish.

**8. Wadman, S.A., K.J. Clark and P.B. Hackett (2005). "Fishing for answers with transposons." *Marine Biotech.* 7: (in press).**

Transposons are one means that nature has used to introduce new genetic material into chromosomes of organisms from every kingdom. They have been extensively used in prokaryotic and lower eukaryotic systems, but until recently there was no transposon that had significant activity in vertebrates. The *Sleeping Beauty* (SB) transposon system was developed to direct the integration of precise DNA sequences into chromosomes. The SB system was derived from salmonid sequences that had been inactive for more than 10 million years. SB transposons have been used for two principle uses – as a vector for transgenesis and as a method for introducing various trap vectors into (gene-trap) or in the neighborhood of (enhancer-trap) genes to identify their functions. Results of these studies show that SB-mediated transgenesis is more efficient than that by injection of simple

plasmids and that expression of transgenes is stable and reliable following passage through the germline. .

**9. Balciunas, D. and S.C. Ekker (2005). “Trapping fish genes with transposons.” Zebrafish (in press).**

Several recent papers describe pilot screens establishing enhancer and gene trap methodologies for use in fish. They have proven these approaches by characterizing genes with novel and sometimes unexpected expression patterns. The resulting fish lines with tissue-specific GFP expression patterns are now being used in further developmental genetics experiments, enhancing the value of fish models for exploring novel biological phenomena. Both Tol2 and *Sleeping Beauty* transposon systems have been successfully adapted for the construction of enhancer and gene trap vectors. In this review, we summarize the results presented in these papers and compare this first generation of trap vectors. We also discuss future challenges and perspectives for wider use of these methodologies.

## E. Other References to the SBTS:

1. **Dawson, A. and D.J. Finnegan (1998). "Sleeping Beauty awakes." Nature Biotech. 16: 20-21.**

In a paper published in *Cell* at the end of last year, Ivics et al. report the reconstruction of the gene for a novel transposase using information from the fossilized remains of transposable elements lying in the genomes of teleost fishes. Not on a par with the creation of dinosaurs from DNA in the stomachs of mosquitoes trapped in amber perhaps, but an impressive achievement nonetheless. Having roused this transposase from a million years or so of inactivity, Ivics et al. have called it "Sleeping Beauty," showing that it can be used to transpose sequences into the chromosomes of mammalian cells in culture.

2. **Li, Z. H., D. P. Liu, J. Wang, Z. C. Guo, W. X. Yin and C. C. Liang (1998). "Inversion and transposition of Tc1 transposon of C. elegans in mammalian cells." Somat. Cell. Mol. Genet. 24: 363-369.**

*Tc1/mariner* transposons are widespread in the eukaryotes. In vitro transposition test indicated that the transposase is the only protein that is needed in transpositions. It was shown later that the reconstructed Tc1-like transposon, "Sleeping Beauty" in fish, and the *Tc1* transposon in *C. elegans* jumps in human cells. This discovery indicates that the *Tc1/mariner* transposon may be engineered as a somatic gene therapy vector if coupled with an efficient gene delivery system. We introduced the *Tc1* transposon from *C. elegans* into different mammalian cell lines and detected the transposition events, indicating that *Tc1* transposon functions in different mammalian cells. Interestingly, a high inversion frequency of the transposon was also detected, suggesting that this type of transposon may add variations to host genome when it is horizontally transferred into a new species.

3. **Plasterk, R.H.A., Z. Izsvak and Z. Ivics (1999). "Resident aliens: the Tc1/mariner superfamily of transposable elements." Trends Genet. 15: 326-332.**

Transgenic technology is currently applied to several animal species of agricultural or medical importance, such as fish, cattle, mosquitoes and parasitic worms. However, the repertoire of genetic tools used for molecular analyses of mice and *Drosophila* is not always applicable to other species. For example, while retroviral enhancer-trap experiments in mice can be based on embryonic stem (ES) cell technology, this is not currently an option with other animals. Similarly, the germline transformation of *Drosophila* depends on the use of the P-element transposon, which does not jump in other genera. This article analyses the main characteristics of *Tc1/mariner* transposable elements, examines some of the factors that have contributed to their evolutionary success, and describes their potential, as well as their limitations, for transgenesis and insertional mutagenesis in diverse animals.

4. **Richardson, P., C. Thoma, B. T. Kren and C. J. Steer (2002). "Strategies for hepatic gene correction." J Drug Target 10: 133-141.**

Gene augmentation has been the paradigm in the majority of gene therapy protocols but in recent years the potential of repairing the mutated gene in situ by targeted gene correction has become a reality. In fact, targeted gene repair has many advantages over conventional replacement strategies, notably the possibility to treat dominant as well as recessive disorders, and the small molecular size of the pharmacologically active agent. Chimeric RNA/DNA oligonucleotides, small fragment homologous replacement, as well as triplex-

forming and single-stranded oligonucleotides are all examples of the growing armamentarium for gene repair, and are the subject of this review. In addition, we have also included a discussion of the reawakened *Sleeping Beauty* (SB) transposon system as a novel non-viral gene replacement strategy.

**5. Richardson, P. D., L. B. Augustin, B. T. Kren and C. J. Steer (2002). "Gene repair and transposon-mediated gene therapy." *Stem Cells* 20: 105-118.**

The main strategy of gene therapy has traditionally been focused on gene augmentation. This approach typically involves the introduction of an expression system designed to express a specific protein in the transfected cell. Both the basic and clinical sciences have generated enough information to suggest that gene therapy would eventually alter the fundamental practice of modern medicine. However, despite progress in the field, widespread clinical applications and success have not been achieved. The myriad deficiencies associated with gene augmentation have resulted in the development of alternative approaches to treat inherited and acquired genetic disorders. One, derived primarily from the pioneering work of homologous recombination, is gene repair. Simply stated, the process involves targeting the mutation in situ for gene correction and a return to normal gene function. Site-specific genetic repair has many advantages over augmentation although it too is associated with significant limitations. This review outlines the advantages and disadvantages of gene correction. In particular, we discuss technologies based on chimeric RNA/DNA oligonucleotides, single-stranded and triplex-forming oligonucleotides, and small fragment homologous replacement. While each of these approaches is different, they all share a number of common characteristics, including the need for efficient delivery of nucleic acids to the nucleus. In addition, we review the potential application of a novel and exciting nonviral gene augmentation strategy--the *Sleeping Beauty* transposon system.

**6. Kren, B.T., N.R. Chowdhury, J.R. Chowdhury and C.J. Steer (2002). "Gene therapy as an alternative to liver transplantation." *Liver Transpl.* 8: 1089-1108.**

Liver transplantation has become a well-recognized therapy for hepatic failure resulting from acute or chronic liver disease. It also plays a role in the treatment of certain inborn errors of metabolism that do not directly injure the liver. In fact, the liver maintains a central role in many inherited and acquired genetic disorders. There has been a considerable effort to develop new and more effective gene therapy approaches, in part, to overcome the need for transplantation as well as the shortage of donor livers. Traditional gene therapy involves the delivery of a piece of DNA to replace the faulty gene. More recently, there has been a growing interest in the use of gene repair to correct certain genetic defects. In fact, targeted gene repair has many advantages over conventional replacement strategies. In this review, we will describe a variety of viral and nonviral strategies that are now available to the liver. The ever-growing list includes viral vectors, antisense and ribozyme technology, and the *Sleeping Beauty* transposon system. In addition, targeted gene repair with RNA/DNA oligonucleotides, small-fragment homologous replacement, and triplex-forming and single-stranded oligonucleotides is a long-awaited and potentially exciting approach. Although each method uses different mechanisms for gene repair and therapy, they all share a basic requirement for the efficient delivery of DNA.

**7. Largaespada, D.A. (2003). "Generating and manipulating transgenic animals using transposable elements." *Reprod. Biol. Endocrinol.* 1: 80.**

Generating and manipulating transgenic animals using transposable elements. Transposable elements, or transposons, have played a significant role in the history of biological research. They have had a major influence on the structure of genomes during evolution, they can cause mutations, and their study led to the concept of so-called "selfish DNA". In addition, transposons have been manipulated as useful gene transfer vectors. While primarily restricted to use in invertebrates, prokaryotes, and plants, it is now clear that transposon technology and biology are just as relevant to the study of vertebrate species. Multiple transposons now have been shown to be active in vertebrates and they can be used for germline transgenesis, somatic cell transgenesis/gene therapy, and random germline insertional mutagenesis. The sophistication of these applications and the number of active elements are likely to increase over the next several years. This review covers the vertebrate-active retrotransposons and transposons that have been well studied and adapted for use as gene transfer agents. General considerations and predictions about the future utility of transposon technology are discussed.

**8. Miskey, C., Z. Izsvak, R.H.A. Plasterk and Z. Ivics (2003). "The *Frog Prince*: a reconstructed transposon from *Rana pipiens* with high transpositional activity in vertebrate cells." *Nucl. Acids Res.* 31: 6873-6881.**

The *Frog Prince*: a reconstructed transposon from *Rana pipiens* with high transpositional activity in vertebrate cells. Members of the *Tc1/mariner* superfamily of transposable elements isolated from vertebrates are transpositionally inactive due to the accumulation of mutations in their transposase genes. A novel open reading frame-trapping method was used to isolate uninterrupted transposase coding regions from the genome of the frog species *Rana pipiens*. The isolated clones were approximately 90% identical to a predicted transposase gene sequence from *Xenopus laevis*, but contained an unpredicted, approximately 180 bp region encoding the N-terminus of the putative transposase. None of these native genes was found to be active. Therefore, a consensus sequence of the transposase gene was derived. This engineered transposase and the transposon inverted repeats together constitute the components of a novel transposon system that we named *Frog Prince* (FP). FP has only approximately 50% sequence similarity to *Sleeping Beauty* (SB), and catalyzes efficient cut-and-paste transposition in fish, amphibian and mammalian cell lines. We demonstrate high-efficiency gene trapping in human cells using FP transposition. FP is the most efficient DNA-based transposon from vertebrates described to date, and shows approximately 70% higher activity in zebrafish cells than SB. *Frog Prince* can greatly extend our possibilities for genetic analyses in vertebrates.

**9. Kawakami, K., H. Takeda, N. Kawakami, M. Kobayashi, N. Matsuda, and M. Mishina, (2004). "A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish.." *Dev. Cell* 7: 133-144.**

A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. We report here development of a novel gene trap method in zebrafish using the Tol2 transposon system. First, we established a highly efficient transgenesis method in which a plasmid DNA containing the Tol2 transposon vector and the transposase mRNA synthesized in vitro were coinjected into one-cell stage embryos. The transposon vector inserted in the genome could be transmitted to the F1 progeny at high frequencies, and regulated gene expression by a specific promoter could be recapitulated in transgenic fish. Then we constructed a transposon-based gene trap vector containing a splice acceptor and

the GFP gene, performed a pilot screen for gene trapping, and obtained fish expressing GFP in temporally and spatially restricted patterns. We confirmed the endogenous transcripts were indeed trapped by the insertions, and the insertion could interfere with expression of the trapped gene. We propose our gene trap approach should facilitate studies of vertebrate development and organogenesis.

**10. Ivics, Z. and Z. Izsvak (2005). "A whole lotta jumpin' goin' on: new transposon tools for vertebrate functional genomics." *Trends Genet.* 21: 8-11.**

Genome sequences of vertebrate model organisms are becoming available, fuelling the next challenging phase of research: annotating all of the genes with functional information. The fundamental annotation of all of the  $\approx 35,000$  genes in mammals will undoubtedly require complementing the technologies of forward and reverse genetics in mutagenesis screens. Two recent articles report on the construction of retrotransposable elements that efficiently 'jump' in mammalian cells. These new elements hold great promise as useful vectors for insertional mutagenesis screens in the mouse.

**11. Miskey, C. Z. Izsvak, K. Kawakami, and Z. Ivics (2005). "DNA transposons in vertebrate functional genomics." *Cell. Mol. Life Sci.* 62: 629-641.**

Genome sequences of many model organisms of developmental or agricultural importance are becoming available. The tremendous amount of sequence data is fuelling the next phases of challenging research: annotating all genes with functional information, and devising new ways for the experimental manipulation of vertebrate genomes. Transposable elements are known to be efficient carriers of foreign DNA into cells. Notably, members of the Tc1/mariner and the hAT transposon families retain their high transpositional activities in species other than their hosts. Indeed, several of these elements have been successfully used for transgenesis and insertional mutagenesis, expanding our abilities in genome manipulations in vertebrate model organisms. Transposon-based genetic tools can help scientists to understand mechanisms of embryonic development and pathogenesis, and will likely contribute to successful human gene therapy. We discuss the possibilities of transposon-based techniques in functional genomics, and review the latest results achieved by the most active DNA transposons in vertebrates. We put emphasis on the evolution and regulation of members of the best-characterized and most widely used Tc1/mariner family.