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Gene Therapy for Melanoma: Progress and Perspectives

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1. Introduction

Gene therapy, the therapeutic transfer of genetic information to a target cell, continues to be a promising alternative in the fight against cancer. In the case of melanoma, the use of an experimental treatment is justified since this disease is incurable in its advanced stages. Is gene therapy a viable option for the treatment of melanoma patients? In this chapter, we will attempt to answer this question by exploring the intersection between the technology of gene therapy and the biology of melanoma, a point at which opportunities for intervention are revealed.

The technology of gene therapy depends on our ability to create a vehicle that will deliver the therapeutic payload to the target cell. Today, a variety of vectors are available that have the capacity to encode a therapeutic gene or sequences that are capable of blocking gene expression, such as RNA interference (RNAi). The technology of viral and non-viral vectors will be discussed. Viral vectors offer the benefit of using existing biological mechanisms in order to enter the target cell. However, when choosing a gene transfer vehicle, viral vectors must be carefully considered since each option will present a unique profile of advantages and disadvantages, especially as related to the efficiency of the virus in reaching the target cell and in the vector's propensity to activate an immune response. Alternatively, non-viral vectors, such as plasmids, offer the advantage of safety and ease of manipulation, but may require the use of physical or chemical agents in order to pass the through the cytoplasmic membrane and enter the cell.

With these gene transfer tools in hand, now we can turn our attention to the options for the therapeutic gene which will be inserted in the vector. Strategies for cancer gene therapy are many, but share the common goal of inhibiting or destroying the tumor cells. This may be accomplished by the use of pro-apoptic genes, tumor suppressor genes, suicide genes or immune-modulating genes (among many other options). In fact, the therapeutic sequence need not be a gene at all, but instead the RNAi system can be employed

to block the expression of critical genes that contribute to tumor progression. Moreover, the gene therapy strategy need not be performed directly in the tumor cells, but may be applied to components of the immune system, such as T-cells or dendritic cells, that will then carry out the anti-tumor activity.

The biology of melanoma presents several opportunities as well as challenges for its treatment by gene therapy approaches. In particular, the high metastatic potential of late stage melanoma represents one of these challenges. Is it realistic to expect that a virus delivered to the primary treatment site will have an effect on distant or even not yet identified metastatic sites? The use of a replicating virus may overcome this barrier since these 'oncolytic' vectors, which multiply only in tumor cells, can spread from cell to cell. In addition, melanoma is considered to be an immunogenic tumor, opening the possibility of generating tumor reactive immune cells with the assistance of gene transfer technology. As an alternative to established modalities, biochemotherapy may be reincarnated in the form of gene transfer of cytokines or interferons. Some cutting edge research efforts include the development of oncolytic viruses that are armed with an immune-modulating gene, creating a double-edged anti-tumor agent.

A growing body of pre-clinical work supports the notion that gene therapy may be a valuable strategy for the treatment of melanoma. To date, quite a few clinical trials of menaloma gene therapy have been performed. With the advances in our understanding of both the technology of gene therapy and the biology of melanoma, we propose that an increasing number of these experimental approaches will reach phase III trials and be offered to a growing number of these patients who have such limited therapeutic options.

2. Gene therapy progress and pitfalls

For more than 20 years now, gene therapy has been applied in more than 1840 clinical trials for the treatment of a great variety of indications (Journal of Gene Medicine's Gene Therapy Clinical Trials Worldwide, http://www.wiley.com/legacy/wileychi/genmed/clinical). Among these trials, cancer represents the disease most commonly treated by therapies that include a gene transfer component. Currently, only a few gene therapy products have been approved for commercialization (Table 1) and the USA has yet to approve any such treatments.

The first gene therapy clinical trial was performed in the USA in 1990. This trial used an *ex vivo* approach where gammaretrovirus was employed to transfer a normal copy of the adenosine deaminase (ADA) gene to peripheral T-cells in two young patients suffering from severe combined immunodeficiency (SCID) due to the lack of ADA function (ADA-SCID) [7]. Neither of the treated patients exhibited any adverse effects and one had clinical benefit lasting well over a decade [8]. Since then, gene therapy for ADA-SCID has evolved and now uses *ex vivo* genetic modification of hematopoietic stem cells resulting in proper immune function, adenosine metabolism and no need for further treatment in 21 of 31 patients, none of whom showed adverse effects [9].

A similar approach for the treatment of SCID-X1 (SCID due to a mutated IL2RG gene, the common IL2 receptor gamma chain) and 18 of 20 patients have had their immune system

restored. Unfortunately, 5 of these children developed leukemia due, in part, to the presence of the therapeutic virus in the patients' genome. Though 4 were successfully treated with chemotherapy, one patient did succumb to the leukemia [9]. Many important lessons were learned from the SCID trials, including the fact that the same vector can be innocuous in one situation (ADA-SCID), yet dangerous in another (SCID-X1). The SCID-X1 trials were more successful in achieving long term benefit (90% vs. 70%) and were less likely to provoke an adverse effect (25% vs. 50%) when comparing the traditional treatment (allogenic bone marrow transplant in particular) and gene therapy, respectively [9]. Efforts are underway to use alternative vectors, such as lentivirus, that should be safer and should not provoke the side effects seen with the gammaretroviral vectors.

Local (year/agency)	Company	Vector	Description	References
China (2003/ SFDA)	Shenzhen Gentech SiBiono	Gendicine (Ad-p53)	Non-replicating adenoviral vector expressing wild-type p53 for the treatment of head and neck cancer.	[1, 2]
China (2005/ SFDA)	Shanghai Sunway Biotech	Oncorine/H101 (Onyx-015)	Conditionally replicating adenovirus containing a mutant E1b protein which confers tumor-specific oncolysis. Approved for treatment of head and neck cancers.	[1, 2]
Phillipines (2007/ BFAD)	Epeius	Rexin-G*	Replication-incompetent retrovector targeted to the tumor matrix (collagen) and expressing a cyclin-g mutant that induces cell death. Approved for treatment of all solid tumors.	[3, 4]
European Union (2012/EMA-CHMP**)	Amsterdam Molecular Therapeutics	Glybera (alipogene tiparvovec)	Adeno-associated virus encoding the Ser(447)X variant of human lipoprotein lipase (LPL) gene for the treatment of familial LPL deficiency.	[5, 6]

*Orphan drug status approved by FDA in 2008 **Approval finalized in late 2012; orphan drug status granted in 2004.SFDA, State Food and Drug Administration of China; BFAD, Bureau of Food and Drugs; EMA-CHMP, European Medicines Agency-Committee for Medicinal Products for Human Use; FDA, Food and Drug Administration, USA

Table 1. Gene therapy products approved for commercial distribution.

Genetic modification of hematopoietic stem cells has also been explored for the treatment of β-thalessemia, Wiskott Aldrich Syndrome, adrenoleukodystrophy and chronic granuloma-tous disease [9, 10]. These trials were generally considered to be successful with respect to clinical benefit of the treatment, yet only the treatment of ALD was free from unwanted cellular proliferation due to the presence of the therapeutic virus. The field continues to evolve and the need for better vectors and a better understanding of their biology is warranted.

Other examples of successful gene therapy have been reported for the treatment of Leber's congenital amaurosis, a genetic alteration that leads to blindness. Several groups have transferred a normal copy of the affected gene, HPE65, to the retina using adeno-associated virus. Of the 18 patients originally treated, many have gained visual acuity and are able to identify shapes and even navigate obstacle courses that were impassable before treatment. In general, these patients first received monocular therapy, but, due to the success of the treatment, several have since been treated in the contralateral eye. The effectiveness of the second treatment was significant since clinical benefit was achieved and re-inoculation of the virus was not blocked by nor did it provoke any unwanted immune response [11].

In 1999, Jessie Gelsinger, a young patient with ornithine transcarbamylase deficiency received a massive dose of adenovirus as part of his gene therapy. Unfortunately, he suffered a strong immune reaction, entered anaphylactic shock and died [10]. Though this case did spark quite a bit of controversy and contributed to the negative public opinion of gene therapy, it does serve as a reminder of how new, experimental and unexplored aspects of gene therapy require continued re-evaluation. In this introduction, we have attempted to present a balanced description of the progress and pitfalls noted in gene therapy trials. Note that of the thousands of patients treated with gene therapy world-wide, we know of only 10 or so cases of serious adverse effects due to the gene therapy agent (the virus). When we consider the cost, time, morbidity and mortality associated with the development of many of the currently accepted ('traditional') treatments, such as bone marrow transplantation and chemotherapy, we see that gene therapy is still in its initial phases, yet many of the benefits do outweigh the risks.

3. Vectors: General concepts

The concept behind gene therapy is really quite simple: introduce genetic material into cells with the aim of inducing a clinically beneficial activity. The process of gene transfer generally requires a vector, or vehicle, to deliver the genetic payload to the target cell. In basic terms, this can be accomplished by either viral or non-viral vectors. Some 65% of clinical trials have relied on viral vectors while 35% used non-viral vectors, including plasmids and RNA (http://www.wiley.com/legacy/wileychi/genmed/clinical/). The principal advantage to using recombinant viral vectors is that their naturally occurring counterparts have already developed mechanisms for reaching and entering cells. This point is also their downfall, since our bodies have also evolved anti-viral defense mechanisms. Since plasmid vectors are essentially free of antigens and since their sequence can be carefully controlled, they are much less likely to provoke an immune response. Many cell types, but especially muscle, are quite efficiently transfected by plasmids.

Selecting a gene transfer vector depends greatly on the therapeutic approach (*ex vivo* or *in situ*), the required duration of transgene expression (for example, long term for immunodeficiencies and metabolic disorders, but short term for cancer), the disease and cell type in question (proliferating or post-mitotic, stem cell or differentiated, etc) and the characteristics of the transgene itself (the length of the cDNA, secretion of the protein product, requirement

for expression in a specific tissue or under a particular physiologic condition). Some of the more popular gene transfer vectors are described in Figure 1 and Table 2.
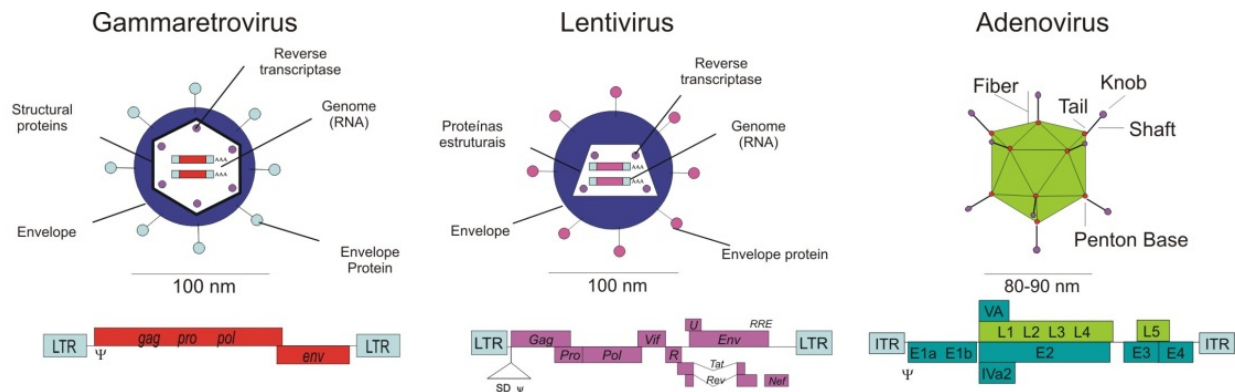
Figure 1. Schematic representation of the wild type counterpart of the typically used recombinant viral vectors. (A) Gammaretroviruses and (B) lentiviruses share similar structures, but differ greatly in their genomes and their impact on cellular function. *Gag, pro, pol* and *env* genes encode structural proteins of the capsid, protease, reverse transcriptase and envelope proteins, respectively. The additional lentiviral genes perform regulatory functions as well as alter cellular function. (C) The serotype 5 adenovirus has a protein capsid (non-enveloped) and a large, complex genome that encodes critical genes for viral replication (E1a, E1b) as well as structural and functional genes that regulate both viral and cellular activities.

In the laboratory, viral vectors are typically manipulated in the form of plasmids that encode the genome to be packaged in the viral progeny (including the gene of interest and regulatory elements that control its expression) as well as information for the generation of a functional virus particle. In general, viral vectors are engineered so that they are incapable of replicating outside of the laboratory. In other words, they can be produced in the lab, but do not form an active infection in patients, thus limiting their horizontal spread. Replication deficient viruses are capable of entering a target cell and expressing the therapeutic gene, but do not carry enough genetic information to form the next generation of virus, a process best referred to as transduction.

Plasmid vectors are much simpler from a technological point of view and come with the benefit of residing in the cell as an episome and being poorly immunogenic. Some considerations when using plasmids include vector design and delivery. Since cells do not actively take up plasmids, it would seem that their utility as a gene transfer vector would be limited. In reality, muscle cells are quite permissive to transfection even when 'naked' plasmid DNA is applied. When complexed to chemical carriers and/or in combination with physical methods of transfection, a great many tissues can be treated with plasmids (or even oligos, siRNA, RNA, etc). Non-viral vectors, therefore, offer certain advantages over recombinant viruses.

Vector targeting can be performed at either the transcriptional or transductional level. In theory, targeting would present the advantage of promoting gene transfer in the cell of interest and avoiding 'off target' effects. In this way, normal healthy cells would be spared and the treatment would be directed to the appropriate cell. Vector targeting should increase safety and efficiency of the treatment. Transductional targeting directs the virus to a specific cell type

and should reduce the number of off target cells that are transduced. In other words, the virus should reach only the desired cell type. Such vectors are engineered so that that viral proteins are able to interact with specific cellular receptors present only (or principally) on the target cell. Transcriptional targeting involves the use a promoter that is active only in the desired cell type or only under specific physiologic conditions. Cancer cells offer many distinctions that benefit transcriptional targeting, such as expression of hTert or survivin, proliferation (which is typically associated with E2F1 activity) and stabilization of HIF1α under hypoxic conditions. Transcriptional targeting does not restrict which cells will be transduced, but does determine where the vector will be allowed to express the transgene. These strategies can also be combined, thus increasing the degree of specificity.

Vector	Gammaretrovirus	Lentivirus	Adenovirus	Adenoassociated Virus	Non-viral (plasmid, naked and lipofection)
Titer, virus particles/ml[a]	10^9	10^9	10^{12}	10^{12}	NA
Route of delivery	Ex vivo	Ex vivo	In situ	In situ	In situ
Integration in the host genome	Yes	Yes	No	Yes/No	No
Long term expression	Yes	Yes	No	Yes	No
Immune response to viral proteins	No	No	Yes	Yes, but less severe	No
Clinical trials[b]	370	55	438	92	456
Typical clinical application	Hematopoietic system	Hematopoietic system	Cancer, vaccines, vascular diseases	Hemophilia, cystic fibrosis	Cardiovascular, cancer, vaccines

a: titer after concentration of virus preparation

b: trials listed on http://www.wiley.com/legacy/wileychi/genmed/clinical/

NA, not applicable

Table 2. Properties of vectors commonly used in clinical gene therapy trials

4. Vectors: Retrovirus

Gene transfer vectors derived from retroviruses have played an important role in the development of current gene therapy approaches. As mentioned above, a retroviral vector (derived from Moloney Murine Leukemia Virus, MoMLV) was the first to be used in an FDA-sanctioned clinical trial, retroviral vectors are the second most popular viral vector and are the vector of choice for *ex vivo* gene transfer approaches (Table 2).

To be clear, we use the term 'retrovirus' to describe all viruses belonging to the retroviridae family, though we will be focusing on gammaretrovirus (the genus which includes MoMLV) and lentivirus (the genus that includes HIV). These wild-type viruses are closely related and share many features, such as a capsid that is enveloped in a lipid bilayer and which carries two copies of the viral genome in the form of single stranded RNA (Figure 1). When the virus envelope protein interacts with its cognate receptor on the cell surface, the viral and cellular membranes fuse, the capsid is internalized and the RNA strands are liberated in the cytoplasm (Figure 2). The process of reverse transcription generates a dsDNA copy of the viral genome which then must gain access to the nucleus before integrating at an essentially random position in one of the host cell's chromosomes. The DNA copy of the gammaretroviral genome gains access to the cell nucleus only upon cell division, when the nuclear envelope is dismantled. For this reason, gammaretroviruses can only infect dividing cells. In contrast, lentiviral DNA passes through the nuclear membrane by an active process that is unrelated to cell division. In other words, lentiviruses are able to infect both dividing and non-dividing cells. Once the retroviral genome has been integrated in the host DNA, it is then referred to as a provirus. Viral gene expression and viral replication result from the information encoded by the provirus. Virus progeny bud from the cell surface, a process that does not harm the host cell [12].

The recombinant versions of retroviral vectors follow the form and function of their wild-type counterparts, but modifications are engineered in the vectors to improve safety and tropism. In general terms, a plasmid (transfer vector) is used to encode the sequence (including the gene of interest) that will be encapsulated in the virus particle and separate plasmids (packaging constructs) are used to encode the proteins that make up the virus structure (Figure 3). A sequence in the viral genome, called ψ, directs encapsidation of the viral RNA. This signal is present in the transfer vector, but not in the packaging constructs, thus the information for generating a new particle is not carried by the progeny, but the gene of interest is. Since the virus progeny do not carry the genes necessary to form a new particle, viral replication does not occur in the transduced cell and serves as a safety mechanism [12].

The tropism of the retroviral vector is determined principally by its envelope protein. Through engineering of the packaging constructs, the native envelope protein can be exchanged for an alternate protein that provides adequate tropism. For gammaretroviral vectors, ample tropism is provided by the 10A1 amphotropic envelope protein, though other options do exist. For lentivirus, the native envelope proteins direct transduction of CD4 and/or CCR5 positive cells. This would be too limited for most gene transfer efforts. Instead, the lentiviral envelope is

typically replaced with the envelope glycoprotein from Vesicular Stomatitis Virus (VSVg). Since VSVg interacts with heparin, a ubiquitous component of the cell membrane, such 'pseudotyped' vectors can transduce most any cell type. The VSVg envelope can also be used to pseudotype gammaretroviral vectors [12, 13].
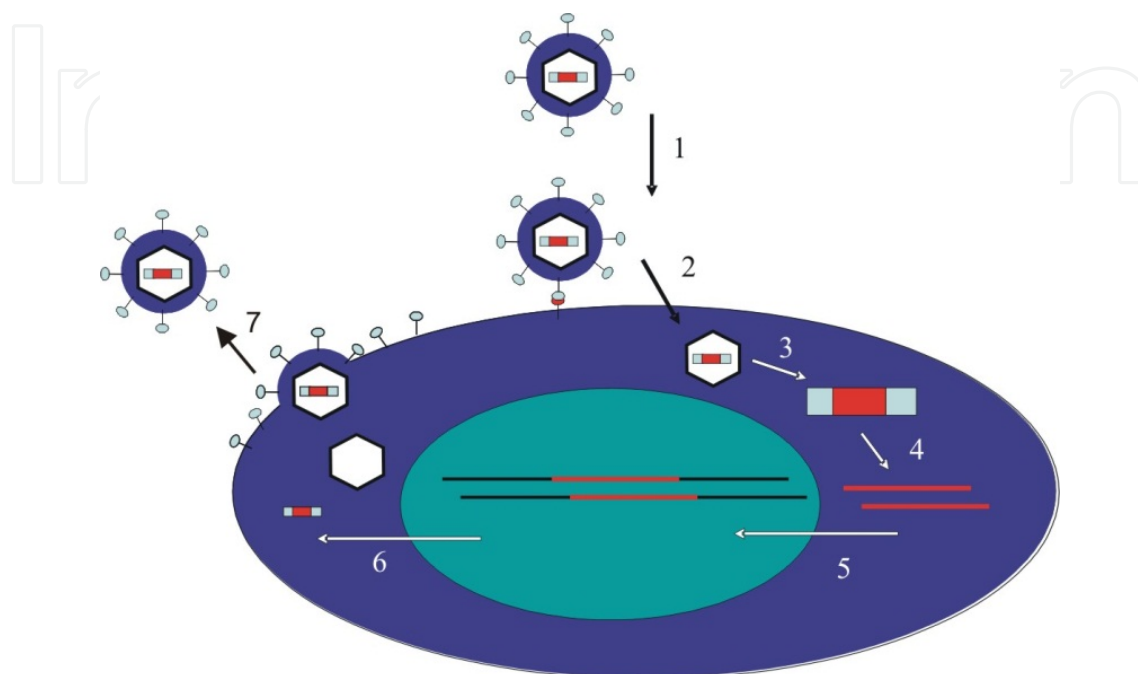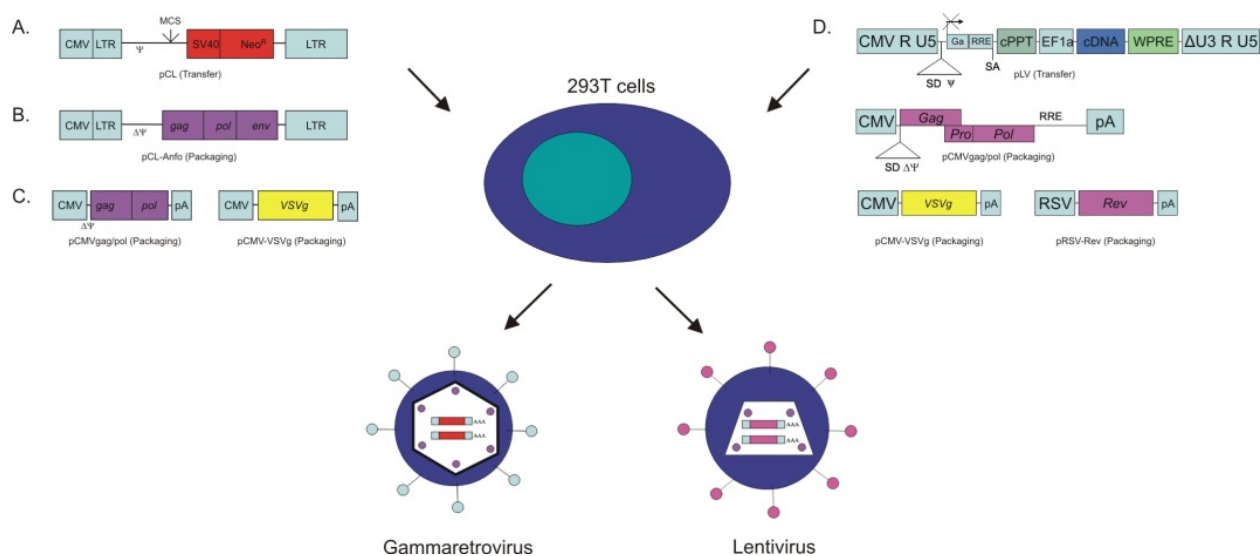

Figure 2. Schematic representation of the retroviral lifecycle. Though a gammaretrovirus is depicted, this process is quite similar for lentiviruses. (1) The retroviral envelope protein interacts with a cellular receptor and membranes are fused. (2) The capsid is internalized and (3) the viral genome, in the form of RNA, is liberated. (4) Reverse transcription results in the generation of a dsDNA copy of the viral genome which then must reach the nucleus (5), a process that for gammaretrovirus requires cell division since the viral DNA is not actively transported across the nuclear membrane, yet lentiviral DNA is and therefore does not require cell division for nuclear import. Once in the nucleus, the viral DNA is inserted in the host genome and is now referred to as a provirus. (6) Expression of the viral genes from the provirus provides all the components necessary for the assembly of progeny which then bud from the cell surface (7).

Retroviral vectors are not efficient vehicles for *in vivo* gene transfer. These vectors are quickly destroyed by complement and have a short half-life in the organism. However, the application of these vectors in cultured cells is an efficient and easy process. In addition, the integration of the provirus ensures that the viral sequence will be passed on to daughter cells after division of the host cell. These characteristics give retroviral vectors an advantage in treatments that require long term expression of the transgene and where *ex vivo* cell manipulation is antici‐ pated, such as the case for treating SCID [13].

Retroviruses have regulatory elements, called long terminal repeats (LTRs) that act as a promoter do drive the expression of viral genes. In recombinant vectors, the LTR can be employed to drive expression of the therapeutic gene, a common practice with gammaretro‐ viral vectors (Figure 3). For safety reasons, the LTR can be inactivated (by deletion of the enhancer sequences contained in the U3 region). Such 'self-inactivating' or 'SIN' vectors require the use of an internal heterologous promoter to drive transgene expression (Figure

3). This practice has long been employed in lentiviral vectors since it offers an additional assurance that recombination between transfer and packaging vectors will not produce a viable, replication competent progeny. An additional safety feature offered by SIN vectors is the decreased chance that the LTR will act as a promoter of host gene expression. The juxta-position of the LTR and a host gene can result in the unwanted expression of that gene (insertional mutagenesis). In the case of a proto-oncogene, the cell would then be at risk of transformation. This is essentially the cause of the cases of leukemia seen in the SCID-X1 trials. Currently, SIN retroviral vectors are favored since they are less likely to activate the expression of cellular genes, especially if the internal promoter does not contain a strong enhancer (for example, the EF1a promoter) [13, 14].

Figure 3. Schematic representation of the retroviral vectors and production. The gene of interest in cloned in a plas-mid (transfer vector) that contains the retroviral regulatory elements (LTR, long terminal repeats) as well as elements to regulate the expression of the transgene. The transfer vector is co-transfected along with the necessary packaging vectors in 293T cells (an easily transfected cell line derived from HEK293) where viral progeny are formed, bud from the cell surface and accumulate in the culture medium (supernatant). For gammaretrovirus, the transfer vector (A) can be transfected along with a single packaging vector (B) or, alternatively, with two additional vectors (C) that result in a pseudotyped virus progeny. For lentivirus, third generation transfer and packaging vectors are shown (D) which are all co-transfected in 293T to form virus progeny. Note that the lentiviral vectors do not contain any viral genes associated with HIV pathogenesis. CMV, cytomegalovirus promoter; SV40, simian virus 40 promoter; NeoR, neomycin resistance gene; *Gag*, group specific antigen (structural proteins); *Pro, pol*, viral enzymes integrase, protease and reverse tran-scriptase; pA, polyadenylation signal; VSVg, envelope glycoprotein of vesicular stomatitis virus; R, U5, repeat and U5 regions of the LTR; ΔU3, U3 region deleted from the LTR; SD, SA, splice donor, splice acceptor; Ψ, Psi encapsidation signal; RRE, rev-responsive element; Rev, lentiviral rev protein that regulates splicing events of the viral RNA; cPPT, cen-tral polypurine tract which facilitates nuclear importation of the lentiviral genome; WPRE, woodchuck hepatitis virus post-transcritptional regulatory element which facilitates nuclear export of the viral transcripts; EF1a, elongation fac-tor 1-alpha promoter which is often employed in retroviral vectors.

Lentiviral vectors are thought to be safer than gammaretroviruses due mainly to their proviral integration preferences. Though neither has a specific integration site, gammaretroviruses have a greater tendency to integrate near the transcriptional start sites of cellular genes than lentivirus [15]. In other words, gammaretroviruses are thought to be more apt to promote

unwanted expression of cellular genes. In the case of SCID-X1, SIN lentiviral vectors present one of the best currently available technologies for the safe transfer of the therapeutic gene [9].

Retroviral vectors play an important role in cancer gene therapy approaches. As will be described in detail, retroviral vectors are frequently used to modify components of the immune system in order to promote an anti-tumor immune response. Though the use of gammaretroviral vectors for this purpose has not met with any unwanted side effects, lentiviral vectors are gaining popularity due to their efficiency and safety.
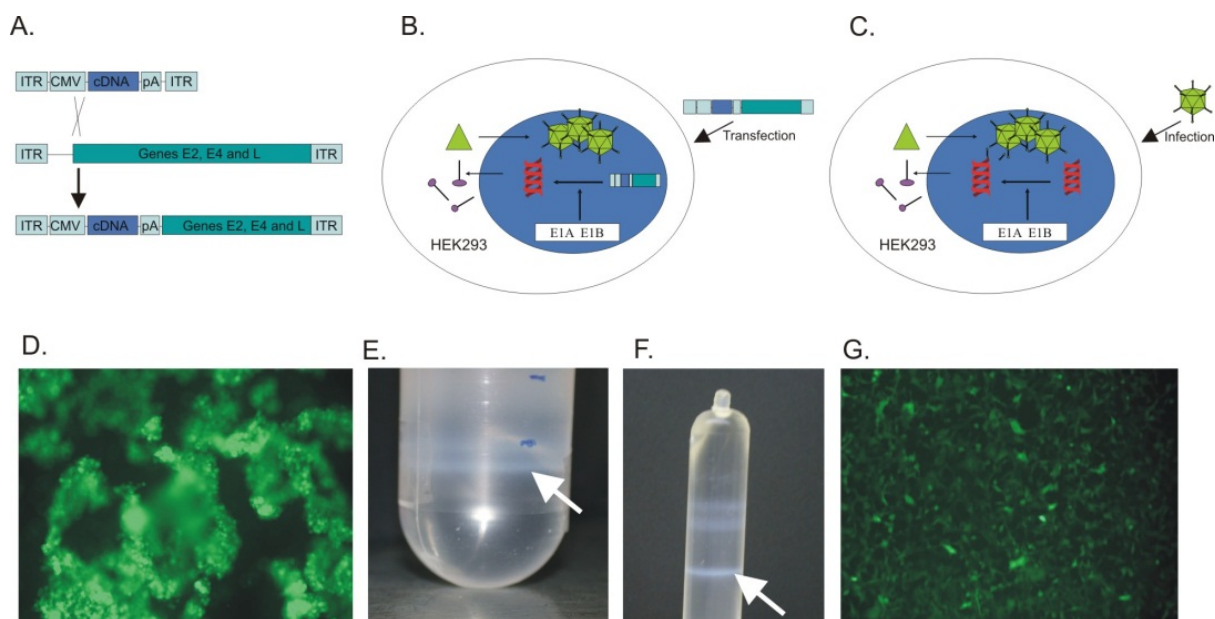
5. Vectors: Adenovirus

Recombinant adenoviral vectors are the vehicle of choice for *in situ* gene therapy of solid tumors (Table 2). Such vectors can be produced at high concentration (10^{12} particles/mL) and are quite well equipped to mediate gene transfer *in vivo*. The genome of the adenoviral vector remains episomal, thus the issues related to provirus integration (discussed above) are rendered moot. However, adenoviral vectors are known for provoking an anti-viral immune response. In the case of cancer gene therapy, the anti-viral immune response has not been associated with serious adverse effects and may even provide a benefit of attracting the immune response to the tumor site. The episomal viral genome and anti-viral immune response limit the duration of vector function, thus making adenoviral vectors inappropriate for the long term treatment of chronic diseases, such as hemophilia or SCID. Yet, the short-lived presence of transduced cells is compatible with cancer gene therapy since the goal is to express a factor that results in the elimination of the tumor cell, a process that does not require long term expression of the transgene [16-19].

The wild-type serotype 5 adenovirus (Ad5) has a linear, dsDNA genome of approximately 36 kb surrounded by a protein capsid from which fiber proteins project (Figure 1). The fiber protein mediates interaction with the Ad5 cognate receptor, CAR (Coxsackie Adenovirus Receptor) (Figure 4). The penton base then interacts with α_v integrins and the virus particle is then internalized, travels to the nucleus via microtubules and, finally, the viral DNA is deposited in the nucleus, a process that is independent of cell division. The viral genome remains episomal while viral transcripts are expressed and progeny are formed. In this case, virus progeny accumulate within the cell eventually resulting in cell lysis and the liberation of viral progeny. Adenoviral replication requires the activity of the viral E1a and E1b proteins which inactivate Rb and p53, respectively, and are involved in transport of viral transcripts and promote the expression of other viral genes [16-19].

In the laboratory, recombinant adenoviral vectors (Ad) are manipulated in the form of plasmids (Figure 5). The transfer vector encodes the gene of interest and the viral vector encodes the remainder of the viral sequence, except that E1a and E1b are deleted. Since the viral vector is quite cumbersome (approximately 40 kb), traditional cloning methods are inefficient for inserting the gene of interest. Alternatively, recombination between the transfer vector and viral vector may be performed or the use of rare restriction sites may be employed, either method resulting in a new plasmid that contains both the gene of interest and the viral sequence.
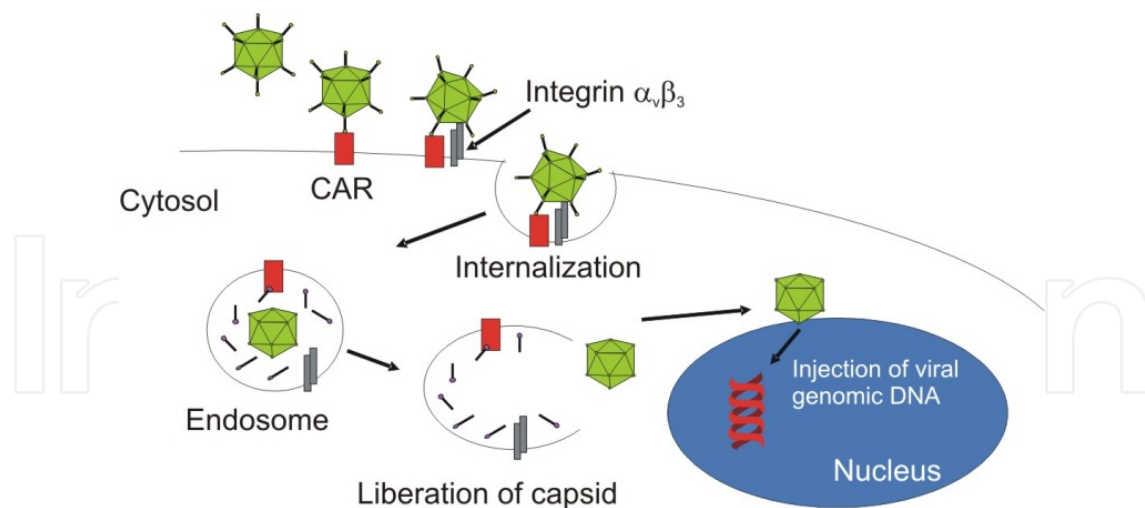
Figure 4. Schematic representation of the adenoviral life cycle. For serotype 5 adenovirus, the knob portion of the fiber protein mediates interaction with the cellular receptor, CAR (Coxsackie Adenovirus Receptor). In a second step of viral/cellular interaction, the penton base then binds with the integrin $\alpha_v\beta_3$. The virus is then internalized, partially disassembled and the capsid is carried to the nucleus my the microtubule network. The viral genome is deposited in the nucleus where expression of the viral genes results in the formation of progeny which accumulate intracellularly, eventually causing lysis and release of progeny.

Figure 5. Schematic representation of recombinant adenoviral vectors and production. (A) The gene of interest is inserted in the small transfer vector which is then recombined with the rest of the viral genome. However, the recombinant genome is devoid of the E1a and E1b genes (thus limiting viral replication). (B) The resulting plasmid is then transfected in HEK293 cells (which harbor the E1a and E1b genes), thus initiating the production of virus. (C) The initial production is then used to infect fresh HEK293 cells, thus amplifying virus production. (D) Photomicrograph of HEK293 cells suffering from the cytopathic effect induced by the replicating adenoviral vector which, in this case, encodes eGFP (enhanced green fluorescent protein). (E) Step and (F) continuous CsCl gradient purification of the virus progeny (indicated by the arrows). (G) Cells transduced with the now purified virus progeny is evidenced by their expression of eGFP.

The new plasmid containing the gene of interest as well as the viral components is transfected into an appropriate cell line in order to initiate the production of virus particles. Since the plasmid is devoid of E1a and E1b, the vector does not provide sufficient information to promote virus production. Instead, the cell line used, HEK293, already contains the E1 genes and provides expression of E1a and E1b in *trans*. In this way, only when the plasmid is introduced in HEK293 cells is virus production initiated. The progeny viruses formed are also deficient for replication since they too lack the E1 gene. However, the progeny virus can then be used to infect fresh HEK293 cells (a process that is much more efficient than transfection) and additional virus progeny will be formed. This 'amplification' of virus is repeated until a suitable volume of viral lysate has been generated. This, in turn, must be purified (at lab scale by CsCl gradient centrifugation, at industrial scale by filtration and chromatography) to rid the virus particles of cellular debris [16-19].

Even after purification, the virus progeny will contain a mixture of viable, infectious particles and defective, incomplete virus capsids. When using the virus to transduce a target cell, only the viable particle will result in transgene expression. However, the defective particles contribute to the load of antigens that the cell/organism receives and, with increased antigen, comes an increased chance of provoking an immune response, much as was seen in the case of Jessie Gelsinger. For this reason, care should be given to distinguish between the total viral load and infectious particles used [20]. Generally, total virus load is expressed as VP/mL (virus particles per milliliter) and infectious virus as IU/mL or PFU/mL (infectious units/mL or plaque forming units/mL). Even in high quality preparations, VP/mL will be 10 to 25 times greater than IU/mL.

Adenoviral vectors have evolved since their initial use was described. First generation vectors have deletions in the E1 and E3 genes, thus limiting replication and eliminating a non-essential viral gene. In an attempt to reduce the anti-viral immune response, second generation vectors with deletion in the E4 region (gene non-critical to viral replication, but which contributes to immune activation) were developed. The third generation of adenoviral vectors is devoid of any gene encoding an adenoviral protein. Though this complicates the strategy for virus production, it does yield virus that are much less immunogenic and provide more prolonged expression of the transgene. Even with these improvements, most cancer gene therapy protocols involving adenovirus use first generation vectors [16, 17].

Expression of the transgene encoded by the adenoviral vector requires the use of an internal heterologous promoter. This provides the opportunity for transcriptional targeting or a strong, constitutively active promoter, such as CMV, may be used. In first generation adenoviral vectors, a total of 7 kb of sequence may be inserted. Especially for cancer gene therapy, this is quite adequate for a promoter, transgene (or two) and polyadenylation signal.

As mentioned above, CAR is the cellular protein that mediates the interaction with Ad5 (Figure 4). In the absence of CAR, Ad5 transduction is quite inefficient. Though many cell types express CAR, some do not, including certain types of cancer (melanoma) and some cancer stem cells. In this case, engineering of the virus may be required so that interaction with CAR is no longer necessary for successful transduction. One such approach involves the insertion of the RGD

tripeptide in the H1 loop of the knob protein. In this case, the principal receptor is now α_v integrins which are widely expressed. By similar methods, the knob can also be adapted so that it interacts with specific cellular receptors of interest. That is to say, transductional targeting is achieved by altering the knob protein [17, 21].

If an adenoviral vector can be targeted at the levels of transcription and transduction, then it becomes tempting to apply the virus systemically with the expectation that it will have specificity for the target cell, such as the tumor. Unfortunately, other components of the virus, such as the fiber and the capsid, mediate interactions with heparin sulfate and factor X, both of which are highly abundant in the liver. In other words, attempts to apply the virus systemically are thwarted by viral sequestration in the liver. Even worse, the virus tends to transduce Kupfer cells (liver macrophages) that, in turn, present viral antigens to the immune system and induce an immune response. This implies that even the treatment of hepatocarcinomas by systemic administration of adenovirus will be undermined by transduction of Kupfer cells. Extensive engineering of the vector is required in order to prevent transduction of liver cells while directing transduction to the correct target [17, 22]. Though a few laboratory studies point to the viability of this approach, for now it remains quite problematic. Successful application of adenoviral vectors is typically performed by *in situ* injection of the virus into the tumor mass.

6. Vectors: Oncolytic adenovirus

Up to this point we have described vectors that are deficient for replication. However, viral replication can also be used to benefit the treatment of cancer. Such oncolytic or virotherapies rely on the viral replication to cause lysis of the tumor cell. The resulting progeny can then infect additional tumor cells and repeat the process, in theory, until all tumor cells have been eliminated. Clearly, such an approach requires extensive engineering of the vector such that replication is permitted only in the tumor cell, thus sparing normal cells [23].

An additional benefit to the oncolytic approach is that cell death does not depend on apoptosis. A fundamental feature of tumor cells is their resistance to cell death even in response to signals that would, in a normal cell, be fatal. Since oncolytic activity is not dependent on cellular genes, there is a greatly reduced chance that the cell would develop resistance to the viral activity. The same cannot be said for many pharmacologic approaches [23].

Several viruses, such as Herpes Simplex, Vaccinia and even retroviral vectors have been developed into oncolytics. However, for simplicity, we will focus on conditionally replicating adenoviruses (CRAds). The key to controlling CRAds lies in the E1 genes. As mentioned above, adenoviral replication requires the function of the E1a and E1b proteins (encoded by the E1 genes). If E1a and/or E1b function is permitted only in tumor cells, then the virus will replicate only in tumor cells. One approach uses a tumor-specific promoter to drive expression of the E1 gene. Promoters such as hTert, E2F1 and survivin are much more active in tumor cells than in differentiated adult tissues. The use of one of these promoters to drive E1 gene expression creates a degree of tumor specificity [23].

Another approach involves the use of a mutant version of the E1a gene. Adenoviral replication is not supported in the presence of wild type Rb and, for this reason, E1a has evolved to interrupt Rb activity. Since Rb is typically inactivated in tumor cells, E1a is no longer required to provide this function. Deletion of the 24 amino acids from E1a which mediate its interaction with Rb results in an altered E1a protein that no longer blocks Rb function. In a normal cell, the persistence of Rb function blocks viral replication. In a tumor cell, the typical loss of Rb activity creates a condition where viral replication is supported. CRAds that combine trans-ductionoal and transcriptional targeting as well as the E1a deletion mutant have been created and tested with promising results [23-25].

A classic example of an oncolytic adenovirus was originally known as Onyx-015. In this vector, the E1b gene was mutated with the intention of permitting viral replication in p53-deficient cells, but normal cells, with wild-type p53, should not allow viral replication. This vector has been widely studied and even approved for commercialization in China (Table 1). However, it was later noted that the underlying mechanism that controls Onyx-015 replication is not related to the p53 status, but instead to the RNA export functions of the E1b protein [26, 27].

In addition to the sophisticated engineering of the CRAds, some these vector also carry a therapeutic gene. Such vectors are referred to as armed CRAds. For example, the expression of GM-CSF or interferon-β together with the activity of the CRAd should promote an anti-tumor immune response. Alternatively, the vector may be armed with additional death-inducing factors, such as the thymidine kinase gene (TK) derived from herpes simplex virus. In this case, the application of the prodrug ganciclovir induces cell killing and augments the effect of the CRAd. Another approach involves the use of the TNF-related apoptosis-inducing ligand (TRAIL) which, when cleaved into a soluble form, induces apoptosis in tumor, but not normal, cells [24, 25, 28].

In all, oncolytic viruses can combine tumor specific activities in order to bring about cell death independent of apoptosis, but can also be armed to induce apoptosis, immune activation and interrupt cellular DNA replication.

7. Vectors: Non-viral

The typical non-viral vector is a recombinant plasmid, though oligos, siRNA and RNA fall into this category. Recombinant plasmids are derived from their wild-type counter-part, a small circle of episomal dsDNA found in a variety of bacteria. In the lab, we need only maintain regulatory sequences that control the replication of the plasmid in their bacterial host as well as an antibiotic resistance gene that facilitates the manipula-tion and maintenance of the plasmid in the lab. The therapeutic gene of interest is then inserted along with appropriate regulatory sequences (such as a promoter and polyade-nylation site). Once constructed, the production of the vector is performed using well es-tablished techniques involving large volumes of bacterial cultures and purification of the plasmid by filtration and chromatography [29-31].

Though the use of plasmid vectors is quite routine and performed in a great many research labs on a daily basis, some considerations of vector design and delivery should be taken into account. The vector itself should not contain extraneous sequences that are not part of the treatment. That is to say, the vector delivered to the patient should not contain antibiotic resistance genes or regulatory sequences of bacterial origin. Such sequences could provide expression of unwanted peptides or even contain GC rich sequences, both known to provoke immune responses. However, production of plasmids requires the presence of just such regulatory sequences. To overcome this, the plasmids are often engineered such that once the bacteria and the plasmids they contain have been expanded to the necessary volume, recombination can be induced such that the unwanted sequences are eliminated and only the small circular DNA containing the gene of interest remains. It is this small circular DNA that is then purified and delivered to the patient, free of non-essential sequences [31].

Plasmid delivery can be performed by direct injection of 'naked' DNA with or without physical methods to promote transfection, such as the gene gun, electroporation or microbubbles. Alternatively, the plasmid may be complexed with chemical compounds that facilitate its passage across the cell membrane. Such complexes may include lipids, polyethylene glycol, polyethyleneimine or other compounds can both secure the DNA molecules as well as mediate cellular uptake. Such approaches are often referred to as 'nanotechnology' since these virus-sized particles can be engineered to achieve high levels of transfection and can even include targeting strategies (such as described for transductional targeting) [30, 32-35].

While non-viral gene transfer may be relatively efficient, simple and safe, the episomal nature of the plasmid and limited half-lives of oligos and mRNA often result in short lived expression of the therapeutic gene. As the cell divides or as the transfected material degrades, the therapeutic gene is lost. Such an approach may be inappropriate for the treatment of chronic illnesses, such as immune deficiencies or metabolic disorders. However, non-viral gene transfer is an interesting option when limited transgene expression is sufficient or desirable, such as for the induction of angiogenesis in ischemic tissues or, in the case of cancer, induction of cell death and/or a tumor-specific immune response.

Recently, a new approach was described for non-viral gene transfer to the skin. This new technology involves the use of an array of very small needles that breakdown over time and has been termed dissolvable protrusion array device (PAD) [36, 37]. For this, an array of pins is dipped in a polyvinyl alcohol polymer solution and slowly raised while drying; essentially creating hollow core stalagmites that can be filled with a solution of nucleic acid, such as siRNA. The array of microneedles can then be applied to the skin, penetrating the stratum corneum barrier and depositing the material where it dissolves and locally releases the therapeutic payload. As a demonstration of this technology, human skin xenografts were treated with PAD loaded with an siRNA againt CD44 and significant reduction in target gene expression was observed [36, 37]. In theory, the PAD can be loaded with more than one therapeutic sequence or even with chemotherapeutic drugs, so it is easy to imagine the administration of genetic and pharmacologic therapies directly to the cutaneous tumor mass.

8. Strategies of melanoma gene therapy

Melanoma has played an important part in the history and development of gene therapy. The first clinical trial to use gene therapy to treat cancer was performed at the US National Institute of Health (NIH) Clinical Center in February of 1991, lead by Steven Rosenberg of the National Cancer Institute (NCI). When the results were published, they affirmed that " attempts at gene therapy for cancer are underway …." [38].

In fact, the milestone study was based on work initiated several years before. Since 1986, Rosenberg had increasing success in treating intractable melanomas with TIL cells (tumor infiltrating lymphocytes) that are surgically removed from a patient's cancer and cultured for 4 to 6 weeks in the laboratory along with interlekin-2 (IL-2), a growth factor that stimulates the immune system, and then the cells are re-infused into the patient. The TILs have a selective affinity for the tumors from which they came, so the TIL-IL2 cells can infiltrate the primary tumors and their metastases [39]. However, as with many new therapies, TIL-IL2 only works a fraction of the time. The question was why? Are the cells reaching their target? A gene transfer experiment was proposed in an attempt to answer that.

The experimental strategy was relatively simple. The first step was to genetically modify human TILs through the introduction of a foreign marker gene in the laboratory (*ex vivo*). The gene for a bacterial enzyme encoding neomycin phosphotransferase (neo^R), which transmits resistance to the antibiotic G418, was chosen. Using a gammaretrovirus to deliver the neo gene, a population of TILs were marked *ex vivo*. Rosenberg, Blaese and Anderson succeeded in getting more than 5% of TILs to take up the neo gene. After administration, these modified TILs can be easy identified in patients' pheripheral blood and tumor biopsies. In 1989, the first of ten patients with end-stage melanoma was treated with infusion of TILs transduced with gammretrovirus and, by following the neo-marker, the research team could follow the TILs. This initial study was designed to determine the long-term traffic patterns and distribution of TILs in the body. It was not expected to offer any medical benefit, yet this clinical experiment demonstrated that gammaretrovirus-mediated transduction could be used to safely modify human lymphocytes which were subsequently administered to the patient. This trial led to additional gene transfer protocols utilizing therapeutic genes with potential for clinical benefit [40].

This and other early gene marking studies suggested that recombinant gene transfer could be achieved in a selected subpopulation of cells. These pioneering trials also helped address important regulatory concerns and stimulated research into delivery vehicles with applications for inherited and acquired diseases. Interestingly, in parallel, M. Blaese and F. Anderson used a similar approach where the gene for adenosine deaminase was transferred by gammaretrovirus into lymphocytes of children with severe combined immunodeficiency disease and is regarded as the first therapeutic gene therapy trial, as mentioned above [7].

Since Rosenberg knew that TILs localize at tumor sites and that TILs can be safely modified with gammaretrovirus, he then attemped to introduce TNF (tumor necrosis factor-alpha) into TILs and use these genetically modified cells as vehicles to deliver TNF, a potent antitumor

protein, directly into the tumor. This trial represents the first time a candidate therapeutic gene was introduced into human cells with the goal of treating cancer [41]. Three late-stage melanoma patients were treated with unselected TILs that had been transduced with the TNF gammaretrovirus, but the subsequent seven patients received modified TILs that were selected for G418 resistance in order to increase the fraction of tranduced cells present in the TIL cultures. The modified TILs were expected to accumulate at the tumor sites and produce local concentrations of TNF high enough to mediate tumor death, yet without exposing the patient to high systemic doses of TNF. The treatment was well tolerated and, as often seen in phase I safety trials, clinical benefit was not profound [38]. However, this trial paved the way for future cancer gene therapy efforts.

To date, some 1186 cancer gene therapy trials have been cited, representing approximately 65% of all gene therapy clinical protocols (http://www.wiley.com/legacy/wileychi/genmed/clinical/). A great many approaches have been attempted with the fundamental goal of eliminating the tumor cells by direct induction of cell death or by induction of an anti-tumor immune response. Specifically in the case of melanoma, some 161 trials have been cited (Figure 6). In fact, melanoma is the most frequent indication seen in cancer gene therapy trials, even more than prostate, lung or breast. Among these melanoma gene therapy trials, 7 phase III protocols have been cited. These include transfer of the B7.1 gene (5 trials) and GM-CSF (2 trial).
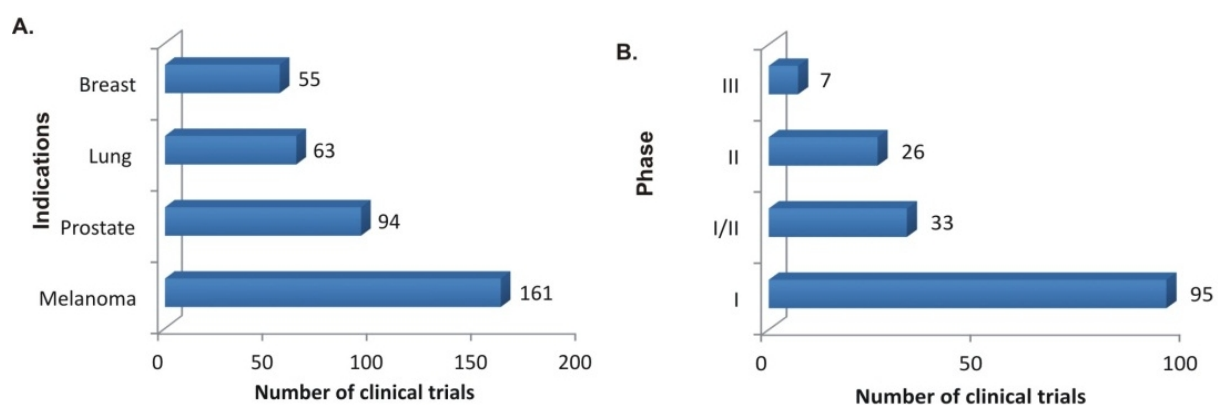
Figure 6. Progress in clinical trials of melanoma gene therapy. (A) Distribution of more frequent indications for cancer gene therapy. (B) Distribution of clinical trials of melanoma gene therapy by phase. Data extracted from Journal of Gene Medicine Clinical Trial Database (current as of June 2012).

These clinical trials highlight the variety of approaches that have been attempted (Figure 7). These include strategies of viral gene transfer (62% of trials), where adenovirus and gammaretrovirus are the most used. Non-viral vectors (38% of trials) have been used as naked DNA or in conjunction with physical and chemical means of improving their efficiency. Strategies have included the transfer of seven principal gene families, including interleukins, tumor associated antigens, suicide genes and genes for the modulation of the immune response (GM-CSF, B7.1 and type I interferon). In the following discussion we present some illustrative examples from pre-clinical to phase III trials.
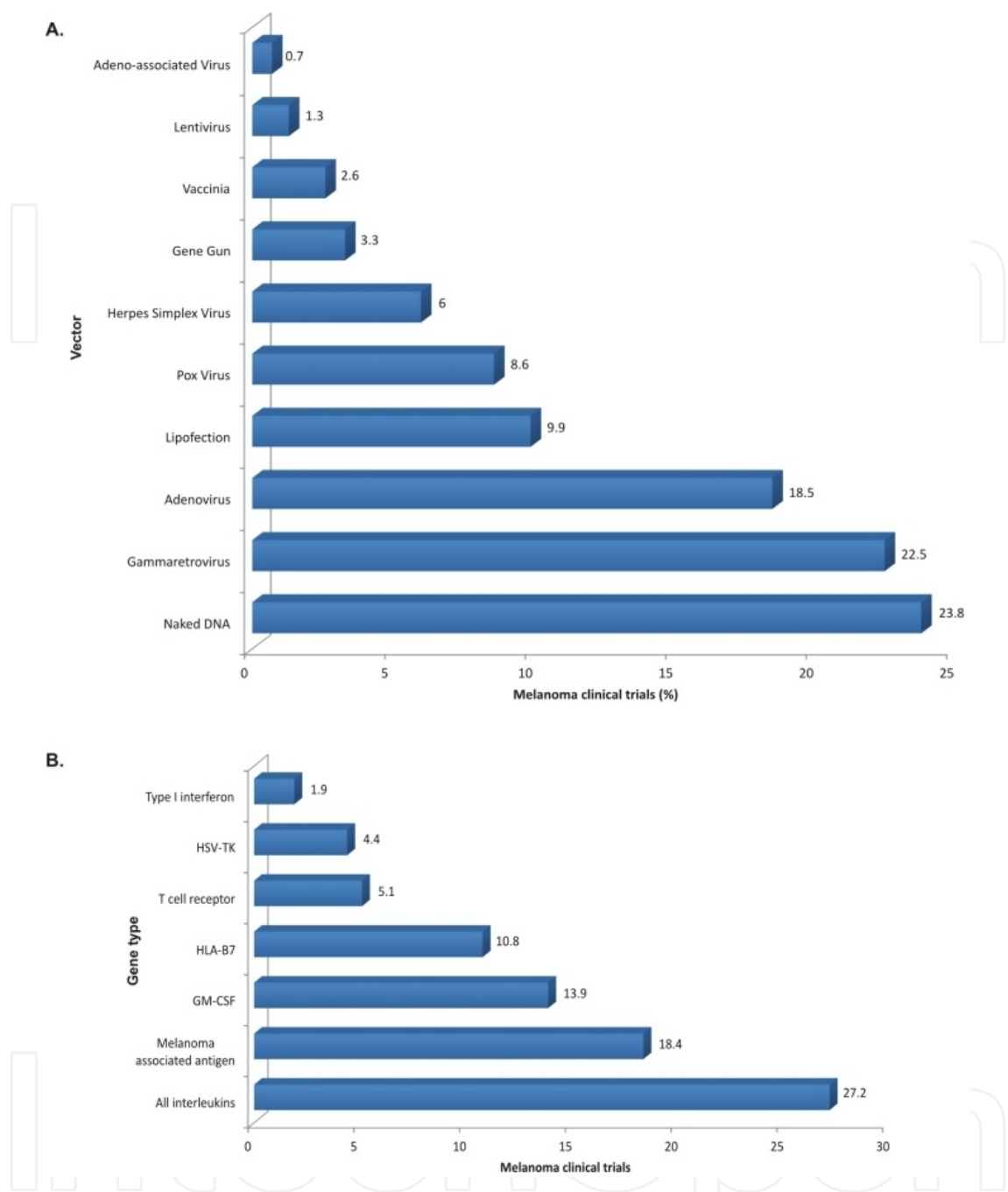
Figure 7. Strategies of melanoma gene therapy tested in the clinic. (A) Frequency of vector usage. (B) Therapeutic gene type used. Data extracted from Journal of Gene Medicine Clinical Trial Database (current as of June 2012).

9. Suicide genes

A classic cancer gene therapy approach involves the transfer of a 'suicide gene' or 'gene directed enzyme prodrug therapy'. In this scenario, an enzyme is introduced in the target cell by gene transfer. This enzyme alone does not have an effect on the cell, but when an appropriate

prodrug is administered, the enzyme converts the innocuous prodrug into an active, death inducing compound. Two of the better known examples are the thymidine kinase gene derived from herpes simplex virus (HSV-TK) which promotes phosphorylation of the prodrug ganciclovir and the bacterial cytosine deaminase (CD) gene which converts the prodrug 5-fluorocytidine (5FC) into the chemotherapeutic agent 5-fluorouracil (5FU). In both cases, the compound produced by the enzyme/prodrug interaction blocks DNA replication and, as a result, induces cell death. The advantages of suicide gene therapy include very limited reaction to the enzyme or prodrug in isolation, yet their combination results in a localized concentration of an anti-neoplastic agent. Since DNA replication is a prerequisite for the action of the modified prodrug, this approach has specificity for dividing cells and spares post-mitotic cells even in the case that they should be transduced. Suicide gene therapy is aided by the 'by-stander' effect where the modified prodrug may be passed from a transduced cell to a non-transduced neighbor, killing it only if it is actively dividing. In addition, at least in the case of HSV-TK/ganciclovir, a significant tumor-specific immune response may be elicited, thus amplifying the effects of the gene therapy even further [42].

Specifically in the case of melanoma, a few examples of clinical application of suicide genes have been reported. For example, a phase I/II study utilized a gammaretroviral vector for the transfer of the HSV-tk gene to melanoma patients. However, these authors actually implanted cells that were actively producing the vector in the patients. In this way, the tumor cells would receive a constant supply of virus until administration of the prodrug which would then bring about the death of both the tumor cells and the virus producing cells. These treatments were associated with well tolerated inflammatory skin reactions and moderate fever. During ganciclovir treatment, the tumors did show signs of shrinkage, but increased in size upon drug withdrawal. These results are quite encouraging given that the treatment was well tolerated and showed signs of clinical benefit, at least during the prodrug treatment [43].

In a separate trial, 13 melanoma patients were treated with gammaretrovirus by direct injection of the virus particles into the tumor mass followed by ganciclovir administration. The gene transfer protocol was well tolerated with only one patient showing grade III pain at the injection site. The treatment with the prodrug was associated with some side effects, including dyspnea, pain and poor appetite. However, no response was detected when comparing injected and non-injected tumors. Though the desired result was no observed, the gene therapy procedure was well tolerated [44].

Laboratory assays using CD have yielded some promising results and also exemplify interesting technologies. For example, Kucerova et al [45] have used mesenchymal stem cells to infiltrate melanoma tumors in a mouse model. In this case, the mesenchymal stem cells offer the advantage of migration to and within the tumor mass, an inherent property of these cells. The human fat-derived mesenchymal cells were transduced with a gammaretrovirus encoding the CD gene and delivered i.v. or i.p. in nude mice bearing a tumor derived from a human melanoma cell line (A375). Significant inhibition of tumor progression was seen with the i.v. injection of the CD-expressing mesenchymal cells plus 5FC treatment [45].

In another example involving CD/5FC, an adenoviral vector was targeted to tumor vascular endothelial cells at both the transductional and transcriptional levels. For this, the adenoviral

vector displayed the RGD tripeptide in the H1 loop of a mutated fiber knob protein (so that transduction depends on $\alpha_v\beta_3$ integrins instead of CAR) and also used the endothelial cell-specific Tie2 receptor promoter to drive expression of the CD gene. B16 tumors were established in nude mice and the modified adenovirus was administered i.v., but only after pre-treatment with hetastarch, a reagent that effectively blocks hepatic uptake of adenovirus. The authors report that only endothelials, pericytes and tumor monocytes were transduced, but not the tumor cells themselves. Administration of 5FC resulted in significant inhibition of tumor progression [46].

10. Adoptive cell transfer

One of the most promising frontiers of cancer therapy is the use of adoptive cell transfer (ACT). Especially in the case of metastatic melanoma, where few options exist, ACT may overcome some of the limitations seen with even the most promising treatments (including high-dose IL-2 and anti-CTLA-4). In addition, a naturally occurring, tumor specific T cell response is seen in a large percentage of melanoma patients, suggesting that these tumors are immunogenic and that T cells can be made to recognize them. ACT utilizes the patients' own T cells where they are activated *ex vivo*, expanded, and returned to the patient to carry out tumor-specific cytolysis. Since gene transfer is the theme of this chapter, we will focus on approaches that involve genetic modification of either the T cells themselves or of the dendritic cells (DC) used to activate them. At its core, ACT is used to generate T cells that are tumor reactive, though several approaches have been described [47, 48].

Tumor infiltrating lymphocytes (TILs) can be isolated from melanoma biopsies and the T cells expanded *ex vivo*. This approach, which does not involve genetic modification, relies on two critical factors, the ability to isolate T cells and expanding them to clinically significant numbers. Over time, alternatives to this approach have been explored, such as preconditioning by lymphodepletion, the use of young TILs (cells that have not undergone extensive expansion *ex vivo*) or enrichment for CD8+ TILs. Isolation of T cell clones from TILs that are specific for melanoma associated antigens (such as MART1/MelanA, NY-ESO-1 or gp100) can also be performed, but this process requires 3 to 5 months and the clinical outcome (50% objective response) does not surpass that seen with polyclonal TILs [47, 48].

The above argument points to considerable success with ACT, however there is room for improvement, especially with respect to tumor regression. Genetic modification of T cells as a form of ACT has been developed and tested clinically. For example, the T cells may be transduced with a retrovirus that provides expression of a cytokine, such as IL-2, IL-12 or IL-15 or may be modified to resist the effects of immunoregulatory factors. The aim here is to preserve the function of the ACT, but does not necessarily assist in tumor reactivity of the T cells. Thus far, IL-12 and IL-15 expressing cells are still being tested, while IL-2 expressing cells did not show any advantage over non-modified ACT [47-50].

Since the role of the T cell receptor (TCR) is to mediate, in part, the recognition of and response to specific antigens, the patients' T cells may be modified by the introduction of a recombinant

TCR with specificity for a tumor associated antigen. This is a time consuming process and the clinical response rate is less than ideal, though this approach is often seen as an alternative for those patients that failed to provide TILs from biopsies. In the ground-breaking work of Morgan et al, peripheral blood lymphocytes from melanoma patients were transduced with a gammaretrovirus encoding the alpha and beta chains of the MART-1-specific TCR. The lymphodelpeted patients received an infusion of the genetically modified, autologous cells. The persistence of these cells was variable and related to experimental conditions of each cohort, but modified cells found even after 90 days. Two (out of 15) patients with progressive metastatic melanoma showed full clinical regression with this treatment [51]. In a later trial conducted by this group, they used improved versions of the TCR and observed a response rate of 30% [52]. Though the response rate was low, these studies show the feasibility of this approach and the importance of fine tuning the TCR.

In a related approach, T cells are modified to express a chimeric antigen receptor (CAR). CARs are engineered molecules that consist of an antigen-recognizing domain derived from an antibody (single-chain variable fragment) fused to a component of the TCR complex (CD3 zeta chain) and, sometimes, also fused to a costimulatory molecule (such as CD28) [47, 48]. This arrangement assures that antigen recognition is sufficient to activate T cell cytolytic function, an interaction that is not HLA restricted and requires only low levels of antigen, thus stream-lining and amplifying the T cell response. The lack of HLA restriction implies that the CAR modified T cells need not be autologous and a supply of cells may be generated and maintained at the ready.

Treatment of melanoma with CAR-modified T cells has been performed only in preclinical models, but these show promising results. For example, a CAR specific for ganglioside GD2 was developed in the Brenner laboratory, inserted in a gammaretroviral vector and used to modify T cells. When these cells were exposed to melanoma cells expressing GD2, cytokines were released and melanoma cells were eliminated both *in vitro* and *in vivo* [53]. In a similar fashion, the Rosenberg and Morgan group has developed a high molecular weight melanoma associated antigen (HMW-MAA or CSPG4)-specific CAR. Both CD4 and CD8 T cells express-ing this recombinant receptor were reactive and induced target cell cytolysis in an HLA-independent manner [54].

Clinical trials with CAR-modified T cells have been performed for other tumor types, in-cluding ovarian cancer, neuroblastoma, leukemia and lymphoma and some clinical bene-fit has been reported [47, 48]. Unfortunately, a patient being treated with ERBB2-targeted, CAR-modified T cells suffered an adverse reaction and died when these cells lodged in the lung due to low level expression of ERBB2 and initiated a cytokine storm [55]. In a separate trial, a patient treated with CD19-targeted cells also succumbed due, it is believed, to a T cell response [56]. These unanticipated events should not detract from the potential of CAR-modified T cells. Clearly, continued study of the CAR design and T cell response is required and, no doubt, is forthcoming. Nor should this event reflect on the field of gene therapy since safety issues related to the vector itself were not involved in the function of the CAR and subsequent cellular responses.

11. Vaccines

Since melanoma is well known to be an immunogenic tumor, there have been a great many efforts to develop vaccines that induce an anti-melanoma response. Many approaches using peptides, whole proteins, whole cell lysates delivered directly to the patient have been attempted. Also, the introduction of GM-CSF in melanoma cells provides a vaccine that promotes the immune response. Alternatively, dendritic cells themselves may be introduced in the patient, a process that relies on the availability of such cells as well as their ability to recruit and activate T cells in the cancer patient. Currently, much emphasis has been given to the target specific approach of blocking CTLA-4 or PD1 with monoclonal antibodies, thus promoting T cell function, for the treatment of melanoma [57]. Future hopes involve the combination of these vaccine and targeted modalities.

GM-CSF acts by recruiting and promoting the maturation of DCs, a process that is crucial to mounting an effective immune response. Many studies have explored the genetic modification of tumor cells with gammaretroviral vectors expressing GM-CSF, irradiation of these cells and subsequent use of these cells as a tumor vaccine. Soiffer et al [58, 59] observed that metastatic lesions in 11 of 16 treated patients had a dense infiltrate of T cells and extensive tumor destruction, a phenomenon not seen in the absence of this treatment. Vaccination sites showed adverse effects, such as erythema and induration and occasional hemorrhaging, otherwise the treatment was well tolerated [58]. In a later trial, this group used adenovirus instead of the gammaretroviral vector for GM-CSF gene transfer to patient-derived metastatic melanoma cells, followed by irradiation and vaccination. The vaccine was successfully produced for 34 of 35 patients and toxicities included only grade 1 to 2 local skin reactions. Again, infiltration and necrosis of metastatic sites was observed (10 of 16 patients). After 36 months of follow-up, 29% of patients were still alive, four of whom showed no evidence of disease [59].

Though the GM-CSF secreting cells provided some measure of improvement, the successful vaccine requires an abundance of functional T cells to carry out cytolysis. One factor that blocks T cell function in many cancer patients is the CTLA-4 gene. By blocking CTLA-4 function, T cell activity is liberated. Ipilumimab, a monoclonal antibody that binds to and blocks CTLA-4 function was recently approved by the FDA for the treatment of late stage melanoma [57]. Studies by the Dranoff group applied ipilumimab in melanoma patients that had previously been treated with the GM-CSF secreting cellular vaccine [60, 61]. The observations included only low grade inflammatory toxicities, though high level ipilumimab treatment is known to cause high grade toxicity in 15 to 25% of melanoma patients. Though not designed to reveal clinical benefit, evidence of tumor shrinkage was observed in some patients. Since ipilumimab also increases the number of Tregs, proper T cell function may require a specific balance between effector and regulatory cells. A future direction may be to combine the vaccine, ipilumimab and an inhibitor of Tregs [60].

As an alternative, DCs can be modified *ex vivo* with antigens or a vector or mRNA encoding tumor associated antigens. These professional antigen presenting cells can then be introduced in the patient as an immunotherapy. For example, the work of Steele et al

[62] describes the transfection of a plasmid encoding both melan-A and gp100 into DC derived from the patients' own PBMCs followed by delivery of these gene-modified cells in the stage IV melanoma patients (n=27). The number of rounds of vaccination was variable, between 1 and 14, though 15 patients received 4 rounds of treatment. In all, partial response or stable disease was seen in seven patients and treatment-related adverse events were quite mild (flu-like symptoms or mild erythema at the injection site). As with many early phase trials, these results show that the treatment was well tolerated and that a positive response was seen in a few patients [62].

12. Oncolytic viruses (virotherapy)

As described above, oncolytic vectors use the lytic virus life cycle in order to destroy cancer cells. These vectors are engineered so that replication can occur only in tumor cells and, in some cases, are armed with a gene to aide in tumor cell killing. The engineering may include transductional and transcriptional targeting, alteration of viral replication genes and the insertion of a functional transgene.

Some interesting examples of conditionally replicating adenoviruses for virotherapy of mouse models of melanoma have been reported. For example, the group of Albert Deisseroth has developed an oncolytic vector where expression of E1a is controlled by the tyrosinase promoter. Tyrosinase, a melanoma associated antigen, is an important factor in melanogenesis and may even have additional regulatory functions [63]. In this way, E1a expression from the vector should be melanoma-specific. In addition, the vector was modified with the insertion of the RGD tripeptide in the H1 loop of the fiber knob protein, much as was discussed above. This vector showed increased infectivity in melanoma cells and suppressed growth of xenograft tumor models [64].

In an alternative approach, an oncolytic adenovirus was developed where the SPARC (secreted protein acidic and rich in cysteines) promoter was used to drive expression of E1a. In this case, the SPARC promoter should support viral replication not only in the tumor cells, but also in tumor associated stromal cells. Though this approach was beneficial for a model of pancreatic carcinoma, the authors observed that the stromal cells in the melanoma model actually hampered virus performance [65]. This study is interesting since it points out the importance and complexity of the tumor microenvironment on the gene therapy approach.

In a final pre-clinical example, an oncolytic adenovirus was developed which was armed with IL-24 (MDA-7, melanoma differentiation-associated gene-7) as well as arrestin. IL-24, a secreted protein, inhibits melanoma by autocrine and paracrine effects, including inhibition of angiogenesis, immune stimulation and radiosensitization. Arrestin also inhibits angiogenesis and tumor growth. With this doubly-armed oncolytic vector, it was shown that melanoma cells were significantly inhibited both *in vitro* and *in vivo*, the latter being associated with reduced angiogenesis and increased apoptosis [66].

Oncolytic vectors have also been tested clinically. We start by describing a second generation HSV (herpes simplex virus) oncolytic that was developed by deleting not only the ICP34.5

gene (typical of first generation vectors), but also the ICP47 gene which impedes antigen expression. In addition, early expression of another viral factor, US11, promotes tumor-specific virus replication. Finally, this vector was armed with GM-CSF (which promotes dendritic cell maturation and, thus, anti-tumor immune response) and termed OncoVEX[GM-CSF]. In a phase I dose escalation trial, viral treatments were generally well tolerated, but side effects were more pronounced in patients who were HSV-seronegative before receiving treatment. Of the 26 evaluable patients, 3 showed stable diseaes (including 2 melanoma cases) and 6 showed flattening of the treated tumors. Some notable aspects of this study were the relatively low frequency of viral treatments, low viral dose and lack of chemotherapy required to induce a response [67]. In phase II trials treating stage IIIc and IV melanoma, treatment with Onco-Vex[GM-CSF] induced tumor specific (MART-1) CD8-positive T cells and reduced Tregs [68] and, in a separate trial, resulted in a 26% response rate [69]. Currently, a phase III trial, 'OPTIM: Oncovex[GM-CSF] pivotal trial in melanoma' is underway [70].

The vaccinia virus, best known for its role in eradicating smallpox, has also been developed into an oncolytic vector [71]. These vectors have a rapid lytic cycle, enter cells by membrane fusion (and not a specific receptor) and the enveloped progeny are protected from immune destruction, all factors that enable poxviruses to spread within the organism. Tumor specific poxviruses rely on reduced type I intereferon response, increased rate of cell division, decreased rate of apoptosis and immune evasion seen in tumor cells. In addition, the epidermal growth factor receptor (EGFR)-Ras signaling pathway, which is typically active in tumor cells, promotes poxvirus replication. Since tumor cells are naturally permissive for poxvirus replication, viral transformation genes can be eliminated from the vector. In this way, the vector will replicate only in tumor cells and leave normal cells unharmed. The oncolytic poxvirus can also be armed, such as with the GM-CSF gene and with the bacterial β-galacto-sidase (LacZ) gene, serving as a marker for transduction as well as an antigen against which antibodies can be generated and measured [71]. The JX-594 poxvirus possesses these features and has been tested clinically.

In a proof of principle trial, 10 melanoma patients were enrolled and treated with no significant side effects, mainly grade 1-2 flu-like symptoms. Post-treatment biopsies showed necrosis and/or intense perivascular lymphocytic infiltration and radiographic tumor assessment, possible in only 5 of the patients, all of which showed stable disease in the treated tumor and 3 patients also showed stable disease at distant, non-treated sites [72].

The oncolytic vectors discussed above were delivered intratumorally. Intravenous delivery, on the other hand, is quite challenging due to the multiple impediments that prevent the virus from successfully reaching its target. However, JX-594 has been delivered i.v. and showed clinical benefit. In this dose escalation study, 23 patients with solid tumors, including mela-noma, were treated and adverse effects were limited to grade 1-2 flu-like symptoms. Staining for the β-galactosidase protein revealed the presence of the virus in tumor tissues. In addition, anti-β-galactosidase antibodies were measured and revealed a dose-dependent relationship with the viral load used to treat the patients. GM-CSF was detected in patient blood samples only after treatment. Evidence of anti-tumor activity was noted, including >25% descrease in

FDG-PET. This is study was the first to show dose-related delivery, replication and transgene expression upon i.v. delivery [73].

13. B7.1

Tumor cells often escape detection by the immune system even when anti-tumor immune cells are generated [74]. Among the many possible mechanisms for this phenomenon, metastatic melanoma cells are quite often deficient for B7-1 and B7-2, factors that are essential for full T cell activation. The lack of even one of these factors leads to T cell anergy, inactivation of the T cell after encountering an antigen, essentially generating self-tolerance. In other words, the lack of these factors actually teaches the T cells to ignore the melanoma. This implies that using gene therapy to replace at least one of these missing factors should initiate tumor specific T cell mediated cytolysis.

In 1993, Gary Nabel showed that a plasmid vector encoding HLA-B7 could be safely introduced into patients' melanoma tumors by means of a liposome. One of the five patients showed reduced tumor mass [75]. A subsequent trial by this group revealed T cell infiltration and TIL reactivity was enhanced in treated patients. In addition, two patients showed local inhibition of tumor growth [76]. The concept was then expanded upon by transfer of the β2-microglobulin gene along with HLA-B7 in a single plasmid vector/liposome complex, a treatment now known as Allovectin-7. In this way, Allovectin-7 provides an allogeneic MHC class I protein, essential for antigen presentation and T cell activation [77].

Several phase II studies of Allovectin-7 have been completed. For example, Stopeck et al [78] reported a regimen where 6 intratumoral injections of 10 μg of Allovectin-7 were performed over a 9 week period. The treatment was well tolerated and regression of the injected lesion was seen in 18% of patients, including a complete response and three partial responses [78]. A few years later, a phase II trial was described where 77 patients were treated with the same Allovectin-7 regimen; 9.1% had complete or partial responses with 4.8 months median duration of the response. Interestingly, this trial showed that the treatment was beneficial even in lesions distant to the treatment site [79].

More recently, a phase II dose escalation study was reported [80]. In this study, 127 patients received a total of 2 mg of plasmid DNA, some 10-times more than was used in the previous trials. Complete response was seen in four patients, 11 achieved partial response and the overall response rate was 11.8%. Stable disease was seen in 32 patients. Interestingly, the duration of response was quite extensive, 13.8 months, and evidence of a systemic effect, such as vitiligo and shrinkage of lesions distant to the treatment site, was seen in some patients. No toxicities above grade 2 were noted, but adverse effects were consistent with the expected pro-inflammatory response triggered by Allovectin-7 [80].

Variations of the HLA-B7 gene transfer strategy have also been explored. For example, the B7.1 cDNA has been inserted in a vaccinia virus. In this way, the virus introduces the HLA-B7 gene to a large number of tumor cells and the vector itself may participate in the anti-tumor immune

response. In a phase I trial involving 12 patients, intratumoral injection of the B7.1-armed vaccinia virus, termed rV-B7.1, yielded no serious adverse effects, 2 patients with vitiligo, partial objective response was seen in 1 patient and disease stabilization in 2. Patients developed an anti-vaccinia antibody response and 5 of 6 patients showed increase in gp100-specific T cells and 4 of 6 showed increase in MART-1-specific T cells. A patient with >59-month survival post-treatment showed an increase in both of the T cell subsets [81].

The Kaufman group also established a vaccinia vector encoding not only B7.1, but also the costimulatory molecules ICAM-1 and LFA-3 (intracellular adhesion molecule-1 and leukocyte function-associated antigen-1, respectively). This new vector was termed rV-TRICOM. In a phase I trial, side effects were limited to low grade reactions at the site of injection, yet there was a 20.7% objective clinical response. One patient showed a complete response over a 22 month period. Due to the lack of HLA-A*0201 patients, anti-tumor immune responses were not measured, yet anti-vaccinia responses were seen [82].

14. Interferon and interleukin gene transfer

Biochemotherapy, such as treatment with recombinant IL-2 or type I interferon, has shown some degree of success. However, these approaches are hampered by the high doses needed to overcome the short half-life of these proteins. Often, these high doses are come with unwanted systemic toxicities [83, 84]. As an alternative approach, gene therapy may offer the advantage of high concentrations of the therapeutic agent, but only localized to the tumor, thus avoiding systemic toxicity.

Clinical trials with adenoviral vectors expressing IL-2 have been performed. For example, Stewart et al [85] reported a lack of conventional clinical response, yet 24% of patients did show incomplete local tumor regression. They also noted increased CD3 and CD8-positive TILs [85]. In another example, a phase I/II trial included 25 metastatic melanoma patients and 10 patients with other solid tumors. Objective clinical responses, including 2 complete responses, were seen in 5 of the melanoma patients who received the higher dosage of vector. Serum levels of IL-2 were increased after treatment and were proportional to the vector dose used. Side effects were limited to flu like symptoms [86].

IL-24 (or MDA-7, melanoma differentiation associated gene-7) has been extensively reviewed and tested in a variety of pre-clinical models. IL-24 was isolated from human metastatic melanoma cells previously treated by the combination of interferon-beta and mezerein, a protein kinase C activator. This treatment induces a differentiated state that is associated with the differential expression of a number of interesting genes, IL-24 among them. IL-24 is a novel member of the IL-10 gene family and has been shown to depress growth and induce apoptosis specifically in transformed, but not normal or immortalized, cells. In addition, IL-24 has been associated with a potent bystander effect, implying that, in a gene therapy scenario, even non-transduced tumor cells may be killed [87, 88].

To the best of our knowledge, two phase I clinical trial has been reported involving IL-24 gene transfer to melanoma patients. In these studies, a non-replicating adenoviral

vector encoding IL-24, termed INGN 241, induced a systemic TH1 cytokine response. Objective responses, including complete response, was seen at higher vector dosages [89, 90]. Despite the melanoma origin of the IL-24 gene, it has been more extensively studied for other tumor types [87].

Gene therapy of melanoma with interferons has not been extensively explored, even though biochemotherapy with type I interferon is well known. One of the few examples includes adenovirus mediated transfer of the interferon-gamma gene. In this trial, 11 patients were treated with increasing vector doses. One grade 3 toxicity was observed and one patient showed stable disease [91]. Matsumoto et al [92] report the use of cationic liposomes complexed with a plasmid encoding the human interferon-beta gene in patients with metastatic melanoma. There were no recognized adverse events and 1 (of 5) patients showed mixed response [92].

Our own laboratories are actively developing novel tools for gene therapy of cancer, including melanoma. Since some 90% of melanoma cases retain wild type p53 [93], we reason that the endogenous p53 protein may be re-activated and induced to participate in the treatment scheme. To this end, we have developed viral vectors where the expression of the transgene is regulated by the transactivation function of p53. We have shown that these vectors offer superior levels of transgene expression [94-96] and the use of p53 or its functional partner, Arf (p14Arf in humans, p19Arf in mice) creates a positive feedback loop [95, 97]. That is to say, transgene function elevates p53 activity which, in turn, promotes expression from the vector and so on. Our published data show that p19Arf, when delivered by our p53-responsive vector, resulted in the sensitization of B16 mouse melanoma cells to pharmacologic agents both *in vitro* and *in vivo* [97]. In our unpublished data, we have combined the activities of the p53/Arf axis with intereferon-β. Though studies are ongoing, we have seen that the combined, but not individual, gene therapies induce massive cell death both *in vitro* and *in vivo*. In addition, immune protection is induced by either *in situ* tumor treatment or by a whole cell vaccine transduced by the gene combination.

15. Conclusion

We have cited several, but certainly not all, examples of how gene therapy is advancing for the treatment of melanoma. Though much has been learned, a long road remains before major successes can be had. As with any experimental approach, progress is incremental and may rely on joining distinct technologies. For example, the history of melanoma gene therapy actually coincides with therapies for immune disorders. But, by daring to test new therapeutic strategies, gene therapy of melanoma has paved the way for cancer gene therapy in general.

The future advances in melanoma gene therapy may come from combining pharmacologic and genetic approaches. For example, the vectors mentioned above may be used in combination with monoclonal antibodies (such as ipilumimab), small molecule inhibitors (such as vermuafenib, PLK4032, the inhibitor of BRAF) and chemotherapies (such as dacarbazine). Though much work remains to be done, it stands to reason that maximizing cell death and the anti-tumor immune response should offer hope for future treatments of melanoma.

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