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Hum Exp Toxicol 2007; 26; 899
DOI: 10.1177/0960327107083556

Human & Experimental Toxicology (2007) **26:** 899–906
http://het.sagepub.com

Exploring DNA damage responses in human cells with recombinant adenoviral vectors

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Recombinant adenoviral vectors provide efficient means for gene transduction in mammalian cells *in vitro* and *in vivo*. We are currently using these vectors to transduce DNA repair genes into repair deficient cells, derived from xeroderma pigmentosum (XP) patients. XP is an autosomal syndrome characterized by a high frequency of skin tumors, especially in areas exposed to sunlight, and, occasionally, developmental and neurological abnormalities. XP cells are deficient in nucleotide excision repair (affecting one of the seven known XP genes, *xpa* to *xpg*) or in DNA replication of DNA lesions (affecting DNA polymerase *eta*, *xpv*). The adenovirus approach allows the investigation of different consequences of DNA lesions in cell genomes. Adenoviral vectors carrying several *xp* and photolyases genes have been constructed and successfully tested in cell culture systems and *in vivo* directly in the skin of knockout model mice. This review summarizes these recent data and proposes the use of recombinant adenoviruses as tools to investigate the mechanisms that provide protection against DNA damage in human cells, as well as to better understand the higher predisposition of XP patients to cancer. *Human & Experimental Toxicology* (2007) **26,** *899–906*

Key words: adenoviral vectors; DNA damage; DNA repair; ultraviolet; xeroderma pigmentosum

Introduction

Sunlight has high-energy ultraviolet (UV) components that reach the Earth surface. These components are known as UVA (320 to 400 nm) and UVB (280 to 320 nm), and the latter is highly harmful to living beings because it generates DNA damage. The major lesions promoted by UV irradiation are cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs). These lesions induce strong distortions in the DNA double helix leading to severe consequences to the cell if not properly removed. Transcription arrest and replication blockage caused by UV lesions disturb cell metabolism, interfere with the cell cycle and induce cell death. DNA mutations are also produced by UV irradiation, as a consequence of misleading DNA processing. At the organism level, these consequences may lead to deleterious events such as aging and cancer. To protect the genetic material from DNA damage, cells are equipped with different DNA repair mechanisms and the removal of major UV-induced lesions is promoted by a mechanism known as nucleotide excision repair (NER).

The importance of NER for humans was first established after the identification of the molecular defect of the autosomic recessive syndrome known as xeroderma pigmentosum (XP). These patients have high sensitivity to sunlight and easily develop skin cancer in exposed areas of the body (1,000 times more frequent than the normal population). Some of the XP patients have also developmental problems and early aging features. In 1968, there was a milestone discovery that cells from these patients were, in general, unable to remove UV-induced lesions from DNA,[1] later identified as a defect in NER. Today, it is

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10.1177/0960327107083556

known that inherited defects in NER are also found in other human syndromes: Cockayne's syndrome (CS), trichothiodystrophy (TTD) and cerebro-oculofacial skeletal syndrome (COFS). Although clinical manifestations of these syndromes differ considerably, varying from aging symptoms to high cancer predisposition, the common feature of the syndromes is the photosensitivity, manifested at different levels.[2] CS and TTD patients do not develop cancer at high frequency, but are highly impaired during development. The genes responsible for these syndromes have been identified and are known as *xpa* to *xpg*, *csa*, *csb* and *ttda*. Intriguingly, mutations in *xpb*, *xpd* and *xpg* genes may also lead to clinical features of CS or TTD diseases. On the other hand, a group of XP patients, known as XP variant, with normal NER, was found to be unable to normally replicate the UV lesions. In fact, later it was shown that they are deficient in the DNA polymerase *eta*, an enzyme responsible for translesion DNA synthesis.[3]

The knowledge of the molecular defects on XP cells was the starting point for understanding how human cells handle lesions in their genome. The NER mechanism is a very versatile repair system, able to remove different types of lesions that cause gross double helix alterations, including those promoted by many chemical genotoxic agents.[4] NER is highly conserved in eukaryotes and requires the action of more than 30 proteins working in a tightly regulated manner (Figure 1). Briefly, the NER pathway consists of six steps: (i) recognition of the lesion carried out basically by the XPC-hHR23B or UV-DDB (UV-damaged DNA binding) complexes [in global genomic repair (GGR)] or RNA pol II stalling [in transcription-coupled repair (TCR)]; (ii) opening of the DNA double helix at the lesion site by the concerted action of the two DNA helicases XPB and XPD (both are components of the TFIIH transcription factor); (iii) delimitation of the damaged area by XPA and RPA proteins; (iv) dual incision of the damaged strand by the XPF and XPG endonucleases; (v) synthesis of DNA in the gap left by the removal of a 24-32 mer oligonucleotides by the replicative DNA polymerases and PCNA (proliferating cell nuclear antigen) and (vi) ligation by DNA ligase I.[4,5]

Research in XP cells has provided enormous amount of information about how mutations originate in human cells and how these mutations can result in a neoplasic phenotype. However, little can still be done to relieve the difficult life of these patients and their families. The complementation of the missing gene through a gene replacement therapy protocol seems a simple approach, but still has many drawbacks. Efforts have been made using

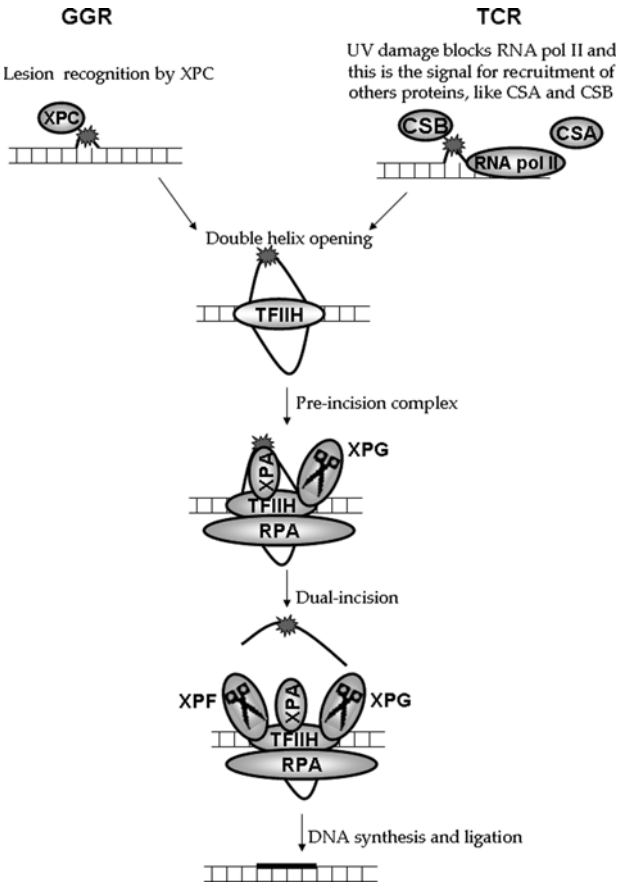

Figure 1 General scheme of nucleotide excision repair of damaged DNA. NER is a very versatile process for the removal of many different types of DNA lesions induced by UV light or several genotoxic agents. This scheme shows the two NER subpathways: GGR on the left and TCR on the right. Check for NER description in the text.

retroviral vectors to promote permanent cell correction by an *ex vivo* integration of the recombinant gene in the genome of XP keratinocytes – precursor cells that are able to generate full skin explants. This approach has been recently reviewed by Magnaldo and Sarasin.[6]

Another potential strategy to transduce XP genes directly to human skin cells is through the use of recombinant adenoviruses. These are efficient vectors that can infect the whole population of a variety of cells, providing excellent means to introduce a DNA repair gene in target cells or skin tissue. Recent data with adenoviral vectors carried out in cell cultures or in XP knockout mice models is presented in this review. Perspectives and limitations of these vectors, as a way to improve our knowledge on the basic mechanisms that cells use to protect their genome from genotoxic agents, are discussed. Finally, the potential applications of these vectors in the treatment of XP patients are evaluated.

In vitro gene transduction with recombinant adenovirus for the correction of DNA repair defect

Recombinant adenoviral vectors were first described in the early days of gene therapy as an efficient way to transduce exogenous genes into mammalian cells.[7,8] Recombinant adenoviruses can be obtained at high titers and do not require antibiotic selection or clone isolation due to the very efficient machinery of infection. For the first generation of adenoviral vectors (which are the ones used for repair genes), viral genes required for DNA replication (*E1A* and *E1B*) are removed from the viral genome backbone. These E1-deleted vectors are able to replicate only in cells that provide these proteins *in trans*.[8] The main cell line employed for this purpose is the HEK293, which was obtained by adenovirus transformation.[9] These viruses infect a wide spectrum of cell lines and tissues, and the transduction of a foreign gene can easily reach 100% of cells exposed to the virus. As infected cells normally do not provide genes that are missing for viral replication, the vector DNA persists as an episome without multiplying or integrating in the cellular genome. Therefore, these viruses are not cytopathic for the cells and do not cause mutations by interrupting a cellular gene. However, the expression of the foreign gene is only transient in dividing cells, due to serial dilutions of virus DNA particles.[10]

Recombinant adenoviruses carrying *xpa*, *xpc* and *xpd* genes were constructed and have been tested for their ability to complement cell lines deficient in these genes,[11,12] as schematically shown in Figure 2. These viruses were employed to infect SV40-transformed and primary fibroblasts derived from non-exposed areas of the XP patients skin with 100% efficiency and low, if any, cytotoxicity. The viruses completely restored UV survival and the DNA repair capacity in defective cell lines, demonstrating that the transduced protein was functional and specific to the XP complementation group. Phenotypic analyses were carried out on different XPA, XPC and XPD deficient cell lines infected or not with the respective viruses. Although XP cells are highly sensitive to UV irradiation, infected cells presented an increased resistance to UV light, comparable with cells proficient in DNA repair. The ability to perform NER was measured through the determination of unscheduled DNA repair synthesis (UDS) after UV irradiation. The UDS activity corresponds to the incorporation of [methyl-^{3}H] thymidine in cells that are not in S-phase and is visualized by the presence of radioactive grains inside nuclei, after autoradiography. UV-irradiated XPA, XPC and XPD deficient cell lines typically exhibit a very low number of grains per nucleus when exposed to UV light. After infection with the corresponding recombinant adenovirus, UDS activity in deficient cells was restored to levels comparable with DNA repair proficient cells. Moreover, it was still possible to detect sustained high expression of XPA and XPC proteins along with phenotypic correction in XP cells for a period of at least two months after infection. It is worthy to note that the recombinant virus was able to complement several cell lines that had mutations in the *xpd* gene, independent if the clinical symptoms were from XP, CS combined with XP or TTD patients. Another interesting feature of these adenoviral vectors is that the amount of the transduced protein in infected cells, determined by western blot and immunocytochemistry, is much higher than the corresponding protein levels normally expressed in NER-proficient cells. This over-expression, however, does not affect UV resistance or UDS of cells that have normal NER. Similar results were also described for cells over-expressing the XPA protein from a chromosomal integrated gene.[13] The simplest explanation for these data is that neither of these proteins (XPA, XPC or XPD) are limiting for NER in human cells. One possibility is that the NER pathway requires a coordinated action of several proteins, so increasing only one of these proteins does not result in speeding up the removal of DNA lesions.

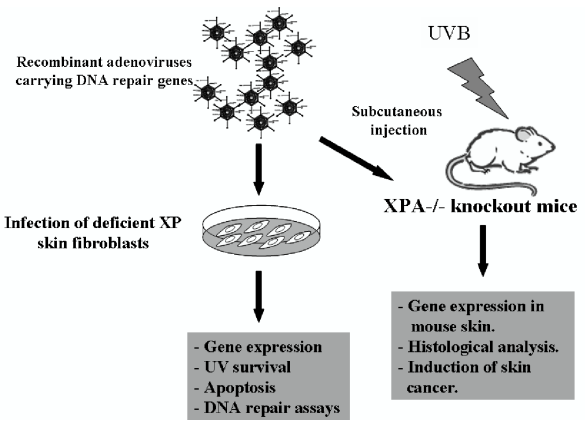

Figure 2 DNA repair gene transduction by recombinant adenoviruses. In gray boxes, potential endpoints for the analysis of *in vitro* (cell culture) or *in vivo* (mice models) phenotypes are indicated.

Use of recombinant adenovirus for correction of translesion synthesis deficiency in XPV cells

In addition to classical XP groups, which are NER defective, another group of patients with XP phenotype, XP variant (XPV), was also described.[14]

Interestingly, they have a normal NER activity. In fact, for XPV patients, the XP phenotype results from a mutation in a gene encoding the translesion synthesis DNA polymerase *eta*, which is responsible for DNA synthesis on UV-irradiated templates.[15,16] In order to understand the roles of DNA polymerase *eta*, an adenovirus carrying polymerase *eta* cDNA was also constructed. As for the other vectors, the recombinant adenovirus has been used to infect SV40-transformed and primary skin fibroblasts from XPV patients. UV-irradiated XPV cells complemented with DNA polymerase *eta* showed survival, cell cycle and elongation of replication products comparable with normal cells.[17] We expect that this recombinant virus will open new avenues for a better understanding of cellular UV responses and cancer development in XPV patients.

Transduction of photolyase genes as a tool to investigate the role of UV-induced lesions

In addition to the NER pathway, nature has evolved a direct, very efficient, light-dependent, mechanism for the specific removal of UV-induced DNA lesions. Photoreactivation is present in a wide variety of species, from bacteria up to marsupial mammals,[18] and involves a single enzyme known as photolyase. This enzyme specifically binds to UV-induced DNA lesions (dipyrimidine lesions) and reverts them by splitting dimers back to undamaged bases, using visible light as energy source.[19] Interestingly, photolyases are specific for the type of UV-induced lesion, CPDs or 6-4PPs.

Considering the deleterious consequences of UV-induced DNA lesions, as well as, both fidelity and efficiency of the photorepair mechanism, it is intriguing to verify the absence of photolyases in placental mammals, including humans. Indeed, in placental mammals, the proteins encoded by the genes homologous to photolyases do not have any photorepair activity but participate as biological circadian photoreceptors.[20] Genes encoding genuine photolyases were probably lost in the course of evolution and the removal of UV-induced lesion is exclusively performed by NER, a much more complex but flexible DNA repair mechanism.[4]

Although UV irradiation has been one of the most studied genotoxic agents, the precise role of each of the DNA lesions in triggering cellular responses, such as apoptosis, mutagenesis and carcinogenesis, is still not completely elucidated. Moreover, CPDs are three-fold more frequent than 6-4PPs after exposure to the same UV dose, making the distinction of the role for these two lesions a difficult task. The use of photolyases may facilitate the investigation of this longstanding issue.[21] Indeed, previous reports have confirmed that heterologous CPD-photolyase expressed in human cells is active, reducing mutagenesis,[22] preventing UV-induced apoptosis[23] and recovering RNA transcription driven by RNA polymerase II.[24] Furthermore, transgenic mice expressing CPD-photolyase and/or 6-4PP-photolyase are capable of removing photolesions in a light-dependent manner.[25,26] Moreover, compared with parental mice, CPD-photolyase transgenic mice have reduced cell apoptosis, epidermal hyperplasia, mutagenesis and carcinogenesis after UVB irradiation. However, mice expressing the 6-4PP-photolyase did not show any improvement, despite the efficient photoremoval of 6-4PPs.[26] These results indicated CPDs as the primary lesions triggering the majority of cell responses after UV irradiation, although cell culture studies pointed to 6-4PPs as important signals triggering cell death and mutagenesis in certain conditions.[27–29]

Adenoviruses carrying a marsupial CPD-photolyase gene (from the rat kangaroo *Potorous tridactylus*) and a plant 6-4PP-photolyase (from *Arabidopsis thaliana*) were recently constructed to address the question of the particular role of these two lesions in UV responses induced in both NER-deficient and NER-proficient human cells. The adenovirus carrying the CPD-photolyase (fused with the reporter protein EGFP) has shown to increase the UV resistance of human primary XPA and XPC cells. Besides, local UV experiments indicated that the fused photolyase migrates fast (<2 min) to regions with damaged DNA in the nuclei and co-localizes with other DNA repair enzymes.[30] Experiments together with the adenovirus carrying the 6-4PP-photolyase are being performed, aiming to discriminate the roles of each of these lesions in UV-induced responses in human cells. The idea is simply photorepair specifically either CPDs or 6-4PPs (or both), in UV-irradiated cells; thereby, one can investigate cellular responses specific to the remaining type of lesion as differential recruitment of repair enzymes, recovery of RNA synthesis and induction of apoptosis (Figure 3). Although apoptosis is an effect opposed to the expected carcinogenic effect in organisms (as dead cells do not generate cancer), it is possible that these two effects may correlate *in vivo*. We expect that experiments with cell culture and XP animal models infected with one of these two adenoviruses will contribute to the understanding of the basis of the deleterious actions of UV irradiation (and, thus, sunlight).

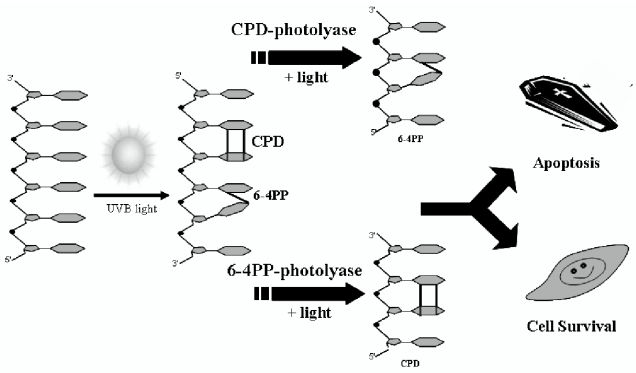

Figure 3 Representation of the lesions induced by UVB irradiation (CPDs and 6–4PPs), with differential repair using specific photolyases, and the influence of DNA damage removal in cell death.

In vivo gene delivery to mice skin

XP knockout mice are attractive models to study XP human syndromes, as they develop UV radiation-induced skin tumors at high rates and also internal tumor by administration of drugs, mimicking the human phenotype.[31] Xpa$^{-/-}$ mice demonstrated skin ulcers five to seven days after 7,12-dimethylbenz[a] anthracene treatment and papilloma development within four weeks prior to promotion. Tumor incidence was also much higher than in heterozygous and wild-type mice. Experiments targeting the lung (using a small dose of benzo[a]pyrene), liver (treatment with aflatoxin B1) and tongue (administration of 4-nitroquinoline-1-oxide in drinking water) provided convincing evidence that XPA mice are also sensitive to carcinogenesis in organs other than the skin.[32] In addition, it was demonstrated that Xpc$^{-/-}$ mutant mice are also highly predisposed to the induction of lung and liver cancers by treatment with 2-acetylaminofluorene (2-AAF) and N–OH-2-AAF.[33]

One of the perspectives of adenovirus carrying DNA repair genes is to use them to investigate their effects *in vivo* (Figure 2). In fact, the recombinant adenovirus carrying the cDNA from *xpa* human gene (AdyXPA) was initially employed for *Xpa*$^{-/-}$ mouse embryo fibroblasts infection (*Xpa*$^{-/-}$ MEF) *in vitro* and afterwards for *in vivo* gene therapy in UVB-irradiated skin of such mice.[34] The AdyXPA efficiently infected *Xpa*$^{-/-}$ MEF (100% transduction), with high levels of transgene expression, as well as complete recovery of UDS and cell survival after UV radiation exposure. Collectively, these results demonstrated that the human XPA protein is able to fully restore defective NER in *Xpa*$^{-/-}$ mutant mice cells.

Subcutaneous injection of AdyXPA led to an extensive expression of the transgene measured indirectly by EGFP fluorescence detection or by XPA protein immunodetection. In skin sections, cells of the epidermis and of the lower layers of the dermis, including basal replicating keratinocytes, expressed the human protein. Keratinocytes are the precursor skin cells, which are believed to be the cell types responsible for tumor development.[35]

Xpa$^{-/-}$ mice infected with the AdyXPA or with an empty adenovirus and wild-ype mice were irradiated on the shaved dorsal skin for four days with a daily UVB dose of 3.4 KJ/m^2. Animals were killed at different periods after treatment and the skin was processed for histological examination. During the progression of the experiment, all mock-infected *Xpa*$^{-/-}$ mice presented persistent skin hyperplasia (measured by the number of cell layers in the epidermis) culminating with skin tumors development (squamous cell carcinomas), five months after the UV irradiation. On the other hand, wild-type animals as well as AdyXPA-infected *Xpa*$^{-/-}$ mice presented only a mild epidermal hyperplasia at early times (24 hours and three weeks) after UV irradiation, restoring the normal epidermis status (1–2 cell layers) at later times without any trace of pre-neoplasic lesions. Therefore, these experiments demonstrated the potential use of adenoviral vectors to efficiently transduce functional genes to skin cells, preventing carcinogenesis caused by a common environmental agent, such as UV.

XP patients: perspectives for gene therapy using recombinant adenoviruses

None of the treatments currently available for XP patients proved to be an enduring successful option with no side effects. Therefore, there is a need to develop new therapeutic strategies that could contribute to the treatment efficacy. An effective gene therapy approach for XP patients should consider the following aspects: vector targeting for skin stem cells (basal membrane keratinocytes), long-term expression and absence of transgene inactivation by promoter silencing or immune system clearance. In fact, the most concerning issue faced by adenovirus gene therapy today is the high host immune response induced after the first application, which may hamper re-administration protocols.[8] Several strategies have been developed aiming to reduce the immune responses and circumvent these difficulties. One approach is to shield the adenoviral vector from the immune recognition, by chemical modification

of the coat proteins with polyethylene glycol (an immunologically inert polymer[36]), or through encapsulation of the adenoviral vector by microspheres composed of sodium alginate or poly(lactic-glycolic) acid or in cationic liposomes.[37–39] In fact, the use of liposome-complexed adenovirus may also improve the ability of gene transfer by such vectors in skin.[40] A second approach is to modulate host immune responses by the use of immunosuppressive drugs.[41] These strategies have been shown to improve the time of gene expression *in vivo*, without substantially reducing the efficiency of adenovirus infection, and should be tested in order to improve the efficiency of XP adenoviruses application in skin cells.

The high surface of the skin as a target tissue raises the search for alternative techniques for the application of adenoviruses. Topical application can be carried out by spreading adenoviral preparations directly on the skin[42,43] or by particle bombardment with a gene gun.[44] The use of epicutaneous patches has been shown to provide effective absorption of an adenovirus-vaccine vector.[45] Recent efforts have been dedicated to the development of high-capacity (gutless) adenoviral vectors that lead to reduced host immunological responses and long-term gene expression.[46,47] However, gutless vector preparations still carry high levels of wild-type adenovirus contamination, hampering their use in human clinical trials.[47] Additionally, a hybrid adenovirus vector carrying a cell-independent DNA replication system, derived from Epstein–Barr virus, has been shown to be useful for sustained gene expression in replicating cells.[48]

In conclusion, the efficient delivery machinery of adenoviral vectors may represent an important strategy for the phenotypic correction of XP cells and represent a good perspective for a corrective therapy in XP patients, improving patient and families life quality.

Adenoviral vectors as a tool for XP complementation group identification

Among eight XP identified genes, *xpa* and *xpc* are responsible for half of all world XP cases.[49] Although *xpc* is one of the usual complementation groups, there are just 24 different mutations described for this gene in XP patients (http://xpmutations.org). Adenoviruses carrying DNA repair genes represent an interesting and easy tool for diagnosis and identification of XP complementation groups. Although this diagnosis has been performed with fibroblasts, recombinant adenoviruses may be used to infect cells present in the blood, such as macrophages.[50] If successful, it will be a much faster and less invasive technique, facilitating the identification of the deficient gene in the particular XP individuals. Besides scientific and epidemiological goals, the identification of the gene defect may help to predict clinical prognostic for the XP patients and guide appropriate genetic counseling for their families.

Concluding remarks

Although recombinant adenoviruses are the most widely used vectors for gene delivery in basic and clinical gene therapy studies, up to now few recombinant adenoviral vectors carrying DNA repair genes have been constructed. These DNA repair adenoviral vectors have been successfully used for complementation assays in cell culture systems. They are also potentially excellent systems for gene delivery and can be used to analyse the dynamics of the repair proteins movement within the UV-irradiated cells, how the over-expression of these proteins may or may not affect the normal repair mechanism, the effect of photo-removal of specific lesions after UV and many other basic questions on cell responses to DNA damage. Complementation tests with adenoviruses may also provide a simple and reliable system to help in patients' diagnosis, being a contribution to applied human genetics. Although *in vivo* experiments have just been launched, the positive results demonstrate that the adenoviruses may help to address basic questions using mice models, especially on the origin of skin cancer in XP patients. As the skin is the most accessible tissue in mammalian organisms, the opportunity to introduce genes through adenoviruses opens new avenues for modifying cell metabolism for health purposes. Moreover, the high susceptibility of XP knockout mice to genotoxic drugs that affect tumorigenesis in organs, such as liver and lung,[32,33] opens the possibility to test recombinant vectors in target tissues other than skin. Therefore, these adenoviral vectors represent the first steps toward a potential gene therapy protocol for gene correction and a hope for improving the quality of life of XP patients.

Acknowledgments

This work was supported by FAPESP (São Paulo, Brazil), CNPq (Brasília, Brazil), CAPES (Brasilia, Brazil) and UNESCO/IBSP (Paris, France). CFMM is a Fellow from the John Simon Guggenheim Memorial Foundation (New York, USA). ARM is supported by the Pew Latin-America.

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