DNA TRANSPOSON BASED GENE DELIVERY IN MAMMALIAN CELLS

Short Ph.D. thesis

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2016

INTRODUCTION

Mobile genetic elements (like viruses and transposons) are long considered to be „parasites" of the genome because their only purpose is to spread their own genetic material. However, their long residence significantly shapes genomes, as they can introduce large scale changes due to their activity and repeating sequences. They contribute to variability, can play a role in adaptation processes and in speciation. Several endogenous proteins are known to have transposase structure which is domesticated for different functions in the cells.

Application of viruses and transposons as genetic tools revolutionized research in the fields of mutagenesis and gene delivery. DNA transposons are extinct from genomes of vertebrates, but retrotransposons can be applied for mutagenesis due to their endogenous ability. However, owing to mechanism of their transposition, they can be remobilised, in addition they can make several partial integrations [1, 2], and like endogenous counterparts their silencing is probable in mammalian cells. These features make retrotransposons inappropriate for gene delivery and gene therapy. Vertebrate application of DNA transposons with their favourable nature was impossible until introduction of *Sleeping Beauty* (SB), which was reconstructed from fish genome, and proved to be active also in human cells [3]. Thanks to completely random integration profile of SB, and to several preclinical studies, SB entered clinical phase trials for *ex vivo* gene therapy of special leukaemia and lymphomas [4], as well as for *in vivo* gene therapy of the most common retina degenerations [5]. With time other DNS transposonons turned out to be applicable in vertebrate animal models. The naturally active *piggyBac* (PB) isolated from an insect genome also shows good efficiency in heterologous systems [6]. With the development of hyperactive transposase variants, the two systems become effective alternatives to viruses, whose tumorigenic potential has made them unfavorable in the field [7, 8].

Characterization as a tool for different cell types is a prerequisite for application of DNA transposons in therapy, especially in terms of safety issues for genetic manipulation of progenitor or embryonic stem cells, which can have higher malignant potential. Before our works few studies addressed to characterise systematically and comparatively DNA transposon gene delivery systems. [9, 10]. These researches pointed out cell type dependent differences, however most of them are limited to a few – often tumorous – cell types, and these works most often are not comparable due to differences in applications. Moreover, efficiency of DNA transposon delivery could be significantly lowered with high dose of transposase by the overproduction inhibition phenomenon (OPI) [11]. Comparative studies of

SB and PB systems showing conflicting results are explained by different susceptibility for inhibition, but it was rarely tested. My primary goal was to investigate and optimise DNA transposon based cellular gene delivery.

AIMS OF THE STUDY

1. Investigation of transgene expression and silencing in cell populations transfected with SB transposon system.

2. Development of methods for measuring the activity of transposon systems during gene delivery (transposon excision, copy number).

3. Identification and characterisation of SB integration sites.

4. Examination of the overproduction inhibition phenomenon, and optimization of cellular gene delivery in different cell types with the two most efficient systems (SB and PB DNS transposon systems).

5. Comparison of gene delivery with different promoters applied in the SB transgene.

METHODS

— We examined the dynamics of retention of GFP transgene expression after SB gene delivery in the long run while transfected cells were cultured in population, which were either enriched by FACS after transfection, or were monitored without enrichment.

— We examined silencing of GFP transgene with treatment of trichostatin A (TSA) and 5-Aza-cytidine. The effects of treatments were monitored by FACS, and were controlled by quantitative PCR (qPCR).

— We established genetically uniform cell clones SB transposon system for development of copy number measurements.

— With the application of the splinkerette PCR technique we identified SB integration sites in transgenic cell clones.

— We characterised these sites by density of TA dinucleotides and by Vstep curve, which is attributable to the deformability of DNA structure.

— For monitoring transgenic cell numbers, we applied puromycin antibiotic resistance gene as a transgene, and transgenic efficiencies were measured by colony forming assays with antibiotic selection. In human embryonic stem cells we determined GFP transgene retention: in cell culture populations, the ratio of transgenic GFP expression was normalised to the GFP ratio measured following transfection.

— For measuring transposase activity in cellular gene delivery we detected events of transposon donor excision, for which we developed a qPCR assay.

— In addition to monitoring excision activity and transgenic efficiency, gene delivery experiments with different conditions were also characterised by the achievable copy numbers.

RESULTS

— In our cell culture gene delivery system for modelling *ex vivo* gene therapy, selection pressure had a great impact on transgene retention. Significant selection disadvantage is attributable to transgene expression regulated by the CMV promoter.
— A ratio of transgene silencing (10% of tranfected cells) occurred immediately after transfection into cells.
— However complete silencing of GFP transgene expression was not detected for 6 weeks after transfection. Nevertheless, in cells expressing GFP, decrease in the intensity of expression was detected when transgenes were regulated by strong promoters, such as CAG or CMV.
— Integration sites found in transgenic cell clones derived from gene delivery experiments executed by active SB transposase, are all results of transpositions and show genomic TA target sites characteristic to SB. These sequences have special Vstep curve character attributable to deformability of the DNA structure.

— We have developed a qPCR assay for monitoring transposase activity during gene delivery, by detecting transposon donor excision events. The excision assay is normalised to the ampicillin resistance gene sequence present in all transposon donor plasmids, thus relative quantification is independent of transfection efficiency, and different cell types or conditions are comparable when the same donor dose was applied.
— We have developed three different assays for reliable measurement of transposon copy numbers, and those specific to left and right transposon sequences of the SB are independent of the transgene content, thus can be utilised in all SB applications.
— We have tested the overproduction inhibition phenomenon in three different cell types, but was detected only in the case of PB systems, and despite the good transfection efficiencies it was measured only in a small extent, when extreme transposase dose was applied. Contrary to literature data, SB100X did not show overproduction inhibition, whereas transgenic cell numbers exhibit saturation like curves in all systems when raising the transposase dose.
— Our results pointed out cell type dependent differences. HEK-293 cell type was the most permissive for the activities of all transposase systems, nonetheless more HeLa cells could become transgenic. In HUES9 embryonic cell line transposase activities were proved to be an order of magnitude lower as compared to other cell types.
— In HeLa and in HEK-293 cells mPB showed lower activity than SB100X, while in embryonic stem cells the two systems can be applied with similarly good efficiencies.
— Hyperactive PB (hyPB) has the highest excision activity in all cell types, however, did not produced more transgenic cells than the mPB, and in embryonic stem cells the two transposases are similarly applicable.
— Transgenic efficiencies in many cases were not correlated with excision activities: as an example SB32 showed higher transgenic rates than of SB100X in HEK-293 cells. Nevertheless, in hard to transfect cell types, like embryonic stem cells mainly the hyperactive variants can produce sufficient gene delivery.
— Using the SB systems, very high copy numbers are achievable (even 40 copies/cell), while mPB and hyPB can reach only on average of few copies.
— Average copy numbers can be effectively reduced by substrate (transposon donor) limitation, which ensures sufficient transgenic cell numbers when high dose of hyperactive transposase variants are applied.

— Transcription activities of different promoters by which transgene is regulated influence the efficiency of SB gene delivery, mainly because of *ab ovo* transposase activity, partly due to limited tolerance of the cells observed in the case of the CMV promoter.

CONCLUSIONS

Transgene expression confers selective disadvantage for transgene carrying cells compared to non-expressing or non-transgenic cells. Initial enrichment of cells expressing the transgene can be beneficial for *ex vivo* gene therapy applications as well. Different transgenes can probably be tolerated differently for the cells, thus expression dose is recommended to be carefully adjusted to eliminate this selection effect. For optimization of transgene expression dose, avoiding the silencing of expression intensity may be important.

In agreement with literature data genomic distribution of SB target sites are completely random, however, due to special characteristics of DNA structure integrations are locally non-random. Half of the target sequences in a particular region prefers only 10% of available TA sites, thus expected positions of SB integrations can be predicted.

Our observations for overproduction inhibition indicates that potential inhibition in the cellular context can be a less well-defined phenomenon, which is not necessarily predictable, thus optimization of gene delivery is recommended in each application.

Comparison of efficiencies of SB and PB systems shows different results depending on cell types, and contrary to general belief it can be not explained by altered susceptibility for overproduction inhibition at least in our experiments. This suggests that cell type dependence of PB might be different than that of SB, and embryonic stem cells ensures more preferential environment for the PB systems, which is likely attributable to specific cellular factors. Interaction of PB with cellular factors is a less studied area. However, well known bias of PB integration into genes makes the system less preferable in gene therapy; in addition, SB100X is applicable with similarly good efficiencies in embryonic stem cells, and even hyPB can not produce much higher transgenic efficiencies.

Transgenic efficiencies either in terms of cell numbers or of copy numbers were not correlated with excision activities of the transposases. Not all of the excisions are followed by integration, and these steps are certainly separated from each other kinetically (even spatially and in time), which idea is supported by the ability of development of integration-defective transposase variants.

Finally, when we would like to give a "user guide" for the application of SB transposon system for cellular gene delivery: as a first issue, it is important to specify whether target cells are hard to transfect (max. 10-15%), or easy to transfect (min. 40-50%) cell types. In hard to transfect cells, high transposon donor dose and the most efficient SB100X transposase will result in sufficient transgenic cell numbers, and can produce only low, on average a few copies per cell. In easy to transfect cells, due to higher transfection efficiency application of strong promoters (like CMV) can be unfavourable for the cells even in the case of relatively harmless GFP transgene, therefore cells protect themselves with silencing the intensity of expression, and can get selective disadvantage. Application of strong promoters in the transgene on easy to transfect cells is recommended only in low transposon donor doses. Using endogenous promoters with moderate or low transcription activity can be beneficial, and these transposon cassettes are mobilised more effectively by the SB, and the level of expression can be optimised with the copy number of integrations. Gene delivery with high transposon donor dose will result in high copy numbers, while at low donor dose few copies are expected. Dose of the transposase might need optimisation to avoid overproduction inhibition, however, because it was not observed in our experiments, transposase can be supplied at high dose in the observed systems.

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