Published in final edited form as:
Curr Opin Mol Ther. 2009 August ; 11(4): 442–447.

Adeno-associated virus vector integration

David R Deyle[1] and **David W Russell**[1,2,*]
David W Russell: drussell@u.washington.edu
[1]University of Washington, Departments of Medicine, Mailstop 357720, 1954 NE Pacific Street, Seattle, WA 98195, USA
[2]University of Washington, Department of Biochemistry, Mailstop 357720, 1954 NE Pacific Street, Seattle, WA 98195, USA

Abstract

Adeno-associated virus (AAV) vectors efficiently transduce various cell types and can produce long-term expression of transgenes in vivo. Although AAV vector genomes can persist within cells as episomes, vector integration has been observed in various experimental settings, either at non-homologous sites where DNA damage may have occurred or by homologous recombination. In some cases, integration is essential for the therapeutic or experimental efficacy of AAV vectors. Recently, insertional mutagenesis resulting from the integration of AAV vectors was associated with tumorigenesis in mice, a consideration that may have relevance for certain clinical applications.

Keywords

Adeno-associated virus; gene integration; homologous recombination; insertional mutagenesis; vector

Introduction

Adeno-associated virus (AAV) vectors are able to transduce a wide range of dividing and non-dividing cell types, which has made these vectors an important tool for gene therapy. A major advantage of AAV vectors is the long-term expression of transgenes that can be obtained after *in vivo* gene delivery. In many cases, AAV vectors integrate into the host cell genome, which can be important for certain applications, but can also have unwanted consequences. Current concepts of AAV vector integration are reviewed, including the mechanisms of integration, the sites of integration and the potential clinical consequences.

The integration of wild-type AAV

AAV is a helper-dependent parvovirus with an approximately 4.7 kb, single-stranded, linear DNA genome that contains two open reading frames encoding the replication and capsid proteins (*rep* and *cap*, respectively) [1,2]. The AAV genome includes inverted terminal repeats (ITRs) that fold into characteristic T-shaped hairpins, which are the only sequences required in cis for the replication and packaging of the AAV genome. Co-infection with a helper virus such as adenovirus or herpesvirus is required for the replication of AAV and for productive infection. In the absence of a helper virus, AAV can produce a latent infection in which the viral genome persists in infected cells. AAV genomes have been recovered from lung tissue [3-5], as might be expected given the respiratory route of infection. Remarkably, AAV has also

*To whom correspondence should be addressed.

been detected in other tissues derived from humans and non-human primates, including bone marrow, brain, spleen, colon, heart, liver, lymph node and kidney [5-7], suggesting that the virus spreads throughout the body during infection. While the presence of these latent genomes is consistent with integrated proviruses, and in some cases Southern blot analysis has suggested the occurrence of integration [6], episomal genomes may also persist for prolonged periods.

An interesting feature of wild-type AAV is that the genome can integrate in a site-specific manner in a region on the long arm of chromosome 19 (19q13-qter), termed the AAVS1 site [1,8,9]. Although large regions of homology do not exist between the AAVS1 site and the AAV genome, AAVS1 contains an active Rep binding element and the AAV Rep protein (a DNA helicase) is required for site-specific integration of the vector. Thus, Rep-mediated replication at AAVS1 may promote DNA repair processes that allow the proviruses to integrate [10]. Approximately 0.1% of infecting wild-type AAV genomes integrate at AAVS1 [1]. Although accounting for the integration of a small fraction of viral DNA, this mechanism could nonetheless play a role in the natural history of the virus, and AAV proviruses have been identified in AAVS1 after natural infection. In two individuals, sequence analysis of the junctions between the vector and chromosome sequences demonstrated that integration had occurred in testicular tissue [11].

Synthetic AAV vectors have also been designed to integrate at the AAVS1 site by the same Rep-dependent mechanism. However, the *rep* gene is toxic and its inclusion in the vector significantly reduces the viral packaging capacity. Progress with rep-containing vectors will not be discussed. The wild-type AAV genome can also integrate at sites in the genome other than AAVS1, as demonstrated by the identification of a provirus on chromosome 1q31.1 in human tonsillar tissue and in multiple different chromosomes *in vitro* [3,12]. These events were presumably Rep-independent and are analogous to the integration of *rep*-deficient vectors described in this review.

Nuclear processing of AAV vector genomes

Most AAV vectors contain a transgene expression cassette between the ITRs that replaces the *rep* and *cap* genes. Vector particles are produced by the co-transfection of cells with a plasmid containing the vector genome and a packaging/helper construct that expresses the *rep* and *cap* proteins in trans [13]. During infection, AAV vector genomes enter the cell nucleus and can persist in multiple molecular states. One common outcome is the conversion of the AAV genome to a double-stranded circular episome by second-strand synthesis or complementary strand pairing [1,2,14-17]. The episomes can be converted to high-molecular-weight concatamers that are observed as tandem repeats [2,18,19]. These multimeric concatamers are believed to be formed by the recombination of monomer genomes and can provide long-term transgene expression, particularly in non-dividing cells [19-22]. AAV vectors can also integrate at non-homologous sites in the host genome, either as single-copy proviruses or concatamers [2,23-25]. Approximately 0.1% of vector genomes that enter cells integrate in this manner [1,26]. Although the frequency of integration by AAV vectors is significantly reduced compared with that of retroviral vectors, integration is sufficiently high to produce many integrated vector proviruses in most experiments. In some cases, both episomal and integrated vector genomes appear as high-molecular-weight DNA, and can be challenging to distinguish. Transgenes can be expressed from either episomal or integrated vector genomes. AAV vectors can also integrate at specific sites in the genome by homologous recombination (see the *Homologous recombination* section) [27].

Non-homologous vector integration

Early studies of AAV vector integration used marker genes to select for cells that contained integrated proviruses. Southern blot, PCR and fluorescence *in situ* hybridization analysis of

transduced human cell lines suggested that AAV vectors integrated at apparently random genomic sites [28-32]. In subsequent studies, AAV shuttle vectors were used to rescue integrated proviruses in the form of bacterial plasmids in order to sequence vector:chromosome junctions. Russell and colleagues analyzed 9 integration sites isolated from HeLa cells [23, 33] and 977 sites from unselected human fibroblasts [34], and Kay and colleagues analyzed 18 sites from the livers of wild-type mice [35] and 347 sites from the livers of mice with hereditary tyrosinemia, in which transduced hepatocytes selectively proliferated [36,37]. More recently, Inagaki *et al* characterized approximately 1000 integration sites from the liver, heart and skeletal muscle of mice [38], and Han *et al* sequenced 5 sites from mouse bone marrow [39]. Taken together, these studies represent the bulk of data on the sequenced AAV vector integration sites and have provided important insights into the AAV vector integration process. PCR and Southern blots also suggested that AAV vector integration occurs in various tissues, including the brain, heart, skeletal muscle, liver, kidney and testicular tissue, although no provirus junctions were identified in these studies [40,41].

The integration of AAV vectors occurred preferentially at specific sites in the genome. Surprisingly, 3 to 8% of integrated vector proviruses were present in the ribosomal DNA repeats that encode ribosomal RNAs [34,37]. This is not a property of other mammalian vectors, but a similar phenomenon has been observed with crustacean and insect transposons. A preference was also noted for the integration of AAV vectors at CpG islands and within 1 kb of transcription start sites [34,37,38], and this preference was also observed with retroviral vectors based on murine leukemia virus [42-44]. Segmental duplications, satellite DNA and palindromes were other preferred integration sites of AAV vectors [34,38]. In addition, integration ‘hot spots’ were identified where multiple independent proviruses were present [34,37]. In mouse hepatocytes, 53 to 62% of AAV vector integrations occurred in active genes [36,37], in contrast to 39% in cultured fibroblasts [34,37]. These data suggest that the non-homologous integration of AAV vectors is not random, and that the preferential integration at certain sites presumably reflects specific mechanisms of integration.

AAV vectors do not provide the proteins required for integration, and therefore the entire mechanism is dependent on host factors. The lack of a vector-encoded nuclease suggests that a key integration step is the availability of free chromosomal ends that can ligate to the AAV genome. AAV vectors were demonstrated to integrate at chromosomal double-strand breaks generated by the endonuclease I-Sce I or by treatment with etoposide and γ -irradiation [45]. Increasing the level of DNA damage increased integration, suggesting that the formation of double-strand breaks was the rate-limiting step. Thus, the preferred integration sites of AAV vectors may represent chromosomal regions prone to double-strand breaks or other forms of DNA damage. For example, CpG islands undergo methylation and mutagenesis that could promote DNA repair [46-48]. In addition, ribosomal DNA repeats, segmental duplications and palindromes may be involved in recombination events that create free chromosomal ends [49-51]. Therefore, integration hot spots may represent regions of genomic instability [34, 37]. Furthermore, transcription start sites are susceptible to damage during the unwinding and exposure of bases that accompany the initiation of transcription [52]. In the case of hepatocytes, in which transcription had a greater effect on integration than in cultured fibroblasts, this may reflect the low proliferation rate of hepatocytes, as the lack of DNA replication would increase the proportion of double-strand breaks that result from transcription. Thus, the available evidence is consistent with a model in which AAV vectors integrate at pre-existing chromosomal breaks that provide free DNA ends for the non-homologous end-joining pathway. Importantly, there is no evidence that the vectors themselves create breaks.

The structure of integrated vector proviruses can vary significantly. Typically, bases are deleted from the vector genome termini and the hairpins of inverted repeats are favored at junction sites [23,24,34,35,37,45]. Rearrangements, deletions and insertions are present in the flanking

chromosomal DNA. Genomic deletions of > 1×10^6 bp and insertions as large as 500 bp have been identified [34,37]. Translocations may also occur at provirus sites in which the left and right termini of the vector are ligated to different chromosomes [34,37]. These translocations are associated with 1 to 2% of all AAV vector provirus insertions [34,37]. While the genetic changes identified at AAV provirus sites are concerning, DNA double-strand breaks are likely to result in mutations and it is possible that similar chromosomal abnormalities would have occurred in the absence of AAV vectors. A key question in the field is whether AAV vectors change the spectrum of chromosomal mutations that arise during normal cellular repair of DNA damage. In one study, AAV vectors did not increase the background mutation frequency of the cellular *HPRT* gene [45], suggesting that AAV vectors do not have a significant mutagenic role.

Consequences of non-homologous integration of AAV vectors

The integration of an AAV vector results in insertional mutagenesis that can alter the expression of chromosomal genes. Possible genotoxic effects include the inactivation of genes at the integration site and the dysregulation of neighboring genes as a consequence of enhancers and promoters present in the vector. The most concerning possibility is the malignant transformation of a transduced cell. The majority of experimental data have demonstrated that AAV vectors are safe in this regard. For example, the incidence of liver tumors in mice (n = 695) that received different AAV vector preparations at between 6 and 8 weeks of age was not increased compared with controls [53]. In addition, there was no increase in tumor formation after infection with AAV vectors in a study in p53-deficient mice that are prone to developing malignancies [54]. However, several studies have reported increased liver tumor incidence in mice that received AAV vectors. In some cases the tumors were likely to have been caused by vector-encoded transgenes, such as the oncogenic HBV X gene present in the woodchuck hepatitis virus post-transcriptional regulatory element [55], or the toxicity of short hairpin RNA that increased hepatocyte division [56]. However, increased tumorigenesis was observed in mice with a deficiency in ornithine transcarbamylase that received an AAV vector expressing *lacZ*, which should not have been an oncogenic transgene [57]; the tumor tissues contained between 0.023 and 23 copies of the vector per cell [57]. Vector:chromosome junctions were not identified in this study so it is unclear if or where the vector genomes integrated.

More convincing evidence for AAV-induced tumorigenesis is derived from experiments with an AAV vector containing a CMV enhancer, a chicken β-actin promoter and a human β-glucuronidase gene [58]. Newborn mice with mucopolysaccharidosis VII (resulting from mutations in β-glucuronidase) injected with the AAV vector had an increased risk of developing tumors, predominantly hepatocellular carcinoma [58]. Although vector genomes were present in tumor tissues, integrated provirus junctions were not identified. A follow-up study demonstrated that non-mutant mice injected with the same vector also had an increased incidence of hepatocellular carcinoma (33 to 56% compared with 8.6% in controls), suggesting that tumor formation was a feature of the AAV vector itself rather than the mutant mouse strain [59]. In this latter study, integrated AAV vector proviruses were observed in four different tumors from four different mice, and all four integrated AAV vectors mapped to a 6-kb region of chromosome 12. Microarray analysis demonstrated nearby chromosomal transcripts were upregulated, in particular one containing several small nucleolar RNAs (Rian) and another containing several microRNAs (Mirg), which could have influenced the expression of target genes responsible for malignant transformation. Although these genes were not previously associated with hepatocellular carcinoma, the occurrence of four independent tumors containing vector proviruses in the same locus (but not in the surrounding normal liver tissue) suggests that insertional mutagenesis by AAV vectors can lead to cancer in some situations [59]. Further research will be required to determine which factors were important for tumorigenesis in this model, including the age and species of the animals used and the types

of vector elements such as the enhancer, promoter or transgene. Whether a similar phenomenon can occur in humans is a crucial question, particularly as a clinical trial is underway with an AAV-based liver-directed gene therapy [60] (ClinicalTrials.gov identifier: NCT00515710). AAV vectors also integrate into tissues other than the liver, including the brain, heart, skeletal muscle, kidneys and testes [38,40,41], thus the possibility that other types of tumors could develop following AAV vector delivery must also be considered.

Homologous recombination

AAV vectors can integrate by homologous recombination, which is termed AAV-mediated gene targeting [27]. In order to promote homologous recombination, a vector that contains homology arms derived from genomic chromosomal DNA that flank the genetic modification being introduced is used. Different types of mutations can arise as a consequence of recombination such as deletions, substitutions and insertions, including the insertion of transgenes and selectable markers [27,61-65]. Under optimal conditions, 0.1 to 1.0% of the total cell population exposed to an AAV targeting vector undergoes gene targeting [27], making this method more efficient than conventional, transfection-based techniques by several logs. Although the mechanism of AAV-mediated gene targeting is not completely understood, the available evidence suggests that the single-stranded vector genome pairs and recombines with homologous chromosomal sequences. Analogous parvoviral vectors based on the mouse minute virus package only one vector strand and exhibit a strand preference in targeting, which argues against a double-stranded vector genome intermediate [66]. Also, double-strand breaks present at the target locus enhance gene targeting by 60- to 100-fold, implicating the double-strand break repair mechanism in recombination [67,68]. For example, RNAi-mediated silencing of *RAD54L* or *RAD54B (*important proteins involved in homologous recombination) reduced gene targeting frequencies, and partial silencing of *XRCC3* (a Rad51-related protein) abolished targeting [69]. In contrast, the silencing of the DNA-dependent protein kinase catalytic subunit, which is involved in non-homologous DNA end-joining, did not significantly reduce the frequency of AAV-mediated gene targeting [69]. These studies suggest that the same homologous recombination mechanisms known to be involved in conventional gene targeting are important for AAV-mediated gene targeting.

The high frequency and accuracy of AAV-mediated gene targeting make this method of gene targeting useful for various different applications. Several investigators have used AAV vectors to introduce mutations into transformed cell lines and study the function of oncogenes [70-81]. AAV vectors have also been used for *in vivo* gene targeting in the mouse liver, including the correction of mutations in a *lacZ* transgene, the β-glucuronidase gene and the fumarylacetoacetate hydrolase (*Fah*) gene [82,83]. Up to 1 in 1×10^4 hepatocytes underwent gene targeting in these experiments, which is a sufficiently high frequency to cure hereditary tyrosinemia in mice with *Fah* mutations, but is too low for most therapeutic applications. *Ex vivo* gene targeting may be a more realistic application at present. AAV vectors have been used to disrupt dominant-negative mutant collagen genes in mesenchymal stem cells from patients with osteogenesis imperfecta. Using a promoter-trap strategy, 30 to 90% of cells containing the selectable marker gene were targeted, and those with the mutant allele disrupted produced healthy collagen [61,62]. In principle, these bone-forming mesenchymal stem cells could be transplanted into patients and produce normal bone. A major advantage of therapeutic gene targeting is the potential to reduce or eliminate the risks of insertional mutagenesis. In the case of AAV-targeting vectors, random integration can still occur, but potential genotoxic effects can be minimized by designing vectors without enhancers or promoters.

Conclusion

Although AAV vectors are often assumed to transduce cells as episomes, chromosomal integration occurs at significant frequencies and can produce the long-term expression of transgenes. Vector integration can occur at non-homologous locations that are sites of DNA damage, or at specific sites via homologous recombination. As with other integrating vectors, insertional mutagenesis is a concern with AAV vectors, and this should be considered in clinical applications.

References

•• of outstanding interest

• of special interest

1. McCarty DM, Young SM Jr, Samulski RJ. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annu Rev Genet 2004;38:819–845. [PubMed: 15568995]
2. Russell DW, Kay MA. Adeno-associated virus vectors and hematology. Blood 1999;94(3):864–874. [PubMed: 10419876]
3. Schnepp BC, Jensen RL, Chen CL, Johnson PR, Clark KR. Characterization of adeno-associated virus genomes isolated from human tissues. J Virol 2005;79(23):14793–14803. [PubMed: 16282479]
4. Chen CL, Jensen RL, Schnepp BC, Connell MJ, Shell R, Sferra TJ, Bartlett JS, Clark KR, Johnson PR. Molecular characterization of adeno-associated viruses infecting children. J Virol 2005;79(23): 14781–14792. [PubMed: 16282478]
5. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM. Clades of adeno-associated viruses are widely disseminated in human tissues. J Virol 2004;78(12):6381–6388. [PubMed: 15163731]
6. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, Calcedo R, Sanmiguel J, Abbas Z, Wilson JM. Adeno-associated viruses undergo substantial evolution in primates during natural infections. Proc Natl Acad Sci USA 2003;100(10):6081–6086. [PubMed: 12716974] •AAV genomes were detected and isolated by PCR in multiple tissues from non-human primates. Southern blot analysis suggested that the AAV genome can persist in cells as both integrated and non-integrated forms. This paper provided unexpected evidence that AAV infections can spread efficiently throughout the body.
7. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci USA 2002;99(18):11854–11859. [PubMed: 12192090]
8. Samulski RJ, Zhu X, Xiao X, Brook JD, Housman DE, Epstein N, Hunter LA. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J 1991;10(12):3941–3950. [PubMed: 1657596]
9. Kotin RM, Siniscalco M, Samulski RJ, Zhu XD, Hunter L, Laughlin CA, McLaughlin S, Muzyczka N, Rocchi M, Berns KI. Site-specific integration by adeno-associated virus. Proc Natl Acad Sci USA 1990;87(6):2211–2215. [PubMed: 2156265]
10. Kotin RM, Linden RM, Berns KI. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO J 1992;11 (13):5071–5078. [PubMed: 1334463]
11. Mehrle S, Rohde V, Schlehofer JR. Evidence of chromosomal integration of AAV DNA in human testis tissue. Virus Genes 2004;28(1):61–69. [PubMed: 14739652] •Two virus:chromosome junctions were isolated from human testicular tissue by ‘genome-walking’ PCR. The isolation of these virus:chromosome junctions demonstrated that AAV can integrate in the testes after infection. These findings raise the possibility that AAV vectors can integrate in spermatogonia and perhaps be transferred by germ-line transmission.
12. Drew HR, Lockett LJ, Both GW. Increased complexity of wild-type adeno-associated virus-chromosomal junctions as determined by analysis of unselected cellular genomes. J Gen Virol 2007;88(Pt 6):1722–1732. [PubMed: 17485532]

13. Grimm D, Kern A, Rittner K, Kleinschmidt JA. Novel tools for production and purification of recombinant adenoassociated virus vectors. Hum Gene Ther 1998;9(18):2745–2760. [PubMed: 9874273]

14. Zhong L, Zhou X, Li Y, Qing K, Xiao X, Samulski RJ, Srivastava A. Single-polarity recombinant adeno-associated virus 2 vector-mediated transgene expression *in vitro* and *in vivo*: Mechanism of transduction. Mol Ther 2008;16(2):290–295. [PubMed: 18087261]

15. Thomas CE, Storm TA, Huang Z, Kay MA. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. J Virol 2004;78(6):3110–3122. [PubMed: 14990730]

16. Zhou X, Zeng X, Fan Z, Li C, McCown T, Samulski RJ, Xiao X. Adeno-associated virus of a single-polarity DNA genome is capable of transduction *in vivo*. Mol Ther 2008;16(3):494–499. [PubMed: 18180769]

17. Nakai H, Storm TA, Kay MA. Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. J Virol 2000;74(20):9451–9463. [PubMed: 11000214]

18. Duan D, Yan Z, Yue Y, Engelhardt JF. Structural analysis of adeno-associated virus transduction circular intermediates. Virology 1999;261(1):8–14. [PubMed: 10484751]

19. Duan D, Sharma P, Yang J, Yue Y, Dudus L, Zhang Y, Fisher KJ, Engelhardt JF. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. J Virol 1998;72(11):8568–8577. [PubMed: 9765395]

20. Nakai H, Yant SR, Storm TA, Fuess S, Meuse L, Kay MA. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. J Virol 2001;75(15):6969–6976. [PubMed: 11435577]

21. Gao G, Lu Y, Calcedo R, Grant RL, Bell P, Wang L, Figueredo J, Lock M, Wilson JM. Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. Mol Ther 2006;13(1): 77–87. [PubMed: 16219492]

22. Grimm D, Pandey K, Nakai H, Storm TA, Kay MA. Liver transduction with recombinant adeno-associated virus is primarily restricted by capsid serotype not vector genotype. J Virol 2006;80(1): 426–439. [PubMed: 16352567]

23. Rutledge EA, Russell DW. Adeno-associated virus vector integration junctions. J Virol 1997;71(11): 8429–8436. [PubMed: 9343199]

24. Yang CC, Xiao X, Zhu X, Ansardi DC, Epstein ND, Frey MR, Matera AG, Samulski PJ. Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration *in vivo* and *in vitro*. J Virol 1997;71(12):9231–9247. [PubMed: 9371582]

25. Miao CH, Snyder RO, Schowalter DB, Patijn GA, Donahue B, Winther B, Kay MA. The kinetics of rAAV integration in the liver. Nat Genet 1998;19(1):13–15. [PubMed: 9590280]

26. Russell DW, Miller AD, Alexander IE. Adeno-associated virus vectors preferentially transduce cells in S phase. Proc Natl Acad Sci USA 1994;91(19):8915–8919. [PubMed: 8090744]

27. Hirata R, Chamberlain J, Dong R, Russell DW. Targeted transgene insertion into human chromosomes by adeno-associated virus vectors. Nat Biotechnol 2002;20(7):735–738. [PubMed: 12089561]

28. Ponnazhagan S, Erikson D, Kearns WG, Zhou SZ, Nahreini P, Wang XS, Srivastava A. Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. Hum Gene Ther 1997;8(3):275–284. [PubMed: 9048194]

29. Kearns WG, Afione SA, Fulmer SB, Pang MC, Erikson D, Egan M, Landrum MJ, Flotte TR, Cutting GR. Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. Gene Ther 1996;3(9):748–755. [PubMed: 8875221]

30. McLaughlin SK, Collis P, Hermonat PL, Muzyczka N. Adeno-associated virus general transduction vectors: Analysis of proviral structures. J Virol 1988;62(6):1963–1973. [PubMed: 2835501]

31. Lebkowski JS, McNally MM, Okarma TB, Lerch LB. Adeno-associated virus: A vector system for efficient introduction and integration of DNA into a variety of mammalian cell types. Mol Cell Biol 1988;8(10):3988–3996. [PubMed: 2847025]

32. Samulski PJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: Normal integration does not require viral gene expression. J Virol 1989;63(9):3822–3828. [PubMed: 2547998]
33. Miller DG, Rutledge EA, Russell DW. Chromosomal effects of adeno-associated virus vector integration. Nat Genet 2002;30(2):147–148. [PubMed: 11799395]
34. Miller DG, Trobridge GD, Petek LM, Jacobs MA, Kaul R, Russell DW. Large-scale analysis of adeno-associated virus vector integration sites in normal human cells. J Virol 2005;79(17):11434–11442. [PubMed: 16103194] •Reported the analysis of 977 AAV vector:chromosome junctions and integration sites in human fibroblasts. This study was the first large-scale analysis of provirus integration sites in human cells and characterization of these junctions provided a representation of insertional mutagenesis by AAV vectors and an improved knowledge of AAV integration.
35. Nakai H, Iwaki Y, Kay MA, Couto LB. Isolation of recombinant adeno-associated virus vector-cellular DNA junctions from mouse liver. J Virol 1999;73(7):5438–5447. [PubMed: 10364291]
36. Nakai H, Montini E, Fuess S, Storm TA, Grompe M, Kay MA. AAV serotype 2 vectors preferentially integrate into active genes in mice. Nat Genet 2003;34(3):297–302. [PubMed: 12778174]
37. Nakai H, Wu X, Fuess S, Storm TA, Munroe D, Montini E, Burgess SM, Grompe M, Kay MA. Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver. J Virol 2005;79(6):3606–3614. [PubMed: 15731255] ••An analysis of 347 AAV vector:chromosome junctions from mouse livers provided definitive evidence for significant in vivo integration by AAV vectors. Integration site preferences were described and integration occurred preferentially in transcribed genes in this study.
38. Inagaki K, Lewis SM, Wu X, Ma C, Munroe DJ, Fuess S, Storm TA, Kay MA, Nakai H. DNA palindromes with a modest arm length of greater, similar 20 base pairs are a significant target for recombinant adeno-associated virus vector integration in the liver, muscles, and heart in mice. J Virol 2007;81(20):11290–11303. [PubMed: 17686840] ••Reported the isolation of multiple independent vector:chromosome junctions from different mouse tissues. An analysis of the integration sites showed that AAV vectors integrate near DNA palindromes. This was the first demonstration of integration in tissues other than the liver after vector delivery.
39. Han Z, Zhong L, Maina N, Hu Z, Li X, Chouthai NS, Bischof D, Weigel-Van Aken KA, Slayton WB, Yoder MC, Srivastava A. Stable integration of recombinant adeno-associated virus vector genomes after transduction of murine hematopoietic stem cells. Hum Gene Ther 2008;19(3):267–278. [PubMed: 18303957]
40. Pachori AS, Melo LG, Zhang L, Loda M, Pratt RE, Dzau VJ. Potential for germ line transmission after intramyocardial gene delivery by adeno-associated virus. Biochem Biophys Res Commun 2004;313(3):528–533. [PubMed: 14697221]
41. Wu P, Phillips MI, Bui J, Terwilliger EF. Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. J Virol 1998;72(7):5919–5926. [PubMed: 9621054]
42. Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol 2004;2(8):E234. [PubMed: 15314653]
43. Hematti P, Hong BK, Ferguson C, Adler R, Hanawa H, Sellers S, Holt IE, Eckfeldt CE, Sharma Y, Schmidt M, von Kalle C. Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. PLoS Biol 2004;2(12):e423. [PubMed: 15550989]
44. Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. Science 2003;300(5626):1749–1751. [PubMed: 12805549]
45. Miller DG, Petek LM, Russell DW. Adeno-associated virus vectors integrate at chromosome breakage sites. Nat Genet 2004;36(7):767–773. [PubMed: 15208627] ••AAV vectors integrated at induced chromosome double-strand breaks. The sequences of vector proviruses in relation to the location of the double-strand breaks were consistent with integration via the non-homologous end-joining repair pathway. This paper provided direct evidence that AAV vectors integrate at pre-existing chromosomal breaks.
46. O'Neill JP, Finette BA. Transition mutations at CpG dinucleotides are the most frequent *in vivo* spontaneous single-based substitution mutation in the human HPRT gene. Environ Mol Mutagen 1998;32(2):188–191. [PubMed: 9776183]

47. Tazi J, Bird A. Alternative chromatin structure at CpG islands. Cell 1990;60(6):909–920. [PubMed: 2317863]

48. Wolf SF, Migeon BR. Clusters of CpG dinucleotides implicated by nuclease hypersensitivity as control elements of housekeeping genes. Nature 1985;314(6010):467–469. [PubMed: 2984578]

49. Kobayashi T. Strategies to maintain the stability of the ribosomal RNA gene repeats – Collaboration of recombination, cohesion, and condensation. Genes Genet Syst 2006;81(3):155–161. [PubMed: 16905869]

50. Bailey JA, Eichler EE. Primate segmental duplications: Crucibles of evolution, diversity and disease. Nat Rev Genet 2006;7(7):552–564. [PubMed: 16770338]

51. Shaw CJ, Lupski JR. Implications of human genome architecture for rearrangement-based disorders: The genomic basis of disease. Hum Mol Genet 2004;13(Suppl 1):R57–R64. [PubMed: 14764619]

52. Aguilera A. The connection between transcription and genomic instability. EMBO J 2002;21(3):195–201. [PubMed: 11823412]

53. Bell P, Wang L, Lebherz C, Flieder DB, Bove MS, Wu D, Gao GP, Wilson JM, Wivel NA. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. Mol Ther 2005;12(2):299–306. [PubMed: 16043099]

54. Schuettrumpf J, Baila S, Khazi F, Liu JH, Bunte R, Arruda V. AAV vectors do not increase the risk of tumor formation in p53 deficient models. Mol Ther 2007;15(Suppl 1):S1. [PubMed: 17522625]

55. Embury JE, Charron CC, Poirier AE, Zori A, Carmichael R, Flotte TR, Laipis PJ. Long term portal vein administration of AAV-WPRE vector results in increased incidence of neoplastic disease and hepatic pathology. Mol Ther 2006;13(Suppl 1) Abs 216.

56. Grimm D, Beer S, Komatsubara K, Lee JS, Koh S, Wang L, Storm TA, Davis CR, Kay MA, Felsher D. Adverse shRNA cytotoxicity can accelerate tumorgenesis in genetically predisposed mice. Mol Ther 2007;15(Suppl 1):S266.

57. Bell P, Moscioni AD, McCarter RJ, Wu D, Gao G, Hoang A, Sanmiguel JC, Sun X, Wivel NA, Raper SE, Furth EE, et al. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. Mol Ther 2006;14(1):34–44. [PubMed: 16682254]

58. Donsante A, Vogler C, Muzyczka N, Crawford JM, Barker J, Flotte T, Campbell-Thompson M, Daly T, Sands MS. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. Gene Ther 2001;8(17):1343–1346. [PubMed: 11571571]

59. Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, Sands MS. AAV vector integration sites in mouse hepatocellular carcinoma. Science 2007;317(5837):477. [PubMed: 17656716]
••Newborn mice injected with an AAV vector developed hepatocellular carcinoma. The region of chromosome 12 where the four integrated proviruses were observed in tumors contains multiple maternally and paternally imprinted genes. Microarray analysis showed upregulation of nearby transcripts. These data provide compelling evidence that AAV integration can be associated with tumorigenesis.

60. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B, Dake M, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 2006;12(3):342–347. [PubMed: 16474400]

61. Chamberlain JR, Deyle DR, Schwarze U, Wang P, Hirata RK, Li Y, Byers PH, Russell DW. Gene targeting of mutant *COL1A2* alleles in mesenchymal stem cells from individuals with osteogenesis imperfecta. Mol Ther 2008;16(1):187–193. [PubMed: 17955022]

62. Chamberlain JR, Schwarze U, Wang PR, Hirata RK, Hankenson KD, Pace JM, Underwood RA, Song KM, Sussman M, Byers PH, Russell DW. Gene targeting in stem cells from individuals with osteogenesis imperfecta. Science 2004;303(5661):1198–1201. [PubMed: 14976317]
••Mesenchymal stem cells from patients with osteogenesis imperfecta were infected with an AAV gene-targeting vector designed to disrupt the COL1A1 gene. After selection, Southern blot analysis demonstrated that 31 to 90% of cells were targeted at the COL1A1 gene. This was the first demonstration of gene targeting in adult human stem cells.

63. Inoue N, Dong R, Hirata RK, Russell DW. Introduction of single base substitutions at homologous chromosomal sequences by adeno-associated virus vectors. Mol Ther 2001;3(4):526–530. [PubMed: 11319913]

64. Inoue N, Hirata RK, Russell DW. High-fidelity correction of mutations at multiple chromosomal positions by adeno-associated virus vectors. J Virol 1999;73(9):7376–7380. [PubMed: 10438827]

65. Kohli M, Rago C, Lengauer C, Kinzler KW, Vogelstein B. Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. Nucleic Acids Res 2004;32 (1):e3. [PubMed: 14704360]

66. Hendrie PC, Hirata RK, Russell DW. Chromosomal integration and homologous gene targeting by replication-incompetent vectors based on the autonomous parvovirus minute virus of mice. J Virol 2003;77(24):13136–13145. [PubMed: 14645570]

67. Miller DG, Petek LM, Russell DW. Human gene targeting by adeno-associated virus vectors is enhanced by DNA double-strand breaks. Mol Cell Biol 2003;23(10):3550–3557. [PubMed: 12724413]

68. Porteus MH, Cathomen T, Weitzman MD, Baltimore D. Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. Mol Cell Biol 2003;23(10):3558–3565. [PubMed: 12724414]

69. Vasileva A, Linden RM, Jessberger R. Homologous recombination is required for AAV-mediated gene targeting. Nucleic Acids Res 2006;34(11):3345–3360. [PubMed: 16822856] •The reduction of the non-homologous end-joining DNA-dependent protein kinase catalytic subunit by RNAi had no effect on gene targeting, while the reduction of homologous recombination proteins RAD54B, RAD54L and XRCC3 decreased or eliminated vector targeting. These results demonstrate which homologous recombination proteins are involved in targeting and a strategy for determining the cellular mechanisms involved.

70. Hurley PJ, Wilsker D, Bunz F. Human cancer cells require ATR for cell cycle progression following exposure to ionizing radiation. Oncogene 2006;26(18):2535–2542. [PubMed: 17043640]

71. Wang P, Yu J, Zhang L. The nuclear function of p53 is required for PUMA-mediated apoptosis induced by DNA damage. Proc Natl Acad Sci USA 2007;104(10):4054–4059. [PubMed: 17360476]

72. Arena S, Isella C, Martini M, de Marco A, Medico E, Bardelli A. Knock-in of oncogenic *Kras* does not transform mouse somatic cells but triggers a transcriptional response that classifies human cancers. Cancer Res 2007;67(18):8468–8476. [PubMed: 17875685]

73. Samuels Y, Diaz LA Jr, Schmidt-Kittler O, Cummins JM, DeLong L, Cheong I, Rago C, Huso DL, Lengauer C, Kinzler KW, Vogelstein B, et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell 2005;7(6):561–573. [PubMed: 15950905]

74. Cummins JM, Rago C, Kohli M, Kinzler KW, Lengauer C, Vogelstein B. Tumour suppression: Disruption of *HAUSP* gene stabilizes p53. Nature 2004;428(6982)•AAV vectors were used to knockout both alleles of the HAUSP gene in human cells by homologous recombination. This study demonstrated that AAV-mediated gene targeting can be a powerful technique for human somatic cell genetics, and that double knockouts can be produced in human cells to determine gene function.

75. Kim JS, Lee C, Bonifant CL, Ressom H, Waldman T. Activation of p53-dependent growth suppression in human cells by mutations in PTEN or PIK3CA. Mol Cell Biol 2007;27(2):662–677. [PubMed: 17060456]

76. Gallmeier E, Calhoun ES, Rago C, Brody JR, Cunningham SC, Hucl T, Gorospe M, Kohli M, Lengauer C, Kern SE. Targeted disruption of *FANCC* and *FANCG* in human cancer provides a preclinical model for specific therapeutic options. Gastroenterology 2006;130(7):2145–2154. [PubMed: 16762635]

77. Dang LH, Chen F, Ying C, Chun SY, Knock SA, Appelman HD, Dang DT. *CDX2* has tumorigenic potential in the human colon cancer cell lines LOVO and SW48. Oncogene 2005;25(15):2264–2272. [PubMed: 16314840]

78. Dang DT, Chen F, Gardner LB, Cummins JM, Rago C, Bunz F, Kantsevoy SV, Dang LH. Hypoxia-inducible factor-1α promotes nonhypoxia-mediated proliferation in colon cancer cells and xenografts. Cancer Res 2006;66(3):1684–1693. [PubMed: 16452228]

79. Cunningham SC, Gallmeier E, Hucl T, Dezentje DA, Calhoun ES, Falco G, Abdelmohsen K, Gorospe M, Kern SE. Targeted deletion of *MKK4* in cancer cells: A detrimental phenotype manifests as decreased experimental metastasis and suggests a counterweight to the evolution of tumor-suppressor loss. Cancer Res 2006;66(11):5560–5564. [PubMed: 16740690]

80. Dang DT, Chen F, Kohli M, Rago C, Cummins JM, Dang LH. Glutathione S-transferase π1 promotes tumorigenicity in HCT116 human colon cancer cells. Cancer Res 2005;65(20):9485–9494. [PubMed: 16230413]

81. Cummins JM, Kohli M, Rago C, Kinzler KW, Vogelstein B, Bunz F. X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Res 2004;64(9):3006–3008. [PubMed: 15126334]

82. Miller DG, Wang PR, Petek LM, Hirata RK, Sands MS, Russell DW. Gene targeting *in vivo* by adeno-associated virus vectors. Nat Biotechnol 2006;24(8):1022–1026. [PubMed: 16878127]

83. Wursthorn K, Storm T, Kay MA, Finegold M, Grompe M. *In vivo* correction of a metabolic liver disease by AAV8-mediated homologous recombination. Mol Ther 2006;13(Suppl 1) Abs 804.