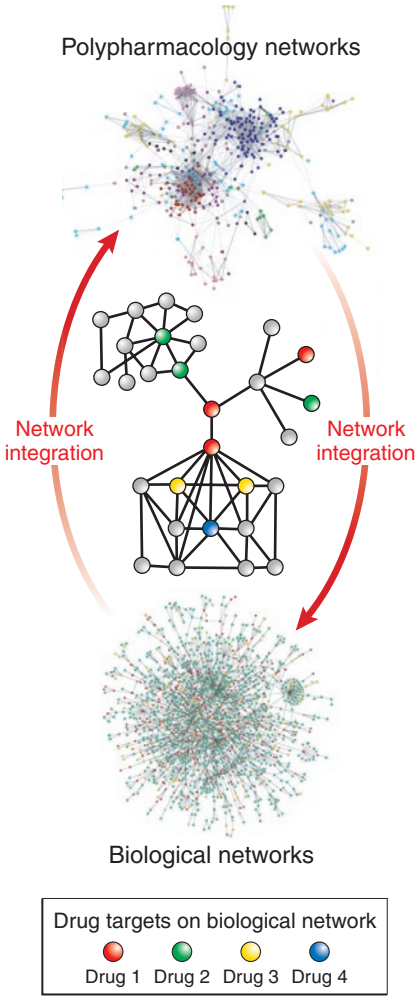

Figure 1 Network pharmacology. A network-centric view of drug action is built by mapping drug-target (polypharmacology) networks (left) onto biological networks (right). The network in the center is a part of the biological network in which nodes (proteins) targeted by the same drug are represented in the same color. Drug efficacy and toxicity can be understood by action at specific nodes and hubs.

and drugs that act directly on disease genes. Interestingly, the network distance metric highlights the trend in recent years towards an increase in drugs that target the genes associated with disease.

Network biology may also play a role in drug-target identification. Is it possible to identify drug targets from their position in a biological network? When the authors mapped drug targets onto human protein interaction data, they found that drug targets tend to have more interactions than average proteins but fewer interactions than essential proteins to a statistically significant degree. These data suggest that drug targets tend be nodes positioned in a 'goldilocks' region of biological networks lying between the essential hubs and redundant peripheral nodes. If drug targets were positioned at nodes that are too highly connected, they would likely be essential proteins, whose perturbation may lead to toxicity. On the other hand, if drug targets were positioned at nodes at the periphery, they would likely be redundant, with little effect on disease phenotype if perturbed. The observation that drug targets, in general, tend to be highly connected but not essential opens up the possibility that statistical network analysis could be a useful tool for prioritization of potential drug targets a priori.

Mapping the polypharmacology network onto the human disease-gene network revealed not only that drugs commonly act on multiple targets but also that drug targets are often involved with multiple diseases. Over 40% of drug targets that map with disease genes mapped to more that one disease. In another study, a network analysis of the OMIM database of genetic associations showed that the genetic origins of most diseases are shared with other diseases: of 1,284 disorders catalogued in OMIM, 867 share at least one gene with another disorder[6]. This finding provides motivation to the growing interest in recent years in drug repurposing or indication-discovery strategies.

Traditionally, medicinal chemists have approached the design of ligands with multiple activities with trepidation, fearing complex structure-activity relationships or conjugated ligands with high molecular weights[7,8]. However, the polypharmacology of approved drugs shows that a more opportunistic approach exploiting multi-target activity may be more attractive. Combining chemogenomics with network biology may enable a new network-pharmacology approach to drug discovery[3,8,9] (**Fig. 1**). Developing methods to aid polypharmacology design[9] can help to both improve efficacy and predict unwanted off-target effects. The recognition, informed by systems biology, that drugs for many disease states may require multiple activities to be efficacious, together with the observed promiscuity of old, small-molecule drugs, may indeed hold the clues to designing a new generation of drugs that perturb biological networks rather than individual targets.

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AAV vectors and tumorigenicity

Mark A Kay

Chromosomal integration of rAAV vectors may induce hepatocellular carcinoma in neonatally treated mice.

Among the many viral vectors under development for gene therapy, recombinant adeno-associated viral (rAAV) vectors have shown special promise because of their efficacy and outstanding safety record in animals. Indeed, this vector system has been used to cure several diseases in animal models and is gaining in popularity for clinical gene-therapy trials. However, a recent study by Donsante *et al.*[1], published in *Science*, has revived a debate on the safety of rAAV vectors[2]. The authors found that neonatal mice treated with a high dose of an rAAV vector showed an increased incidence of hepatocellular carcinoma. Moreover, they discovered a common vector-integration site near a microRNA (miRNA) cluster in 4/4 independent tumors, suggesting a mechanism based on insertional mutagenesis.

Several lines of evidence indicated that the rAAV vectors were contributing to malignancy. rAAV vector fragments were not detected in surrounding normal liver tissue. Genes close to the rAAV vector proviruses were overexpressed. About 10% of the ~400 known mouse microRNA sequences as well as some of the small nucleolar RNA genes are

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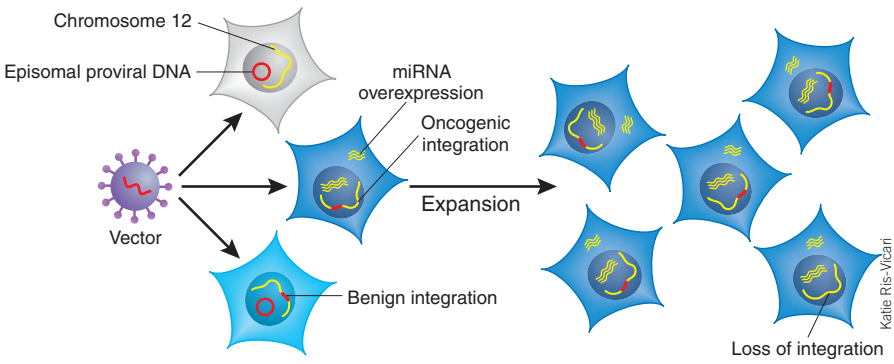

Figure 1 Model of AAV-mediated oncogenesis. AVV vector transduction normally results in maintenance of episomal vectors without integration into the host genome (white hepatocyte). Rare integration is usually benign (light-blue hepatocyte). However, when vectors integrate near the miRNA locus mir-143 on chromosome 12 (dark-blue hepatocyte), dysregulated miRNA and miRNA-target expression can result in a selective growth advantage that contributes in combination with accumulating secondary mutations to cellular expansion and oncogenesis. Loss of the original integration event may or may not modulate cancer cell properties.

located in or near the common integration site (a 6-kb region of chromosome 12 within or near the mir-341 miRNA transcript), and the finding that many of these genes were dysregulated in all analyzed tumors from the rAAV-treated mice suggested that they may have contributed to tumor formation. There is a syntenic region on human chromosome 14 that has been linked to human cancers. However, because microarray analyses were not performed on the hepatocellular carcinoma tumors isolated from the non-rAAV treated animals and because microRNA dysregulation is common, and likely involved in oncogenesis, it cannot be rigorously concluded that the dysregulation was due to rAAV integration. The authors did establish that at least one microRNA locus was not upregulated in the tumors. Nonetheless, taken together, their findings represent a compelling argument for the tumorigenicity of rAAV vectors.

Safety concerns related to rAAV vectors are not new. A 2001 study showed that mice with the lethal lysosomal storage disease mucopolysaccharidosis type VII (MPSVII) caused by deficiencies in acid hydrolase β-glucuronidase (GUSB) develop a high incidence of hepatocellular carcinoma when treated with rAAV-GUSB as neonates[2]. This result stimulated a joint review of the oncogenic risk of rAAV at a March 2001 meeting of the US Food and Drug Administration and the National Institutes of Health Recombinant DNA Advisory Committee[3]. Numerous presentations concluded that there was no evidence of enhanced tumorigenicity in studies examining rAAV administration in hundreds of mice followed for a year or more.

Moreover, alternate explanations of the MPSVII mouse study were proposed: the MPSVII mouse was on an unusual genetic background; high amounts (from a small number of cells indicative of AAV-2 transduction) of the transgene product interfered with the function of the enzyme's receptor (which itself had been shown to contribute to cellular transformation); or an undetermined pathogen was present in the mouse colony. In addition, two laboratories showed independently that there was no evidence for vector integration in three separate tumor tissues from MPSVII mice with hepatocellular carcinoma, suggesting that if rAAV did play a role in generating the tumors, the mechanism was not insertional mutagenesis. The general consensus was that there was no strong evidence for the tumorigenicity of rAAV vectors. More recent studies have reached a similar conclusion[4]. In the current study, both the wild-type and MPSVII rAAV-treated mice that developed hepatocellular carcinoma were on the same genetic background, eliminating the disease itself as contributing to oncogenesis.

rAAV vectors are believed to integrate into the genome at low frequency. Early experiments suggested that stable rAAV-mediated transgene expression was due to proviral integration, as detected by large-molecular-weight double-stranded genomes found in animal tissues after vector administration. It turned out that most of these large-molecular-weight genomes are concatemeric episomal DNA molecules and that most of the transcriptionally active genomes are circular monomers, with only a fraction—perhaps a few percent—of the genomes integrating into the host genome[5] (**Fig. 1**). However, the integration events are not random and tend to favor transcriptionally active regions of the chromosome[6,7] and/or already-broken portions of the chromosome[8,9]. Moreover, even if a small percentage of the rAAV proviral transduction events resulted in chromosomal integrations, these would likely amount to millions of integrations, a number lower than that expected with an equivalent number of transduction events of recombinant retroviral vectors, but higher than that obtained with adenoviral vectors. Although one might assume that the miRNA locus region identified by the authors might be a hotspot for integration, a recent study has shown no preference for this or other miRNA loci[9]. This suggests that integration into this oncogenic miRNA site will result in a selective growth advantage.

In the present study, the authors could not determine the exact composition of the vector DNA sequence contained within the integration site. This, coupled with differences in the techniques used for detecting integration in the tumors evaluated in the earlier MPSVII study[2], might explain why vector integration was not detected in previous hepatocellular carcinomas. However, several issues remain unresolved. First, the authors claim that in each tumor there were between 3 and 27 vector genomes per 100 diploid equivalents, because many hepatocytes are polyploid in the mouse. Although this is true in adult animals, all hepatocytes are diploid in the neonate. Assuming that the integration is haploid, and even with a substantial number of nonmalignant cells mixed in the tumor, it is hard to reconcile the generation of tumors with integration into only 3% of host genomes as the causative event. Perhaps rAAV integration is a dynamic process that continues over time (after hepatocytes are polyploid), although this was shown not to occur at any appreciable level in adult liver over a period of one year[5]. In addition, it is conceivable that the oncogenesis mechanism is related to a hit-and-run phenomenon in which integration is followed by a deletion of vector DNA and surrounding chromosomal DNA.

A second unresolved question raised by the study is that the incidence of hepatocellular carcinoma in nontreated normal control animals was higher than expected[1,2]. This suggests that an additional event (such as an environmental factor) might be contributing to oncogenesis. Finally, in contrast to most other studies, which evaluated rAAV administration in mature animals, the present study used neonatal mice. It is possible

that the developing liver is more susceptible to rAAV-mediated events that enhance the generation of tumors, and this will need to be worked out in the future.

What are the implications of the study of Donsante *et al.* for current rAAV gene therapy efforts, especially clinical trials? In my opinion, this result alone should not affect the approval of rAAV trials, but patients who are considering participation should be informed of it—and of other preclinical studies that do not support an oncogenic risk. As with many clinical trials, patients will have to weigh the risks even when experts cannot explain or extrapolate the animal data to humans.

Nonetheless, the present study is important and should stimulate new investigation. The mechanism of tumor formation should be further elucidated not only as a scientific curiosity but with the aim of minimizing any potential hazard. There are enormous complexities in designing preclinical tumor-risk studies with enough animals to be statistically meaningful. Simple parameters, such as genetic background, route and dose of vector administration, age and sex, length of monitoring, and transgene product and promoter sequences, may all influence the outcome. Moreover, advances in rAAV vector development will introduce additional parameters that could influence safety, including production methods, new rAAV serotypes and the structure of the vector DNA, which might be engineered to reduce promiscuous integration. No matter what results are obtained in animals, we must remember that a mouse is not a man, and extrapolation is not necessarily precise.

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