ApoE gene therapy to treat hyperlipidemia and atherosclerosis

Julian D Harris*, Vanessa Evans & James S Owen

Address
Royal Free & University College Medical School
The UCL Institute of Hepatology
Hampstead Campus
Upper 3rd Floor
Rowland Hill Street
London NW3 2PF
UK
Email: j.harris@medsch.ucl.ac.uk

*To whom correspondence should be addressed

Current Opinion in Molecular Therapeutics 2006 **8**(4):
 ISSN 1464-8431

Atherosclerosis is the leading cause of death in industrialized countries and is becoming an increasingly worldwide risk to health. Apolipoprotein E (ApoE) is a blood circulating protein with pleiotropic atheroprotective properties that has emerged as a strong candidate for treating hypercholesterolemia and cardiovascular disease. In this review, we discuss the major developments in both viral and non-viral vectors aimed at achieving efficient delivery and sustained expression of an ApoE transgene. The technological advances in engineering viruses include cross-packaging to generate different serotypes of recombinant adeno-associated virus, and the use of multiple-deleted and helper-dependent recombinant adenovirus vectors to minimize immune responses and to package genomic loci. Non-viral ApoE delivery systems are also described in this review, including plasmids and cell-based therapy. Finally, a radical, alternative to gene addition that has the potential for permanent cure in many genetic diseases – 'targeted gene editing' – is reviewed. This technology uses synthetic oligonucleotides to correct underlying point mutations in situ and has been evaluated for repairing dysfunctional APOE genes.

Keywords Adeno-associated virus, adenovirus, ApoE, cardiovascular disease, gene repair, hypercholesterolemia, lipoprotein

Introduction

One-third of deaths worldwide are from cardiovascular disease (CVD), totaling 16.7 million deaths per year in contrast to just 5.7 million deaths from AIDS, malaria and tuberculosis combined [1]. Significantly, only approximately 20% of CVD deaths are in high-income countries; the vast majority of CVD deaths occur in low- and middle-income countries. Atherosclerosis is a progressive inflammatory response to complex interactions between cells endogenous to the arterial wall, and plasma lipoproteins. Such cells include endothelial cells and smooth muscle cells, blood monocytes, lymphocytes and platelets, [2]. Early atherosclerotic lesions are lipid streaks, characterized by cholesterol-loaded foam cells within the vascular endothelium. Foam cells originate from blood monocyte macrophages. These cells are recruited into the arterial intima by upregulated adhesion molecules on activated endothelium, where they relentlessly ingest oxidized low-density lipoprotein (LDL) or cholesterol-rich remnant lipoprotein particles via unregulated scavenger receptors. Later, smooth muscle cells infiltrate the arterial intima and their proliferation within the intima fosters the development of advanced lesions.

Unsurprisingly, a significant risk factor for the development of atherosclerosis is raised plasma LDL, which is prone to oxidation and is the main source of cholesterol accumulating in the arteries during endothelium injury. The statin class of drugs, often in combination with the cholesterol absorption inhibitor ezetimibe, helps prevent early morbidity or death by lowering plasma cholesterol [3•]. However, these drugs do not rectify low levels of atheroprotective high-density lipoproteins (HDL), an equally important risk factor for the development of this condition, and rarely regress atherosclerotic lesions. Alternative strategies to combat occlusive CVD are still urgently needed for many patients [4•].

Somatic gene therapy promises a treatment and cure for many genetic, endocrine and metabolic diseases, including atherosclerosis, where a gene is defective or where its overexpression is therapeutic. Circulating proteins are attractive targets for genetic manipulation. Plasma apolipoprotein E (ApoE) is a 34-kDa polymorphic glycoprotein largely secreted by liver (~ 90%), although other tissues including macrophages contribute to its secretion. The rarest isoform, ApoE2 differs from wild-type ApoE3 by an Arg158Cys substitution and causes recessive Type III hyperlipidemia, while ApoE4 (Cys112Arg) produces a dominant hyperlipidemia and is a significant risk factor for CVD [5•,6•]. The present review will discuss ApoE3 as a candidate gene therapeutic to counteract dysfunctional ApoE isoforms and also, importantly, for the widespread treatment of hyperlipidemia and atherosclerosis. The integral part that ApoE3 plays in maintaining cholesterol and lipoprotein homeostasis will be summarized, before highlighting its diverse lipid-independent functions, which help protect against atherogenesis. Mouse models of ApoE deficiency will be described together with their potential for the preclinical testing of ApoE3 gene therapeutics, before summarizing the indirect evidence for the benefits of ApoE gene therapy. Finally, the viral and non-viral delivery systems that have been used for ApoE gene transfer will be described, as well as gene repair strategies for correcting the dysfunctional ε2 and ε4 alleles.

Atheroprotective functions of ApoE related to lipid metabolism

ApoE is intimately involved in the metabolism of dietary lipids, which enter the circulation as large triglyceride-rich chylomicron particles. Following lipolysis, cholesterol-rich and potentially atherogenic remnants are left, which depend on ApoE for rapid hepatic clearance via the LDL receptor (LDLR) and LDL-receptor-related protein (LRP). Although ApoE3 and ApoE4 have similar binding capabilities, ApoE2 is defective with only 2 and 40% binding activity to the LDLR and LRP, respectively. This function is verified by the gross hypercholesterolemia and premature atheroma seen in ApoE-deficient (ApoE$^{-/-}$) mice, or in transgenic mice expressing dysfunctional ApoE [7•]. ApoE also has several contributory roles in 'reverse cholesterol transport', the atheroprotective HDL-dependent pathway through which

excess cholesterol in peripheral tissues, including arteries, is carried to the liver for biliary excretion. Such maintenance of cholesterol homeostasis in cells, particularly macrophages, is fundamental to preventing foam cell formation and atherogenesis. Efficient efflux from cells depends on the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, but 'lipid-poor' ApoAI- and ApoE-containing particles (pre-β1 HDL and γ-LpE, respectively) are avid initial acceptors of cellular cholesterol. As macrophages secrete ApoE, but not ApoAI, at lesion sites, ApoE may be the dominant acceptor in clearing cholesterol from arteries. Furthermore, ApoE can facilitate HDL maturation by activating lecithin-cholesterol acyltransferase, cholesteryl ester transfer protein (CETP) and hepatic lipase, and may also stimulate the selective uptake of HDL-C esters via the hepatic scavenger receptor B1 [6•].

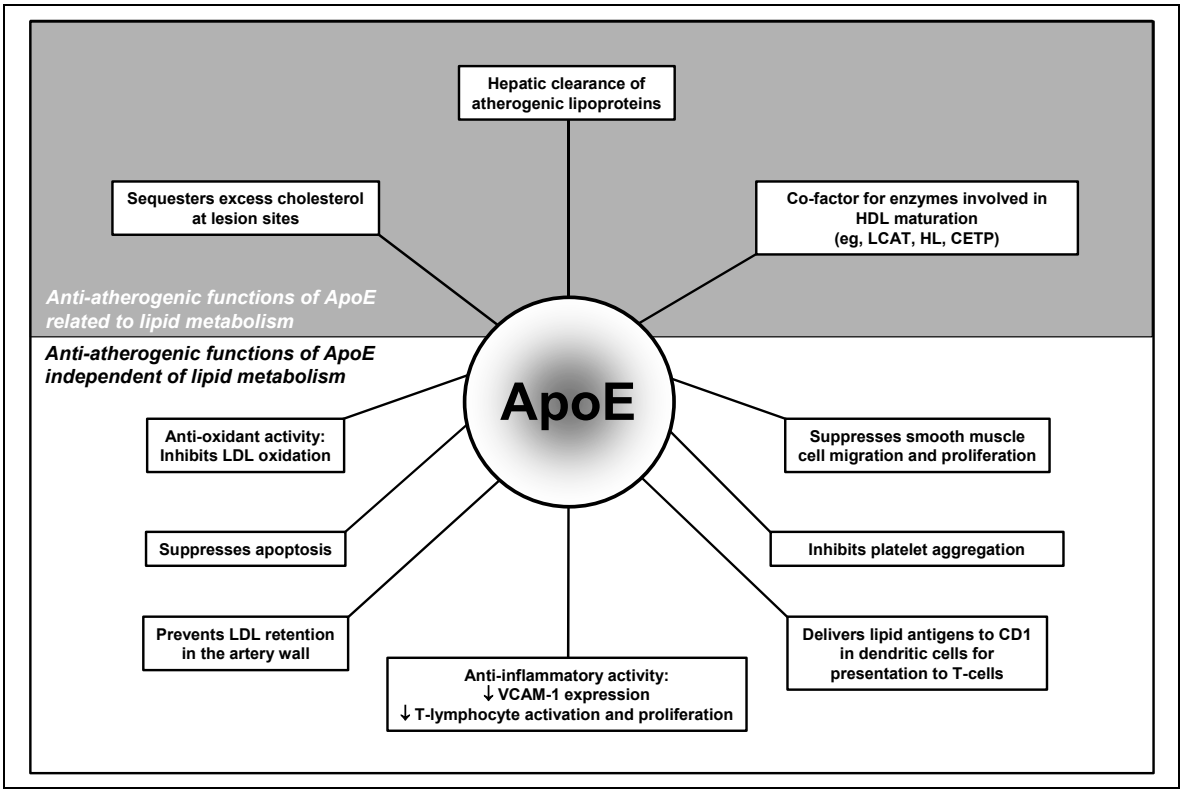
Atheroprotective functions of ApoE unrelated to lipid metabolism

Several biological functions of ApoE unrelated to lipid transport have been identified, which are increasingly recognized as making significant contributions to its anti-atherogenic activity [5•,6•,8•] (Figure 1). These include ApoE-mediated inhibition of platelet aggregation [9] and smooth-muscle-cell migration and proliferation [10•], as well as diverse actions to suppress different stages of the inflammatory response [11-13]. ApoE is also reported to impede subendothelial LDL retention, which is an early pro-atherogenic event [14], and to protect against oxidative stresses [15] and apoptosis [16]. The pathophysiological significance of these observations is supported by transgenic mice studies in which low-level ApoE expression provides atheroprotection in ApoE$^{-/-}$ animals without reducing hyperlipidemia [8•,17•]. Importantly, Raffai *et al* have provided compelling evidence that ApoE also induces regression of pre-existing atherosclerotic lesions, independently of lowering plasma cholesterol [18••].

Mouse models of ApoE deficiency

Mice resist atherosclerosis, and the most susceptible strain, C57BL/6J, develops only small immature fatty streak lesions with largely lipid-laden foam cells, when fed a pro-atherogenic diet [7•]. Hence, despite their many advantages, the use of mice in atherosclerosis research remained limited until the availability of genetically modified animals. A key impetus was the generation of ApoE$^{-/-}$ mice, which are grossly hypercholesterolemic on a normal chow diet and spontaneously develop widespread fibroproliferative lesions [19••]. These early lesions evolve into advanced complex plaques with smooth muscle cell caps and necrotic cores – a pathological progression that parallels the human disease. Indeed, on moderately fatty diets ApoE$^{-/-}$ mice exhibit atherosclerotic plaque rupture [20•], the principal cause of coronary artery thrombotic occlusion and subsequent myocardial infarction in humans. This animal has emerged,

Figure 1. The multiple atheroprotective functions of ApoE.



The anti-atherogenic properties of ApoE are divided into those related (shaded area) and those unrelated to lipid metabolism (unshaded area). **CETP** cholesteryl ester transfer protein, **HDL** high-density lipoprotein, **HL** hepatic lipase, **LCAT** lecithin-cholesterol acyltransferase, **LDL** low-density lipoprotein, **VCAM** vascular cell adhesion molecule.

therefore, as the premier model of human atherogenesis, most recently for gene expression profiling during plaque progression [21] and for proteomic/metabolomic analyses of atherosclerotic vessels [22]. In addition, as described in the following section, ApoE$^{-/-}$ mice are an excellent preclinical model for evaluating the therapeutic potential of ApoE gene transfer in human genetic dyslipoproteinemias.

Transgenic mice that overexpress common and rare isoforms of human ApoE have been produced [7•]. Although recessive Type III hyperlipoproteinemia is seen in humans homozygous for the *APOE2* gene, dominant inheritance occurs with several rare ApoE variants, including ApoE3$_{Leiden}$ where a single defective allele is sufficient for development of the condition. Like ApoE2, ApoE3$_{Leiden}$ is binding-defective because an extra seven-amino-acid tandem repeat sequence at residues 121 to 127 causes a conformational change in the protein. However, while ApoE3$_{Leiden}$ transgenic mice are hyperlipidemic and very responsive to high-fat diets, ApoE2 transgenics exhibit the hyperlipidemic phenotype only in the absence of endogenous mouse ApoE. Thus, in mice, the ApoE2 and ApoE3$_{Leiden}$ mutations behave as recessive and dominant inheritances, respectively, which parallels the situation in patients [7•]. Knock-in mice in which the endogenous mouse ApoE gene has been replaced with one of the common human ApoE isoforms have also been created. Human ApoE3 and ApoE4 homozygous mice were essentially normolipidemic, though less able to resist diet-induced atherosclerosis than wild-type mice [23,24]. However, mice expressing human ApoE2 had many characteristics of Type III hyperlipoproteinemia, including defective clearance of remnant lipoproteins and spontaneous development of atherosclerosis on a normal chow diet [25].

ApoE gene therapeutics: Gene transfer vector systems

Bolus infusions of plasma-purified or recombinant ApoE protein markedly reduced plasma cholesterol levels in rabbits with genetic or diet-induced hypercholesterolemia, while a longer-term study prevented progression of atherosclerosis [26•]. This early preclinical evidence endorses the human *APOE* gene as a strong candidate for therapeutic manipulation. Similarly, synthetic peptide mimics of the ApoE-binding region cleared cholesterol-rich lipoproteins in ApoE$^{-/-}$ mice [27•], while transgenic mice overexpressing ApoE were protected from diet-induced or diabetic hyperlipidemia [28,29]. In addition, macrophage-restricted expression of ApoE in transgenic mice [30], or in ApoE$^{-/-}$ mice following transplantation of wild-type bone marrow [31••,32••], has also been shown to inhibit atherogenesis. Taken together, these data provide indirect support for developing systems that could allow for the therapeutic gene delivery of ApoE to treat hyperlipidemia and atherosclerosis.

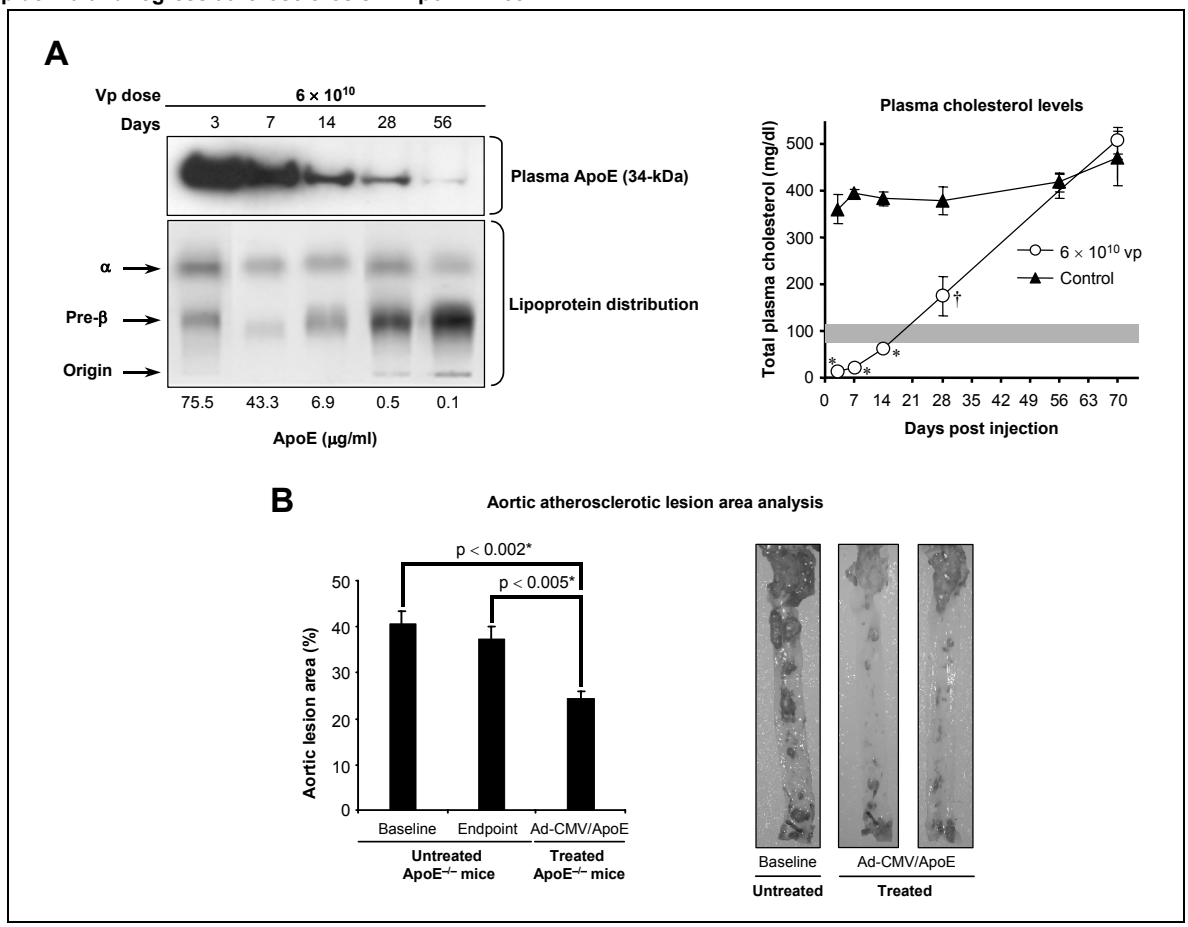
Recombinant adenovirus vectors

The first ApoE gene transfer studies were reported ten years ago using recombinant adenovirus (rAd) serotype 5 vectors. These vectors produced substantial amounts of plasma ApoE following intravenous injection into ApoE$^{-/-}$ mice, which lowered plasma cholesterol and after 1 month had slowed aortic atherogenesis [33•]. Adenoviruses consist of a non-enveloped, icosahedral capsid containing a linear double-stranded DNA genome that exists and propagates predominantly as extrachromosomal episomes. Although rAd vectors have wide tissue tropism, and infect both dividing and quiescent cells, they routinely deliver foreign DNA sequences into mammalian liver with near 100% efficiency following systemic administration. Unfortunately, the therapeutic effect of these first-generation vectors, which only had deletions in no more than two early genes (E1 and E3), was transient; a strong cytotoxic T-cell immunological response was triggered by residual low-level viral gene expression. This immune response led to clearance of transduced hepatocytes and precluded repeat vector administration. To circumvent this barrier, ApoE$^{-/-}$ and immunodeficient mice were cross-bred and then injected with a rAd vector expressing human ApoE3; not only was high plasma cholesterol normalized and atherogenesis halted, but 6 months after treatment the atherosclerotic plaque was virtually absent [34••]. This study confirmed the potential of ApoE gene therapy to regress advanced atherosclerosis.

Second-generation rAd vectors contain additional viral genome sequences deleted or inactivated in other early genes (E2 and/or E4) and show reduced toxicity and prolonged transgene expression. One such vector has a temperature-sensitive mutation in the E2A region and has been shown to allow sustained human ApoE expression, which normalizes the plasma lipoprotein profile in ApoE$^{-/-}$ mice [35]. Significantly, such liver-secreted ApoE gains access to the arterial intima, where it can potentially sequester excess cellular cholesterol and exert its diverse non-lipid atheroprotective actions [36•]. Indeed, in a subsequent study, hepatic expression of human ApoE3 using this second-generation rAd vector directly induced regression of pre-existing atherosclerotic lesions without reducing plasma cholesterol or altering lipoprotein distribution. As this ApoE gene transfer strikingly reduced isoprostane levels, which are reliable markers of lipid peroxidation and oxidative stress *in vivo*, it was concluded that the intrinsic antioxidant properties of ApoE were important contributors to its atheroprotective action [37•].

Deletion of the adenoviral polymerase gene is reported to minimize virus-derived gene expression [38••,39••] and further refinements of this second-generation vector include an additional deletion of the pre-terminal protein (pTP) gene from the rAd genome [40•]. We have used the [E1-, E3-, polymerase-] rAd vector with the strong cytomegalovirus (CMV) promoter to drive human ApoE3 expression [38]. High levels of plasma ApoE were achieved initially, which normalized the hyperlipidemia in ApoE$^{-/-}$ mice (Figure 2A) and retarded early aortic lesions. Advanced atheroma in older animals could also be acutely regressed (Figure 2B). Nevertheless, ApoE transgene expression remained transient, as reflected in a rebound of the plasma cholesterol concentration and a return to a hyperlipidemic phenotype (Figure 2A). This effect was not due to an immune response, but to shutdown of the CMV promoter [41]. When the ubiquitous elongation factor-1α mammalian promoter was used [42], or a liver-specific promoter to drive human ApoE3 expression in the [E1-, E3-, polymerase-, pTP-] rAd vector [43], no attenuation of plasma ApoE was noted and, though the concentrations reached were much lower, this sustained transgene expression produced significant retardation of atherosclerosis. These findings support the therapeutic potential of polymerase-deleted or polymerase/pTP-deleted rAd vectors.

Figure 2. Ability of liver-directed administration of a [E1-, E3-, polymerase-] rAd vector expressing human ApoE to reduce hyperlipidemia and regress atherosclerosis in ApoE$^{-/-}$ mice.



(**A**) Young mice (6 to 8 weeks) were injected intravenously with Ad-CMV/ApoE (6×10^{10} virus particles [**vp**]; n = 4) and their plasma was analyzed at varying times for ApoE (Western blotting and ELISA), lipoprotein distribution (agarose gel electrophoresis) and total cholesterol. Lipoprotein distribution represents the electrophoretic separation, from the origin, of lipoprotein subclasses α and pre-β by their electrophoretic migration properties. The shaded horizontal bar in the graph indicates the range of plasma cholesterol levels found in wild-type mice (90 ± 13 mg/dl; mean ± SD), while differences between the Ad-CMV/ApoE-treated group and untreated ApoE$^{-/-}$ mice were analyzed by Student's unpaired *t*-test: **†** = $p < 0.05$; * = $p < 0.001$. (**B**) Old ApoE$^{-/-}$ mice (10.5 months) were similarly injected (2×10^{10} vp; n = 4) and 4 weeks later their aortae stained *en face* for lipid deposition using Oil Red O. The percentage lesion area (mean ± SEM) was compared with untreated mice at baseline (n = 5) and endpoint (n = 4) by Student's unpaired *t*-test; representative stained aortae are also shown, to the right of the bar chart. Additional details may be found in reference [41]. **Ad** adenovirus, **ApoE** apolipoprotein E, **CMV** cytomegalovirus.

Nevertheless, the introduction of helper-dependent rAd (HD-rAd) vectors [44•], in which all viral genes are deleted, has been the most significant advance to reduce rAd vector-related toxicity and immunogenicity *in vivo* [45•,46•,47••]. Although these HD-rAd vectors, sometimes termed 'last generation' or 'gutless' rAd vectors, still induce innate immune responses against the vector capsid, they display prolonged transgene expression because they are devoid of all viral coding sequences. This feature removes the trigger for the adaptive immune response that would otherwise lead to complete clearance of rAd-transduced cells. In addition, HD-rAd vectors can accommodate 36 kb of DNA, allowing for the incorporation of transgene cassettes containing genomic loci, which generally outperform cDNA expression constructs [45•,46•,47••]. Thus, a single injection of an HD-rAd vector carrying the mouse ApoE genomic locus into ApoE$^{-/-}$ mice gave high-level stable expression of ApoE and provided lifelong (~ 2.5 years) correction of genetic deficiency, whereas an HD-rAd carrying an ApoE cDNA expression cassette was less effective, giving lower plasma ApoE which slowly declined over time, resulting in partial rebound to the hypercholesterolemic phenotype [47••]. Intriguingly, HD-rAd vectors also show episomal persistence compared with first-generation vectors, while their administration by systemic hydrodynamic high-volume injection increases hepatic transduction with minimal dissemination to other tissues/organs and gives a less severe innate inflammatory response [48]. This observation suggests that liver-directed gene therapy may be improved if vector dissemination is preferentially, if not exclusively, restricted to liver. Although systemic hydrodynamic delivery to the liver is not feasible in a clinical setting owing to a cardiovascular risk, it may be possible to restrict vector delivery by surgically isolating the

liver (49), or by direct administration via branches of the portal vein [50•]. As yet, however, clinical trials are not possible until HD-rAd vectors can be produced completely free of helper virus, which is currently required to supply *in trans* the essential viral proteins needed for genome replication, packaging and capsid formation [44•].

Recombinant adeno-associated virus vectors

Adeno-associated viruses (AAV) are a family of replication-defective parvoviruses containing a linear single-stranded DNA genome. Wild-type AAV integrates into the AAVS1 site located on human chromosome 19q using the nicking activity of viral Rep68/78 proteins [51]. Recombinant AAV (rAAV) is considered the safest viral vector. Wild-type AAV is not associated with human disease and rAAV rarely integrates and forms in target cells intermolecular concatemers that are responsible for long-term episomal persistence and stable transgene expression [52,53]. Indeed, in muscle, high and stable transgene expression is reported without an immune response. Nonetheless, when we injected a rAAV vector expressing human ApoE3 into the TA muscle of ApoE$^{-/-}$ mice, we were unable to detect ApoE in plasma, although antibodies against the protein were evoked. This gene transfer did not affect the hyperlipidemia, but nevertheless did significantly reduce atherosclerotic plaque density [54•]. These findings suggest that low levels of circulating ApoE can protect against atherosclerosis progression. This study utilized a rAAV vector derived from AAV serotype 2, which transduces cells very efficiently *in vitro*. More recently, rAAV vectors based on alternative AAV serotypes have been evaluated and show great promise to significantly improve transduction efficiencies *in vivo*.

To date, 11 different serotypes (designated AAV1 to AAV11) and over one hundred genomic variants of AAV from human and non-human primate samples have been identified. Their isolation has led to the development of new pseudotyped rAAV vectors; specifically, capsids of alternative AAV serotypes harboring a rAAV serotype 2 genome. These vectors have strikingly improved performances *in vivo* and tissue tropisms are largely different from AAV serotype 2. Such vectors have allowed effective transduction of therapeutically relevant targets, including rAAV2/8 to transduce liver [55••,56,57] and rAAV2/1, rAAV2/6, rAAV2/7 and rAAV2/8 to transduce muscle cells [55••,58-60]. For example, liver-directed administration of an rAAV2/8 vector encoding human ApoE into ApoE$^{-/-}$ mice produced normal human levels (50 to 80 μg/ml) of plasma ApoE [12]. In contrast to serotype 2, these different AAV serotypes uncoat more rapidly, a feature thought to account for their greater transduction efficiencies, as it enhances annealing of single-stranded plus and minus rAAV genomes into stable, transcriptionally active double-stranded DNA molecules [61]. Similarly, the introduction of self-complementary rAAV (scAAV) vectors, which circumvent the need for annealing of rAAV vector genomes (Figure 4), allows a more rapid and higher level of transgene expression [62••,63•].

These important advances, and the realization that neutralizing antibodies against the newly discovered AAV serotypes occur less frequently than against serotype 2 [55••], have provided the impetus to instigate clinical trials using different serotypes. Anion-exchange chromatography has been developed to purify the different AAV serotypes [64•]. This process produces biologically potent rAAV stocks and is readily scaled-up, which facilitates the transition to extensive *in vivo* studies (including larger animals and non-human primates) that are a prerequisite to well-designed clinical trials. The use of insect cells, rather than human HEK293T cells, has also been suggested to produce pseudotyped rAAV vectors, as this system is amenable to large-scale clinical manufacturing [65•]. However, one concern prior to widespread clinical assessment, is the possibility of rAAV chromosomal integration. This event occurs at very low frequency compared with episomal retention, but in mouse liver rAAV2/2 is reported to preferentially integrate into active genes [66]. Whether this holds true for other serotypes or other animals is uncertain, although interestingly, rAAV integrants could not be detected following rAAV2/2-mediated transduction of skeletal muscle [67].

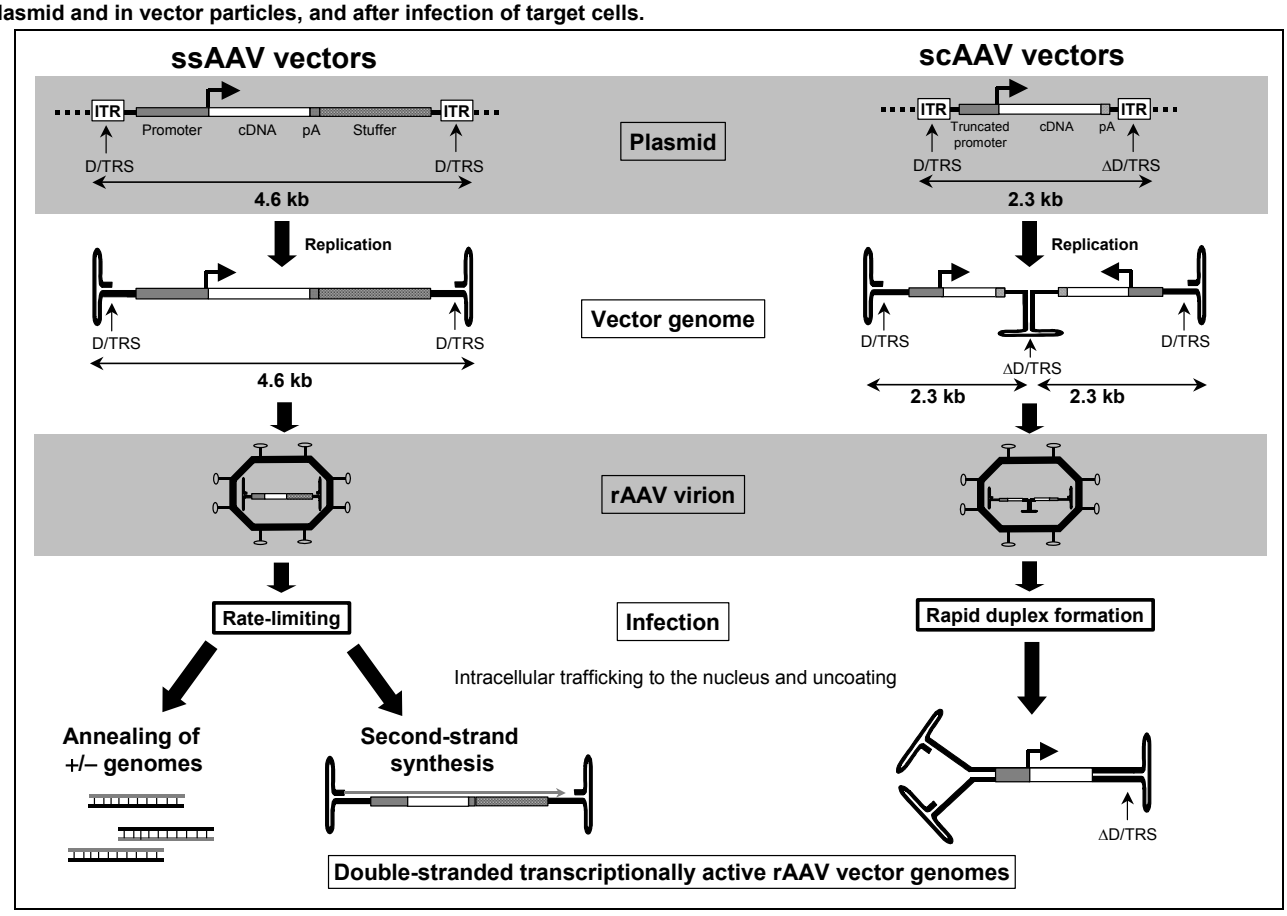
Non-viral gene transfer

Plasmids

Plasmids have low immunogenicity, can be manufactured in quantity and to high purity, and are expressed episomally, thereby making insertional mutagenesis highly unlikely. Skeletal muscle is highly vascularized and is an effective secretory platform for circulating proteins by gene transfer; it represents an alternative therapeutic target to liver, being an accessible and stable tissue with little nuclear turnover. Although muscle does not normally secrete ApoE, non-hepatic-, non-macrophage-derived ApoE is known to be atheroprotective [17•,68]. Indeed, intramuscular injection of a naked plasmid DNA vector expressing human ApoE3 into ApoE$^{-/-}$ mice, gave modest, but sustained lowering of plasma cholesterol [69]. Moreover, our own plasmid injections inhibited formation of xanthoma and atherosclerotic plaque, although we found no reduction in hyperlipidemia [70].

Skeletal muscle fibers are surrounded by several structures, including an extracellular matrix and connective tissue, which form barriers against efficient plasmid transfer. However, hyaluronidase pre-treatment of muscle in conjunction with electropulsing (electroporation) can markedly improve plasmid transfection [71]. We assessed this combination by injecting a plasmid vector containing a CMV enhancer/chicken β-actin (CAG) promoter to drive human ApoE3 expression into tibialis anterior (TA) muscles of ApoE$^{-/-}$ mice. Western blotting revealed ApoE protein only in electropulsed muscle (Figure 3A). Two muscle-specific promoters (CK6 and C512) were also tested, but produced significantly less ApoE than the CAG vector (Figure 3B). Moreover, the hyperlipidemia was not ameliorated in these preliminary experiments, despite enhanced ApoE expression by electrotransfer, suggesting that a more efficient delivery vector is needed to reach full therapeutic levels of plasma ApoE.

Figure 3. Comparison of recombinant self-complementary AAV and conventional single-stranded AAV vector genomes in the plasmid and in vector particles, and after infection of target cells.



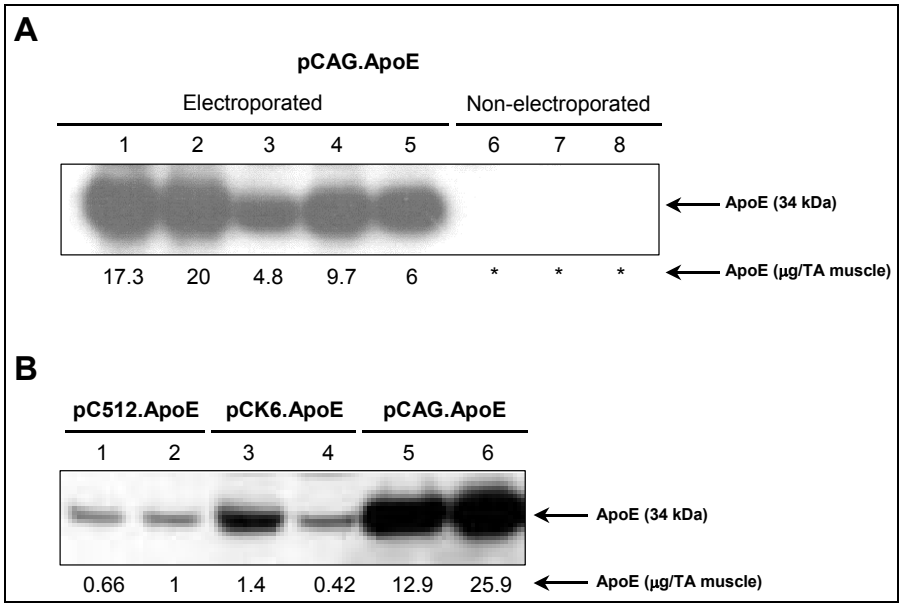
The schematic illustrates how single-stranded adeno-associated virus (**ssAAV**) and self-complementary adeno-associated virus (**scAAV**) vectors differ in the size of their transgene cassettes, in replication and packaging into viral particles, and in the formation of transcriptionally active recombinant AAV (**rAAV**) genomes. Both vectors are flanked by the AAV inverted terminal repeats (**ITR**), which assume a 'T'-shaped configuration through base-pairing of palindromic sequences upon replication of rAAV vector genomes. The right-hand ITR of scAAV vectors is mutated by deleting the terminal resolution site (**TRS**) and the D-sequence (**ΔD/TRS**). Replication initiates, therefore, from the left-hand ITR, proceeds through the mutant end without terminal resolution and, using the opposite strand as template, continues back across the genome to create a dimer. The end result is a self-complementary genome with a central mutant ITR, but two wild-type ITRs at each end, which is replicated and packaged in the normal way [62••]. However, the scAAV transgene cassette must be half the size of conventional ssAAV vectors, which is achieved by using truncated promoters [63•] and/or removing non-coding sequences (such 'stuffer' DNA is often introduced into ssAAV vectors to ensure an optimal ~ 4.6 kb packaging size). Following infection, the released vector genomes must avoid degradation and be converted into double-stranded transcriptionally active DNA. Annealing of plus and minus genomes, and perhaps second-strand synthesis, is required for ssAAV-mediated transduction with both pathways considered rate limiting. However, for scAAV vectors the complementary sequences rapidly hybridize to form stable DNA duplexes.

Cell-based therapy

Restoration of ApoE expression by transplanting normal (ApoE+/+) bone marrow cells into ApoE$^{-/-}$ mice reverses their hypercholesterolemia and prevents atherosclerosis progression [31••,32••]. Conversely, irradiated wild-type mice transplanted with bone marrow cells from ApoE$^{-/-}$ mice develop more severe atherosclerosis on a high-fat diet than animals transplanted with normal bone marrow. Studies in which ApoE$^{+/+}$ bone marrow was mixed with ApoE$^{-/-}$ bone marrow in increasing amounts and transplanted into ApoE$^{-/-}$ recipient mice, indicate that only approximately 1 μg ApoE/ml plasma (2.5% of normal levels) is required to fully reduce plasma cholesterol [72•]. This observation has encouraged cell-based therapeutic approaches, most notably using ApoE-expressing retroviruses to transduce ApoE$^{-/-}$ bone marrow cells *ex vivo* for transplantation into ApoE$^{-/-}$ recipient mice. Initial studies have shown ApoE expression from arterial macrophages and protection from early atherosclerosis [73,74], while an improved self-inactivating retroviral vector with macrophage-restricted expression produced sufficient ApoE to reverse both hypercholesterolemia and atherosclerotic lesion progression [75].

A different strategy employs *in vivo* ApoE-secreting mini-organs. One study embedded recombinant endothelial cells secreting human ApoE3 in Matrigel (a solubilized basement membrane preparation) for intradermal injection into ApoE$^{-/-}$ mice; plasma cholesterol was halved for over 3 months and atherosclerotic plaque was reduced [76]. In another

Figure 4. Intramuscular electrotransfer of plasmids expressing human ApoE in ApoE$^{-/-}$ mice.



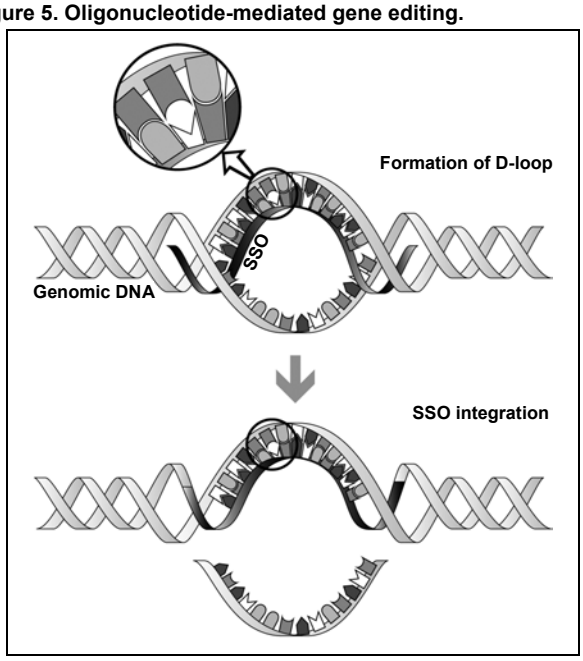
Tibialis anterior (**TA**) muscles of apolipoprotein E (**ApoE**)$^{-/-}$ mice were pre-injected with bovine hyaluronidase and then were injected with an ApoE expression plasmid (20 µg) immediately followed by an electrical pulse (electroporation). One week later, each muscle was excised and proteins extracted for ApoE analysis by Western blotting and ELISA. (**A**) Mice were injected with pCAG.ApoE3 with (lanes 1 to 5) and without (lanes 6 to 8) electroporation. * Muscle ApoE below the sensitivity limit of the ELISA for accurate quantification. (**B**) Expression of ApoE following intramuscular electrotransfer of pCK6.ApoE3 and pC512.ApoE3 compared with pCAG.ApoE3.

investigation, we encapsulated engineered Chinese hamster ovary (CHO) cells expressing human ApoE3 into porous alginate-based microspheres, which allow nutrients to enter and ApoE to egress, but protect the cells from immune surveillance. These microspheres were implanted into the peritoneum of ApoE$^{-/-}$ mice and gave circulating levels of ApoE that in the first few days reduced total plasma cholesterol, whilst increasing atheroprotective HDL [77•]. While we were encouraged by these findings, it was clear that the technology must be improved and refined before routine use, particularly for long-term treatment to retard or to regress atherosclerosis.

Gene editing

During the last decade, a radical alternative to gene transfer has emerged with the prospect of permanent cure in many genetic diseases: *targeted gene repair*. This elegant strategy aims to correct underlying point mutations *in situ* and initially involved the use of synthetic RNA:DNA oligonucleotides, termed RDOs or chimeraplasts. These oligonucleotides were designed to hybridize specifically to defective DNA, enabling the cell's own mismatch machinery to recognize and repair the faulty DNA (Figure 5). We pioneered the technology for CVD, showing that 68-mer RDOs efficiently converted dysfunctional ApoE2 to wild-type ApoE3 in recombinant CHO cells by directing a C→T nucleotide substitution. The correction was verified by gene sequencing and by isoelectric focusing analysis of secreted protein, while prolonged passaging and cloning of cells showed that the corrected allele was stably maintained [78••].

Figure 5. Oligonucleotide-mediated gene editing.



A synthetic single-stranded all-DNA oligonucleotide (**SSO**) is designed to hybridize perfectly to the wild-type sequence of the mutated gene. The resulting mismatch (**D-loop**) with the point mutation (circled inset) allows the cell's own repair activity to recognize and correct the genomic defect. Several mechanisms have been proposed, but, as illustrated here, physical incorporation of SSO sequences into the genomic site to 'replace' the region around the mutant base is favored as the initial step [101]. As yet, subsequent events to carry out the genomic DNA repair remain poorly understood, although there is evidence that specific pathways are involved.

However, despite these encouraging findings, our attempts to extend the work and correct the dysfunctional human ε4 allele to the ε3 allele, and vice-versa, in recombinant CHO cells and in EBV-transformed lymphocytes from patients have disappointed. Conversions were noted by polymerase chain reaction (PCR)/restriction fragment length polymorphism analyses, but were unstable and corrected clones could not be isolated [79,80]. Although we ruled out PCR artifacts for the initial repair [79], we cannot yet explain our failure; as noted elsewhere [81], gene-editing technology remains inconsistent and difficult to reproduce. Our initial conclusion was that manufacturing changes had produced poorer quality reagents [79,82]; in turn, this led to the use of higher amounts of RDO and delivery vehicle to achieve repair, opening the possibility of cytotoxic/apoptotic actions. An intriguing alternative, or co-possibility, is that cells have effective defense mechanisms that counteract targeted genome sequence alterations by blocking the cell-cycle progression, in this case of corrected cells [83]. Notwithstanding, many workers now use single-stranded all-DNA oligonucleotides (SSOs) and studies targeting a sensitive reporter gene suggest that 1 to 2% of corrected cells are able to escape G2/M arrest and go on to produce viable colonies [83]. If this finding is reproducible and viable, edited cells can be isolated, then the technology will have great value for manipulating cells *in vitro*, including the generation of new clonal cell lines.

Conclusion

Despite improved cardiovascular pharmaceuticals, there is still no drug that efficiently reverses atherosclerotic pathology. In this article we review the potential of human ApoE3 gene therapy as a generic treatment for hyperlipidemia and atherosclerosis, as well as its ability to counteract ApoE dysfunction or deficiency, though inferences must be tempered with the recognition that only preclinical data are available and that CVD is a chronic disease of multifaceted origin. There are also important practical considerations. Efficient delivery of the human ApoE3 transgene to the target tissue or organ is needed, without widespread vector dissemination or induction of harmful side effects. The liver, which secretes 90% of plasma ApoE, would seem the preferred target, particularly as liver-derived ApoE is known to infiltrate the arterial wall [36•,37•]. However, the recent development of macrophage-restricted vectors [58] makes *ex vivo* transduction of hematopoietic stem cells (HSCs) an attractive option, while muscle is an accessible and stable tissue. Choosing the optimal vector is also problematic; as we reiterate below, each has inherent characteristics that confer specific advantages and disadvantages.

Adenovirus-based therapies remain attractive, despite a tragic death in 1999 resulting from an acute phase immune response [84]. HD-rAd vectors have low immunogenicity and much potential [47••], provided current limitations of inefficient production and helper virus contamination can be overcome. Recent innovative protocols show promise, improving yields and reducing helper-virus and replication competent adenovirus (RCA) contaminants in HD-rAd vector stocks [44•]. The new AAV isolates are also attracting much attention for safe, specific and effective gene transfer; in particular, rAAV-7 for muscle and rAAV-8 to transduce liver or muscle cells. Indeed, the most recent report demonstrates that intravenous injection of rAAV2/8 or rAAV2/7 vectors expressing human ApoE3 produce sustained therapeutic levels of liver-directed ApoE3 in plasma, which normalizes the plasma lipoprotein profile in $ApoE^{-/-}$ mice and completely prevents atherosclerosis at one year [85••]. Moreover, it appears possible to select for rAAV vectors with specific, enhanced properties by screening a library of capsid mutants [86], while the 4.7-kb packaging constraint of rAAV can be overcome by using trans-splicing and overlapping vector constructs, which then re-assemble a split gene within the target cell [87]. Hybrid AAV/Ad vectors also have high capacity and combine efficient rAd transduction with the sustained transgene expression of rAAV [88].

New techniques for viral gene purification and delivery are in development. Laboratory-scale production of rAd and rAAV using commercial membrane-based kits give a similar yield and purity to standard density-gradient ultracentrifugation using $CsCl_2$ (rAd or rAAV) or iodixanol (rAAV) [89•], while large-scale manufacture by anion-exchange chromatography gives a high-purity recombinant virus with increased biological potency [90•]. Polymeric coatings are proposed for shielding viral vectors from neutralizing antibodies and also permit incorporation of targeting ligands to give cell-specificity [91]. Alternative approaches to circumvent pre-existing immunity to human rAd vectors include the generation of animal-derived adenoviral vectors [92,93] and the engineering of key immunogenic epitopes on the hexon capsid protein of rAd serotype 5 vectors to evade neutralizing antibodies [94••]. Specific retargeting of rAAV or rAd vectors has been achieved by inserting phage-display-derived peptides into appropriate surface-exposed sites of the capsid. This process permits efficient and selective transduction of human vascular endothelial cells and smooth muscle cells [95•], while a mix of genetic and chemical capsid modifications are also possible, allowing coupling of protein as well as non-protein ligands [96,97].

Safety considerations have revived interest in non-viral DNA ('naked' DNA) delivery. Noteworthy advances include the use of minicircle DNA vectors devoid of bacterial sequences that give robust and persistent transgene expression [98], and the design of plasmids that carry a scaffold/matrix-attached region for long-term episomal gene expression [99]. Of equal importance is improved delivery efficiency, including to the liver, by rapid, high-volume injection (ie, hydrodynamic transfection) via systemic [100] or regional routes [50•], or to muscle by electric and/or ultrasound pulsing of injection sites [101].

Although the above gene addition strategies hold significant promise, they nevertheless have inherent shortcomings compared to the ideal therapeutic of safe and efficient gene repair or site-specific, targeted gene integration. Oligonucleotide-directed gene editing has under-performed ever since encouraging preliminary reports were published,

but systematic studies are underway to investigate basic repair mechanisms [102] and the causes of conversion instability. These investigations should clarify whether the technology can develop into a viable therapeutic. Integrating retroviral vectors have been widely used for stable gene transfer in proliferating cells, often HSCs, although there are concerns regarding insertional mutagenesis. Relevant to this review are lentiviral vectors for direct *in vivo* delivery because, unlike other retroviruses, they can transduce a high percentage of non-dividing cells, such as hepatocytes. Indeed, recombinant lentiviruses have delivered human ApoE2, E3 and E4 to a mouse model of Alzheimer's disease using direct intracerebral administration [103]. Other systems to direct naked DNA integration and stable transgene expression include transposons or phage integrases [104]. However, the most exciting development is targeted integration, which introduces transgenes using double-strand break technology to stimulate homologous recombination. Potentially, this recombination has specificity via engineered zinc-finger nucleases and hence can repair or modify endogenous genes efficiently. However, as emphasized elsewhere [105,106], significant challenges remain before the *in situ* repair of genetic mutations becomes a clinical reality.

Optimization of ApoE gene transfer has largely been achieved in ApoE$^{-/-}$ mice, a convenient preclinical model of ApoE deficiency. However, to assess whether ApoE gene augmentation can be a generic treatment for hyperlipidemia and atherosclerosis (as suggested by infusion of ApoE protein [26•] and studies in transgenic animals [28,29]) alternative models are required. Such models might include the LDLR$^{-/-}$ mouse, which shows significant reductions in aortic plaque following treatment with a second-generation rAd expressing human ApoE3 [37•,107•], and also fat-fed hamsters and the Watanabe heritable-hyperlipidemic (WHHL) rabbit. Hamsters are widely used to test hypolipidemic drugs because, unlike mice and rats, they synthesize CETP and have a similar lipoprotein profile to humans. The WHHL rabbit lacks functional LDLRs and develops a spontaneous atherosclerosis that resembles the pathology in human familial hypercholesterolemia. Such models can help evaluate treatment regimes. For example, low but sustained levels of plasma ApoE3 may protect against atherogenesis and combat hypercholesterolemia or ApoE deficiency, whereas high transient levels could be employed to acutely regress atherosclerotic lesions. ApoE3 gene transfer might also complement surgical procedures, such as coronary angioplasty for preventing restenosis and carotid endarterectomy for removing harmful plaque. However, it is important to monitor ApoE3 transgene expression because very low levels will fail to reverse hypercholesterolemia, while paradoxically supraphysiological levels promote hypertriglyceridemia [108]. Therefore, a relatively narrow therapeutic range of plasma ApoE3 is required for normolipidemia, suggesting that it will be prudent to construct expression vectors controlled by regulatable promoters. Alhough it is clear that there is much work ahead – in particular, regarding the optimizing of vectors and their delivery, and to critically test them in more rigorous models of CVD – the long-term aim of bringing ApoE gene therapeutics into the clinic remains firmly on track.

Acknowledgements

We gratefully acknowledge the British Heart Foundation in funding our own studies cited herein, and the help of David Woods in preparing Figure 5.

References

•• of outstanding interest
• of special interest

1. Poole-Wilson P: **The prevention of cardiovascular disease worldwide: Whose task and WHO's task?** *Clin Med* (2005) **5**(4):379-384.

2. Ross R: **Atherosclerosis – an inflammatory disease.** *N Engl J Med* (1999) **340**(2):115-126.

3. Mikhailidis DP, Wierzbicki AS, Daskalopoulou SS, Al-Saady N, Griffiths H, Hamilton G, Monkman D, Patel V, Pittard J, Schachter M: **The use of ezetimibe in achieving low density lipoprotein lowering goals in clinical practice: Position statement of a United Kingdom consensus panel.** *Curr Med Res Opin* (2005) **21**(6):959-969.
• *A review describing the success of ezetimibe as a combination therapy with statins or as a monotherapy, for reducing LDL cholesterol.*

4. Linsel-Nitschke P, Tall AR: **HDL as a target in the treatment of atherosclerotic cardiovascular disease.** *Nat Rev Drug Discov* (2005) **4**(3):193-205.
• *A review that discusses the mechanisms that underlie the protective effect of HDL cholesterol and then describes several strategies to increase HDL cholesterol levels to treat cardiovascular disease.*

5. Davignon J: **Apolipoprotein E and atherosclerosis: Beyond lipid effect.** *Arterioscler Thromb Vasc Biol* (2005) **25**(2):267-269.
• *An editorial describing the many pleiotropic properties of ApoE.*

6. Greenow K, Pearce NJ, Ramji DP: **The key role of apolipoprotein E in atherosclerosis.** *J Mol Med* (2005) **83**(5):329-342.
• *This review describes the molecular and cell biology of ApoE, as well as its many anti-atherogenic properties and its regulation by factors implicated in the pathogenesis of cardiovascular disease.*

7. Wouters K, Shiri-Sverdlov R, van Gorp PJ, van BM, Hofker MH: **Understanding hyperlipidemia and atherosclerosis: Lessons from genetically modified apoe and ldlr mice.** *Clin Chem Lab Med* (2005) **43**(5):470-479.
• *A good review on mouse models of hyperlipidemia and atherosclerosis including detailed accounts on the ApoE$^{-/-}$ mouse anas well as transgenic mice expressing common and rare ApoE isoforms.*

8. Curtiss LK: **ApoE in atherosclerosis : A protein with multiple hats.** *Arterioscler Thromb Vasc Biol* (2000) **20**(8):1852-1853.
• *An editorial that discusses the findings that low levels of plasma ApoE are anti-atherogenic in the absence of changes in plasma cholesterol levels.*

9. Riddell DR, Graham A, Owen JS: **Apolipoprotein E inhibits platelet aggregation through the L-arginine:nitric oxide pathway. Implications for vascular disease.** *J Biol Chem* (1997) **272**(1):89-95.
10. Hui DY, Basford JE: **Distinct signaling mechanisms for apoE inhibition of cell migration and proliferation.** *Neurobiol Aging* (2005) **26**(3):317-323.
• *A review that summarizes data demonstrating that ApoE protects against atherosclerotic lesion progression by inhibiting smooth-muscle cell migration and proliferation.*

11. Stannard AK, Riddell DR, Sacre SM, Tagalakis AD, Langer C, von Eckardstein A, Cullen P, Athanasopoulos T, Dickson G, Owen JS: **Cell-derived apolipoprotein E (ApoE) particles inhibit vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells.** *J Biol Chem* (2001) **276**(49):46011-46016.

12. Ali K, Middleton M, Pure E, Rader DJ: **Apolipoprotein E suppresses the type I inflammatory response *in vivo*.** *Circ Res* (2005) **97**(9):922-927.

13. van den EP, Garg S, Leon L, Brigl M, Leadbetter EA, Gumperz JE, Dascher CC, Cheng TY, Sacks FM, Illarionov PA, Besra GS, Kent SC, Moody DB, Brenner MB: **Apolipoprotein-mediated pathways of lipid antigen presentation.** *Nature* (2005) **437**(7060):906-910.

14. Skalen K, Gustafsson M, Rydberg EK, Hulten LM, Wiklund O, Innerarity TL, Boren J: **Subendothelial retention of atherogenic lipoproteins in early atherosclerosis.** *Nature* (2002) **417**(6890):750-754.

15. Pham T, Kodvawala A, Hui DY: **The receptor-binding domain of apolipoprotein E is responsible for its antioxidant activity.** *Biochemistry* (2005) **44**(20): 7577-7582.

16. Grainger DJ, Reckless J, McKilligin E: **Apolipoprotein E modulates clearance of apoptotic bodies *in vitro* and *in vivo*, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice.** *J Immunol* (2004) **173**(10):6366-6375.

17. Thorngate FE, Rudel LL, Walzem RL, Williams DL: **Low levels of extrahepatic nonmacrophage ApoE inhibit atherosclerosis without correcting hypercholesterolemia in ApoE-deficient mice.** *Arterioscler Thromb Vasc Biol* (2000) **20**(8):1939-1945.

• *A paper suggesting that low levels of ApoE are anti-atherogenic without lowering plasma cholesterol levels.*

18. Raffai RL, Loeb SM, Weisgraber KH: **Apolipoprotein E promotes the regression of atherosclerosis independently of lowering plasma cholesterol levels.** *Arterioscler Thromb Vasc Biol* (2005) **25**(2):436-441.

•• *A paper demonstrating for the first time that ApoE promotes regression of atherosclerosis independently of lowering plasma cholesterol.*

19. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL: **Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells.** *Cell* (1992) **71**(2):343-353.

•• *A report describing the generation of the ApoE$^{-/-}$ mouse, an important landmark in animal models to study atherosclerosis, as the mice spontaneously develop atherosclerotic lesions on a normal chow diet with a pathology similar to advanced human atherosclerotic lesions.*

20. Williams H, Johnson JL, Carson KG, Jackson CL: **Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice.** *Arterioscler Thromb Vasc Biol* (2002) **22**(5):788-792.

• *A paper strengthening the relevance of the ApoE$^{-/-}$ mouse to atherosclerosis research; the authors show that atherosclerotic lesions in this model undergo spontaneous plaque rupture as noted for the human disease.*

21. Lutgens E, Faber B, Schapira K, Evelo CT, van HR, Heeneman S, Cleutjens KB, Bijnens AP, Beckers L, Porter JG, Mackay CR, Rennert P, Bailly V, Jarpe M, Dolinski B, Koteliansky V, de FT, Daemen MJ: **Gene profiling in atherosclerosis reveals a key role for small inducible cytokines: Validation using a novel monocyte chemoattractant protein monoclonal antibody.** *Circulation* (2005) **111**(25):3443-3452.

22. Mayr M, Chung YL, Mayr U, Yin X, Ly L, Troy H, Fredericks S, Hu Y, Griffiths JR, Xu Q: **Proteomic and metabolomic analyses of atherosclerotic vessels from apolipoprotein E-deficient mice reveal alterations in inflammation, oxidative stress, and energy metabolism.** *Arterioscler Thromb Vasc Biol* (2005) **25**(10):2135-2142.

23. Sullivan PM, Mezdour H, Aratani Y, Knouff C, Najib J, Reddick RL, Quarfordt SH, Maeda N: **Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis.** *J Biol Chem* (1997) **272**(29):17972-17980.

24. Knouff C, Hinsdale ME, Mezdour H, Altenburg MK, Watanabe M, Quarfordt SH, Sullivan PM, Maeda N: **Apo E structure determines VLDL clearance and atherosclerosis risk in mice.** *J Clin Invest* (1999) **103**(11):1579-1586.

25. Sullivan PM, Mezdour H, Quarfordt SH, Maeda N: **Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human Apoe*2.** *J Clin Invest* (1998) **102**(1):130-135.

26. Yamada N, Inoue I, Kawamura M, Harada K, Watanabe Y, Shimano H, Gotoda T, Shimada M, Kohzaki K, Tsukada T, Shiomi M, Watanabe Y, Yazaki Y: **Apolipoprotein E prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits.** *J Clin Invest* (1992) **89**(2):706-711.

• *A paper demonstrating that augmentation of plasma ApoE levels by intravenous infusion of ApoE protein can prevent progression of atherosclerosis in conditions other than ApoE deficiency.*

27. Nikoulin IR, Curtiss LK: **An apolipoprotein E synthetic peptide targets to lipoproteins in plasma and mediates both cellular lipoprotein interactions *in vitro* and acute clearance of cholesterol-rich lipoproteins *in vivo*.** *J Clin Invest* (1998) **101**(1):223-234.

• *This paper reports that intravenous injection of a synthetic ApoE peptide encompassing its receptor-binding domain (residues 141 to 155) results in acute clearance of cholesterol-rich lipoproteins from the circulation of ApoE$^{-/-}$ mice.*

28. Shimano H, Yamada N, Katsuki M, Shimada M, Gotoda T, Harada K, Murase T, Fukazawa C, Takaku F, Yazaki Y: **Overexpression of apolipoprotein E in transgenic mice: Marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia.** *Proc Natl Acad Sci USA* (1992) **89**(5):1750-1754.

29. Yamamoto K, Shimano H, Shimada M, Kawamura M, Gotoda T, Harada K, Ohsuga J, Yazaki Y, Yamada N: **Overexpression of apolipoprotein E prevents development of diabetic hyperlipidemia in transgenic mice.** *Diabetes* (1995) **44**(5):580-585.

30. Bellosta S, Mahley RW, Sanan DA, Murata J, Newland DL, Taylor JM, Pitas RE: **Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice.** *J Clin Invest* (1995) **96**(5):2170-2179.

31. Linton MF, Atkinson JB, Fazio S: **Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation.** *Science* (1995) **267**(5200):1034-1037.

•• *This paper demonstrates that transplantation of normal bone marrow into ApoE$^{-/-}$ mice results in correction of the hypercholesterolemia and protects against aortic and coronary atherosclerosis.*

32. Shi W, Wang X, Wang NJ, McBride WH, Lusis AJ: **Effect of macrophage-derived apolipoprotein E on established atherosclerosis in apolipoprotein E-deficient mice.** *Arterioscler Thromb Vasc Biol* (2000) **20**(10):2261-2266.

•• See [28••].

33. Kashyap VS, Santamarina-Fojo S, Brown DR, Parrott CL, Applebaum-Bowden D, Meyn S, Talley G, Paigen B, Maeda N, Brewer HB Jr: **Apolipoprotein E deficiency in mice: Gene replacement and prevention of atherosclerosis using adenovirus vectors.** *J Clin Invest* (1995) **96**(3):1612-1620.

• *An early report showing that a first-generation rAd vector for liver-directed human ApoE3 gene transfer can reverse the hypercholesterolemia in ApoE$^{-/-}$ mice and, over a 4-week period, significantly retard atherosclerotic lesion progression.*

34. Desurmont C, Caillaud JM, Emmanuel F, Benoit P, Fruchart JC, Castro G, Branellec D, Heard JM, Duverger N: **Complete atherosclerosis regression after human ApoE gene transfer in ApoE-deficient/nude mice.** *Arterioscler Thromb Vasc Biol* (2000) **20**(2):435-442.

•• *This paper describes the first study to demonstrate complete atherosclerotic lesion regression, six months after liver-directed rAd-mediated ApoE gene transfer in immunodeficient athymic ApoE$^{-/-}$ mice, with normalization of plasma cholesterol and lipoprotein distribution.*

35. Tsukamoto K, Smith P, Glick JM, Rader DJ: **Liver-directed gene transfer and prolonged expression of three major human ApoE isoforms in ApoE-deficient mice.** *J Clin Invest* (1997) **100**(1):107-114.

36. Tsukamoto K, Tangirala R, Chun SH, Pure E, Rader DJ: **Rapid regression of atherosclerosis induced by liver-directed gene transfer of ApoE in ApoE-deficient mice.** *Arterioscler Thromb Vasc Biol* (1999) **19**(9):2162-2170.

• *This paper describes the use of second-generation rAd vectors for liver-directed gene transfer and reports ApoE isoform-specific effects on atherosclerosis in ApoE$^{-/-}$ mice. The study demonstrates that liver-derived ApoE gains access to arterial lesions via the circulation, reduces plasma cholesterol and regresses pre-existing atherosclerotic lesions.*

37. Tangirala RK, Pratico D, FitzGerald GA, Chun S, Tsukamoto K, Maugeais C, Usher DC, Pure E, Rader DJ: **Reduction of isoprostanes and regression of advanced atherosclerosis by apolipoprotein E.** *J Biol Chem* (2001) **276**(1):261-266.

• *This study demonstrates regression of advanced atherosclerotic lesions by using a second-generation rAd vector for liver-directed human ApoE3 gene transfer in LDLR$^{-/-}$ mice. The authors also showe that ApoE gene transfer significantly reduced isoprostane levels, which are reliable markers of lipid peroxidation and oxidative stress in vivo, and speculate that the intrinsic anti-oxidant properties of ApoE may have contributed directly to these anti-atherogenic effects.*

38. Amalfitano A, Hauser MA, Hu HM, Serra D, Begy CR, Chamberlain JS: **Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted.** *J Virol* (1998) **72**(2):926-933.

•• *A paper describing the development of a second-generation rAd vector with deletions in the E1, E3, and polymerase genes. These new vectors have several improvements compared to first-generation rAd vectors, including increased cloning capacity, decreased risk of generating replication competent adenovirus (RCA) during vector production, and significant reduction in adenoviral late gene expression.*

39. Hu H, Serra D, Amalfitano A: **Persistence of an [E1-, polymerase-] adenovirus vector despite transduction of a neoantigen into immune-competent mice.** *Hum Gene Ther* (1999) **10**(3):355-364.

•• *A report demonstrating that liver-directed gene transfer using a [E1$^-$, E3$^-$, polymerase$^-$] rAd vector results in persistent transgene expression and reduced hepatotoxicity. The paper suggests a 2-hit hypothesis for immune clearance of rAd-transduced cells: the first hit is due to adenoviral gene expression while the second hit is due to transgene immunogenicity. The attenuation of the first hit by using these improved second-generation rAd vectors allows for extended persistence, despite the continued presence of the transgene-derived stimulus.*

40. Hodges BL, Serra D, Hu H, Begy CA, Chamberlain JS, Amalfitano A: **Multiply deleted [E1, polymerase-, and pTP-] adenovirus vector persists despite deletion of the preterminal protein.** *J Gene Med* (2000) **2**(4):250-259.

• *This paper describes a further development of polymerase-deleted rAd vectors, where the pre-terminal protein (pTP) gene is also removed. This extends the cloning capacity, further reduces the risk of generating RCA during vector production, and improves the in vivo performance of these rAd vectors as they cause reduced hepatotoxicity.*

41. Harris JD, Graham IR, Schepelmann S, Stannard AK, Roberts ML, Hodges BL, Hill V, Amalfitano A, Hassall DG, Owen JS, Dickson G: **Acute regression of advanced and retardation of early aortic atheroma in immunocompetent apolipoprotein-E (apoE) deficient mice by administration of a second generation [E1(-), E3(-), polymerase(-)] adenovirus vector expressing human apoE.** *Hum Mol Genet* (2002) **11**(1):43-58.

42. Harris JD, Graham IR, Schepelmann S, Stannard A, Amalfitano A, Owen JS, Dickson G: **Retardation of atherosclerosis in immunocompetent apolipoprotein (apo) E-deficient mice following liver-directed administration of a [E1, E3, polymerase] adenovirus vector containing the elongation factor-1 promoter driving expression of human apoE cDNA.** *Gene Ther Mol Biol* (2005) **9**(A):23-32.

43. Harris JD, Graham IR, Amalfitano A, Owen JS, Dickson G: **Delivery of human apolipoprotein (apo) E to liver by an [E1-, E3-, polymerase-, pTP-] adenovirus vector containing a liver-specific promoter inhibits atherogenesis in immunocompetent apoE-deficient mice.** *Gene Ther Mol Biol* (2006) **10**(A):17-30.

44. Alba R, Bosch A, Chillon M: **Gutless adenovirus: Last-generation adenovirus for gene therapy.** *Gene Ther* (2005) **12**(Suppl 1):S18-S27.

• *A review on the development of HD-rAd vectors.*

45. Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S: **Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity.** *Nat Genet* (1998) **18**(2):180-183.

• *A paper describing the use of a HD-rAd vector containing the complete human α_1-antitrypsin (hAAT) locus. This hAAT-based rAD outperformed a first-generation rAd vector following liver-directed delivery, giving tissue-specific, high-level- and long-term-transgene expression without detectable toxicity.*

46. Schiedner G, Hertel S, Johnston M, Biermann V, Dries V, Kochanek S: **Variables affecting *in vivo* performance of high-capacity adenovirus vectors.** *J Virol* (2002) **76**(4):1600-1609.

• *A paper that evaluates factors which are important in transgene expression via HD-rAd vectors. A series of vectors were generated that carried different combinations of human human α_1-antitrypsin (hAAT) expression constructs and stuffer DNA. Levels of hAAT obtained from genomic DNA were significantly higher than levels with small cDNA expression cassettes. In addition, the type of promoter used to drive transgene expression influences whether antibodies against the transgene product are generated.*

47. Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L: **Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector.** *Proc Natl Acad Sci USA* (2001) **98**(23):13282-13287.

•• *This study establishes that a single intravenous injection of a HD-rAd vector carrying the complete murine ApoE genomic locus provides complete lifetime phenotypic protection against hyperlipidemia and atherosclerosis in the ApoE$^{-/-}$ mouse. In contrast, a HD-rAd carrying an ApoE-cDNA-expression cassette gave lower levels of plasma ApoE that declined significantly after one and a half years, resulting in a rebound to the hypercholesterolemic phenotype. The authors also showed that effective repeat administration was feasible by re-treating with a HD-rAd of a different serotype.*

48. Brunetti-Pierri N, Palmer DJ, Mane V, Finegold M, Beaudet AL, Ng P: **Increased hepatic transduction with reduced systemic dissemination and proinflammatory cytokines following hydrodynamic injection of helper-dependent adenoviral vectors.** *Mol Ther* (2005) **12**(1):99-106.

49. Brunetti-Pierri N, Ng T, Iannitti DA, Palmer DJ, Beaudet AL, Finegold MJ, Carey KD, Cioffi WG, Ng P**: Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates.** *Hum Gene Ther* (2006) **17**(4):391-404.

50. Zhang X, Dong X, Sawyer GJ, Collins L, Fabre JW: **Regional hydrodynamic gene delivery to the rat liver with physiological volumes of DNA solution.** *J Gene Med* (2004) **6**(6):693-703.

• *This paper demonstrates that liver-directed gene transfer can be achieved with minimal dissemination to other organs and tissues without the need for hydrodynamic injection. Plasmid DNA delivery to specific segments of the liver was achieved by administrating the vector dose via branches of the portal vein.*

51. Jang MY, Yarborough OH, III, Conyers GB, McPhie P, Owens RA: **Stable secondary structure near the nicking site for adeno-associated virus type 2 Rep proteins on human chromosome 19.** *J Virol* (2005) **79**(6):3544-3556.

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

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

54. Harris JD, Schepelmann S, Athanasopoulos T, Graham IR, Stannard AK, Mohri Z, Hill V, Hassall DG, Owen JS, Dickson G: **Inhibition of atherosclerosis in apolipoprotein-E-deficient mice following muscle transduction with adeno-associated virus vectors encoding human apolipoprotein-E.** *Gene Ther* (2002) **9**(1):21-29.

• *This study establishes that a serotype 2 rAAV vector for muscle-directed human ApoE3 gene transfer in the ApoE$^{-/-}$ mouse can reduce atherosclerotic plaque density.*

55. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM: **Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy.** *Proc Natl Acad Sci USA* (2002) **99**(18):11854-11859.

•• *This paper describes the isolation of AAV serotypes 7 and 8 from nonhuman primate tissue by a PCR-based molecular rescue method, and the use of these serotypes as vectors for somatic gene transfer.*

56. Lebherz C, Gao G, Louboutin JP, Millar J, Rader D, Wilson JM: **Gene therapy with novel adeno-associated virus vectors substantially diminishes atherosclerosis in a murine model of familial hypercholesterolemia.** *J Gene Med* (2004) **6**(6):663-672.

57. Davidoff AM, Gray JT, Ng CY, Zhang Y, Zhou J, Spence Y, Bakar Y, Nathwani AC: **Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models.** *Mol Ther* (2005) **11**(6):875-888.

58. Chao H, Monahan PE, Liu Y, Samulski RJ, Walsh CE: **Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors.** *Mol Ther* (2001) **4**(3):217-222.

59. Blankinship MJ, Gregorevic P, Allen JM, Harper SQ, Harper H, Halbert CL, Miller DA, Chamberlain JS: **Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6.** *Mol Ther* (2004) **10**(4):671-678.

60. Louboutin JP, Wang L, Wilson JM: **Gene transfer into skeletal muscle using novel AAV serotypes.** *J Gene Med* (2005) **7**(4):442-451.

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

62. McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ: **Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*.** *Gene Ther* (2003) **10**(26):2112-2118.

•• *A paper reporting the development of scAAV vectors, which show a faster onset of transgene expression and higher transduction efficiency compared to conventional ssAAV vectors.*

63. Nathwani AC, Gray JT, Ng CY, Zhou J, Spence Y, Waddington SN, Tuddenham EG, Kemball-Cook G, McIntosh J, Boon-Spijker M, Mertens K, Davidoff AM: **Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver.** *Blood* (2005) **107**(7):2653-2661.

• *A description of scAAV vectors for liver-directed gene transfer in mice and nonhuman primates. Using hemophilia B gene therapy as a model, the authors demonstrate that lower scAAV-vector doses achieve therapeutic levels of transgene product compared to conventional ssAAV vectors.*

64. Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr., Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder RO: **Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors.** *Methods* (2002) **28**(2):158-167.

• *A report showing that anion-exchange chromatography can purify rAAV serotypes, which, unlike serotype 2, lack heparin-binding properties.*

65. Kohlbrenner E, Aslanidi G, Nash K, Shklyaev S, Campbell-Thompson M, Byrne BJ, Snyder RO, Muzyczka N, Warrington KH, Jr., Zolotukhin S: **Successful production of pseudotyped rAAV vectors using a modified baculovirus expression system.** *Mol Ther* (2005) **12**(6):1217-1225.

• *A paper proposing production of rAAV vectors in insect cells using a recombinant baculovirus system, which potentially allows ease of scale-up to clinical manufacturing of vector stocks.*

66. Nakai H, Wu X, Fuess S, Storm TA, Munroe D, Montini E, Burgess SM, Grompe M, Kay MA: **Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver.** *J Virol* (2005) **79**(6):3606-3614.

67. Schnepp BC, Clark KR, Klemanski DL, Pacak CA, Johnson PR: **Genetic fate of recombinant adeno-associated virus vector genomes in muscle.** *J Virol* (2003) **77**(6):3495-3504.

68. Wientgen H, Thorngate FE, Omerhodzic S, Rolnitzky L, Fallon JT, Williams DL, Fisher EA: **Subphysiologic apolipoprotein E (ApoE) plasma levels inhibit neointimal formation after arterial injury in ApoE-deficient mice.** *Arterioscler Thromb Vasc Biol* (2004) **24**(8):1460-1465.

69. Rinaldi M, Catapano AL, Parrella P, Ciafre SA, Signori E, Seripa D, Uboldi P, Antonini R, Ricci G, Farace MG, Fazio VM: **Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by intramuscular injection of plasmid DNA.** *Gene Ther* (2000) **7**(21):1795-1801.

70. Athanasopoulos T, Owen JS, Hassall D, Dunckley MG, Drew J, Goodman J, Tagalakis AD, Riddell DR, Dickson G: **Intramuscular injection of a plasmid vector expressing human apolipoprotein E limits progression of xanthoma and aortic atheroma in apoE-deficient mice.** *Hum Mol Genet* (2000) **9**(17):2545-2551.

71. McMahon JM, Signori E, Wells KE, Fazio VM, Wells DJ: **Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase – increased expression with reduced muscle damage.** *Gene Ther* (2001) **8**(16):1264-1270.

72. Hasty AH, Linton MF, Swift LL, Fazio S: **Determination of the lower threshold of apolipoprotein E resulting in remnant lipoprotein clearance.** *J Lipid Res* (1999) **40**(8):1529-1538.

• *Here, mixing ApoE$^{-/+}$ bone marrow with ApoE$^{-/-}$ marrow in increasing amounts and transplanting into ApoE$^{-/-}$ recipient mice, revealed that a threshold of ~ 1 μg ApoE/ml plasma is required for the hepatic clearance of remnant lipoproteins.*

73. Hasty AH, Linton MF, Brandt SJ, Babaev VR, Gleaves LA, Fazio S: **Retroviral gene therapy in ApoE-deficient mice: ApoE expression in the artery wall reduces early foam cell lesion formation.** *Circulation* (1999) **99**(19):2571-2576.

74. Yoshida H, Hasty AH, Major AS, Ishiguro H, Su YR, Gleaves LA, Babaev VR, Linton MF, Fazio S: **Isoform-specific effects of apolipoprotein E on atherogenesis: Gene transduction studies in mice.** *Circulation* (2001) **104**(23):2820-2825.

75. Gough PJ, Raines EW: **Gene therapy of apolipoprotein E-deficient mice using a novel macrophage-specific retroviral vector.** *Blood* (2003) **101**(2):485-491.

76. Cioffi L, Sturtz FG, Wittmer S, Barut B, Smith-Gbur J, Moore V, Zupancic T, Gilligan B, Auerbach R, Gomez F, Chauvin F, Antczak M, Platika D, Snodgrass HR: **A novel endothelial cell-based gene therapy platform for the *in vivo* delivery of apolipoprotein E.** *Gene Ther* (1999) **6**(6):1153-1159.

77. Tagalakis AD, Diakonov IA, Graham IR, Heald KA, Harris JD, Mulcahy JV, Dickson G, Owen JS: **Apolipoprotein E delivery by peritoneal implantation of encapsulated recombinant cells improves the hyperlipidaemic profile in apoE-deficient mice.** *Biochim Biophys Acta* (2005) **1686**(3):190-199.

• *In this study, recombinant Chinese hamster ovary cells expressing human ApoE3 were encapsulated into alginate-based microspheres and implanted into the peritoneum of ApoE$^{-/-}$ mice. ApoE was readily detected in plasma and reduced the plasma cholesterol levels, while increasing anti-atherogenic HDL.*

78. Tagalakis AD, Graham IR, Riddell DR, Dickson JG, Owen JS: **Gene correction of the apolipoprotein (Apo) E2 phenotype to wild-type ApoE3 by *in situ* chimeraplasty.** *J Biol Chem* (2001) **276**(16):13226-13230.

•• *The first report of gene editing in the cardiovascular field. A synthetic DNA-RNA oligonucleotide (chimeraplast) was shown to convert the dysfunctional APOE2 gene into wild-type APOE3 in recombinant CHO cells. The repair was stable and permanent at the DNA, mRNA, and protein levels. Successful targeting of APOE2 was also achieved in-vivo, using transgenic mice overexpressing human ApoE2, and in lymphocytes from a patient homozygous for the ε2 allele.*

79. Tagalakis AD, Dickson JG, Owen JS, Simons JP: **Correction of the neuropathogenic human apolipoprotein E4 (APOE4) gene to APOE3 *in vitro* using synthetic RNA/DNA oligonucleotides (chimeraplasts).** *J Mol Neurosci* (2005) **25**(1):95-103.

80. Mohri Z, Sperber G, Manzano A, Tagalakis AD, Dickson G, Owen JS: **Gene editing of the wild-type APOE3 gene to the dysfunctional variants APOE2 or APOE4 using synthetic RNA-DNA oligonucleotides (chimeroplasty).** *Gene Ther Mol Biol* (2005) **9**(B):143-152.

81. Taubes G: Gene therapy. **The strange case of chimeraplasty.** *Science* (2002) **298**(5601):2116-2120.

82. Manzano A, Mohri Z, Sperber G, Ogris M, Graham I, Dickson G, Owen JS: **Failure to generate atheroprotective apolipoprotein AI phenotypes using synthetic RNA/DNA oligonucleotides (chimeraplasts).** *J Gene Med* (2003) **5**(9):795-802.

83. Olsen PA, Randol M, Krauss S: **Implications of cell cycle progression on functional sequence correction by short single-stranded DNA oligonucleotides.** *Gene Ther* (2005) **12**(6):546-551.

84. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML: **Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer.** *Mol Genet Metab* (2003) **80**(1-2):148-158.

85. Kitajima K, Marchadier DHL, Miller GC, Gao GP, Wilson JM, Rader DJ: **Complete prevention of atherosclerosis in apoE-deficient mice by hepatic human apoE gene transfer with adeno-associated virus serotypes 7 and 8.** *Arterioscler Thromb Vasc Biol* (2006): Epub ahead of print

••*This recent report describes the use of rAAV2/7 and rAAV2/8 vectors to mediate liver-directed human ApoE3 gene transfer in the ApoE$^{-/-}$ mouse, providing complete protection against hyperlipidemia and atherosclerosis.*

86. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV: **Directed evolution of adeno-associated virus yields enhanced gene delivery vectors.** *Nat Biotechnol* (2006) **24**(2):198-204.

87. Ghosh A, Yue Y, Duan D: **Viral serotype and the transgene sequence influence overlapping adeno-associated viral (AAV) vector-mediated gene transfer in skeletal muscle.** *J Gene Med* (2006) **8**(3):298-305.

88. Goncalves MA, van dV, I, Knaan-Shanzer S, Valerio D, de Vries AA: **Stable transduction of large DNA by high-capacity adeno-associated virus/adenovirus hybrid vectors.** *Virology* 2004, **321**(2):287-296.

89. Duffy AM, O'Doherty AM, O'Brien T, Strappe PM: **Purification of adenovirus and adeno-associated virus: comparison of novel membrane-based technology to conventional techniques.** *Gene Ther* (2005) **12**(Suppl 1):S62-S72.
• *This paper evaluates the yield, transgene expression and purity of rAd and rAAV vectors using commercial kits for small-scale production and concludes that they are comparable to conventional methods.*

90. Burova E, Ioffe E: **Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: Methods and implications.** *Gene Ther* (2005) **12**(Suppl 1):S5-S17.
• *A detailed review describing different chromatographic methods for rAd and rAAV vector production and purification.*

91. Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW: **Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies.** *Gene Ther* (2001) **8**(5):341-348.

92. Hofmann C, Loser P, Cichon G, Arnold W, Both GW, Strauss M: **Ovine adenovirus vectors overcome preexisting humoral immunity against human adenoviruses *in vivo*.** *J Virol* (1999) **73**(8):6930-6936.

93. Farina SF, Gao GP, Xiang ZQ, Rux JJ, Burnett RM, Alvira MR, Marsh J, Ertl HC, Wilson JM: **Replication-defective vector based on a chimpanzee adenovirus.** *J Virol* (2001) **75**(23):11603-11613.

94. Roberts DM, Nanda A, Havenga MJ, Abbink P, Lynch DM, Ewald BA, Liu J, Thorner AR, Swanson PE, Gorgone DA, Lifton MA, Lemckert AA, Holterman L, Chen B, Dilraj A, Carville A, Mansfield KG, Goudsmit J, Barouch DH: **Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity.** *Nature* (2006) **441**(7090):239-243.
•• *A paper describing a way of avoiding rAd-serotype-5 immunity by exchanging the seven hypervariable regions (HVRs) of the hexon – a likely target for antibody binding – with those of the rare adenovirus serotype 48. This chimeric rAd vector was able to circumvent pre-existing anti-adenovirus-serotype-5 immunity.*

95. Baker AH, Kritz A, Work LM, Nicklin SA: **Cell-selective viral gene delivery vectors for the vasculature.** *Exp Physiol* (2005) **90**(1):27-31.
• *A review describing recent advances in the targeting of rAd and rAAV vectors to the vasculature, including specific gene transfer to endothelial cells and smooth-muscle cells.*

96. Kreppel F, Gackowski J, Schmidt E, Kochanek S: **Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors.** *Mol Ther* (2005) **12**(1):107-117.

97. Stachler MD, Bartlett JS: **Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells.** *Gene Ther* (2006) **13**(11):926-931.

98. Chen ZY, He CY, Kay MA: **Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression *in vivo*.** *Hum Gene Ther* (2005) **16**(1):126-131.

99. Jenke AC, Stehle IM, Herrmann F, Eisenberger T, Baiker A, Bode J, Fackelmayer FO, Lipps HJ: **Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome.** *Proc Natl Acad Sci USA* (2004) **101**(31):11322-11327.

100. Liu F, Song Y, Liu D: **Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA.** *Gene Ther* (1999) **6**(7):1258-1266.

101. Habib N, Havlik R, Jiao L, Kiri A, Jensen S, Nicholls J, Sarraf C, Goldspink G, Davies A, Slavik L, Wood C, Tiraby M, Drocourt D, Reynes JP, Smadja C, Tiraby J: **Combination of optisona with ultrasound and electroporation increases albumin and thrompoietin transgene expression whilst elongation factor promoter prolongs its duration.** *Gene Ther Mol Biol* (2004) **8**(A):1-8.

102. Radecke S, Radecke F, Peter I, Schwarz K: **Physical incorporation of a single-stranded oligodeoxynucleotide during targeted repair of a human chromosomal locus.** *J Gene Med* (2006) **8**(2):217-228.

103. Dodart JC, Marr RA, Koistinaho M, Gregersen BM, Malkani S, Verma IM, Paul SM: **Gene delivery of human apolipoprotein E alters brain Abeta burden in a mouse model of Alzheimer's disease.** *Proc Natl Acad Sci USA* (2005) **102**(4):1211-1216.

104. Ehrhardt A, Xu H, Huang Z, Engler JA, Kay MA: **A direct comparison of two nonviral gene therapy vectors for somatic integration: *In vivo* evaluation of the bacteriophage integrase phiC31 and the Sleeping Beauty transposase.** *Mol Ther* (2005) **11**(5):695-706.

105. Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC: **Highly efficient endogenous human gene correction using designed zinc-finger nucleases.** *Nature* (2005) **435**(7042):646-651.

106. Porteus MH, Carroll D: **Gene targeting using zinc finger nucleases.** *Nat Biotechnol* (2005) 23(8):967-973.

107. Tsukamoto K, Tangirala RK, Chun S, Usher D, Pure E, Rader DJ: **Hepatic expression of apolipoprotein E inhibits progression of atherosclerosis without reducing cholesterol levels in LDL receptor-deficient mice.** *Mol Ther* (2000) **1**(2):189-194.
• *This study uses a second-generation rAd vector expressing human ApoE3 for liver-directed gene transfer in LDLR$^{-/-}$ mice. Despite having little effect on plasma cholesterol, hepatic ApoE expression significantly reduced the progression of atherosclerosis, implying that ApoE gene augmentation will protect against atherosclerosis progression in conditions other than ApoE deficiency.*

108. Kypreos KE, van Dijk KW, Havekes LM, Zannis VI: **Generation of a recombinant apolipoprotein E variant with improved biological functions: Hydrophobic residues (LEU-261, TRP-264, PHE-265, LEU-268, VAL-269) of apoE can account for the apoE-induced hypertriglyceridemia.** *J Biol Chem* (2005) **280**(8):6276-6284.