Gene therapy: too much splice can spoil the dish

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The use of integrating vectors for gene therapy — required for stable correction of gene expression — carries the risk of insertional mutagenesis, which can lead to activation of a tumorigenic program. In this issue of the *JCI*, Moiani et al. and Cesana et al. investigate how viral vectors can induce aberrant splicing, resulting in chimeric cellular-viral transcripts. The finding that this is a general phenomenon is concerning, but some of their results do suggest approaches for the development of safeguards in gene therapy vector design.

Gene therapy is coming of age, with a growing number of successes finally fulfilling promises long heralded but without much to show in the clinic. Particularly inspiring is the demonstration that stem cell–targeted ex vivo gene therapy can cure inherited hematological disorders such as congenital immunodeficiencies and thalassemia (1–6). Because this requires the life-long expression of a therapeutic transgene in a cell lineage constantly replenished from the differentiation of self-renewing precursors, these need to be stably modified, a feat that so far can be reliably achieved only with integrating viral vectors. This carries a price, including the risk that a growth-promoting gene in the neighborhood of the transgenic integrant could be unduly activated and promote the expansion of cells thereby selected, culminating in an oncogenic process. The secondary development of acute leukemias in patients initially cured of their severe combined immunodeficiency (commonly called "bubble boys") by autotransplantation of retrovirally corrected HSCs was an early and cruel reminder of this dramatic manifestation of insertional mutagenesis (7). Similar complications have plagued gene therapy trials for chronic granulomatous disease (8) and Wiskott-Aldrich syndrome (9).

Insertional mutagenesis most commonly results from the stimulation of a cellular promoter through *cis*-acting influences exerted by transcriptional elements present in the vector provirus integrated nearby. For instance, all cases of leukemia in the cohort of retrovirally treated patients with severe combined immunodeficiency resulted from the transcriptional activation of *LMO2*, a known proto-oncogene, by enhancer sequences contained in the long terminal repeat (LTR) of the murine leukemia virus–derived (MLV-derived) therapeutic vector (10). In the clinic, MLV-based gene delivery systems are being progressively supplanted by HIV-derived lentiviral vectors, which are far more efficient in nondividing or slowly dividing cells, including minimally stimulated HSCs. As a lucky bonus, lentiviral vectors appear to carry a lower risk of insertional mutagenesis (11), probably because they tend to integrate within the transcribed region of genes, whereas MLV and derived vectors land in and around promoters (12). Furthermore, the design of self-inactivating (SIN) vectors, in which LTR-containing transcriptional elements are deleted during reverse transcription, further minimizes the risk of proto-oncogene activation (11).

Nature's insolent unpredictability

Retroviruses have long been known to have more than one trick in their bag to perturb gene expression. Accordingly, it was no surprise to learn that a patient successfully treated for β-thalassaemia by lentiviral vector–mediated HSC transduction owed his newly gained transfusion independence to the emergence of a dominant myeloid clone, in which the growth-promoting *HMGA2* gene was activated not only transcriptionally but also posttranscriptionally (3). The latter effect occurred via vector-triggered aberrant splicing, which generated a truncated *HMGA2* transcript that escaped regulation by a microRNA (miRNA) directed at the 3′ end of the full-length mRNA (Figure 1).

In this issue of the *JCI*, the teams of Fulvio Mavilio and Eugenio Montini, who have had a long-standing interest in assessing the genotoxicity of integrating vectors, follow up on this observation by reporting large-scale explorations of provirus-induced aberrant splicing (13, 14). Both studies were performed using lentiviral vectors and human cells, notably HSCs and primary T lymphocytes. While distinct in their methodological approaches, both analyses led to the same conclusion: vector-induced aberrant splicing, in which transcripts emanating from upstream cellular promoters are spliced into provirus-derived RNAs, is a general phenomenon. Through highly sensitive yet very specific techniques, these chimeric ("read-through") transcripts were systematically detected in populations of transduced cells. While neither study could claim a strong quantitative power, the examination by Mavilio and colleagues of a limited set of integrants revealed read-through transcripts for more than half of the targeted genes in all cell types tested (13).

Levels of chimeric transcripts were most often low, in part due to nonsense-mediated mRNA degradation, a process triggered by abnormally long 3′ noncoding regions. However, in about 10% of cases, read-through mRNAs matched their physiological counterparts in abundance (13), which, considering that retroviral integration is monoallelic, suggests complete subversion of transcripts produced by the targeted locus.

Sequence analyses of a high number of chimeric transcripts cumulatively confirmed that they originated from bona fide aberrant splicing and pointed to vector elements more likely to precipitate this event. However, many of these elements were cryptic splice sites that were not predictable. Montini and colleagues went on to demonstrate that mutating some of these sequences could reduce the rate of read-through transcription, but this was accompanied by a drop in vector titer, which might make this approach untenable for many applications (14). They also found that the presence of a wild-type LTR increased the incidence of aberrant splicing,

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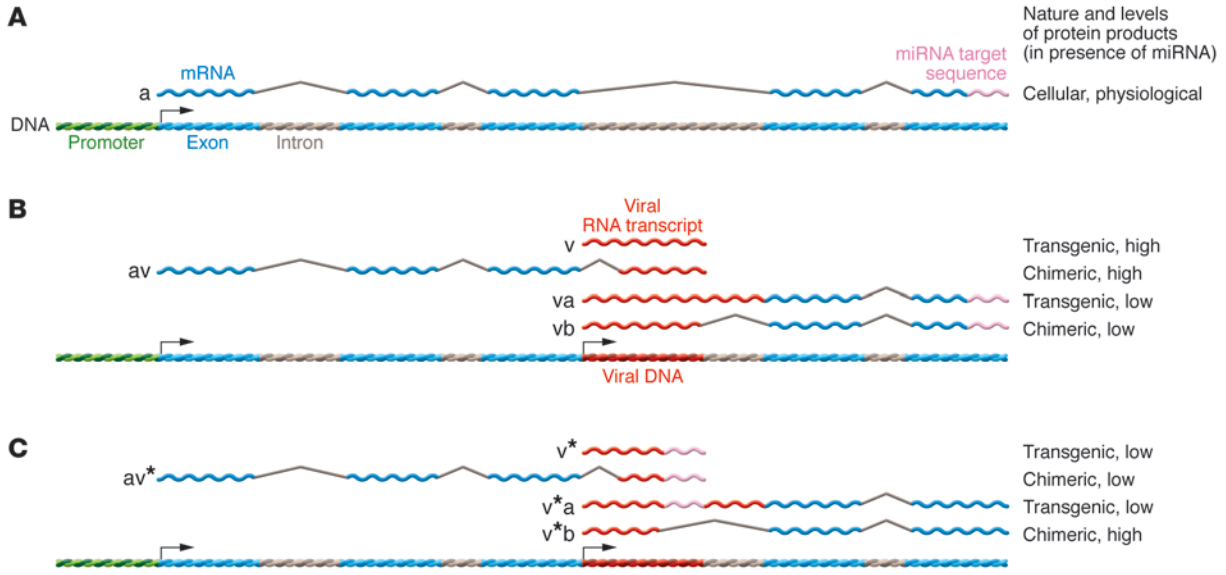

Figure 1
Vector-induced chimeric transcripts. (**A**) A cellular gene producing an mRNA endowed with a regulatory miRNA target sequence at its 3′ end. Protein product is described at right. (**B**) The same gene, with a vector provirus integrated between two exons in the sense orientation. Two general categories of aberrant mRNAs are depicted as either 5′ (av) or 3′ (va/vb) fusions between vector (v) and cellular transcripts. Compared with its physiological counterpart (a), av mRNA yields a truncated cellular protein (potentially fused to a fragment of the transgenic protein) at high levels, owing to the loss of 3′ miRNA target sequences. va results from proviral transcriptional read-through, and vb results from the use of a cryptic splice donor in the vector. Only the transgenic protein is produced at significant levels from va, as translation of the cellular part of this transcript would require reinitiation, a very inefficient process. The resulting transcript is predicted to be expressed at low levels, irrespective of the presence of an miRNA target sequence, due to nonsense-mediated degradation. (**C**) The provirus-harboring locus, with insertion of target sequences for a stage-specific miRNA in the vector transcript as a safeguard. Both vector-derived (v*) and cellular-viral fusion (av*, v*a) mRNAs will be degraded in cells expressing the miRNA, e.g., transformation-prone stem cells, resulting in very low levels of abnormal protein. However, a vb-like mRNA devoid of miRNA target sequence owing to aberrant splicing would escape downregulation, as would av-like transcripts generated from a provirus integrated in the antisense orientation.

but since Mavilio and colleagues performed all of their analyses with SIN lentiviral vectors, this nuance gives no real comfort. These findings mirror the recent report of proviral transcriptional read-through transcripts in keratinocytes derived from skin stem cells transduced with SIN lentiviral vectors (15). It is thus likely that some degree of vector-induced aberrant splicing always occurs within a population of retrovirally transduced cells and at least a fraction of these cells harbor RNAs generated by 5′ or 3′ fusion of viral and cellular transcripts (Figure 1, A and B).

The thin line between curing disease and inducing disease

What are the clinical implications of this phenomenon? Because current gene therapy protocols involve the genetic modification of populations of cells, rather than the replacement of abnormal tissues by expansion of a single corrected cell clone, the only phenotypes of medical relevance will be those conferring a selective advantage to serendipitously modified cells. For instance, a fusion transcript that led to the death of its rare host cell would have no impact at the level of a mixed population. In contrast, a proliferation-promoting event will result in a dominant phenotype, with selective expansion of the corresponding clone over its uncorrected and physiologically corrected counterparts. This can classically occur by overexpression of a growth factor or by production of a dominant-negative mutant, for instance, one in which a C-terminal regulator domain is truncated (Figure 1B). Sometimes, such clonal expansion can have, at least transiently, a beneficial impact. This was the case in the lentivirally cured patient with thalassemia, for whom sufficient levels of hemoglobin would most likely not have been obtained without the generation of a β-globin–producing *HMGA2*-activated clone, considering the low levels of stem cell gene modification achieved in this type of protocol and the absence of intrinsic growth advantage of corrected erythroblasts (3). However, emergence of a dominant cell clone should be, as a rule, considered as the likely prelude of a multistep oncogenic process, the most fearsome long-term complication of gene therapy with integrating vectors.

Lessons for vector design

Can the risk of aberrant splicing be predicted for a given vector? The risk of aberrant splicing can only be predicted for a given vector to an extent through the types of in vitro analyses described in this issue of the *JCI* (13, 14). However, observations collected so far point to the frequent use of noncanonical cryptic splice donor or acceptor sites, in some cases generated by reverse transcription–induced mutations, and to the critical influence of integrated locus-specific elements (3, 13, 14). Nevertheless, the finding that in most cases levels of viral-cellular fusion transcripts are low, whether due to weak rates of aberrant splicing or to missense-mediated RNA degradation, is reassuring, as many growth-promoting factors only

dose-dependently trigger cell proliferation. The stability of a lentiviral vector–induced, truncated *HMGA2* mRNA that was deleteriously increased by loss of miRNA target sequences is a sobering counterexample (3), yet it suggests approaches for the development of safeguards. For instance, the inclusion of cell type– and stage-specific miRNA target sequences in vector-derived transcripts can elegantly restrict transgene expression to particular targets (16). Properly tailored, it could similarly serve to destabilize harmful fusion transcripts in cells particularly susceptible to transformation, namely stem cells and early precursors. In situations in which only differentiated cells require phenotypic correction for disease to be prevented, the safety margins of integrating gene therapy vectors could thus be significantly increased by combining stage- and lineage-specific promoters, to avoid proto-oncogene activation in stem cells and early precursors (17), and sequences targeted by miRNAs expressed in these cells, in which they would promote the degradation of dangerous cellular-viral fusion transcripts (Figure 1C and ref. 18). Pending the advent of efficient techniques for site-specific integration and clonal stem cell expansion (19), such tricks may significantly improve the safety of tools currently available for gene- and cell-based therapies.

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Epidermal Langerhans cells tune skin reactivity to contact allergens

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Allergic contact dermatitis is a common disorder that has fascinated dermatologists and immunologists for decades. Extensive studies of contact sensitivity reactions in mice established a mechanistic paradigm that has been revisited in recent years, and the involvement of Langerhans cells (LCs), a population of epidermal dendritic cells, in immune responses to epicutaneously applied antigens has been questioned. In this issue of the *JCI*, Gomez de Agüero et al. describe an elegant series of experiments that implicate LCs in tolerance induction, positioning these cells as key regulators of immunologic barrier function.

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Skin represents a dynamic, responsive interface that separates organism and environment (reviewed in ref. 1). Epidermal keratinocytes that are capped by a nonvital cornified layer and joined by a network of tight junctions constitute a physical barrier that, under normal circumstances, prevents entry of many environmental agents. An increasingly well-defined constellation of immune and inflammatory cells creates an immunologic barrier that is poised to respond to environmental insults that breach the skin's physical barrier. Reactivity of this immunologic barrier is fine tuned. Pathogenic microbes trigger responses that are sufficiently vigorous and sustained so that offending organisms are contained and ultimately cleared without