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Alpha-1 Antitrypsin Deficiency: Recent Developments in Gene Therapy Research

Catherine M. Greene and Noel G. McElvaney
Royal College of Surgeons in Ireland, Education and Research Centre,
Beaumont Hospital, Dublin 9.
Ireland

1. Introduction

Alpha-1 antitrypsin (AAT) deficiency is a hereditary disorder associated with mutations in the *SERPINA1* gene (Kelly et al., 2008; Greene et al., 2008). Over 100 different alleles have been identified however the most common disease-causing mutation, termed Z, encodes a glutamic acid to lysine substitution at position 342 of the mature AAT protein. As a member of the serine proteinase inhibitor family, the role of AAT is to inhibit serine proteases throughout the body but principally in the lung. The ZAAT protein fails to adopt the correct protein conformation and polymerises and accumulates intracellularly in AAT-producing cells. The liver is the major source of the body's pool of AAT. The major consequences of ZAAT accumulation in hepatocytes are toxic gain of function events leading to endoplasmic reticulum (ER) expansion and dilation and activation of multiple ER stress signalling pathways (Lomas et al., 1992; Teckman & Perlmutter, 2000; Lawless et al., 2004; Hidvegi et al., 2005; Hidvegi et al., 2007; Miller et al., 2007). These predispose to liver failure. The second major clinical consequence of ZAAT deficiency is a lower than normal antiprotease protective screen throughout the body, but most importantly in the lung (Lomas et al., 1993). ZAAT deficient individuals can develop emphysematous lung disease as early as in their 4th decade.

Gene therapies to treat both aspects of the disease are currently at various stages of development. For the liver disease approaches that can be considered include ribozymes, antisense, peptide nucleic acids and small-interfering RNAs; all designed to inhibit expression of the mutant gene (recently reviewed in McLean et al., 2009). For the lung disease gene therapies using non-viral, lentiviral and adeno-associated viral approaches to express the normal gene either locally or intramuscularly have been reported (Chulay et al., 2011; Brantly et al., 2006; Flotte et al., 2007; Argyros et al., 2008; Brantly et al., 2009; Liqun Wang et al., 2009); all aim to increase AAT levels in the circulation above the deficiency threshold of 11 M. New approaches are focused on coupling haematopoietic stem cell therapy with AAT-lentiviral gene therapy (Ghaedi et al., 2010; Argyros et al., 2008). This chapter will review the history and current state-of-the-art in these areas.

2. Gene therapies targeting ZAAT-related liver disease

There are currently no available treatments for AAT deficiency-related liver disease other than transplantation. The 5-year survival is 83% for adults and 90% for children post

transplant (Kemmer et al., 2008). As an alternative to transplantation gene therapy approaches aimed at inhibiting ZAAT expression in the liver can potentially be used to stop the production of the mutant Z protein, hence prohibiting the accumulation of the protein in the liver and providing protection against liver disease. Such approaches include the use of ribozymes, small interfering RNA (siRNA) and small DNA fragments (SDF), and a number of these genetic approaches designed to downregulate ZAAT expression have been tested in animals as therapies for liver disease in AAT deficiency. To date there have been no reports on the use of zinc-finger nucleases (ZFN) or peptide nucleic acids (PNA) to treat ZAAT-related liver disease (Figure 1). ZFNs are artificial restriction enzymes that can be engineered to target specific DNA sequences and exploit a cell's DNA repair machinery to precisely alter the genome; PNAs are synthetic DNA analogues that hybridize to complementary DNA or RNA to facilitate anti-gene or antisense inhibition, respectively (Jensen et al., 1997; Pellestor & Paulasova, 2004). It has been suggested that if ZAAT levels can be decreased to those similar or lower than MZ AAT heterozygotes there may be clinical benefit (Cruz et al., 2007) as heterozygous individuals rarely develop severe liver disease, particularly in childhood.

2.1 Ribozymes

Ribozymes are catalytic RNA molecules capable of cleaving RNA with high specificity. Their catalytic properties were discovered almost 30 years ago (Kruger et al., 1982) and they are now known to contain both a catalytic RNA domain that cleaves a target mRNA and a substrate-binding domain that contains an antisense sequence to the target mRNA sequence that enables them to bind to their target mRNA sequence through Watson–Crick base pairing (Trang et al., 2004). The best characterised ribozymes have hammerhead or hairpin shaped active centres; both are promising gene-targeting reagents.

Hammerhead ribozymes capable of cleaving AAT mRNA have been constructed and were shown to be effective at inhibiting ZAAT expression in a human hepatoma cell line (Zern et al., 1999). In this study the hepatoma cells were also stably transduced with a modified AAT cDNA capable of producing wildtype AAT protein, but resistant to cleavage by the ZAAT-targetted ribozyme. Later a bi-functional vector was constructed, which contained both the ribozyme and the ribozyme-resistant AAT gene. Once transduced into hepatoma cells, the cells showed effective expression of the transduced AAT under conditions where the endogenous AAT gene was inhibited (Ozaki et al., 1999). Effective gene therapy for ZAAT deficiency requires stable transduction of resting hepatocytes, ideally to deliver both wild-type AAT and to inhibit production of ZAAT. Duan *et al.* treated transgenic mice expressing the human ZAAT allele with a simian virus 40 (SV40)-derived vector carrying a ribozyme designed to target the human transcript. The mice showed a marked decrease of human AAT mRNA and protein in the liver, and serum levels of human AAT were decreased by up to 50% 3-16 weeks after transduction (Duan et al., 2004).

Together these data show promise for the use of ribozymes for ZAAT-related liver disease. Importantly ribozymes bind to their targets with high specificity however there can be problems with their use to knockdown gene expression *in vivo* as their activity *in vivo* is not usually sufficient to achieve the desired effects.

2.2 Small interfering RNA

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing. It is initiated by short double-stranded RNA (dsRNA) sequences called small
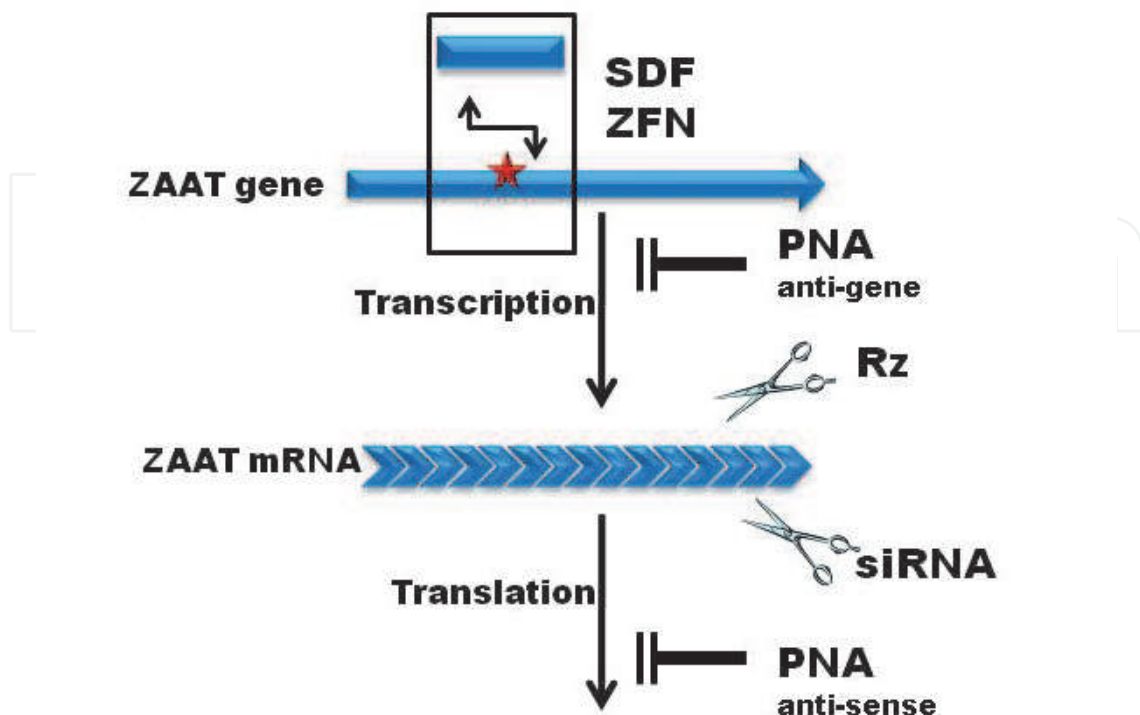
Fig. 1. Gene therapies for ZAAT-related liver disease. The Z mutation (shown by the red star) can be repaired using small DNA fragments (SDF) or zinc finger nucleases. Peptide nucleic acids (PNA) can inhibit the processes of transcription or translation whilst ribozymes (Rz) and siRNA target the mRNA and inhibit translation.

interfering RNA (siRNA) that are generated from longer transcripts by the enzyme Dicer. Each siRNA has specificity for a target RNA via its homology to sequences within the target gene (Elbashir et al., 2001). siRNAs form part of the RNA-induced silencing complex (RISC), a multi-component nuclease containing RNAse III, that enables the destruction of target mRNAs (Hammond et al., 2000). The argonaute protein within RISC incorporates one strand of an siRNA, known as the guide strand, and uses this as a template for recognizing complementary mRNA. Once found the target mRNA is cleaved by activation of the RNAse activity within RISC.

The delivery *in vivo* of siRNAs by liposomes or cationic lipids has been described. Other strategies for local or systemic administration of siRNA include electropulsation, delivery by viral vector, chemical modifications of siRNA molecules or polyamine or other encapsulation technologies. Targetting siRNAs directly to the liver can be achieved by local administration via injection through the hepatic portal vein and can prevent unwanted effects on normal host tissues. In 2007 studies describing the use of siRNAs designed to downregulate endogenous AAT within hepatocytes were reported. Using an adeno-associated virus (AAV) backbone three separate siRNA sequences were transduced singly or as a trifunctional construct into cell culture models. Whilst each siRNA had activity independently, the levels of AAT expression showed the greatest decrease, up to five-fold lower than controls, with the trifunctional construct. Following packaging of this construct

into an AAV serotype 8 capsid, and transduction into the livers of transgenic mice overexpressing human ZAAT, a decrease in total and monomer ZAAT levels coupled with reversal of ZAAT accumulation was evident within the liver up to 3 weeks after vector injection (Cruz et al., 2007).

2.3 Small DNA fragments

The use of small DNA fragments (SDF) for homologous replacement of mutant genes allows targets to be directly altered by insertion, deletion or replacement. Theoretically direct conversion of the mutant sequence to a wild-type genotype ensues, thereby restoring the normal phenotype. The basis of this technology involves the introduction of small DNA fragments into cells that recombine with the genomic DNA at a targeted site, thereby producing a specific change in the sequence. The feasibility of using SDF targeting the AAT gene has been reported. SDFs encoding the normal AAT sequence were generated and transfected *ex vivo* into peripheral blood monocytes from control and ZAAT deficient individuals. The SDFs corrected the defective gene in ZAAT monocytes and treatment was associated with an increase in AAT secretion. A similar methodology to repair the ZAAT gene defect in hepatocytes should have beneficial effects on secretion, and could ultimately lead to protection of both the lung and liver in ZAAT deficient individuals (McNab et al., 2007).

3. Gene therapies to target ZAAT-related lung disease

There have been a number of gene therapy trials for the treatment of AAT deficiency-related lung disease. The first used cationic liposomes encapsulating a plasmid carrying the human AAT cDNA (Brigham et al., 2000). In this open-label study the liposomes were administered intranasally to 5 ZAAT deficient individuals. The results showed that AAT levels were increased at day 5 following administration and returned to baseline by day 14. Subsequent studies have improved on this by using non-pathogenic human parvovirus recombinant AAV vectors that are more efficient at transgene delivery and are more capable of extended transgene expression.

3.1 Intramuscular adeno-associated viral gene delivery

In 2006 Brantly *et al.* reported their results from a phase I trial of intramuscular injection of a recombinant adeno-associated virus serotype 2 vector (rAAV2) carrying the human AAT gene into 12 AAT deficient adults (Brantly et al., 2006). Subjects were divided into groups of three and allocated to one of four dose cohorts ranging from 2.1×10^{12} to 6.9×10^{13} vector genomes. Each was injected into the deltoid muscle of their non-dominant arm sequentially in a dose-escalating fashion. No vector-related adverse events occurred in any of the participants although anti-rAAV2 capsid antibodies were present and rose after vector injection. In this study just one subject exhibited low-level expression of wild-type AAT in their serum (82 nM) up to 30 days after receiving an injection of 2.1×10^{13} vector genomes. Residual AAT levels from previous AAT protein replacement therapy was blamed for interfering with the detection of any possible vector expression in most of the other individuals.

Later in another phase 1, open-label, dose-escalation clinical trial sustained AAT expression was achieved using a rAAV serotype 1 vector (Brantly et al., 2009). rAAV1 is substantially more efficient than rAAV2 in transducing skeletal muscle (Cruz et al., 2007). Once again subjects were dosed via intramuscular (i.m.) injection and those subjects who had been

receiving protein therapy discontinued its use for 28 or 56 days prior to vector administration. In those who received 2.2×10^{13} and 6.0×10^{13} vector genome particles normal AAT was expressed above background in all subjects. AAT expression was sustained at levels 0.1% of normal for at least 1 year in the highest dosage level cohort. Vector administration was well tolerated and there were no changes in hematology or clinical chemistry parameters however neutralizing antibody and IFN-gamma enzyme-linked immunospot responses to rAAV1 capsid were evident in all subjects at day 14.

The most recent report on this gene therapy approach describes the results of a preclinical evaluation of a rAAV1 vector expressing human AAT made using a recombinant herpes simplex virus production method that can achieve much higher yields enabling a substantial increase in dosage in clinical studies (Chulay et al., 2011). The toxicology study in mice treated i.m. with this vector showed that the HSV-produced vector had favorable characteristics in terms of purity, efficiency of transduction, and human AAT expression.
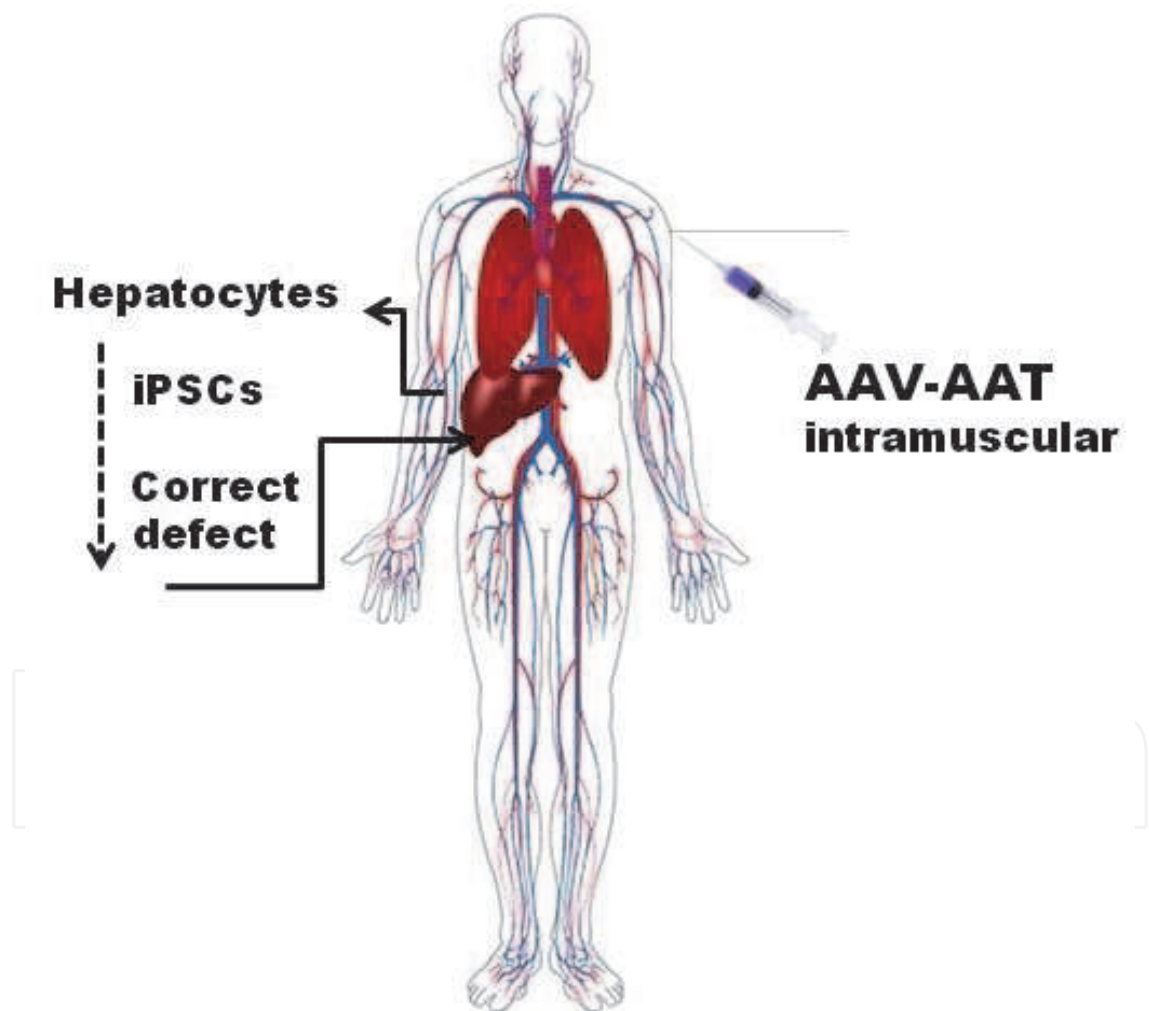

Fig. 2. Gene therapy strategies for ZAAT-related lung disease. The normal AAT gene can be introduced via intramuscular injection of an AAT-expressing adeno-associated virus (AAV) leading to production of normal AAT protein. Alternatively a patient's own cells can be harvested, induced to pluripotency (iPSCs) and the defect corrected *ex vivo* prior to autologous cell therapy.

Administration with this vector led to no significant differences in clinical findings or hematology and no gross changes in pathology although there were mild changes in skeletal muscle at the injection site. These consisted of focal chronic interstitial inflammation and muscle degeneration, regeneration and vacuolization in vector-injected animals. Vectors were detectable in blood 24 hours after dosing and declined thereafter, with no copies detectable 90 days after dosing. Antibodies to human AAT were detected in almost all treated animals, with antibodies to HSV detectable in most animals that received the highest vector dose. With higher doses of HSV-produced vector, the increase in serum human AAT levels was dose-dependent in females and, interestingly, greater than dose-proportional in males. Together all of these studies support continued development of rAAV1-AAT vectors for i.m. gene delivery for the treatment of AAT deficiency (Figure 2).

3.2 Viral gene delivery to the lung

Transduction of cells within the lung is an attractive approach for AAT gene therapy. Since somatic tissues are comprised of heterogeneous, differentiated cell lineages that can be difficult to specifically transfect (although this may have been largely overcome by the work of Flotte using i.m. injection (section 3.1)), methods have been tested to deliver gene therapies directly to cells within the lung. These include AAV and other viral methods to deliver the AAT gene directly to cells within the lung for local expression of AAT at the site where it is most needed to provide an antiprotease protective screen. Both integrating lentiviral systems and non-integrating AAV vectors are capable of gene transfer and expression *in vivo* in non-dividing cells and approaches using both viruses have been undertaken. Using recombinant AAV vectors Liqun Wang *et al.* evaluated the transduction and expression efficiencies of several rAAV serotypes and rAAV2 capsid mutants with specific pulmonary targeting ligands (Liqun Wang et al., 2009). Noninvasive intranasal delivery was compared with intratracheal lung delivery into the mouse lung. Of all the vectors tested they found that recombinant rAAV8 was the most efficient serotype at expressing AAT in the lung. Intratracheal administration was superior to intranasal delivery for most vectors. Limberis *et al.* also characterized a wide range of vectors and nine different serotypes of rAAV for their ability to transduce mouse and human ciliated airway epithelium (Limberis et al., 2009). In their studies rAAV5 and rAAV6 were found to be the most efficient at transducing mouse epithelium whilst only rAAV6 was as effective in human cells. Variants of AAV6 with targeted mutations were also tested. One of these, rAAV6.2, was more efficient than all the other rAAV vectors tested and shows promise for development and preclinical testing. In a separate AAT-rAAV6 study involving delivery to the lungs of mice and dogs, administrations led to increased and extended intrapulmonary AAT levels, however the animals developed immune responses including a lymphoproliferative response to the AAV capsid (Halbert et al., 2010). Another approach using intratracheal instillation of an AAT lentiviral system led to successful selective delivery of the AAT transgene to alveolar macrophages in the mouse lung (Wilson et al., 2010). These cells persisted in the alveoli and expressed AAT for the lifetime of the adult mouse and achieved localized secretion of therapeutic levels of AAT protein into the airway epithelial lining fluid. Using a mouse model of emphysema this approach ameliorated the progression of emphysema without inducing humoral or cellular immune responses.

3.3 Stem cell therapy and other approaches to gene delivery

An ideal gene therapy approach should enable persistent transgene expression without limitations of safety and reproducibility. Using an individual's own cells, cultured *in vitro* as

the delivery platform for gene therapy is a new approach that can potentially circumvent these problems. The use of stem cells, either the induced pluripotent form or hematopoietic/mesencymal derived, has been considered for AAT gene therapy. For example, lentivirally transduced murine hematopoietic stem cells (HSCs) expressing a human AAT transgene have been developed (Wilson et al., 2008). Once transplanted into irradiated mouse recipients these HSCs maintained their multipotency, self-renewal potential, and could reconstitute the hematopoietic systems of both primary and secondary recipients. Furthermore these lentivirally-modified cells generated sustained and systemic expression of AAT *in vivo* for over 30 weeks. Other genetically modified mesenchymal stem cells (MSCs) have been used that, once differentiated into hepatocyte-like cells, offer a potentially unlimited source of cells for autologous transplant procedures (Ghaedi et al., 2010). These human MSCs were transduced with a lentiviral AAT expression vector and were shown to express major hepatocyte marker genes and AAT. This technology has potential as an *in vitro* source of cells for transplantation therapy of liver diseases in AAT-deficient patients. Another angle on this approach is being developed by Li *et al.* (Li et al., 2010). They assessed the feasibility of bone marrow (BM) cell-based liver gene delivery of human AAT. Their *in vitro* studies showed that both lentiviral and rAAV vectors expressing AAT can be transduced into these cells and transplanted into the liver where they differentiate into hepatocytes and express AAT, resulting in sustained levels of circulating AAT in the recipient mice. In another study they used adipose tissue as an abundant, accessible and replenishable source of adult stem cells (Li et al., 2011). Adipose tissue-derived mesenchymal stem cells (AT-MSCs) resemble bone marrow-derived mesenchymal stem cells (BM-MSCs). Having investigated the feasibility of AT-MSC-based liver gene delivery for the treatment of AAT deficiency by transducing mouse AT-MSCs with an rAAV1 vector carrying the human AAT gene, it was shown that when the cells were then transplanted into the mouse liver that they expressed human AAT. Importantly, no anti-human AAT antibodies were detected.

Human induced pluripotent stem cells (iPSC) also hold great promise for cell-based therapy and in cell studies of genetic diseases. A strategy using dermal fibroblasts from AAT-deficient individuals has generated a library of patient-specific human iPSC cell lines (Rashid et al., 2010). Once differentiated into hepatocytes the resulting cells exhibit properties of mature hepatocytes and recapitulate key pathological features of AAT deficiency including accumulation of misfolded AAT in the endoplasmic reticulum. Using an approach such as this it may be possible to generate iPSC hepatocytes from an AAT-deficient individual, repair the defect *in vitro* with for example SDFs, ribozymes or zinc finger nucleases and transplant these 'corrected' cells back into the original donor (Figure 2). The next step in advancing this technology will be to achieve this without the worry of endogenous effects mediated by reprogramming transgenes. A number of methods already exist for generating murine or neonatal iPSCs free of reprogramming transgenes however, until recently this was not the case for disease-specific iPSC from humans with inherited or degenerative diseases. Now a humanized version of a single lentiviral "stem cell cassette" vector to accomplish efficient reprogramming of normal or diseased skin fibroblasts obtained from humans has been developed (Somers et al., 2010). The human iPSCs generated using this vector contain a single viral integration that can excise to generate human iPSCs free of integrated transgenes. As a proof of principle, this strategy has been

used to generate lung disease-specific iPSC lines from individuals with AAT deficiency-related emphysema. These cells have the ability to differentiate into developmental precursor tissue of lung epithelia and will prove invaluable for AAT-deficiency related investigations. However the most exciting aspect of this technology is its potential to generate safer autologous 'corrected' cell therapies similar to those described above.

It is sometimes suggested that non-viral episomal plasmid DNA (pDNA) vectors may offer some advantages over viral vectors in that they can be produced cheaply and in large quantities. Argyros *et al*. developed such a vector using the human AAT promoter to drive expression of a luciferase gene and achieve long-term expression in murine liver (Argyros et al., 2008). Over the course of the study the vector was maintained as an episome and did not replicate. Luciferase expression was detectable up to 6 months and this is believed to be by means of a unique scaffold/matrix attachment region (S/MAR) element that inhibits methylation of the AAT promoter.

4. Conclusion

Since the first human gene therapy trials using lymphocytes for the cellular delivery of the adenosine deaminase gene to patients with severe combined immune deficiency (SCID) and as tumour-infiltrating vehicles there has been a remarkable expansion in the development of gene therapy strategies (Culver et al., 1991a; 1991b; 1991c). The most promising data have come from therapies targeting monogenic disorders. With respect to ZAAT deficiency, rAAV2 vectors, and more recently rAAV1 due to its higher tropism for muscle cells, have shown great promise for delivery of the AAT gene. The advantages of AAV vectors include not only their lack of acute pathology but also their episomal nature. Occasional problems with adaptive immune responses to its capsid proteins have been encountered in some contexts (Manno et al., 2006), however newer generation vectors with greater safety and efficiency of gene transfer are constantly under development. Non-AAV gene therapy strategies for AAT-related lung disease, although less advanced, also show potential. Genetic therapies designed to down-regulate expression of ZAAT in hepatocytes to reverse the toxic gain of function in the liver are also less well developed, however the next decade is likely to see huge progress in this area. Finally the recent advent of various stem cell technologies can only enhance efforts in ZAAT deficiency and gene therapy research.

5. Acknowledgments

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Gene Therapy Applications

Edited by Prof. Chunsheng Kang

The aim of our book is to provide a detailed discussion of gene therapy application in human diseases. The book brings together major approaches: (1) Gene therapy in blood and vascular system, (2) Gene therapy in orthopedics, (3) Gene therapy in genitourinary system, (4) Gene therapy in other diseases. This source will make clinicians and researchers comfortable with the potential and problems of gene therapy application.

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