

Assays to Evaluate the Genotoxicity of Retroviral Vectors

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Proto-oncogene activation by the retroviral enhancer and/or promoter of replication-competent viruses or disruption of tumor suppressor genes upon vector integration are well-described mutational mechanisms during infection with replication-competent retroviruses and have led to the identification of many genes that are relevant to cancer pathogenesis [1]. The risk of insertional mutagenesis with therapeutic vectors first became evident in a gene therapy trial for the X-linked inherited form of severe combined immunodeficiency (X-SCID) when two [2], and subsequently a third [3] successfully treated patient developed leukemia. A striking example of clonal dominance emerged in a gene therapy trial for chronic granulomatous disease when the majority of hematopoietic cells in two patients who otherwise were successfully treated were found to have insertions into, or near one or more of the *MDS1-EVII*, *PRDM16* or *SETBP1* genes [4]. Transduced target cell populations contain cells with insertional mutations that may alter gene expression thereby conferring a growth advantage that potentially leads to clonal dominance, immortalization or neoplastic transformation during *in vitro* culture or *in vivo* following transplantation. The experience in clinical trials and accumulating evidence in cell culture and animal models [reviewed in 5] underscores the potential importance of vector design in improving the safety of therapeutic gene transfer into hematopoietic cells with retroviral vectors.

Gene therapists have many options to consider that might improve the safety profile of a therapeutic vector [reviewed in 5]. Utilization of a self-inactivating (SIN) design eliminates the retroviral enhancer/promoter from the integrated long-terminal repeats (LTR). Expression of the therapeutic transgene in a SIN vector depends on an internal promoter and many are available that, in principle, have variable probabilities of influencing the expression of nearby genes. Flanking the therapeutic transgene cassette with insulator elements [6], either within the vector or within the integrated LTRs, may improve transgene expression by reducing integration position effects and also block interactions between regulatory elements in the promoter of the vector transgene and surrounding gene sequences. Given the many variables that may be tested, there is a definite need for reliable, sensitive and reproducible assay systems to evaluate vectors with respect to their potential safety profile.

A recent report from the Baum laboratory describes the development of a cell culture assay to evaluate the relative genotoxicity of conventional oncoretroviral and SIN

oncoretroviral vectors [7]. This work builds on a prior report from the Copeland laboratory [8] in which evidence was presented that transduction of primary murine bone marrow cells with a replication-incompetent, marker vector resulted in a high frequency of emergence of immortalized cell populations. These populations remained cytokine dependent and were nontumorigenic in murine models. Interestingly, many of the populations contained vector integrations into or near the *MDS1/EVII* or *PDRM* loci. Retroviral enforced expression of the *EVII* coding sequences seemed sufficient to achieve cell immortalization [8]. In the new work reported in Blood from the Baum laboratory [7], the immortalization assay was modified in several ways in an effort to make it more quantitative. Vector preparations of equivalent titer were loaded onto retronectin-coated plates and used to transduce fixed numbers of cells in serum-free medium. After fourteen days of expansion in culture, one hundred cells were plated into individual wells. The frequency of wells which gave rise to proliferating cell populations provided an estimate of the immortalization frequency in the transduced population.

Importantly, the SIN vector was capable of generating immortalized cells in this assay. Although the investigators estimated that the SIN vector was twelve-fold less efficient than the conventional vector in achieving immortalization, when expressed per vector copy number, several factors mitigate against a simple quantitative interpretation of this difference with respect to relative vector safety. The authors note that the replating frequency reflects a combination of the number of independent mutants as well as their competitive growth in the initial bulk culture. Integration site analysis disclosed that many of the clones that expanded sufficiently to allow analysis contained 3-10 vector insertions and were often genetically identical or shared many of the same insertions. Six of eight clones contained an insertion upstream and in a reverse orientation from the *EVII* coding sequences resulting in production of an *EVII* protein lacking the PR domain, which has been implicated as a negative regulator of tumorigenesis. Overall, this work unequivocally shows that a SIN vector with an internal oncoretroviral promoter exhibits genotoxicity and represents a first step toward a quantitative evaluation of two vectors of differing design.

The Sorrentino laboratory has taken a different approach in searching for unique risk factors for insertional mutagenesis in a mouse model of X-SCID gene therapy [9]. Mouse

models of X-SCID have been derived by knock-out of the γ_c gene and correction of these immunodeficient mice has been achieved by retroviral vector-mediated gene transfer. No evidence of an enhanced risk of a lymphoproliferative process occurred in the context of the initial trials of gene therapy in these models [reviewed in 5 and 9]. Accordingly, the Sorrentino laboratory derived a mouse strain that is also deficient in the *Arf* tumor suppressor gene, the function of which is lost in the majority of patients with T-cell leukemia. *Arf*^{-/-} animals were bred to the γ_c -deficient animal to derive *Arf*^{-/-}, γ_c -deficient animals. Correction of the γ_c deficiency by retroviral vector-mediated gene transfer was associated with a markedly increased incidence of a malignant lymphoproliferative process with a latency of approximately four to six months. The bone marrow of the *Arf*^{-/-}, γ_c -deficient animals contained an expanded population of primitive hematopoietic cells that seem likely to be committed lymphoid progenitors arrested in development by virtue of the γ_c deficiency. This primitive population, when rapidly expanding following gene correction, may create the opportunity for the generation of neoplastic cells both because of the oncogenic potential of specific retroviral integration events and from other spontaneously occurring mutations. Integration site analysis demonstrated insertion near or within known proto-oncogenes and common integration sites with two integration events near the same proto-oncogene were found. In addition, clonal expansion during *in vitro* culture was evident, in that tumors having an identical integration site were found in two or three animals. On-going experiments have been designed to evaluate various features of retroviral vector design with respect to the risk of lymphomagenesis following gene correction in this model.

The Naldini laboratory has also used a tumor-prone mouse model lacking two tumor suppressor genes (*Cdkn2a*^{-/-}) to compare the genotoxicity of a SIN lentiviral vector with an internal cellular promoter to a conventional MLV oncoretroviral vector with intact LTRs [10]. *Cdkn2a*^{-/-} bone marrow cells, when infused into irradiated wild-type recipients, form hematopoietic tumors within 150-300 days following transplantation. Transduction of *Cdkn2a*^{-/-} cells with the MLV vector accelerated the occurrence specifically of myeloid tumors in this model whereas the SIN lentiviral vector did not accelerate the rate of tumor formation. This apparent low genotoxicity of the lentiviral vector occurred despite a higher vector copy number than that observed with the MLV vector. The improved safety profile of the lentiviral vector could reflect either or both the SIN design or the use of an internal cellular promoter rather than an oncoretroviral promoter. The SIN vector utilized in the Baum laboratory's experiments contained an internal oncoretroviral promoter.

My laboratory has taken yet another approach to evaluating the relative genotoxicity of various vector designs. We have used recombinant adeno-associated viral vector gene targeting to precisely recreate the insertion into the first intron of the *LMO2* gene present in one of the X-SCID

patients that developed leukemia. Insertion of an expression cassette having a single retroviral LTR promoter resulted in more than a one thousand-fold increase in the expression of the *LMO2* gene in individual Jurkat T-cell clones [B. Ryu and A. Nienhuis, unpublished observations] in agreement with the results from the Baum laboratory that a single LTR promoter is genotoxic [7]. The expression cassette is flanked by LoxP sites permitting Cre-mediated exchange with substitution of expression cassettes having differing designs. Current studies are focused on evaluating the ability of an insulator element to prevent oncoretroviral LTR-mediated, increased *LMO2* gene expression.

Progress has been rapid in devising various assays to evaluate specific aspects of the vector design although there are many heretofore unappreciated complexities. For example, the insulator element used in most gene therapy vectors (5'CHS4) includes both chromatin boundary forming and enhancer blocking functions [11]. However, these functions have been defined, for the most part, in erythroid cells. Little evidence is available to allow generalization that the boundary-forming and enhancer-blocking functions of the 5'CHS4 element will be evident in other cell types. Also these two distinct functions will be needed at different stages of hematopoiesis. The enhancer blocking activity is required to prevent proto-oncogene activation in primitive hematopoietic cells that are susceptible to immortalization. In contrast, the chromatin boundary forming function that prevents position effects on transgene expression is required in terminally differentiated cells in which the transgene functions. A single insulator element may not provide these two distinct functions in the appropriate cellular contexts. Efforts to sort out these complexities will be well served by the newly developed assays for assessing the relative genotoxicity of various vector designs.

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