cause defective recognition of cell surface antigens involved in migration, extravasation, or fusion. Finally, the combination of the exogenous human α-sarcoglycan with the murine sarcoglycans β, γ, δ leads to the formation of a tetrameric but chimeric complex of unknown functionality.

An ultimate goal of cell therapy approaches is to generate biological products amenable to clinical trials, which impose several restrictions regarding production, characterization, quality control, and delivery to prevent the worsening of the pathologies by embolisms, immune reactions, unexpected differentiations, or fibroses. With this in mind the authors have tested the robustness of their protocols using iPS cell lines certified to be free of viral integrations, and the cells proposed by Tedesco and collaborators may constitute the first generation of a new category of progenitors that, despite not being completely equivalent to their natural *in situ* counterparts, may share on demand some angiogenic and/or myogenic capacities. Further work will be necessary to (i) assess their safety and stability, (ii) document the colonization of specific muscles affected in several myopathies (diaphragm, intercostal muscles, heart), (iii) compare HIDEMs derived from various initial cell types, and (iv) assess their potential immunogenicity, even in an autologous context.[27] The production of HIDEMS may benefit from ongoing progress in our understanding of the biology of pluripotent stem cells, which are emerging as important weapons in the armamentarium of cell, gene, and molecular therapy.

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rAAV-Mediated Tumorigenesis: Still Unresolved After an AAV Assault

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The risk of oncogenesis mediated by vector-induced insertional mutagenesis during therapeutic gene transfer has received much attention in recent years. Any nucleic acid, regardless of how it is delivered, can cause insertional mutagenesis if it integrates into the genome. The two parameters that define this risk are integration site preference and frequency of integration. Recombinant adeno-associated viral (rAAV) vectors have been shown to be safe and efficacious in early gene therapy clinical trials,[1–4] although such vectors do integrate into the genome at a low but measureable rate (0.1 to 1% of transduction events) in animal models.[5,6] In this issue of *Molecular Therapy*, Rosas and colleagues test various conditions that are hypothesized to facilitate rAAV integration so as to determine whether these events can lead to an increased rate of oncogenesis.[7]

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The issue of oncogenesis in rAAV-mediated gene therapy became more than just a theoretical concern when tumors were identified in neonatal β-glucuronidase-deficient mice treated with rAAV.[8] Four of these tumors were found to contain integration sites within the imprinted *Dlk1-Dio3* locus on mouse chromosome 12qF1, which led to the development of hepatocellular carcinoma (HCC) in a manner independent of β-glucuronidase status.[9] To confirm causality, an rAAV vector designed to integrate into the *Dlk1-Dio3* locus via homologous recombination was shown to be able to replicate the HCC phenotype.[10]

Although rAAV-mediated integration is much lower than that seen with either retroviral/lentiviral or transposon vectors, it is much greater than that observed with plasmid or adenoviral vectors. Moreover, it has been shown that rAAV proviral genomes preferentially integrate near or within transcriptionally active genes.[11–13] There is a long-standing debate regarding whether these integrations significantly enhance the risk of oncogenesis,[8,9,11,14–16] which has been exacerbated by the existence of a number of confounding variables in previous studies that have yet to be fully controlled for (for discussion see ref. 14).

In the new study, the authors generated a self-complementary AAV (scAAV) vector that included promoter and enhancer sequences but lacked coding or polyadenylation sequences (CBA-null), which was specifically designed to enhance readthrough transcription into neighboring genes. Integration of this vector was compared to that of a more typical gene transfer vector that included coding and polyadenylation sequences (scAAV-CMV-GFP-pA). To further promote vector-induced oncogenesis, a subset of mice was treated with camptothecin and/or underwent partial hepatectomy. Camptothecin is a DNA double-strand break–inducing agent that should increase proviral DNA integration,[17] whereas a surgical partial hepatectomy drives hepatocellular regeneration, which has several implications for potential integration (described below). Two murine strains were evaluated: C3H/HeJ mice, which develop HCC at a high frequency (30–50% of males)[18]; and severe combined immunodeficient (SCID) mice, which lack the catalytic subunit of DNA-PK.[19] The latter mice exhibit impaired B- and T-cell maturation resulting in a lack of cell-mediated and humoral-adaptive immune responses, as well as impaired DNA double-strand break repair. Both of these parameters have been shown to increase the rate of AAV integration.[20] This combination of factors is akin to a pilot in a flight simulator trying to land a plane while various safety overrides are systematically inactivated.

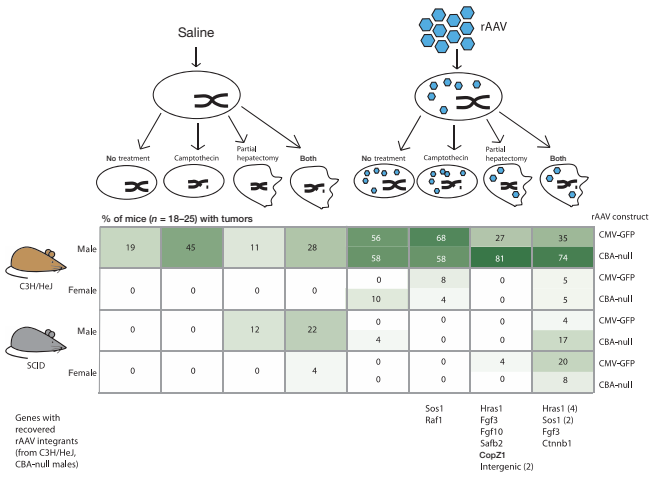

Figure 1 Overview of the rate of oncogenesis and the identified integrated genes. rAAV, recombinant adeno-associated virus; SCID, severe combined immunodeficient.

So, was there a safe landing? In short, yes, although perhaps with some turbulence. In all but one condition, rAAV sequences did little to promote tumorigenesis (**Figure 1**). A large number of mice ($n \approx 25$ per group) were used to deduce significant increases in tumor incidence in each test group. SCID mice produced very few tumors; the same can be said for female C3H mice and mice treated with camptothecin. However, male C3H mice, which are prone to develop HCC, did show an accelerated and statistically significant increase in tumor incidence in the liver following delivery of either scAAV-CMV-GFP-pA or CBA-null rAAV vectors. Tumors from CBA-null-treated animals contained identifiable rAAV integration sites, suggesting that rAAV accelerated the tumor phenotype by inducing additional oncogenic events. Importantly—and boding well for prevention of toxicity—no tumors were identified in any of the 12 additional tissues examined. This was consistent with previous studies in which tumor sites were limited to the liver.[10] However, the absence of tumors in nonhepatic tissues may reflect low levels of transduction in other tissues following intravenous infusion of rAAV8 vectors.

Another arm of the study yielded inconsistent and inconclusive results as to whether partial hepatectomy had an effect on tumor progression. Partial hepatectomy can enhance tumorigenesis both by enabling clonal expansion of cells with oncogenic integrations, and by inducing cell division and corresponding DNA replication that could promote viral integration. However, a two-thirds partial hepatectomy results in all hepatocytes undergoing one or two cell divisions resulting in the loss of ~90–95% of rAAV genomes.[21] If performed at an early time point (16 hours post-rAAV infusion in this case), a two-thirds partial hepatectomy might decrease the total number of integration events. However, it should be noted that in the current study the degree of liver regeneration was probably less robust, as less than 50% of the liver was removed. Nonetheless, in control mice injected with either saline or AAV-CMV-GFP, partial hepatectomy led to a decrease in tumor incidence, whereas a small increase in tumor frequency was observed in CBA-null–treated animals.

These conflicting results are also reflected in the vector genome analysis. The mean vector copy number (per diploid genome) in tumor samples was higher in tumors from post-hepatectomy CBA-null–treated animals than in tumors from the AAV-CMV-GFP–treated control groups, suggesting that CBA-null integration was more often associated with tumor formation. However, the mean vector copy number in tumors from AAV-CMV-GFP

control mice post-hepatectomy and post–camptothecin + hepatectomy indicated less than one copy of vector genome per cell, possibly due to a "hit-and-run" phenomenon. In a hit-and-run event the evidence of an integration event can disappear if the region surrounding the proviral AAV genome is lost for any reason (e.g., recombination). Second, the number of nonmalignant cells contained within the tumor will affect the integration signal within a malignancy. Interestingly, the vector copy number reported at 2 weeks post-transduction was ~100 times higher than the amount at the end of the study, suggesting that the C3H/HeJ liver undergoes substantial regeneration and loss of episomal DNA relative to a normal liver even without surgical partial hepatectomy. This effect is therefore partly redundant with the partial hepatectomy, where vector copy number decreased ~10 times from control mice treated with the same vectors but without induced liver regeneration.

The integration locations are also of particular interest. Four integration events occurred in the *Hras* promoter region, whereas three were in intron 8 of *Sos1*, leading to activation of downstream, but not upstream, *Sos1* exons. Since the transactivation domain is at the beginning of the *Sos1* gene, the presumed novel gene product would be devoid of this regulatory domain and remain constitutively active. Two events occurred at the *Fgf3* gene promoter in an antisense orientation, nevertheless leading to *Fgf3* activation. Finally, single integration events took place near or within the *Fgf10*, *Raf1*, *Safb2*, *Ctnnb1*, and *CopZ1* genes. The induction of cell division could influence genes such as *Fgf3* and *Fgf10*, which are typically silenced in the adult liver and presumably have a closed chromatin state, to become actively expressed and have the potential to be exposed to rAAV integration events.

Does this mean that these are hot spots of oncogenesis? Considering that the authors did not detect multiple integration sites per tumor, it appears that these sites are particularly prone to integration events. However, the C3H mice, which already commonly develop HCC, do so through activating mutations at codon 61 of the *Hras* gene.[22] Thus, it would be relevant in this study to sequence *Hras* in these tumor samples to determine if compounding mutations were present or required for transformative growth. The appearance of *Hras* mutations in C3H mice further suggests that *Hras* is permissive in this model to DNA damage, and that integration at this site is less likely to be a function of the viral vector sequence. Without a comprehensive evaluation of the effect of gene activation, it remains unclear whether the integration sites identified in this study are indeed oncogenic, or whether additional integration events took place that were not detected by linear amplification–mediated polymerase chain reaction. Furthermore, some integration events may have prompted local genomic rearrangements during DNA repair. This could disrupt endogenous genes while eliminating any trace of the offending viral vector. This situation may account for the lack of a detectable integration event in the majority of tumors (85 of 102) isolated from rAAV-infected mice. The advent of RNA sequencing technologies and their diminishing cost will help enable discovery of some of these "missing" integration events in the future.

Does this study settle the question of whether rAAV is carcinogenic? In some respects, it diminishes the fear of rAAV-mediated toxicity. The only mice that exhibited an increased tumor burden were those that were already predisposed and those that have been shown to develop tumors in up to 80% of males, depending on additional treatments.[23] This raises the issue of whether rAAV should be used to treat disorders for which the disease pathogenesis is already oncogenic (e.g., viral hepatitis). Several variables can potentially influence the rate of oncogenesis, most notably the sex and age of the mice (e.g., neonates vs. adults); the transgene sequence and choice of promoter; the vector serotype, dose, and delivery route; the genetic background and environmental living conditions. Even if one could control for these variables, it is not clear how transferable these results are to human clinical studies. However, this study encourages the continued study of "safe harbor" sites where integration events have little effect on the transcription of neighboring genes[24] or are directed into known benign genomic locations such as the widespread rRNA loci.[25,26] Additionally, this study has added to the list of genomic locations at which integration sites should be monitored and avoided.

It is estimated that there are 300 billion hepatocytes in the human liver, and if one assumes a vector dose in which 10% of the hepatocytes are transduced, even with an integration rate of 0.1%, a single individual will have ~30 million hepatocytes with at least one integration event—not a trivial number. However, there is no way, at present, to correlate the number of these events with the risk of oncogenesis. Overall, it seems logical that proviral integration will provide some risk, but overall animal studies continue to suggest that this risk is relatively low. It appears that additional flight simulations are required to ensure a safe landing for rAAV delivery in the future.

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A New Vision of Mesenchymal Stromal Cells

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Although corneal grafts can restore vision to damaged and opaque eyes, these grafts, like any other transplanted tissue, may be rejected by the host so that blindness returns. In this issue of *Molecular Therapy*, Oh *et al.* report that the infusion of human mesenchymal stromal cells (MSCs) prevents allogeneic corneal rejection in a murine model and—crucially—that these benefits may be mediated by a soluble, MSC-derived anti-inflammatory protein, tumor necrosis factor-α (TNF-α) stimulated gene/protein 6 (TSG-6).[1] If similar effects can be produced in humans, injection of the recombinant protein may greatly increase the success and survival of corneal grafts.

Infection, injury, or inborn errors of metabolism can all damage the cornea, the transparent tissue covering the pupil in the eye, producing an opaque scar that leaves the person functionally blind. Corneal transplants were among the first successful human tissue transplants and have been very effective in restoring vision to affected individuals. The unique environment of the eye supports a phenomenon termed anterior chamber acquired immune deviance that usually allows even mismatched corneal transplants to survive without rejection.[2,3] This same immune tolerance mechanism may help to explain why the ocular environment has allowed recent gene therapy advances for retinal degenerative diseases and ocular malignancies.[4–8] Because of this corneal immune privilege, low-risk corneal transplants have an 80% chance of being rejection-free after 5 years, even without the immunosuppressive therapy needed for most other human tissue grafts. Although outcomes are generally good, there is an increased risk of graft failure that may be as high as 50% over 5 years if inflammation is present at the time of transplant, if the endothelial layer is disrupted during surgery, or if there has been a previous rejection episode. Systemic immunosuppression has not been proven useful in preventing this complication, and although prolonged administration of topical steroids may help salvage corneal rejection, this therapy is commonly associated with cataract and glaucoma. More recent efforts have therefore focused on improving surgical techniques to enable salvaging of the endothelial cell layer of the host (if this layer is not diseased), allowing transplantation of only the anterior layer of the donor cornea. Technically this is a difficult procedure, and it remains uncertain whether graft survival is truly increased.

The report by Oh *et al.*[1] offers an alternative to the above approaches. Recent studies had already suggested that the administration of (allogeneic) MSCs can decrease the incidence of organ rejection in a variety of animal transplant models through mechanisms that were not elucidated.[9] Oh *et al.* now show that the same approach can prevent the rejection of corneal grafts in a murine model. They show that MSC infusion suppressed early surgically induced inflammation and reduced the activation of antigen-presenting cells in the cornea and draining lymph nodes. The subsequent risk of immune rejection was decreased and allograft survival prolonged. Importantly, these benefits did not require the local presence of the MSCs, which did not infiltrate the transplanted cornea but instead appeared to remain in the lung vasculature. This paradox prompted the investigators to seek an indirect mechanism of immune modulation. Previous investigations had shown that MSCs secrete the soluble anti-inflammatory protein TSG-6 after tissue injury and that TSG-6 prevented subsequent inflammation.[10,11] This evidence prompted the authors to investigate the cytokine messages upregulated in human MSCs injected into mice in conjunction with corneal engraftment, and they found that TSG-6 messenger RNA was increased more than any of the other cytokines tested. TSG-6 is a 30-kDa secreted protein that contains a hyaluronan-binding domain and, as its name suggests, is expressed in response to the presence of TNF-α as well as interleukin-1 (ref. 12). As a member of the hyaluronan-binding

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