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The Role of DNA Repair Pathways in Adeno-AssOCIated Virus Infection and Viral Genome Replication / Recombination / Integration

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1. Introduction

Cellular DNA is constantly being damaged not only by extrinsic factors such as ionizing radiation and environmental carcinogens but also by intrinsic agents such as reactive oxygen species arising during normal cellular metabolism. Of the myriad of DNA lesions, inflicted by extrinsic and intrinsic genome damaging agents, DNA double strand break (DSB) is the most threatening. Replication fork arrest at DNA lesions could also be a threat since stalled replication forks, if fail to restart appropriately, induce DNA strand breaks. When cells encounter such strand breaks and other types of DNA damage, they mount a DNA damage response (DDR) (Harper & Elledge, 2007) that senses DNA damage and initiates a cascade of signal transduction pathways consequently culminating in cell cycle arrest, DNA repair and/or apoptosis when the DNA lesions become irreparable. Although cells are equipped with such DNA damage sensing and repair machinery primarily to handle damaged cellular DNA, triggers and receivers of DDR are not necessarily the cells’ own genetic materials. DDR can also be provoked by essentially non-damaged DNA exogenously introduced into cells, most commonly viral genetic materials in nature and recombinant DNA (e.g., viral vectors for gene delivery) in laboratory. During virus-host interaction, viruses manipulate DDR upon infection of cells in a way that benefits their life cycles, while host cells fight against them to eliminate the invaders. DDR is detrimental to viral life cycles in many instances; therefore, DDR is often viewed as an innate antiviral host defense mechanism. For example, adenoviruses express viral proteins that block...
the cellular non-homologous end joining (NHEJ) pathway, which, unless inactivated, concatemerizes viral genomes and prohibits viral genome packaging into virions (Evans & Hearing, 2005; Stracker et al., 2002). Viruses may also take advantage of DDR in their life cycle as seen in retroviruses, which exploit the NHEJ pathway to complete insertion of their genomic materials into host cellular DNA (Daniel et al., 1999; Li et al., 2001). Similar but distinct types of “intervention” by viruses on DDR have been found in many other viruses (Lilley et al., 2007; Weitzman et al., 2004, 2010). Thus, understanding DDR and DNA repair machinery is imperative for elucidating the biology of viruses and viral vectors, and conversely, studying virus biology provides new insights into fundamental biological processes elicited by DNA damage. In this context, interactions between viruses and host DDR and DNA repair machinery have recently gained attention and established a new area of basic research. Importantly, this field of study is relevant to gene therapy research in overcoming its limitations and drawbacks and improving the current molecular therapy approaches.

Adeno-associated virus (AAV) represents a good example for exploring this new research field, which studies the interactions between viruses and DNA repair machinery. AAV has become increasingly popular as a promising gene delivery vehicle. Wild type AAV (wtAAV) is replication defective and recombinant AAV (rAAV) is devoid of virally encoded genes. Despite their replication-defective nature and/or lack of expression of viral proteins, there are significant interactions between virus and host DNA repair machinery, which determine the fates of the virus and the host cells following infection. In this chapter, we provide an overview of how wtAAV and rAAV alter the fate of the host cells through DDR, and how DDR processes the viral genomic DNA by exerting DNA repair machinery to establish the lytic and latent life cycles of wtAAV and transduction of rAAV.

2. Adeno-associated virus (AAV)

Adeno-associated virus (AAV) is a non-enveloped replication-defective animal virus of approximately 20 nm in diameter (Figure 1a). It belongs to Dependovirus, a genus of the family Parvoviridae, which has a viral capsid in the simplest icosahedral shape composed of 60 units of viral structural proteins. Productive AAV replication requires co-infection of a helper virus such as adenoviruses and herpesviruses. A virion has an approximately 5-kb single-stranded DNA genome of either plus or minus polarity at an equal probability. AAV serotype 2 and many other serotypes are prevalent in human populations worldwide and up to 80% of adult humans have been infected with AAV in their childhood (Boutin et al., 2010; Calcedo et al., 2009; Erles et al., 1999). AAV is generally considered as a non-pathogenic virus, and clinical relevance of AAV infection in humans appears to be limited to male infertility (Erles et al., 2001), early miscarriage in pregnant women (Burguete et al., 1999; Pereira et al., 2010) and protection against cervical cancer (Su & Wu, 1996; Walz & Schlehofer, 1992) although some studies have shown negative results (Strickler et al., 1999). The current relevance of AAV in biological and medical research primarily stems from its benefits as a tool for gene delivery and genetic engineering of the cellular genome and as a refined agent for inducing DDR without damaging the cellular genome (Table 1).

2.1 Wild type adeno-associated virus serotype 2 (wtAAV2)

2.1.1 Structural organization of wtAAV2

AAV was first identified as a contaminant in adenovirus stocks in early 1960s (Atchison et al., 1965). Since infectious wild type AAV2 (wtAAV2) clones were generated from
Table 1. AAV as biological agents and tools

<table>
<thead>
<tr>
<th>AAV-derived agents and tools</th>
<th>Applications</th>
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| rAAV of any serotypes       | - Delivery of exogenous genetic materials to cells with no toxicity  
                             | - Targeted genetic manipulation of cells (i.e., precise introduction of insertion, deletion or a small mutation at a defined location in the cellular genome in a predicted manner)  
                             | - Introduction of DDR without damaging the cellular genome  
                             | - Identification of DNA breakage sites in the cellular genome |
| wtAAV2                      | - Introduction of DDR without damaging the cellular genome  
                             | - Tumor cell-specific killing |
| Rep68/78                    | - Site-specific insertion of exogenous genetic materials at the AAVS1 site in the human chromosome 19q13.42 |

Fig. 1. Wild-type AAV (wtAAV) and recombinant AAV (rAAV). (a) Structural organization of wtAAV and rAAV. wtAAV genome is a single-stranded DNA with ITRs at the both ends. The two viral genes (the rep and cap genes) encode five non-structural (Rep and AAP) and three structural (VP) proteins, and they are controlled by the three viral promoters (p5, p19, and p40). AAV virion particle consists of VP1, VP2, and VP3. AAP supports assembly of VP proteins. rAAV genome is devoid of the viral components except for the ITRs, and contains an exogenous DNA of interest. (b) Representative photomicrographs of XGal-stained sections of the murine liver and heart transduced AAV8-EF1α-nls lacZ and AAV8-CMV-lacZ vectors at a high dose (7.2 x 10^12 viral particles per mouse), respectively. All the hepatocytes and cardiomyocytes express the lacZ marker gene product, which turns transduced cells blue by the XGal staining.
recombinant plasmids (Samulski et al., 1982), AAV2 has been most extensively studied for viral capsid structure, genome organization, virally encoded protein functions, AAV life cycle and infection pathways (reviewed in Berns & Parrish, 2007; Carter et al., 2009; Smith & Kotin, 2002). WtAAV2 has a single-stranded DNA genome of 4679 nucleotides (nt) in length (GenBank accession no. AF043303) that comprises the region encoding 2 viral genes (e.g., the rep and the cap genes), their promoters (p5, p19 and p40 promoters), a polyadenylation signal, and two 145-nt inverted terminal repeats (ITR) forming T-shaped DNA hairpins at each viral genome terminus (Figure 1a). WtAAV2 expresses a total of 3 structural proteins (VP1, VP2 and VP3 from the cap gene) and 5 non-structural proteins (Rep40, Rep52, Rep68, and Rep78 from the rep gene and AAP from an alternative open reading frame from the cap gene). VP proteins form the AAV viral capsids while Rep proteins play roles in viral genome replication, packaging and site-specific viral genome integration at the AAVS1 site in the human chromosome 19 (Kotin et al., 1990, 1992). AAP protein (AAP stands for assembly-activating protein), which was identified in 2010, plays a role in directing VP proteins to nucleolus, the organelle where new AAV virions assemble (Sonntag et al., 2010).

2.1.2 The viral life cycle of wtAAV2

The life cycle of wtAAV2 consists of the lytic and the latent phases. Following infection through its surface receptors including heparan sulfate proteoglycan (Summerford & Samulski, 1998), wtAAV2 viral particles are carried to the nucleus where single-stranded viral genomes are released from virions into nucleoplasm. When an adenovirus co- or super-infects cells that are infected with wtAAV2, adenovirus helper functions are supplied and wtAAV2 enters the lytic phase where productive viral genome replication takes place. Adenoviral E1a, E1b55k, E4orf6, DNA-binding protein (DPB), and virus-associated RNA I (VAI-RNA) have been identified as the helper functions required for the growth of wtAAV2 and rAAV (Berns & Parrish, 2007; Geoffroy & Salvetti, 2005). In the lytic cycle, there are significant interactions between viral components and host DDR mediated by adenoviral E1 and E4 gene products and AAV large Rep proteins (i.e., Rep68/78). The interactions are primarily aimed at blocking the cell cycle and suppressing the NHEJ DNA repair pathway (more details are described in 3.2.).

In the absence of helper virus co-infection, most of the viral genomes are lost during cell division in dividing cells because they do not replicate or segregate together with the cellular genome into daughter cells. However, a certain proportion of AAV genomes establishes a latent phase by integration into the cellular genome, particularly at the AAVS1 site located within the human chromosome 19q13.42 (Kotin et al., 1992; Samulski et al., 1991). The AAVS1 site has a 33-nt DNA sequence within the myosin binding subunit (MBS) 85 gene and this short DNA sequence serves as the target for the site-specific integration (Linden et al., 1996b; Tan et al., 2001). The site-specific integration process requires expression of Rep68/78, which binds to a GCTC repeat element termed Rep binding site (RBS) and creates a nick at a nearby 3'-CCGGT/TG-5', designated terminal resolution site (trs). A set of these two recognition sequences is located within the AAV-ITR and the AAVS1 site (Brister & Muzyczka, 1999; McCarty et al., 1994). Several cellular factors have been shown to modulate Rep68/78-mediated site-specific integration (Figure 2b), although the experimental observations appear to be in conflict in some aspects. High mobility group protein 1 (HMG1) binds to Rep78,
enhances its RBS binding and nicking activities, and promotes site-specific integration at the AAVS1 site (Costello et al., 1997). In addition, the human immunodeficiency virus type 1 (HIV-1) TAR RNA binding protein 185 (TRP-185) binds to the RBS within the AAVS1 site, interact with Rep68, enhances Rep68's helicase activity, and controls selection of AAV genome integration sites within the AAVS1 locus (Figure 2b) (Yamamoto et al., 2007). This mode of latency is unique to wtAAV2 among the animal viruses, and is the case at least in cultured cells. In latently-infected human tissues, a majority of wtAAV2 genomes persist as circular genomes and no site-specific integration has been demonstrated even by sensitive PCR-based assays (Schnepp et al., 2005); therefore, the significance of the site-specific integration of wtAAV2 in natural infection in humans remains elusive.

2.1.3 wtAAV2 viral components that evoke DDR
Among the viral components, large Rep proteins, the cis-acting replication element (CARE) within the p5 promoter (Fragkos et al., 2008; Francois et al., 2005; Nony et al., 2001; Tullis & Shenk, 2000), AAV-ITR, and the unusual single-stranded nature of the viral genome are particularly important in AAV-evoked DDR and interaction with DNA repair machinery. These elements could potentially activate DDR without AAV viral genome replication.

2.2 Recombinant AAV (rAAV) vectors
Recombinant AAV (rAAV) vectors are genetically-engineered viral agents that carry heterologous DNA to be delivered to target cells and are devoid of all the viral genome sequence except for the 145-nt (ITR) at each genome terminus. Until early 2000s, rAAV vectors were primarily derived from AAV2 due to the limited availability of alternative serotypes at that time. rAAV2 vectors have a broad host range and outstanding ability to deliver genes of interest to both dividing and non-dividing cells of various types in vitro. However, rAAV2 exhibits limited transduction efficiency in tissues and organs in vivo when administered in experimental animals. This drawback of rAAV2 has recently been overcome by the discovery of new serotypes exemplified by AAV serotypes 8 and 9 (AAV8 and AAV9) (Gao et al., 2002, 2004). rAAV vectors derived from serotypes alternative to AAV2 have become widely available at present and been shown to exhibit unprecedented robust transduction in various tissues and organs by intravascular injection of the vector (Figure 1b) (Foust et al., 2009; Ghosh et al., 2007; Inagaki et al., 2006; Nakai et al., 2005a; Sarkar et al., 2006; Vandendriesche et al., 2007; Wang et al., 2005). It should be noted that alternative serotype rAAV vectors are in general those containing a rAAV2 viral genome encapsidated with an alternative serotype viral coat (i.e., pseudosерotyped rAAV2 vectors) (Rabinowitz et al., 2002). Therefore, they are often referred to AAV2/8 and AAV2/9 (genotype/serotype) when rAAV2 genome is contained in AAV8 and AAV9 viral coats, respectively. In addition to the exploitation of AAV capsids derived from various serotypes and variants present in nature, recent advances in genetic engineering of viral capsids aiming for the creation of specific cell type/tissue-targeting vectors have significantly broadened the utility of this vector system (Asokan et al., 2010; Excoffon et al., 2009; Koerber et al., 2009; Yang et al., 2009). Double-stranded (ds) rAAV vectors and gene targeting rAAV vectors are also worthy of note. Ds rAAV contains a ds viral genome in place of a single-stranded (ss) DNA
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(McCarty, 2008). Because ds rAAV vectors overcome the rate-limiting step in transduction, i.e., conversion from ss to ds DNA in infected cells, they exhibit a 1-2 log higher transduction efficiency than that achievable with the conventional ss vectors (McCarty et al., 2003; Wang et al., 2003). Gene-targeting rAAV vectors have the ability to introduce genomic alterations precisely and site-specifically at high frequencies of up to 1% (Russell & Hirata, 1998; Vasileva & Jessberger, 2005), which is several logs higher than that achievable by the conventional homologous recombination (HR) approaches (Thomas & Capecchi, 1987) (please refer to 3.5 for more details). Nonetheless, even if rAAV vectors are devoid of several key components that trigger DDR (i.e., large Rep expression and the CARE), establishment of rAAV transduction heavily relies on the interactions between rAAV viral genomes and DNA repair machinery irrespective of serotypes or nature of viral genomes (i.e., ss rAAV or ds rAAV).

3. AAV and DNA repair pathways

An overview of AAV and DNA repair pathways is summarized in Figure 2.

3.1 AAV-evoked DDR

3.1.1 Earlier evidence for the role of DDR in the AAV genome processing

Although the interplay between virus and DDR is a relatively new area of research, earlier studies indicated potential roles of DDR in the AAV life cycle or viral genome processing. The first indicative evidence came from the observation that cells treated with a wide variety of genotoxic agents including UV irradiation and carcinogens such as hydroxyurea could support wtAAV2 genome replication in the absence of helper virus co-infection (Yakinoglu et al., 1988; Yakobson et al., 1987, 1989). Subsequently, such treatment was found to augment rAAV2 transduction efficiency in both dividing and non-dividing cells with the latter showing a more dramatic enhancing effect (Alexander et al., 1994; Russell et al., 1995). These earlier observations suggested that activated DNA repair pathways following DNA damage induced by genotoxic treatment somehow facilitated the conversion of rAAV genomes from ss to ds DNA by second-strand synthesis (Figure 2f, g and h) (Ferrari et al., 1996; Fisher et al., 1996). As mentioned earlier, the formation of ds AAV genomes is a critical step for wtAAV to initiate productive infection and for rAAV to undergo abortive infection and express transgene products. A better response to the treatment in non-dividing cells conforms to the idea that DNA repair pathways are constitutively activated to a greater extent in dividing cells. Such activation is required to repair DNA replication errors that occur naturally and unavoidably. Although the underlying mechanism of this effect still remains elusive, one can speculate that up-regulation of DNA repair pathways increases the pool of cellular factors required for AAV genome processing. Alternatively, factors inhibitory for wtAAV genome replication or rAAV transduction may become sequestered from AAV genomes to multiple DNA repair foci formed on the damaged cellular genome (Figure 2f and g). A recent observation that the MRN complex and ATM, the major DDR proteins, have an inhibitory effect on rAAV transduction supports the latter model (Cataldi & McCarty 2010; Cervelli et al., 2008; Choi et al., 2006; Sanlioglu et al., 2000; Schwartz et al., 2007).
Fig. 2. A hypothetical model of the interactions between DNA damage responses (DDRs), DNA repair proteins and adenovirus co-infection, adenoviral E1B55k/E4orf6 degrades and E4orf3 mislocates the MRN complex and activates the ATM pathway. Over-expression of Rep68/78 supplied in trans, mediates site-specific integration of AAV genomes, allowing processing of viral genomes toward wtAAV2 replication and rAAV transduction. ATM may also have an inhibitory effect on the S phase. (d, e) Activation of the ATR pathway by wtAAV2 or rAAV genome results in the G2/M arrest. (f, g) Artemis/DNA-PKcs promote generation of circular monomers via intramolecular recombination of rAAV genomes in pre-existing breaks in the cellular genome via NHEJ pathway(s). DNA-PKcs is dispensable but it could influence rAAV gene targeting. In the pathway extending from AAV-ITR indicate leading strands generated by a second-strand synthesis mechanism. In the pathway extending from AAV-ITR indicate leading strands generated by a second-strand synthesis mechanism.
3.1.2 DNA repair proteins associated with AAV genomes in cells

AAV is composed of only two elements, VP proteins that form viral capsids and a single-stranded viral genomic DNA. VP proteins primarily determine biological properties of various AAV serotypes; i.e., how AAV particles reach cells, enter cells, traffic in cytoplasm and nucleoplasm, and uncoat virion shells to release viral genomes. At present there is no evidence indicating that the above-mentioned AAV infection pathways driven by the capsid trigger DDR. AAV-evoked DDR is all about the cellular responses against AAV viral genomes except for wtAAV2, which expresses Rep68/78 proteins that also trigger DDR. It is plausible that the structure of single-stranded DNA with T-shaped hairpin termini, which is unusual and is not present in the cellular genome, is recognized as damaged DNA and triggers DDR. Direct evidence for the association of AAV genomes with DNA repair machinery has obtained in chromatin immunoprecipitation (ChIP) studies where AAV genomes and their associated cellular factors were crosslinked by formalin and precipitated together using antibodies specific to DNA repair proteins. To date, the MRN complex, Ku86, Rad52, RPA and DNA polymerase delta have been identified as factors bound to ss AAV genomes (Cervelli et al., 2008; Jurvansuu et al., 2005; Zentilin et al., 2001). In addition, immunofluorescence microscopy has revealed that the MRN complex, ATR, TopBP1, BLM, Brca1, Rad17, RPA, and Rad51 are recruited to the discrete nuclear foci where AAV genomes accumulate (Cervelli et al., 2008; Jurvansuu et al., 2005). Table 2 summarizes the roles of DNA repair proteins in AAV infection/transduction, AAV genome self-circularization, and AAV genome integration into the host genome.

3.1.3 AAV genome activates ATR-mediated DDR

Although not exclusive, the DNA repair proteins found to be associated with AAV genomes described in 3.1.2 are those involved in the ATR-mediated DDR that is triggered by stalled replication forks (Figure 2d and e) (reviewed in Branzei & Foiani, 2010 and Shiotani & Zou, 2009). At stalled replication forks, ss DNA regions become coated with RPA. RPA then recruits ATR-ATRIP and the Rad17 complex to the damaged site. The Rad17 complex subsequently recruits the ring-shaped trimeric Rad9/Rad1/Hus1 (9-1-1) complex, and finally ATR-ATRIP kinase becomes activated by TopBP1 recruited to the site and sends a DNA damage checkpoint signal (Figure 2d and e). In addition, Mre11 has also been reported to relocalize to stalled replication forks to a limited extent (Mirzoeva & Petrini, 2003). AAV-ITR exhibits a close structural similarity to stalled replication forks in that it contains both ss DNA regions and ss DNA-ds DNA junctions. This strongly supports a model in which AAV-ITR is recognized as a stalled replication fork and triggers the checkpoint response via ATR kinase. The actual activation of the ATR pathway by AAV genomes has been confirmed by the demonstration that the ATR-downstream effector proteins; i.e., Chk1 and RPA, become phosphorylated in cells infected with wtAAV2 or UV-irradiated wtAAV2, both of which are devoid of the ability to replicate or express viral genes in the system used for the experiment (Fragkos et al., 2008; Ingemarsdotter et al., 2010; Jurvansuu et al., 2005, 2007). Interestingly, rAAV2 genome devoid of the 55-nt CARE within the p5 promoter does not evoke the ATR-mediated checkpoint signal, and it has been shown that co-existence of both ITR and CARE in an AAV genome is essential for the activation (Fragkos et al., 2008). The consequence of the AAV genome-evoked ATR-mediated DDR is G2/M cell cycle arrest in wild type cells, while it leads to cell death in p53-deficient cells (Ingemarsdotter et al., 2010; Jurvansuu et al., 2007) (please see 3.1.4. for more details). Cell cycle arrest in the late S and/or G2 phases following infection of wtAAV2 or UV-irradiated wtAAV2 was observed.
Table 2. DNA repair and AAV

<table>
<thead>
<tr>
<th>Protein</th>
<th>Effects of deficiency²</th>
<th>Note</th>
<th>References³</th>
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<tr>
<td></td>
<td>T C I</td>
<td>In vitro</td>
<td>In vivo</td>
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<tr>
<td>Artemis</td>
<td>↓</td>
<td></td>
<td>↓ ↓</td>
</tr>
<tr>
<td>ATM</td>
<td>↑ / / ↑ / ↑</td>
<td>wtAAV2-evoked signal ↑</td>
<td>1</td>
</tr>
<tr>
<td>ATR</td>
<td>↑ / / → →</td>
<td>rAAV2 genome replication ↓ when</td>
<td>2, 3, 4, 5</td>
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<tr>
<td></td>
<td></td>
<td>deficient</td>
<td></td>
</tr>
<tr>
<td>BLM</td>
<td>↓</td>
<td>wtAAV2 genome replication ↑ when</td>
<td>6</td>
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<td></td>
<td></td>
<td>deficient</td>
<td></td>
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<tr>
<td>Chkl</td>
<td>↓ / / ↑ / ↑ / → /</td>
<td>rAAV2 genome replication ↓ when</td>
<td>14, 15</td>
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<td></td>
<td></td>
<td>deficient</td>
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<tr>
<td>DNA-PKcs</td>
<td>↓</td>
<td>Targeting efficiency ↑ when deficient</td>
<td>3, 4</td>
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<td></td>
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<tr>
<td>Ku70/80(86)</td>
<td>↑</td>
<td>wtAAV2 genome replication ↓ when deficient</td>
<td>3, 4</td>
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<td>ligation IV</td>
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<tr>
<td>MDC1</td>
<td>↑</td>
<td>Targeting efficiency ↓ when deficient</td>
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<tr>
<td>MRN</td>
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<td>Rad54L</td>
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<tr>
<td>WRN</td>
<td>↓</td>
<td>Induction of cell death when deficient</td>
<td>4</td>
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<tr>
<td>X RCC3</td>
<td>→</td>
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<tr>
<td>pRb</td>
<td></td>
<td>Induction of cell death when deficient</td>
<td>12, 13</td>
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<tr>
<td>p21</td>
<td></td>
<td>Induction of cell death when deficient</td>
<td>12, 13</td>
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<tr>
<td>p53</td>
<td></td>
<td>Induction of cell death when deficient</td>
<td>12, 13</td>
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</tbody>
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² Observed effects caused by deficiency or knockdown are summarized. T, transduction efficiency; C, AAV genome self- circularization efficiency; I, AAV genome integration efficiency. In the table, arrows indicate an increase (↑), a decrease (↓) or no change (→).

³ References cited are as follows: 1, Inagaki et al., 2007b; 2, Sanlioglu et al., 2000; 3, Zentilin et al., 2001; 4, Choi et al., 2006; 5, Cataldi et al., 2010; 6, Song et al., 2006; 7, Choi et al., 2010; 8, Daya et al., 2009; 9, Cervelli et al., 2008; 10, Schwartz et al., 2007; 11, Vasileva et al., 2006; 12, Garner et al., 2007; 13, Raj et al., 2001; 14, Schwartz et al., 2009; 15, Collaco et al., 2009; 16, Nakai, unpublished.

3.1.4 AAV genome-evoked DDR leading to cell death

A unique aspect of AAV-evoked DDR is the ability to induce cell death without productive viral genome amplification, viral gene expression or cellular DNA damage, when cells are devoid of p53 expression. In 2001, Raj et al. reported an unexpected experimental observation that wtAAV2 infection of an osteosarcoma cell line that lacks expression of functional p53 leads to cell death through apoptosis or mitotic catastrophe, whereas the wild type control cells merely undergo a transient cell cycle arrest in the G2 phase (Raj et al., 2001). Mitotic catastrophe is an ill-defined term describing an apoptosis-like cell death during mitosis that takes place even in the presence of unrepaired DNA damage (Castedo et al., 2004; Vakifahmetoglu et al., 2008). This p53 deficiency-dependent cell killing effect was observed in an earlier study although how and what DDR is involved was not known at that time (Winocour et al., 1988).
also observed when cells were infected with UV-irradiated wtAAV2 or microinjected with a 145-nt AAV2-ITR oligonucleotide, demonstrating that the unusual structure of the AAV2-ITR sequence itself is the culprit (Raj et al., 2001). Initially it was presumed that infection of wtAAV2 activates the ATM-p53-mediated DDR, which in turn increases and decreases the levels of p21 and CDC25C, respectively, resulting in the G2 arrest (Raj et al., 2001). Although the mechanism of p53 deficiency-dependent cell killing by AAV genomes still remains elusive, a series of subsequent studies on this phenomenon has revealed at least three potentially independent AAV-evoked pathways leading to cell death: the pathways involving (1) p53-p21-pRb, (2) p84N5 via caspase 6, and (3) ATR-Chk1. In the first mechanism, AAV-evoked DDR signal is transduced to a potent antiapoptotic proteins, pRb, via p53 and p21. Therefore, cells defective in this pathway fail to transduce the DDR signal to pRb, leading to apoptosis (Garner et al., 2007). In the second mechanism, functional defect of the p53-p21-pRb pathway allows activation of the nuclear death domain protein p84N5, which otherwise is inhibited by association with pRb (Doostzadeh-Cizeron et al., 1999). The activated p84N5 then induces apoptosis via caspase-6 (Garner et al., 2007). In the third mechanism, AAV genomes activate ATR, which in turn phosphorylates Chk1, causing a transient cell cycle arrest in the G2 phase. In the absence of p53, cells fail to sustain the G2 arrest following degradation of the unstable Chk1, progress suicidally into mitosis, and die via mitotic catastrophe associated with centriole overduplication and the subsequent formation of multipolar mitotic spindles (Ingemarsdotter et al., 2010; Jurvansuu et al., 2007). Whether all of the pathways or only some of them are triggered by AAV genomes remains unknown at present.

3.1.5 AAV2 Rep68/78-evoked DDR
In addition to AAV genome as a trigger of DDR, AAV2 large Rep proteins (i.e., Rep68/78) themselves also evoke DDR independent of AAV genome. In the lytic phase of the AAV life cycle where cells are co-infected with a helper virus, Rep proteins are strongly expressed and exert many functions in the network of cellular proteins and viral factors derived from adenovirus or other helpers. Rep proteins can also be expressed without helper virus infection but only to a limited extent due in part to the large Reps' ability to negatively regulate their own promoter (p5) and the promoter for the small Rep proteins (p19) (Beaton et al., 1989; Kyostio et al., 1994). The significance of Rep68/78 expression in the absence of helper viruses reside in a series of the AAVS1-targeting approaches that exploit wtAAV2's ability to introduce exogenously derived DNA into the AAVS1 site in a site-specific manner (Figure 2c) (Henckaerts & Linden, 2010; Linden et al., 1996a). In these approaches, a donor vector in any context (e.g., plasmid DNA, adenoviral vectors or rAAV) containing a gene of interest and RBS is delivered to human cells where AAV2 large Rep expression is supplied by the same vector or a separate one. The AAV2-ITR sequence is commonly used as an RBS-containing cis element; however, the p5 promoter also serves as an alternative (Philpott et al., 2002).

It has been known that Rep68/78 shows significant cellular toxicity due to the strong antiproliferative action of the protein (Yang et al., 1994). Rep78 completely blocks the cell cycle in the S phase (Saudan et al., 2000). Studies have shown that Rep78 exerts two independent but complementing DDR-associated cellular signal transduction pathways to arrest the cell cycle. The two pathways are the pRb pathway and the ATM-Chk2 pathway (Berthet et al., 2005). In the first pRb pathway, Rep78 expression leads to an increased level of the cyclin-dependent kinase inhibitor p21 and accumulation of hypophosphorylated pRb,
the active form of pRb protein (Berthet et al., 2005; Saudan et al., 2000) (Figure 2c). Consequently, cellular proteins that control cell cycle progression such as cyclin A, cyclin B1, and Cdc2, are down-regulated, resulting in slowing down the cell cycle (Saudan et al., 2000). Supporting this model, this effect is substantially attenuated in pRb-deficient mouse embryonic fibroblasts (Saudan et al., 2000). An increased amount of p21 might explain the inability to phosphorylate pRb upon large Rep expression, but the transcriptional activation of p21 has been shown to occur via a p53-independent pathway (Hermanns et al., 1997). In the second ATM-Chk2 pathway, the DNA-nicking activity of Rep68/78 creates multiple damaged sites in the cellular genome, which activates the ATM-Chk2 pathway and arrests the cell cycle (Figure 2c) (Berthet et al., 2005). Large Rep proteins create a break in only one strand of two-stranded DNA, which is not a type of damage that usually activates ATM. It is currently unknown how Rep68/78-induced DNA damage triggers this pathway. Worthy to note, activation of either one of the above-mentioned two pathways by itself is not sufficient for the complete block of the cell cycle, which is attainable by Rep68/78 expression (Berthet et al., 2005). It appears that there would be many other Rep68/78-associated DNA repair pathways that have yet to be identified. This is because a recent study using a tandem affinity purification (TAP) approach has demonstrated physical interaction of Rep78 with many DNA repair-associated proteins including DNA-dependent protein kinase catalytic subunit (DNA-PKcs), minichromosome maintenance (MCM) proteins, Ku70/80, proliferating cell nuclear antigen (PCNA), RPA, and structural maintenance of chromosome 2 (SMC2) (Nash et al., 2009).

3.2 wtAAV2 genome replication and DNA repair pathways

In the lytic phase of the wtAAV2 life cycle, viral genome replication requires co-infection of a helper virus. Since human adenoviruses have been most extensively studied in the context of AAV virology, this section specifically focuses on the interplay between wtAAV2, human adenoviruses, and DNA repair machinery. The adenoviral components required in the lytic infection are E1a, E1b55k, E4orf6, DBP, and VAI. A series of adenovirus/AAV co-infection studies has provided significant insights into how DDR and DNA repair machinery play roles in AAV genome replication in the presence of adenovirus helper functions (Collaco et al., 2009; Schwartz et al., 2009). It has been shown that adenoviral E1b55k/E4orf6 degrades the MRN complex via the ubiquitin-proteasome pathway and E4orf3 mislocalizes the MRN complex to aggresome, abrogating the MRN function in triggering the ATM and ATR pathways (Figure 2a) (Collaco et al., 2009). In addition, E4orf6 dissociates ligase IV from ligase IV/XRCC complex and degrades it (Jayaram et al., 2008). The main consequence of this adenoviral manipulation of DDR is inhibition on NHEJ, which prevents concatemeric adenoviral genome formation and promotes adenoviral genome replication and packaging. Although it remains elusive how beneficial the inhibition of NHEJ is in the AAV lytic life cycle, the significance of E1b/E4-mediated degradation of MRN complex in the wtAAV2 lytic cycle has been revealed by the observation that the MRN complex binds to AAV-ITR and inhibits wtAAV2 genome replication and rAAV vector transduction (Cervelli et al., 2008; Schwartz et al., 2007). Along the same line, the observation that cells deficient in ATM exhibit a higher rAAV transduction efficiency (Sanlioglu et al., 2000) might be explainable on the assumption that lack of ATM would be an equivalent to inactivation of the MRN complex because the MRN complex serves as a damage sensor that activates the ATM pathway (Carson et al., 2003). It is tempting to propose that dislocation of the MRN complex and other inhibitory factors from AAV genomes to the sites in the cellular genome where
genome integrity is more severely threatened, is the mechanism for the augmentation of wtAAV2 genome replication and rAAV vector transduction by genotoxic treatment (Figure 2g). Suppression of the NHEJ pathway that involves DNA-PKcs and Ku proteins, however, may or may not be beneficial, because one study has shown that deficiency of these proteins both resulted in impaired rAAV2 genome replication (Choi et al., 2010) whereas another study has reported that siRNA-mediated knockdown of DNA-PKcs enhanced wtAAV2 genome replication (Collaco et al., 2009).

In addition to the adenovirus-evoked DDR, productive wtAAV2 viral genome replication triggers DDR distinct from that observed in adenovirus only infection (Collaco et al., 2009; Schwartz et al., 2009). Adenovirus-wtAAV2 co-infection results in much more pronounced activation of ATM and the checkpoint kinases, Chk1 and Chk2. This activation occurs independently of the MRN complex; therefore, the activation sustains even if MRN complex starts being degraded by adenoviral E1b/E4 proteins (Collaco et al., 2009). Other DDR substrate proteins RPA, NBS1 and H2AX become phosphorylated as the lytic phase progresses (Collaco et al., 2009; Schwartz et al., 2009). It has been shown that AAV genome replication is essential and sufficient to induce the DDR signal transduction cascade observed in the adenovirus co-infection, and Rep proteins does not play a role in the activation of DDR (Collaco et al., 2009; Schwartz et al., 2009). Among the three phosphatidylinositol 3-kinase-like kinases (PIKKs) that initiate signal transduction (i.e., ATM, ATR and DNA-PKcs), ATM and DNA-PKcs are the primary kinases that phosphorylate downstream DDR substrates, and ATR appears to play only a minor role in the lytic phase of the AAV life cycle (Collaco et al., 2009; Schwartz et al., 2009). Although the significance of the DDR in the AAV lytic cycle remains unclear, the activation of the ATM pathway appears to be beneficial for AAV genome replication (Collaco et al., 2009).

3.3 rAAV genome recombination and DNA repair pathways

3.3.1 rAAV genome processing is mediated solely by DNA repair machinery

After entering nuclei, rAAV virion shells break down, releasing single-stranded (ss) vector genomes into nucleoplasm, which subsequently convert to various forms of double-stranded (ds) genomes (Deyle & Russell, 2009; Schultz & Chamberlain, 2008). It should be noted that rAAV does not express any viral gene products that can process viral genomes such as recombinases and integrases; therefore the processing of viral genomes must heavily depend on DNA repair machinery. In addition, unlike the battle between adenovirus and the host DNA repair systems as described in 3.2, rAAV has no means to manipulate DNA repair pathways once viral genomes evoke DDR. Unless rAAV genomes have been processed to completion into stable ds DNA with no free ends, DDR would remain activated due to the continued presence of viral DNA in an unusual structure presenting a single strand with free ends. In mammalian cells, extrachromosomal free DNA ends at ds rAAV genome termini as well as those in ds linear plasmid DNA, when exogenously delivered, appear to be removed primarily by ligating two free ends and making a single continuous ds DNA strand via NHEJ and/or occasionally HR rather than by DNA degradation (Nakai et al., 2003b; Nakai, unpublished observation). In this sense, the rAAV genomes processed into various forms in their latency could be viewed as byproducts that have been created and disposed of by a cellular defense mechanism against potentially toxic exogenous agents.
3.3.2 Single-to-double-stranded rAAV genome conversion and DNA repair machinery
How ss rAAV genomes become ds DNA is not completely understood but the process involves the following two mechanisms; second-strand synthesis (Ferrari et al., 1996; Fisher et al., 1996; Zhong et al., 2008; Zhou et al., 2008) and annealing of plus and minus strands (Hauck et al., 2004; Nakai et al., 2000). It has been shown that, upon rAAV infection, the MRN complex becomes activated, physically associates with AAV2-ITR and inhibits wtAAV2 replication and rAAV transduction (Figure 2a and h) (Cervelli et al., 2008; Schwartz et al., 2007); therefore, MRN appears to have some role in the conversion of ss to ds DNA. ATM has also been suggested to be a cellular factor that inhibits the single-to-double-stranded genome conversion because transduction efficiency with ss rAAV is significantly enhanced in ATM-deficient cells in vitro (Figure 2h) (Sanlioglu et al., 2000). However, a recent study has proposed an ATM-mediated gene silencing model rather than the mechanism involving the second-strand synthesis to explain the ATM's inhibitory effect. This model stems from the observation that, in the absence of ATM, ds rAAV transduction was enhanced as well, indicating that an alternative mechanism other than second-strand synthesis is involved (Cataldi & McCarty, 2010). Another factor that is known to inhibit this process is tyrosine-phosphorylated FKBP52, which binds to AAV-ITR and inhibits second-strand synthesis (Qing et al., 2001). Its dephosphorylation by T-cell protein tyrosine phosphatase (TC-PTP) dissociates FKBP52 from AAV-ITR, allowing the formation of ds genomes (Qing et al., 2003). In vitro AAV replication studies have identified the DNA polymerase that catalyzes second-strand synthesis as DNA polymerase δ (Nash et al., 2007), which is a polymerase that fills a single-stranded DNA gap created during the nuclear excision repair (Torres-Ramos et al., 1997). Physical association of DNA polymerase δ and AAV genome has also been demonstrated (Jurvansuu et al., 2005). At present it remains elusive whether and how the above-mentioned signal kinases (i.e., MRN and ATM) and effectors (FKBP52 and DNA polymerase δ) are linked in the rAAV-evoked DDR.

3.3.3 Extrachromosomal rAAV genome recombination and DNA repair machinery
In addition to the above-mentioned single-to-double-stranded genome conversion, rAAV genomes are further processed into the following stable ds forms by intra- or inter-molecular DNA recombination mediated solely by DNA repair machinery, and establish the latent infection. The viral genome forms in the latent phase include ds circular monomers, large concatemers (circular and/or linear), and rAAV proviral genomes that are stably integrated into the host cellular genome at low frequencies (Deyle & Russell, 2009; Schultz & Chamberlain, 2008). It has not been determined when the rAAV genome recombination takes place, which may be either before, at, or after completion of the single-to-double-stranded genome conversion. In dividing cells, extrachromosomal genomes are lost because they do not replicate episomally, whereas they can be stabilized and maintained as chromatin in quiescent cells in animal tissues (Penaud-Budloo et al., 2008). Earlier studies indicated that the formation of large concatemeric rAAV genomes is important for transgene expression; however, accumulated observations might favor a model in which extrachromosomal circular monomer genomes, not large concatemers or integrated forms, are primarily responsible for persistent and stable transgene expression in rAAV-transduced animal tissues (Nakai et al., 2001; Nakai et al., 2002; Nathwani et al., 2011). In extrachromosomal rAAV genome recombination, AAV-ITR plays a pivotal role in mediating recombination. Although it has yet to be elucidated how DDR is evoked by rAAV.
In the context of rAAV genome recombination, it is not unreasonable to speculate that the T-shaped hairpin structure within the AAV-ITR and/or ss DNA-ds DNA junctions in the stem of the hairpin DNA trigger DDR. A set of DNA repair proteins, which includes DNA-PKcs, Artemis, ATM, MRN, BLM, and WRN (Figure 2h), has been found to be involved in rAAV genome recombination (Cataldi & McCarty, 2010; Choi et al., 2006; Duan et al., 2003; Inagaki et al., 2007b; Nakai et al., 2003b; Sanlioglu et al., 2000; Song et al., 2001). Deficiency of these proteins impairs intramolecular recombination of ss rAAV and/or ds rAAV genomes via the AAV-ITR sequence. DNA-PKcs and Artemis are the two major components in the classical NHEJ pathway of DSB repair. Artemis, when activated by DNA-PKcs, possesses an endonuclease activity and resolves DNA hairpin loops and flaps formed at broken DNA ends to facilitate ds DNA end joining (Ma et al., 2005). BLM and WRN are members of the RecQ family of DNA helicases. They unwind ds DNA to ensure the formation of proper recombination intermediates, and mediate a variety of DNA transactions, mainly HR (Bernstein et al., 2010). MRN is a multifaceted protein complex that functions as a primary sensor of DSB, binds DNA lesion, recruits ATM, and processes DNA ends by utilizing the Mre11 endo- and exo-nuclease activity that creates recombinogenic 3’ single-stranded tails (Williams et al., 2010). The initial study of the structure of ITR-ITR junction sequences revealed that the majority of the recombination junctions in ds circular monomer genomes exhibited a 165-nt double-D ITR structure, the hallmark of HR (Duan et al., 1999; Xiao et al., 1997). This indicates that HR is the major pathway for intra- and intermolecular genome recombination events. Supporting this view, Rad52, which is a key player in HR, was identified as a protein that binds to rAAV genomes in cultured cells (Zentilin et al., 2001). Interestingly, deficiency of Rad52 does not affect rAAV transduction efficiency or genome processing in murine liver (Nakai, unpublished observation). It remains possible that HR plays a major role in rAAV genome recombination at least under certain cellular environment; however, accumulated observations by us and others rather support a model in which NHEJ is the major pathway for extrachromosomal rAAV genome recombination. In the absence of DNA-PKcs or Artemis, intramolecular recombination is significantly impaired in cultured cells and animal tissues (Cataldi & McCarty, 2010; Duan et al., 2003; Inagaki et al., 2007b; Nakai et al., 2003b; Song et al., 2001), and the footprints on junction DNA are quite consistent with NHEJ-mediated recombination, showing nucleotide deletions of various degrees with occasional microhomology at junctions (Inagaki et al., 2007b). Interestingly, intra- and inter-molecular recombination events that form ds circular monomers and ds concatemers, respectively, are differentially regulated by different DNA repair pathways (Figure 2h). Intramolecular recombination heavily depends on the Artemis/DNA-PKcs-dependent NHEJ pathway, while the NHEJ pathways that mediate intermolecular recombination are redundant because intermolecular recombination occurs efficiently in the absence of DNA-PKcs or Artemis (Inagaki et al., 2007b). The DNA-PKcs or Artemis-independent NHEJ might be those involving ATM and/or MRN (Cataldi & McCarty, 2010; Choi et al., 2006; Duan et al., 2003; Inagaki et al., 2007b; Nakai et al., 2003b; Sanlioglu et al., 2000; Song et al., 2001). Alternatively, HR might be the major pathway for intermolecular rAAV recombination. This model stems from the observation that recombination between two homologous AAV-ITRs derived from the same serotype is preferred to that between two non-homologous AAV-ITRs derived from different serotypes (Yan et al., 2007). The ATR pathway does not appear to be involved in extrachromosomal rAAV genome recombination (Cataldi & McCarty, 2010).
How DNA-PKcs and Artemis process rAAV genome termini and mediate recombination has been extensively studied in the context of murine tissues. In DNA-PKcs or Artemis-deficient SCID mice, ds linear rAAV genomes with covalently closed hairpin caps at genome termini accumulate in rAAV-transduced tissues (Figure 3b). In SCID mouse thymi, V(D)J recombination is impaired resulting in accumulation of covalently-sealed hairpin intermediates at V(D)J coding ends in the T cell receptor gene (Rooney et al., 2002; Roth et al., 1992) (Figure 3a). These two phenomena are essentially the same in that if hairpin structures at DNA ends are not cleaved by the Artemis/DNA-PKcs endonuclease activity, covalently closed DNA ends accumulate without undergoing further recombination. Therefore, intramolecular recombination most likely uses the same Artemis/DNA-PKcs-dependent NHEJ pathway used for V(D)J recombination. It is not easy to determine GC-rich AAV-ITR hairpin DNA structures at sequencing levels; however this shortcoming has been overcome by exploiting the bisulfite PCR technique. Utilizing this method, the primary cleavage site by the Artemis/DNA-PKcs endonuclease activity has been mapped to the 5′ end of the 3-base AAA loop at the AAV-ITR hairpin tips (Figure 3b) (Inagaki et al., 2007b). In DNA-PKcs-deficient SCID mouse tissues, the relative proportion of rAAV genome recombination junctions exhibiting the hallmark of HR increases, indicating compensatory activation of HR in the absence of DNA-PKcs in quiescent cells in animal tissues (Nakai, unpublished observation). In this regard, worthy of note are the following observations made by us and others that DNA repair pathways might somehow be linked to epigenetic modifications of rAAV genomes. We have found that the cytomegalovirus (CMV) immediately early gene promoter in rAAV genome can be significantly silenced in Artemis- or DNA-PKcs-deficient mouse muscle (Nakai, unpublished observation). Recently, Cataldi et al. reported that the CMV promoter is somewhat silenced in ATM-proficient murine fibroblasts compared to that in ATM-deficient cells (Cataldi & McCarty, 2010). These observations imply that rAAV genome recombination via NHEJ generates more functionally active genomes than HR presumably due to a difference in epigenetic modifications of rAAV genomes (Cataldi & McCarty, 2010).

### 3.4 rAAV genome integration and DNA repair pathways

rAAV is devoid of Rep68/78 expression; therefore, it lacks the ability to integrate into the cellular genome site specifically. In addition, rAAV does not harness machinery designed specifically for integration into the cellular genome. rAAV vectors are generally considered as episomal vectors, but they do integrate into the cellular genome of both dividing and non-dividing cells at low frequencies (Deyle & Russell, 2009; McCarty et al., 2004). This process is entirely dependent on the host cellular DNA repair machinery. Although it is not easy to determine the frequency of rAAV genome integration in each case and it may vary depending on the amount of rAAV genomes delivered to cells, integration has been reported to occur at approximately ~0.1% of total input rAAV genomes (Russell et al., 1994) or up to ~4% of cell population in rAAV-infected cultured cells (Cataldi & McCarty, 2010), or at approximately 0.1% of rAAV-transduced hepatocytes when rAAV is injected into newborn mice (Inagaki et al., 2008). rAAV genome integration occurs at nonrandom sites in both cultured cells and somatic cells in animals. The preferred genomic sites for integration include the 45s pre-ribosomal RNA gene, transcriptionally active genes, DNA palindromes, CpG islands, and the neighborhood of transcription start sites (Inagaki et al., 2007a; Miller et al., 2005; Nakai et al., 2003a). Although the mechanism of integration remains largely unknown, it has been presumed that input rAAV genomes are fortuitously captured at pre-
Fig. 3. A similarity of Artemis / DNA-PKcs-mediated hairpin cleavage in V(D)J recombination and AAV-ITR recombination. (a) During V(D)J recombination, the recombination activating gene products, Rag1 and Rag2 endonucleases, cleave the immunoglobulin and T cell receptor genes, forming covalently closed hairpin loops at cleaved DNA ends. Artemis/DNA-PKcs complex resolves the hairpin loops, which triggers the subsequent recombination between the two coding ends via the classical NHEJ DNA repair pathway. In cells deficient in either Artemis or DNA-PKcs (SCID phenotype), hairpin coding ends remain unrecombined and accumulate. (b) The same Artemis/DNA-PKcs-dependent NHEJ pathway mediates intramolecular AAV-ITR recombination, forming circular monomer genomes. Intermolecular AAV-ITR recombination occurs independently of Artemis/DNA-PKcs. A red arrowhead indicates the primary target for Artemis/DNA-PKcs-mediated cleavage. In SCID mouse tissues, ds linear rAAV genomes with covalently closed AAV-ITR hairpin caps accumulate.

existing breaks in the cellular genome when the DNA breaks are repaired by DNA repair machinery, which establishes rAAV integration. This model has been supported by the observations that rAAV genome integrations are frequently found at I-SceI-induced DSBs in the cellular genome (Miller et al., 2004) and genotoxic treatments can increase integration rates (Russell et al., 1995). Clinically, rAAV vectors are generally considered to be safe; however, one study has shown that vector genome integration could cause insertional mutagenesis leading to hepatocarcinogenesis in a mouse model (Donsante et al., 2007).

The detailed analyses of rAAV vector genome-cellular genome junction sequences in cultured cells and murine tissues have provided significant insights into which and how DNA repair pathways play roles in rAAV integration (Inagaki et al., 2007a; Miller et al., 2005; Nakai et al., 2005b). rAAV integration does not take place in a neat cut-and-paste fashion and always accompanies various degrees of deletions in rAAV genome terminal sequences and the cellular genomes around integration sites. Complex genomic
rearrangements are not rare and integration often causes a chromosomal translocation. All of these observations fit very well with a model in which NHEJ mediates rAAV integration. A series of studies has shown that DNA-PKcs has negative or positive effects on integration depending on the experimental systems used (Figure 2i). In a cell-free in vitro rAAV integration system, ss rAAV integration frequency increases and decreases by the addition of DNA-PKcs antibody and purified DNA-PKcs, respectively, leading to a conclusion that DNA-PKcs inhibits rAAV integration (Song et al., 2004). Whereas, in a cell culture system using DNA-PKcs-proficient M059K and deficient M059J cells, DNA-PKcs has been shown to enhance integration of both ss rAAV and ds rAAV (Cataldi & McCarty, 2010; Daya et al., 2009). In the context of animal experiment, Song et al. have exploited a two-thirds partial hepatectomy approach and shown that rAAV genomes integrate in DNA-PKcs-deficient SCID mouse livers at a significantly greater frequency than that of wild type control animals (i.e., >50% in SCID versus <10% in wild type mice) (Song et al., 2004). Whereas, our most recent study has indicated that this effect could be observed at a limited range of liver transduction levels, and deficiency of DNA-PKcs may not have a generalized effect on rAAV integration frequency (Adachi & Nakai, unpublished observation). Nonetheless, a high-throughput ss rAAV integration site analysis in mouse liver, muscle and heart has successfully identified many rAAV integration sites in both wild type and SCID mouse tissues. This indicates that DNA-PKcs itself does not play a direct role in the process of rAAV genome integration (Figure 2i) (Inagaki et al., 2007a). Other DNA repair proteins that might participate in the rAAV genome integration process include ATM, which also shows varying effects on integration in cell culture experiments depending on the types of cells and rAAV (i.e., ss versus ds rAAV) (Cataldi & McCarty, 2010; Sanlioglu et al., 2000). Interestingly, our study has implied that rAAV genomes more preferably integrate in the cellular genome than remain as extrachromosomal genomes when murine hepatocytes receive a minimum rAAV dose to establish latency (Adachi & Nakai, unpublished observation). This observation indicates that different DDRs are evoked and recruit different DNA repair machinery depending on the amount of DDR triggers in a cell. Collectively, at this point, there is no consensus model that explains which and how DNA repair pathways mediate rAAV integration. As for the integration of rAAV at the AAVS1 site in the presence of Rep68/78 expression, DNA-PKcs enhances site-specific integration of ss rAAV but not ds rAAV, indicating differential effects of DNA repair proteins in the Rep-mediated integration (Daya et al., 2009).

3.5 rAAV-mediated gene targeting and DNA repair pathways

HR mediated by the conventional vector systems occurs with efficiencies of a range of 10^-6 to 10^-7. In this regard, rAAV has become increasingly popular as the most efficient tool to precisely introduce defined DNA modifications at the target site in the cellular genome with remarkably high efficiencies of up to 1% in the cell population (Hendrie & Russell, 2005; Khan et al., 2011; Russell & Hirata, 1998; Vasileva & Jessberger, 2005). Targeting efficiencies could be increased further by 60-100 fold or more by introducing a DSB at the target site with a site-specific endonuclease (Miller et al., 2003; Porteus et al., 2003). This system, named the gene targeting rAAV vector system, has been applied in various disciplines, not only for gene therapy (Chamberlain et al., 2004) but also for generating knockout animals (Sun et al., 2008) and other types of basic research (Khan et al., 2011). Gene targeting rAAV serves as a donor vector that carries a DNA segment homologous to the chromosomal target sequence with a desired modification being introduced. The length of the homology arms can be 1.7
kb or potentially shorter, which is an advantage over the conventional targeting vectors that require a longer homologous DNA sequence (Hirata & Russell, 2000). Despite significant advance in the applications of the system, the underlying mechanism for rAAV-mediated gene targeting is poorly understood. As described above, rAAV does not harness any machinery designed specifically for mediating highly efficient gene targeting. The unusual structure of viral genome DNA is the only element that makes the system much more efficient than the conventional approaches.

The mechanism of rAAV-mediated gene targeting has just begun to be partly elucidated. Studies have indicated that the single-stranded nature of gene-targeting rAAV is key to efficient gene targeting reactions. Experimental evidence has come from the observation that, when mixtures of gene-targeting ss rAAV and ds rAAV vectors were used, gene correction rates correlated with the amounts of ss rAAV but not ds rAAV within the mixtures (Hirata & Russell, 2000). Another study took advantage of recombinant minute virus of mice (rMVM), a rAAV-like parvovirus-based vector that predominantly packages viral genomes of minus polarity and does rarely undergo second-strand synthesis to form ds viral genomes. When reporter cells were infected with gene-targeting rMVM vectors containing either the coding or noncoding strand of a transgene cassette, a significant difference in targeting efficiencies was revealed between the two, indicating that ss viral genomes are the substrate (Hendrie et al., 2003). However, a recent study points out limitations in the previously used assay systems and argues against the above model because ds rAAV has also been found to mediate gene targeting at a higher level compared with the ss rAAV control (Hirsch et al., 2010). Although the nature of gene targeting substrates may be a subject of debate, it is clear that rAAV genome integration and rAAV-mediated gene targeting use different DNA repair pathways. Genotoxic treatment, which significantly augments rAAV genome integrations, does not affect gene targeting efficiency (Hirata & Russell, 2000). In addition, rAAV gene targeting occurs preferentially in S-phase cells and does not take place at an appreciable level in terminally differentiated murine skeletal muscle fibers (Liu et al., 2004; Trobridge et al., 2005). Moreover, the cell cycle dependence has not clearly been demonstrated in rAAV integration and a study has demonstrated a readily appreciable level of rAAV integration in terminally differentiated cardiomyocytes and skeletal myofibers (Inagaki et al., 2007a). Collectively, NHEJ appears to be the major DNA repair pathway involved in rAAV integration while rAAV-mediated gene targeting uses HR. It has been demonstrated that RAD51/RAD54 pathway of HR is required for efficient rAAV-mediated gene targeting (Figure 2j), and deficiency of either of the NHEJ proteins, DNA-PKcs and Ku70, enhances the targeting rates (Fattah et al., 2008; Vasileva et al., 2006). Although the DNA-PKcs effect appears to be a cell-type dependent phenomenon (Fattah et al., 2008), the observations underscore the significant contribution of the HR pathways in rAAV-mediated gene targeting. Manipulation of HR and NHEJ pathways with small molecules will offer a novel and effective means to further improve rAAV-mediated gene targeting approaches to genetically engineer cellular genomes.

4. AAV as a tool for studying damaged DNA sites, DDR, and DNA repair pathways

AAV has provided the most powerful means to deliver genetic materials to a broad range of cell and tissue/organ types without toxicity and to introduce sequence modifications at defined locations. What has made AAV more attractive is its utility as an unprecedented research tool to study molecular and cellular biology, where gene delivery is not a primary
goal. As described in 3.1.3 and 3.1.4, AAV has been successfully exploited as a refined agent that can trigger DDR toward cell cycle arrest and apoptosis. AAV can deliver an element that triggers DDR (e.g., stalled replication forks) extrachromosomally with minimal transcriptional responses (McCaffrey et al., 2008) and without damaging the cellular genome. Although the phenomena observed in the AAV-based system may not necessarily recapitulate what takes place when the cellular DNA is damaged, it is assumed that molecularly defined extrachromosomal DDR triggers would provide a simple and less complicated means to study cellular responses to DNA damage. In addition, AAV has been exploited to study potential differences in DNA repair pathways among various tissues in the context of living animals. This type of study has demonstrated that, in hepatocytes, there is significant redundancy of Artemis/DNA-PKcs-independent NHEJ pathways that process hairpin DNA ends, while such redundancy is not observed in skeletal myofibers or cardiomyocytes in mice (Inagaki et al., 2007b). Moreover, AAV has recently emerged as a powerful tool to identify DNA sites damaged either endogenously or exogenously by genotoxic treatment or agents. Using rAAV as a tool to label pre-existing damaged DNA sites, a study has shown that DNA palindromes with an arm length of ≥ 20 base pairs in the cellular genome represent the sites susceptible to breakage in mouse tissues (Inagaki et al., 2007a). Another study has taken a similar AAV-based labeling approach and demonstrated frequent off-target cleavage of the cellular genome by a rare cutting endonuclease, I-SceI, following expression of I-SceI in cells (Petek et al., 2010). Perhaps applications of AAV in biological and medical research will not be limited to the disciplines described above and will continue to expand with the advent of novel rAAV vector technologies.

5. Conclusions

The virus-host interaction from a viewpoint of viral components and DNA repair machinery is an emerging research area that would offer unprecedented means to study both virology and molecular and cellular biology. The interaction in this aspect is most studied with adenoviruses, herpesviruses, and retroviruses including human immunodeficiency virus. These viruses have evolved sophisticated machinery to benefit them by manipulating or controlling DDR, DNA repair machinery, and the cell cycle. In this regard, AAV (i.e., wtAAV and rAAV) represents a unique viral agent in that Rep proteins are the sole viral components that interact with DNA repair machinery and rAAV expresses no such component. Despite the seemingly simple nature of AAV, there are significant virus-host interactions that involve DDR and DNA repair machinery in AAV infection, and we have just begun to appreciate them as summarized in this chapter. There has been an increasing interest in AAV primarily as a promising gene delivery vector and more recently as a new tool to study DNA damage, DDR, and DNA repair machinery. Studying AAV from various scientific aspects including virology, immunology, physiology, gene therapy, DNA damage, DDR, DNA repair, genomic instability, carcinogenesis, and so on, would significantly advance our knowledge about AAV and could solve unanswered fundamental biological questions that are difficult to address by the conventional approaches.

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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