

Gene editing of the wild-type *APOE3* gene to the dysfunctional variants *APOE2* or *APOE4* using synthetic RNA-DNA oligonucleotides (chimeraplasts)

Research Article

Zahra Mohri¹, Galia Sperber¹, Anna Manzano^{1,2}, Aristides D. Tagalakis¹, George Dickson², James S. Owen^{1,*}

¹Department of Medicine, Royal Free & University College Medical School, London, UK

²School of Biological Sciences, Royal Holloway University of London, Egham, UK

*Correspondence: James S. Owen, PhD, Department of Medicine (Upper 3rd Floor), Royal Free & University College Medical School, University College London, Hampstead Campus, Rowland Hill Street, London NW3 2PF, UK. Telephone: +44-20-7433-2853; fax: +44-20-7433-2852; e-mail: j.owen@medsch.ucl.ac.uk

Keywords: Apolipoprotein E; atherosclerosis; chimeraplasty; transfection

Abbreviations: apolipoprotein E, (apoE); asialoglycoprotein receptor, (ASGR); Chinese hamster ovary, (CHO); Dulbecco's modified Eagle's medium, (DMEM); fetal bovine serum, (FBS); galactotetraose-PEI, (Gal4-PEI); human hepatoblastoma, (HepG2); low-density lipoprotein, (LDL); phosphate-buffered saline, (PBS); polyethyleneimine, (PEI); polymerase chain reaction-restriction fragment length polymorphism, (PCR-RFLP); single nucleotide polymorphisms, (SNPs)

Received: 21 April 2005; Revised: 19 May 2005

Accepted: 14 July 2005; electronically published: July 2005

Summary

Plasma apolipoprotein E (apoE) is secreted by liver (>90%) and macrophages, and protects against atherosclerosis by contributing to cholesterol homeostasis and by locally restricting lesion development. Three common isoforms arise from single nucleotide polymorphisms (SNPs): apoE2, the rarest variant, differs from wild-type apoE3 by an R158C substitution and causes recessive Type III hyperlipidaemia, whereas apoE4 (C112R) produces a dominant hyperlipidaemia. Molecular explanations for these relationships are poorly defined, but most likely reflect differences in receptor binding and/or intracellular trafficking. Potentially, single-base mutations can be corrected in genes by using synthetic RNA-DNA oligonucleotides (chimeraplasts), which harness cellular mismatch repair mechanisms. Here, we evaluate gene editing of the *APOE3* gene in HepG2 hepatoblastoma cells and THP-1 monocyte-macrophages, with the aim of producing new human clonal cell lines that secrete apoE2 or apoE4 for subsequent biological investigations. Initially, we used polyethyleneimine (PEI) to transfer chimeraplasts and showed that brief centrifugation improved conversion efficiency. A dose-dependent conversion was seen by PCR-RFLP analysis in HepG2 cells and confirmed by direct sequencing. Additional increases in conversion efficiency were also noted when receptor-dependent uptake was exploited, using galactotetraose-PEI and mannose-PEI for HepG2 and THP-1 cells, respectively. Unexpectedly, however, the conversions appeared unstable as analysing 50 clones isolated by limiting dilution from chimeraplast-treated THP-1 and HepG2 cells consistently revealed no genotypic changes. Whether this instability is due to cytotoxic or apoptotic effects of the transfection complex, or to a recent suggestion that cells have effective defence mechanisms that counteract targeted genome sequence alterations, remains to be established.

I. Introduction

Apolipoprotein E (apoE) is a circulating 34-kDa polymorphic protein that mediates receptor-dependent clearance of atherogenic lipoproteins from plasma (Mahley and Ji, 1999). Single nucleotide polymorphisms

(SNPs) of the *APOE* gene were reported over 20 years ago (Rees et al, 1983). Three common alleles (2, 3, 4) lead to six homozygous and heterozygous phenotypes: apoE2/E2, E3/E3, E4/E4, E2/E3, E2/E4, and E3/E4. Although variation is evident in different populations, the

3 allelic frequency is the most common (~0.72) while those of 2 and 4 are estimated at 0.11 and 0.17, respectively (Corbo and Scacchi, 1999). ApoE2 (R158C) has ~1% of normal binding for the low-density lipoprotein (LDL)-receptor, and is the least cationic isoform differing from apoE3 and apoE4 (C112R) by one and two charge units, respectively. The 2 allele potentially protects against atherosclerosis (Weisgraber, 1994), but paradoxically causes recessive type III hyperlipidaemia (Mahley et al, 1999). ApoE4 has normal receptor binding ability (Siest et al, 1995), but nevertheless produces a dominant hyperlipidaemia and is a significant risk factor for cardiovascular disease, as verified by meta-analysis (Song et al, 2004).

Most plasma apoE (>90%) is secreted by liver (Kraft et al, 1989), but macrophages and other tissues also contribute. Indeed, macrophage-restricted expression of apoE in transgenic mice (Bellosta et al, 1995), or in apoE-deficient mice following transplantation of wild-type bone marrow (Linton et al, 1995), is able to inhibit atherogenesis. This action has highlighted multiple additional atheroprotective properties of apoE, independent of cholesterol transport (Davignon, 2005), which suggest that apoE acts locally to restrict atherosclerotic lesion development. These include antioxidant (Miyata and Smith, 1996) and anti-inflammatory (Sacre et al, 2003) activities and inhibition of vascular smooth muscle cell proliferation and migration (Zeleny et al, 2002); importantly, each of these biological activities was found to be isoform-dependent with apoE4 providing least atheroprotection.

An emerging technology, gene editing, has the potential to correct point mutations *in-situ*. In a landmark study, Yoon et al, (1996) reported episomal correction of a mutated alkaline phosphatase gene using synthetic RNA-DNA oligonucleotides (chimeraplasts) to direct the repair. Since then, the broad applicability of chimeraplast-mediated gene editing in genomes of cultured mammalian cells (Cole-Strauss et al, 1996; Kren et al, 1998; Alexeev et al, 2000), animals (Bandyopadhyay et al, 1999; Rando et al, 2000), plant cells (Beetham et al, 1999; Zhu et al, 2000), cell-free systems (Rice et al, 2000), and yeast (Rice et al, 2001) has been demonstrated by several groups worldwide. These reports include our own on the successful repair of the defective 2 allele to wild-type 3 in recombinant cells and human lymphocytes (Tagalakis et al, 2001). Here, we report the feasibility of gene editing in cultured HepG2 hepatoblastoma cells and THP-1 monocyte-macrophages (both derived from homozygous apoE3/E3 donors), with the aim of producing new apoE2- and apoE4-secreting human clonal cell lines for subsequent biological investigations. Despite optimizing conditions to achieve efficient conversions, the nucleotide substitutions proved unstable in both cell types, as judged by cloning and routine polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

II. Materials and Methods

A. Cell culture

Human hepatoblastoma (HepG2), monocyte-macrophage (THP-1), or embryonic kidney (HEK-293) cells were cultured in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% of 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Recombinant Chinese hamster ovary (CHO) cells stably-secreting apoE3 (Tagalakis et al, 2001) were cultured in Iscove's modified DMEM supplemented as described for human cells, and with 1% non-essential amino acids.

B. Design of RNA-DNA oligonucleotides (chimeraplasts)

The sequence of human genomic apoE3 was used to design 68-mer chimeraplasts, termed apoE3>E2 and apoE3>E4, that potentially would convert *APOE3 APOE2* (C T) and *APOE3 APOE4* (T C), respectively (Figure 1). Chimeraplasts were synthesized by MWG Biotech. The top chimeric strand of the double-stranded molecules contained modified RNA residues, which provide strong RNA-DNA hybridisation as well as protection against RNase H-mediated degradation. The DNA in the bottom all-DNA strand is complementary to the *APOE3* sequence with the exception of a central mismatched base. The complementary base to this mismatch is in the middle of a pentameric DNA region in the chimeric strand.

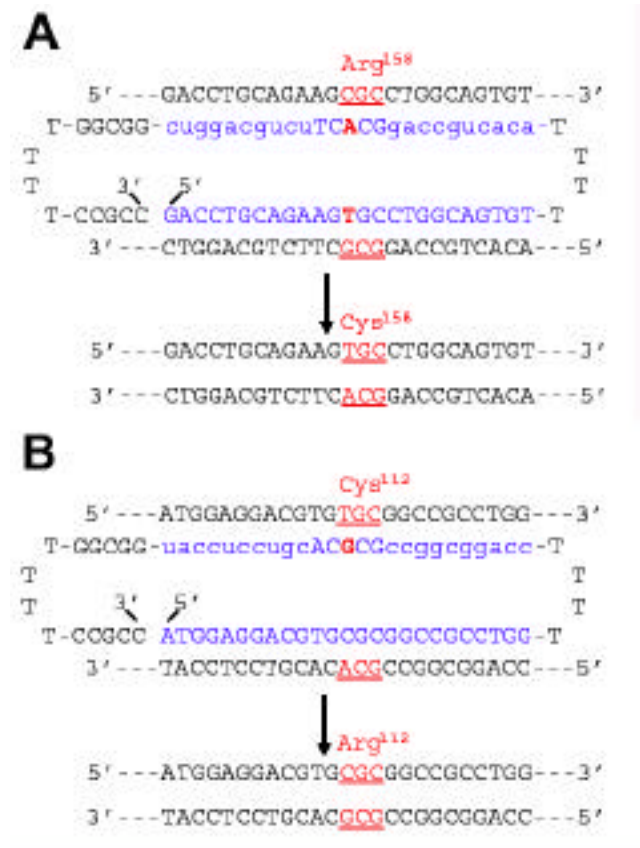


Figure 1. Chimeraplast design and structure. (A) The sequence of the self-complementary apoE3>E2 chimeric oligonucleotide is shown aligned and intertwined to the target human *APOE3* gene, with the homologous sequence shown in blue and the bases mismatched with the target (and corresponding to the intended gene editing) are shown underlined in red. The DNA residues of the chimeraplast are in uppercase and 2'-O-methyl RNA residues in lowercase, while the G:C clamp and the poly (T) hairpin loops are shown in black. The target codons of the *APOE3* gene are shown in red, before and after editing. (B) The apoE3>E4 chimeraplast and its target sequence are illustrated in an identical manner.

C. Transfection experiments

Cells were seeded in 6-well or 12-well plates to reach 50-70% confluency the following day. The transfection complex was freshly prepared by mixing 22-kDa linear polyethyleneimine (PEI) (ExGen 500; TCS Biologicals Ltd., Botolph Claydon, UK) with increasing concentrations of chimeraplasts (200-1000 nM) and, most commonly, at a phosphate to amine molar ratio of 1:5. Complexes were allowed to form for 10 min at room temperature with addition of 150 mM NaCl. In some experiments, PEI was mixed with, or replaced by, galactotetraose-PEI (Gal4-PEI) or mannose-PEI (Man-PEI). These reagents are derived by chemical modification of branched PEI and serve as ligands for cell-surface receptors (Bettinger et al, 1999; Sato et al, 2001). Prior to transfection, the cells were washed with phosphate-buffered saline (PBS) followed by addition of 0.5 ml serum-free medium to each well. Complexes (50 μ l) were then added to the cells. Four-to-eight hours post-transfection, 1.5 ml of fresh full medium was added to each well and the cells incubated for a further 24-48 h. Cellular DNA was then extracted (DNeasy kit, Qiagen) for PCR-RFLP analysis.

D. Analysis of gene editing by PCR-RFLP and sequencing

The extracted DNA was amplified by PCR using sense (5'-TCCAAGGAGCTGCAGGCGG CGCA-3') and anti-sense (5'-ACAGAATTCCGCCCCGGCCTGGTACACTGCCA-3') primer

pairs (Hixson et al, 1990). The 227 bp product was digested with *Cfo*1 and the fragments separated on 4% agarose gels, or 20% Tris-buffered EDTA polyacrylamide gels (Novex, Gröningen, The Netherlands). Each apoE genotype was distinguished by a unique combination of *Cfo*1 fragment sizes (Figure 2). The sense primer was used for automated DNA sequencing (Babraham Institute, Cambridge, UK) of purified PCR products (Wizard PCR Preps DNA Purification System, Promega).

E. Analysis of chimeraplast uptake by FACS

Cells were seeded and treated with a 5'-fluorescein-labelled chimeraplast, targeting the *APOE* gene as described above. Six hours post-transfection, cells were harvested and nuclei isolated by suspension in 0.1% (v/v) Igepal CA-630 (Sigma-Aldrich) in PBS on ice, followed by separation from membranes by aspiration through a 26G needle. Nuclei were then suspended and fixed in ice-cold 50% ethanol. Fluorescent nuclei were counted using a FACSCalibur flow cytometer (BD Biosciences) equipped with an argon ion laser. Excitation of the fluorescein was at 488nm (530/30nm emission bandpass filter) and 10,000 events were counted per sample. Data were obtained from three independent experiments

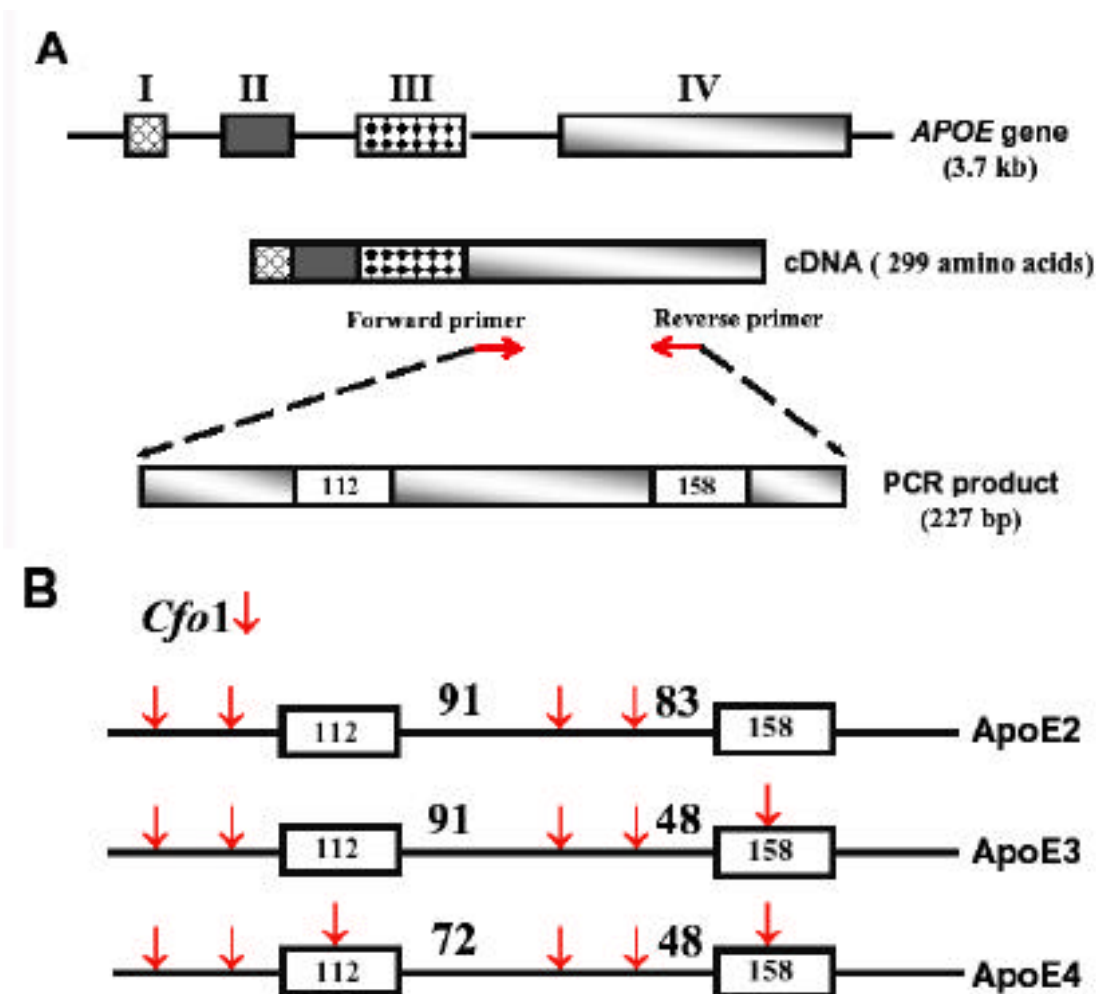


Figure 2. ApoE genotyping by PCR-RFLP analysis. (A) PCR was used to amplify a region from exon IV of the human *APOE* gene. (B) The 227 bp PCR was isotypied by digestion with *Cfo*1 to give characteristic cleavage fragments for *APOE2*, *APOE3*, and *APOE4* as depicted by the arrows, with the diagnostic band sizes also indicated.

III. Results

A. *APOE3* to *APOE2* conversions in CHOE3 and HEK-293, but not HepG2, cells

In preliminary experiments, CHOE3, HEK-293 and HepG2 cells were transfected with 400 nM of apoE3>E2 chimeraplast complexed to PEI at a 1:5 phosphate:amine molar ratio. Clear conversions were observed in the readily transfected cell lines, CHOE3 and HEK-293 (Figure 3A and 3B). This validates the activity of the chimeraplast to edit both cDNA and genomic *APOE3*. However, under identical conditions, no conversion was detected in HepG2 cells.

B. Modifying chimeraplast delivery gives detectable *APOE3* to *APOE2/4* conversions in HepG2 cells

As the apoE3>E2 chimeraplast appeared inactive towards HepG2 cells, the culture plates were briefly centrifuged (3 min, 400 x g) after addition of the chimeraplast-PEI complex to improve contact with the cells. When analysed by PCR-RFLP the diagnostic 83 bp band was clearly evident, whereas without centrifugation no conversion was seen (Figure 4A).

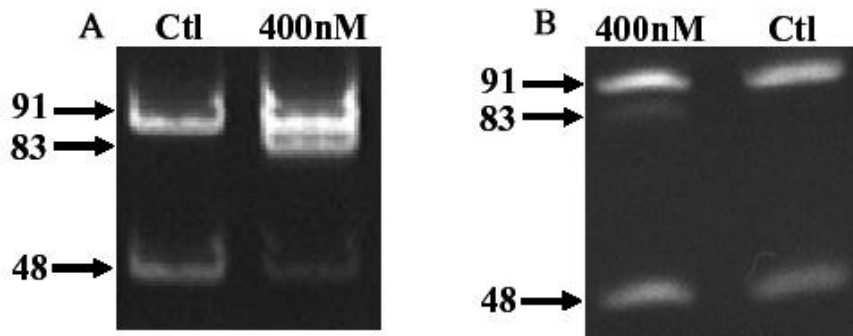


Figure 3. Conversion of *APOE3* to *APOE2* in CHOE3 and HEK-293 cells by chimeraplasty. (A) CHOE3 cells were treated with 400 nM of apoE3>E2 chimeraplast complexed with PEI at a phosphate:amine molar ratio of 1:5. DNA was extracted after 24 h, followed by PCR-RFLP analysis with separation of the *Cfo*I digestion fragments on 20% TBE gels. (B) Human HEK-293 cells were treated and analysed in an identical manner. Successful conversions were detectable in both cell lines, as judged by the appearance of the diagnostic 83 bp band. Ctl, control mock-transfected cells.

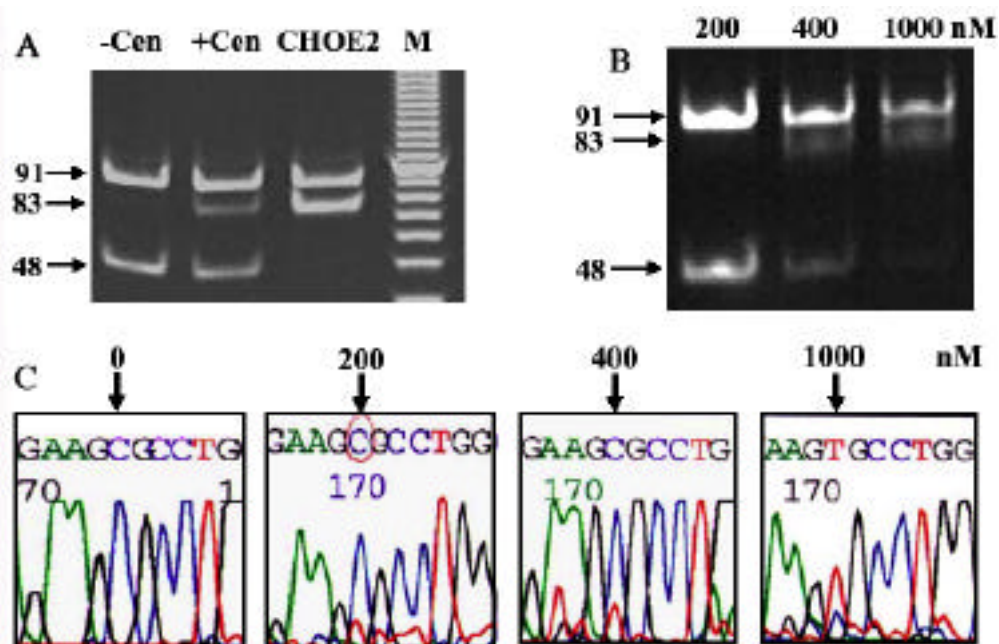


Figure 4. A centrifugation step facilitates gene conversion of *APOE3* to *APOE2* in HepG2 cells. HepG2 cells were seeded into two 12-well plates and the following day both plates were transfected with the apoE3>E2 chimeraplast (400 nM) complexed with PEI. One plate was incubated as before, whereas the other plate was briefly centrifuged prior to incubation. (A) Conversion to *APOE2* was detectable only in cells subjected to centrifugation. (B) In a separate experiment, HepG2 cells were transfected with increasing concentrations (200-1000) of the apoE3>E2 chimeraplast plus the centrifugation step. A dose-dependent conversion was detected by PCR-RFLP analysis, as indicated by emergence of the 83 bp diagnostic band and disappearance of the 48 bp fragment. (C) This gene conversion of C → T was confirmed by direct sequencing. The targeted base is arrowed and at 1000 nM chimeraplast is outweighed by the substituted T. -Cen, cells not centrifuged; +Cen, cells subjected to centrifugation; CHOE2, reference PCR-RFLP fragments from CHOE2 DNA; M, 10 bp ladder

Furthermore, when the HepG2 cells were treated with increasing amounts of apoE3>E2 chimeraplast (200-1000 nM) the conversion was dose-dependent, both by PCR-RFLP analysis (Figure 4B) and direct sequencing of the purified PCR product (Figure 4C). Indeed, sequence analysis indicated a conversion well in excess of 50% using 1000 nM (Figure 4C).

By contrast, identical transfections of HepG2 cells with the apoE3>E4 chimeraplast (200-1000 nM; PEI plus centrifugation) failed to give detectable conversions (Figure 5A); nor did complexing the chimeraplast with Gal4-PEI, which is a ligand for the asialoglycoprotein receptor on hepatocytes, induce the expected T C substitution (data not shown). However, when the chimeraplast (800 nM) was complexed with a 1:1 mix of Gal4-PEI and PEI, the transfected HepG2 cells showed a clear conversion to the apoE4 genotype (Figure 5A), which was also detected by direct sequencing (Figure 5B). This difference between the two carriers appeared to reflect delivery efficiency to the nucleus, as studies with a fluorescently-labelled chimeraplast showed almost a 2-fold increase in uptake into the nuclei when the mixed

PEI/Gal4-PEI carrier was used ($18.8 \pm 1.5\%$ [mean \pm SD; $n=3$] compared to $10.5 \pm 1.6\%$ for PEI alone; Figure 5C).

C. Transfection of THP-1 cells with both chimeraplasts

Unlike HepG2 cells, addition of the centrifugation step when treating THP-1 cells with the apoE3>E2 chimeraplast (400 nM complexed with PEI at a 1:5 phosphate:amine molar ratio) did not produce a conversion as judged by PCR-RFLP analysis. Negative results were also obtained with Man-PEI, which binds to the endocytic mannose receptor on THP-1 cells, when used at 200 or 400 nM, but did show a conversion at 800 nM (Figure 6A). This conversion was confirmed in a second independent experiment (Figure 6B). On the other hand, no *APOE3* to *APOE4* conversions were detected using the apoE3>E4 chimeraplast even though the THP-1 cells were subjected to a variety of treatments, including a range of PEI or Man-PEI concentrations (200-1000 nM) plus centrifugation, and varying the phosphate:amine molar ratios (1:3-1:9) (data not shown).

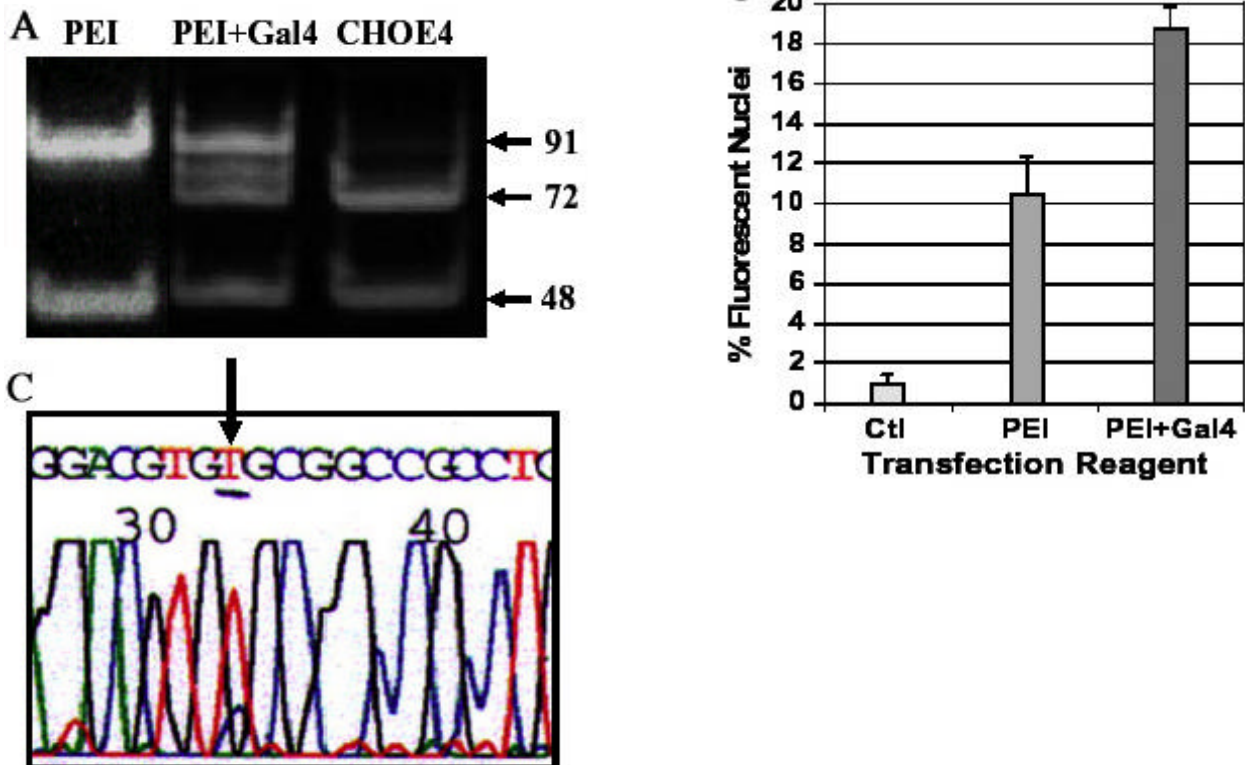


Figure 5. Chimeraplast delivery by galactose-modified PEI is required for *APOE3* to *APOE4* gene conversion in HepG2 cells. (A) HepG2 cells were transfected with apoE3>E4 chimeraplast (800nM) complexed with PEI or a 1:1 mix of PEI/Gal4-PEI, followed by brief centrifugation. When analysed by PCR-RFLP, the chimeraplast delivered by PEI alone failed to produce a conversion, whereas the PEI/Gal4-PEI combination was successful as indicated by appearance of the diagnostic 72 bp band (arrow). Note, that the analysis is complicated by an additional band at ~80 bp, which though unexpected has been observed previously during routine genotyping of apoE4/E3 or apoE4/E4 patients (Wu et al, 2000). (B) Substitution of T by C was verified by direct sequencing, which revealed colocalization of the two nucleotides (arrow). (C) The nuclear uptake of fluorescently-labelled chimeraplast was analysed by FACS in three independent experiments. The data are shown as mean \pm SD and demonstrate that the 1:1 mix of PEI/Gal4-PEI is a more efficient carrier for chimeraplast delivery than PEI alone ($P<0.05$ by Student's *t*-test). PEI, carrier was PEI alone; PEI+Gal4, carrier was PEI mixed 1:1 with Gal4-PEI; CHO E4, reference PCR-RFLP fragments from CHO E4 DNA; Ctl, control mock-transfected cells.

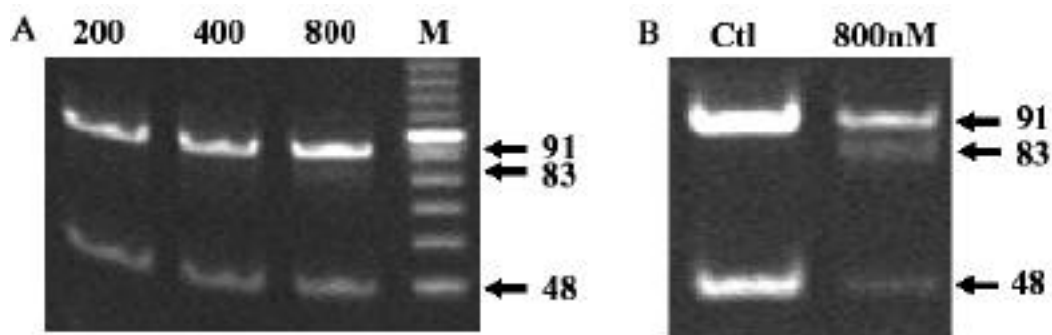


Figure 6. Editing of the *APOE* gene in THP-1 cells using chimeraplasts. (A) THP-1 cells were transfected with varying amounts of the apoE3>E2 chimeraplast (200-800 nM) complexed with Man-PEI at a phosphate:amine molar ratio of 1:5, followed by brief centrifugation. The diagnostic 83 bp was not evident at 200 and 400 nM, but was detected in cells treated with 800 nM of the chimeraplast. (B) This finding was confirmed in a second independent experiment when PCR-RFLP analysis again showed clear emergence of the 83 bp digestion fragment. M, 10 bp ladder; Ctl, control mock-transfected cells.

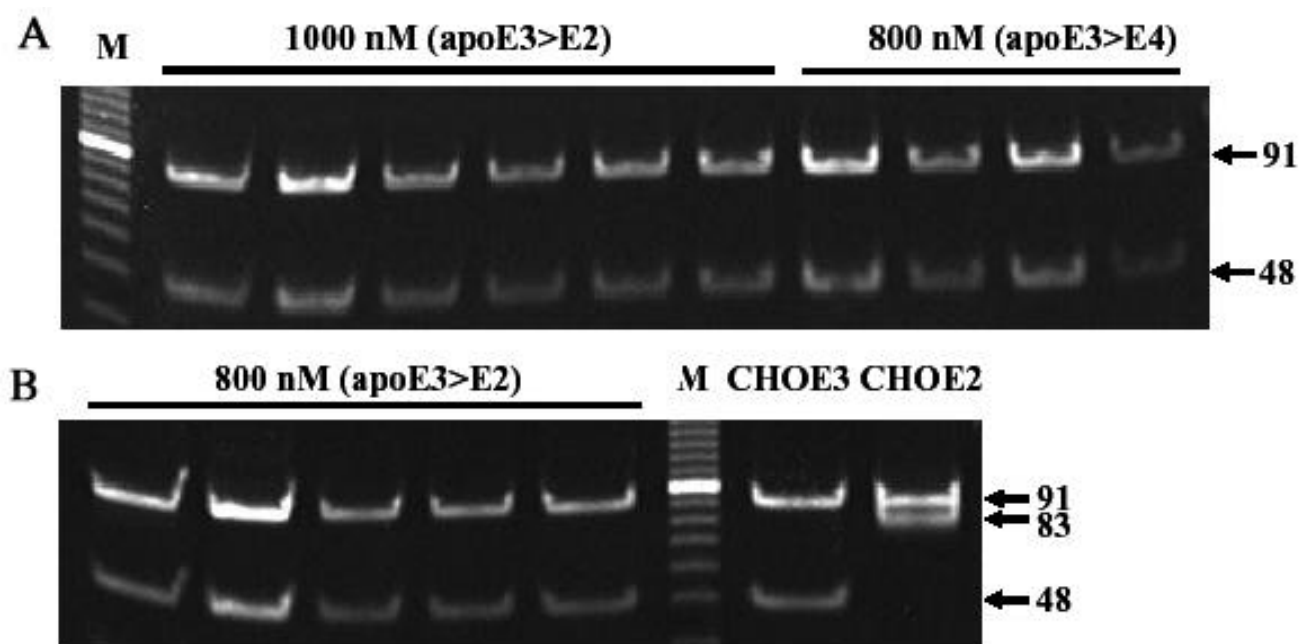


Figure 7. Cloning of chimeraplast-treated HepG2 and THP-1 cells. Samples of the HepG2 (1000 nM or 400 nM) and THP-1 (800 nM) cells that were successfully treated with apoE3>E2 chimeraplast, as well as HepG2 cells that had been converted with apoE3>E4 chimeraplast (800 nM) were passaged four times and cloned by limiting dilution. Fifty clones of each cell line were then analysed by PCR-RFLP. (A) Some of the analysed HepG2 clones are shown, but no conversion could be detected in any of the lines. (B) Similarly, when the THP-1 clones were analysed the diagnostic 83 bp band could.

D. Cloning of chimeraplast-treated HepG2 and THP-1 cells

Samples of the HepG2 and THP-1 cells that were successfully treated were maintained in culture, passaged four times and then cloned by limiting dilution in 96-well plates. DNA was extracted from 50 clones of each (HepG2 cells treated with 400 nM or 1000 nM of apoE3>E2 chimeraplast, THP-1 cells treated with 800 nM of apoE3>E2, and HepG2 cells treated with 800 nM apoE3>E4) and was analysed by PCR-RFLP. Unexpectedly, all the clones were of *APOE3* genotype (Figure 7).

IV. Discussion

The pleiotropic atheroprotective effects of apoE encompass cholesterol transport — sequestration from arteries (Huang et al, 1994) and disposal of cholesterol-rich remnants in liver (Mahley and Ji, 1999) — as well as diverse beneficial actions at sites of atherosclerotic lesions (Davignon, 2005). Molecular explanations for these activities, however, are often incomplete, while differential effects of the common apoE isoforms are not fully documented. To further such research endeavours, we have sought to generate new human hepatocyte and macrophage cell lines that secrete dysfunctional apoE2 or apoE4. As plasma-purified apoE has reduced biological activity compared to the native, lipidated apoE particles secreted by cells (LaDu et al, 1995; Stannard et al, 2001),

we believe that such cell lines will be invaluable research tools. For example, they will be a significant resource in which to study intracellular trafficking of the individual isoforms (Cullen et al, 1998), while the biological and physicochemical characteristics of the secreted particles can be compared (DeMattos et al, 2001; Sacre et al, 2003). The technology will also be of interest to researchers working in different areas. Thus, apoE4 is a risk factor for Alzheimer's disease and other neurodegenerative disorders (Thilakawardhana et al, 2005), while apoE alleles moderate damage caused by viral infections, including hepatitis C (Wozniak et al, 2002), herpes simplex virus (Dobson and Itzhaki, 1999), and HIV (Corder et al, 1998), or by the malaria protozoon (Wozniak et al, 2003).

In an initial reagent-validation experiment, we used a 68-mer apoE3>E2 chimeraplast (400 nM) to edit *APOE3* to *APOE2* in recombinant CHO cells. This was the reverse of a previous study in which we repaired the *APOE2* gene in CHO2 cells (Tagalakis et al, 2001). We also showed that genomic DNA in human HEK-293 cells could be targeted, whereas under identical conditions no conversion could be detected in HepG2 cells. As *APOE3* is not transcribed in HEK-293 cells, this argues against the suggestion that efficient conversions are transcription-coupled (Liu et al, 2002; Igoucheva et al, 2003). Positive conversions, however, were readily achieved by brief centrifugation of HepG2 cell monolayers; presumably, improved sedimentation of the chimeraplast-PEI complexes gave greater interaction with the cell surface and facilitated their uptake. But this additional step did not give detectable conversion in HepG2 cells when the apoE3>E4 chimeraplast was used. Whether this represents a locus- or sequence-specific difference between the two SNPs or, more likely, a difference in purity between the two long 68-mer chimeraplast molecules (Manzano et al, 2003) was not investigated further.

Rather, we sought to improve delivery of the chimeraplast-vehicle complex by exploiting receptor-mediated endocytosis via the asialoglycoprotein receptor (ASGR) present on hepatocytes. Transfer of DNA to hepatocytes through the ASGR initially used asialoorosomucoid-polylysine as the vehicle (Wu et al, 1989), while subsequent work established PEI harbouring galactosyl residues as an effective agent (Zanta et al, 1997). Indeed, PEI modified with lactose (galactose- 1,4-glucose) efficiently delivers chimeraplasts to primary cultured hepatocytes, and to rat liver *in vivo* (Bandyopadhyay et al, 1999; Kren et al, 1998). Nevertheless, substituting primary amino groups of PEI with sugars strongly affects properties of the DNA-polymer (polyplex), including charge, size, and cytotoxicity (Zanta et al, 1997; Kunath et al, 2003; Kichler, 2004). Thus, although Gal4-PEI (PEI bearing linear tetragalactose) delivers DNA more efficiently than Gal-PEI (Bettinger et al, 1999), the apoE3>E4 chimeraplast still failed to produce a detectable nucleotide substitution in HepG2 cells. However, by mixing Gal4-PEI with PEI in a 1:1 ratio a successful *APOE3* to *APOE4* conversion was demonstrated, albeit using a high concentration of chimeraplast (800 nM). It appears that this mixed reagent, which combines receptor binding

(Gal4-PEI) and effective DNA condensing (PEI) properties, is superior to the individual polymers.

The *APOE3* gene proved more difficult to target in THP-1 cells, perhaps because of their inherent low transfection efficiency. Thus, as both apoE3>E2 and apoE3>E4 chimeraplasts gave no obvious conversions using PEI plus centrifugation for delivery, we investigated glycotargeting, this time via the surface-bound mannose receptors expressed by THP-1 cells (Suzuki et al, 1998). Mannose receptors recognize mannose, fucose and N-acetylglucosamine residues (Achord et al, 1977), but not galactose-terminal glycoproteins (Lennartz et al, 1987). Moreover, PEI modified with mannose delivers DNA into macrophages (Sato et al, 2001) or dendritic cells (Diebold et al, 1999) with high efficiency. Here, we detected gene conversion in THP-1 cells when a high level (800 nM) of the apoE3>E2 chimeraplast was transferred with Man-PEI as carrier. However, the apoE3>E4 reagent produced no change, conceivably because glycotargeting is less efficient in THP-1 cells than HepG2 cells; alternatively, mismatch repair activity may be low in monocyte-macrophages. Whatever factors are important, it is clear that each cell line differs and requires its own specific optimization protocol. For example, in the present study the ease of apoE3>E2 chimeraplast-mediated gene conversion was CHO2 or HEK-293 (PEI alone) > HepG2 (PEI + centrifugation) > THP-1 (Man-PEI).

When some of the successfully converted HepG2 and THP-1 cells were passaged several times and cloned, no conversion was detected when 50 clones from each of four batches (three HepG2; one THP-1) of treated cells were analysed. This was unexpected, since the nucleotide substitutions were prominent 24 h post-transfection, and most unlikely to be false positives. Thus, although there has been speculation that PCR-based assays might produce artefacts (Thomas and Capecchi, 1997; Zhang et al, 1998), this early criticism was not supported by experimental evidence and was later discounted by corroborative PCR-independent techniques, including Southern blotting, reporter gene assays and phenotypic analyses (protein and enzyme assays), which verified the reality of gene editing (Graham et al, 2001; Yin et al, 2005). Moreover, 'spiking' experiments with intact chimeraplast or potentially degraded fragments failed to show artefactual conversions (Tagalakis et al, 2005). Finally, the findings reported herein are difficult to reconcile with false positives, as we also report negative data and differences between the apoE3>E2 and apoE3>E4 reagents.

One explanation for unstable conversions might be a general cytotoxic action of the polyplexes, due to PEI or its modified versions (Gebhart and Kabanov, 2001). An alternative possibility is that converted cells are triggered to undergo apoptosis, perhaps in response to high cellular levels of oligonucleotides, as fragmented or single-stranded DNA can induce cell cycle arrest and promote cell death (Nur-E-Kamal et al, 2003). Consistent with our findings, Strauss and colleagues reported selective apoptosis in gene-converted cells 4-6 days post-transfection, a time-frame which was thought to exclude any general toxicity factors (Olsen et al, 2003). More recently, this group has used recombinant cells expressing

integrated mutated eGFP (enhanced green fluorescent protein) to show that nearly all converted cells arrest in the G2/M cell cycle phase, though they also report that a small percentage escape from, or do not enter, the arrest and can form viable corrected colonies (Olsen et al, 2005). Intriguingly, the authors speculate that cells may have effective defence mechanisms that counteract targeted genome sequence alterations by blocking cell cycle progression of corrected cells.

In summary, our findings establish the feasibility of editing the endogenous *APOE3* gene in HepG2 and THP-1 cells using synthetic oligonucleotides. However, the conversions were unstable and mutated clones were not isolated. Notwithstanding, a recent report using a sensitive reporter gene assay suggests that 1-2% of converted cells are stable and viable (Olsen et al, 2005). This implies that screening much larger numbers of individual clones will enable new apoE2- and apoE4-secreting THP-1 and HepG2 cell lines to be isolated. Such a possibility, together with systematic studies to determine the timing and cause of conversion instability, merit further investigation in future research. Several immediate avenues suggest themselves: use of low-toxicity vehicles such as atelocollagen (Nakamura et al, 2004) and of high-purity single-stranded oligonucleotides, perhaps lacking protective chemical modifications as these can lower conversion efficiency (Dekker et al, 2003); and the selective manipulation of elements within gene repair pathways (Parekh-Olmedo et al, 2005), including suppressing MSH2 (Dekker et al, 2003) or enhancing RAD51 and/or XRCC2 (Thorpe et al, 2002; Olsen et al, 2003) activities. It is to be hoped that refinements and changes to the technology in these key areas will lead to efficient and robust gene editing, and eventually to the treatment of disease caused by point mutations.

Acknowledgements

This research was supported by British Heart Foundation PhD studentships to Z.M (FS/99014) and G.S. (FS/2000011), while A.M was supported by a Marie-Curie Fellowship. We thank Dr Thierry Bettinger for the generous provision of Gal4-PEI and Man-PEI.

References

- Achord DT, Brot FE and Sly WS (1977) Inhibition of the rat clearance system for agalacto-orosomucoid by yeast mannans and by mannose. **Biochem Biophys Res Commun** 77, 409-415.
- Alexeev V, Igoucheva O, Domashenko A, Cotsarelis G and Yoon K (2000) Localized in vivo genotypic and phenotypic correction of the albino mutation in skin by RNA-DNA oligonucleotide. **Nat Biotechnol** 18, 43-47.
- Bandyopadhyay P, Ma X, Linehan-Stieers C, Kren BT and Steer CJ (1999) Nucleotide exchange in genomic DNA of rat hepatocytes using RNA/DNA oligonucleotides. Targeted delivery of liposomes and polyethylenimine to the asialoglycoprotein receptor. **J Biol Chem** 274, 10163-10172.
- Beetham PR, Kipp PB, Sawycky XL, Arntzen CJ and May GD (1999) A tool for functional plant genomics: chimeric RNA/DNA oligonucleotides cause in vivo gene-specific mutations. **Proc Natl Acad Sci USA** 96, 8774-8778.
- Bellosta S, Mahley RW, Sanan DA, Murata J, Newland DL, Taylor JM and Pitas RE (1995) Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. **J Clin Invest** 96, 2170-2179.
- Bettinger T, Remy JS and Erbacher P (1999) Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. **Bioconjug Chem** 10, 558-561.
- Cole-Strauss A, Yoon K, Xiang Y, Byrne BC, Rice MC, Gryn J, Holloman WK and Kmiec EB (1996) Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. **Science** 273, 1386-1389.
- Corbo RM and Scacchi R (1999) Apolipoprotein E (APOE) allele distribution in the world. Is APOE*4 a 'thrifty' allele? **Ann Hum Genet** 63, 301-310.
- Corder EH, Robertson K, Lannfelt L, Bogdanovic N, Eggertsen G, Wilkins J and Hall C (1998) HIV-infected subjects with the E4 allele for APOE have excess dementia and peripheral neuropathy. **Nat Med** 4, 1182-1184.
- Cullen P, Cignarella A, Brennhansen B, Mohr S, Assmann G and von Eckardstein A (1998) Phenotype-dependent differences in apolipoprotein E metabolism and in cholesterol homeostasis in human monocyte-derived macrophages. **J Clin Invest** 101, 1670-1677.
- Davignon (2005) Apolipoprotein E and atherosclerosis: beyond lipid effect. **Arterioscler Thromb Vasc Biol** 25, 267-269.
- Dekker M, Brouwers C and te Riele H (2003) Targeted gene modification in mismatch-repair-deficient embryonic stem cells by single-stranded DNA oligonucleotides. **Nucleic Acids Res** 31, e27.
- DeMattos RB, Rudel LL and Williams DL (2001) Biochemical analysis of cell-derived apoE3 particles active in stimulating neurite outgrowth. **J Lipid Res** 42, 976-987.
- Diebold SS, Lehrmann H, Kurska M, Wagner E, Cotten M and Zenke M (1999) Efficient gene delivery into human dendritic cells by adenovirus polyethylenimine and mannose polyethylenimine transfection. **Hum Gene Ther** 10, 775-786.
- Dobson CB and Itzhaki RF (1999) Herpes simplex virus type 1 and Alzheimer's disease. **Neurobiol Aging** 20, 457-465.
- Gebhart CL and Kabanov AV (2001) Evaluation of polyplexes as gene transfer agents. **J Control Release** 73, 401-416.
- Graham IR, Manzano A, Tagalakis AD, Mohri Z, Sperber G, Hill V, Beattie S, Schepelmann S, Dickson G and Owen JS (2001) Gene repair validation. **Nat Biotechnol** 19, 507-508.
- Hixson JE and Vernier DT (1990) Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. **J Lipid Res** 31, 545-548.
- Huang Y, von Eckardstein A, Wu S, Maeda N and Assmann G (1994) A plasma lipoprotein containing only apolipoprotein E and with gamma mobility on electrophoresis releases cholesterol from cells. **Proc Natl Acad Sci USA** 91, 1834-1838.
- Igoucheva O, Alexeev A, Pryce M and Yoon K (2003) Transcription affects formation and processing of intermediates in oligonucleotide-mediated gene alteration. **Nucleic Acids Res** 31, 2659-2670.
- Kichler A (2004) Gene transfer with modified polyethylenimines. **J Gene Med** 6, S3-S10.
- Kraft HG, Menzel HJ, Hoppichler F, Vogel W and Utermann G (1989) Changes of genetic apolipoprotein phenotypes caused by liver transplantation. Implications for apolipoprotein synthesis. **J Clin Invest** 83, 137-142.
- Kren BT, Bandyopadhyay P and Steer CJ (1998) In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. **Nat Med** 4, 285-290.

- Kunath K, von Harpe A, Fischer D and Kissel T (2003) Galactose-PEI-DNA complexes for targeted gene delivery: degree of substitution affects complex size and transfection efficiency. **J Control Release** 88, 159-172.
- LaDu M, Pederson TM, Fraill DE, Reardon CA, Getz GS and Falduto MT (1995) Purification of apolipoprotein E attenuates isoform-specific binding to beta-amyloid. **J Biol Chem** 270, 9039-9042.
- Lennartz MR, Wileman TE and Stahl PD (1987) Isolation and characterization of a mannose-specific endocytosis receptor from rabbit alveolar macrophages. **Biochem J** 245, 705-711.
- Linton MF, Atkinson JB and Fazio S (1995) Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. **Science** 267, 1034-1037.
- Liu L, Rice MC, Drury M, Cheng S, Gamper H and Kmiec EB (2002) Strand bias in targeted gene repair is influenced by transcriptional activity. **Mol Cell Biol** 22, 3852-3863.
- Mahley RW and Ji ZS (1999) Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. **J Lipid Res** 40, 1-16.
- Mahley RW, Huang Y and Rall SC Jr (1999) Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia) Questions, quandaries, and paradoxes. **J Lipid Res** 40, 1933-1949.
- Manzano A, Mohri Z, Sperber G, Ogris M, Graham I, Dickson G and Owen JS (2003) Failure to generate atheroprotective apolipoprotein AI phenotypes using synthetic RNA/DNA oligonucleotides chimeraplasts. **J Gene Med** 5, 795-802.
- Miyata M and Smith JD (1996) Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. **Nat Genet** 14, 55-61.
- Nakamura M, Ando Y, Nagahara S, Sano A, Ochiya T, Maeda S, Kawaji T, Ogawa M, Hirata A, Terazaki H, Haraoka K, Tanihara H, Ueda M, Uchino M and Yamamura K (2004) Targeted conversion of the transthyretin gene in vitro and in vivo. **Gene Ther** 11, 838-846.
- Nur-E-Kamal A, Li TK, Zhang A, Qi H, Hars ES and Liu LF (2003) Single-stranded DNA induces ataxia telangiectasia mutant (ATM)/p53-dependent DNA damage and apoptotic signals. **J Biol Chem** 278, 12475-12481.
- Olsen PA, McKeen C and Krauss S (2003) Branched oligonucleotides induce in vivo gene conversion of a mutated EGFP reporter. **Gene Ther** 10, 1830-1840.
- Olsen PA, Randol M and Krauss S (2005) Implications of cell cycle progression on functional sequence correction by short single-stranded DNA oligonucleotides. **Gene Ther** 12, 546-551.
- Parekh-Olmedo H, Ferrara L, Brachman E and Kmiec EB (2005) Gene therapy progress and prospects: targeted gene repair. **Gene Ther** 12, 639-646.
- Rando TA, Disatnik MH and Zhou LZ (2000) Rescue of dystrophin expression in mdx mouse muscle by RNA/DNA oligonucleotides. **Proc Natl Acad Sci USA** 97, 5363-5368.
- Rees A, Shoulders CC, Stocks J, Galton DJ and Baralle FE (1983) DNA polymorphism adjacent to human apoprotein A-1 gene: relation to hypertriglyceridaemia. **Lancet** 1, 444-446.
- Rice MC, May GD, Kipp PB, Parekh H and Kmiec EB (2000) Genetic repair of mutations in plant cell-free extracts directed by specific chimeric oligonucleotides. **Plant Physiol** 123, 427-438.
- Rice MC, Bruner M, Czymbek K and Kmiec EB (2001) In vitro and in vivo nucleotide exchange directed by chimeric RNA/DNA oligonucleotides in *Saccharomyces cerevisiae*. **Mol Microbiol** 40, 857-868.
- Sacre S, Stannard AK and Owen JS (2003) Apolipoprotein E (apoE) isoforms differentially induce nitric oxide production in endothelial cells. **FEBS Lett** 540, 181-187.
- Sato A, Kawakami S, Yamada M, Yamashita F and Hashida M (2001) Enhanced gene transfection in macrophages using mannoseylated cationic liposome-polyethylenimine-plasmid DNA complexes. **J Drug Target** 9, 201-207.
- Siest G, Pillot T, Regis-Bailly A, Leininger-Muller B, Steinmetz J, Galteau MM and Visvikis S (1995) Apolipoprotein E: an important gene and protein to follow in laboratory medicine. **Clin Chem** 41, 1068-1086.
- Song Y, Stampfer MJ and Liu S (2004) Meta-analysis: apolipoprotein E genotypes and risk for coronary heart disease. **Ann Intern Med** 2004 141, 137-147.
- Stannard AK, Riddell DR, Sacre SM, Tagalakis AD, Langer C, von Eckardstein A, Cullen P, Athanasopoulos T, Dickson G and Owen JS (2001) Cell-derived apolipoprotein E (ApoE) particles inhibit vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells. **J Biol Chem** 276, 46011-46016.
- Suzuki T, Ohno N, Ohshima Y and Yadomae T (1998) Soluble mannann and beta-glucan inhibit the uptake of *Malassezia furfur* by human monocytic cell line, THP-1. **FEMS Immunol Med Microbiol** 21, 223-230.
- Tagalakis AD, Graham IR, Riddell DR, Dickson JG and Owen JS (2001) Gene correction of the apolipoprotein (Apo) E2 phenotype to wild-type ApoE3 by in situ chimeraplasty. **J Biol Chem** 276, 13226-13230.
- Tagalakis AD, Dickson G, Owen JS and Simons JP (2005) Correction of the neuropathogenic human apolipoprotein E4 (APOE4) gene to APOE3 in vitro using synthetic RNA/DNA oligonucleotides (chimeraplasts). **J Mol Neurosci** 25, 95-104.
- Thilakawardhana S, Everett DM, Murdock PR, Dingwall C and Owen JS (2005) Quantification of apolipoprotein E receptors in human brain-derived cell lines by real-time polymerase chain reaction. **Neurobiol Aging** 26, 813-823.
- Thomas KR and Capocchi MR (1997) Recombinant DNA technique and sickle cell anemia research. **Science** 275, 1404-1405.
- Thorpe PH, Stevenson BJ and Porteous DJ (2002) Functional correction of episomal mutations with short DNA fragments and RNA-DNA oligonucleotides. **J Gene Med** 4, 195-204.
- Weisgraber KH (1994) Apolipoprotein E: structure-function relationships. **Adv Protein Chem** 45, 249-302.
- Wozniak MA, Itzhaki RF, Faragher EB, James MW, Ryder SD and Irving WL (2002) Apolipoprotein E-epsilon 4 protects against severe liver disease caused by hepatitis C virus. **Hepatology** 36, 456-463.
- Wozniak MA, Faragher EB, Todd JA, Koram KA, Riley EM and Itzhaki RF (2003) Does apolipoprotein E polymorphism influence susceptibility to malaria? **J Med Genet** 40, 348-51.
- Wu CH, Wilson JM and Wu GY (1989) Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo. **J Biol Chem** 264, 16985-16987.
- Wu YY, Delgado R, Costello R, Sunderland T, Dukoff R and Csako G (2000) Quantitative assessment of apolipoprotein E genotypes by image analysis of PCR-RFLP fragments. **Clin Chim Acta** 293, 213-221.
- Yin W, Kren BT and Steer CJ (2005) Site-specific base changes in the coding or promoter region of the human beta and gamma globin genes by single-stranded oligonucleotides. **Biochem J** 290, 253-261.
- Yoon K, Cole-Strauss A and Kmiec EB (1996) Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA-DNA oligonucleotide. **Proc Natl Acad Sci USA** 93, 2071-2076.
- Zanta MA, Boussif O, Adib A and Behr JP (1997) In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. **Bioconjug Chem** 8, 839-844.
- Zeleny M, Swertfeger DK, Weisgraber KH and Hui DY (2002) Distinct apolipoprotein E isoform preference for inhibition of

smooth muscle cell migration and proliferation.
Biochemistry 41, 11820-11823.

Zhang Z, Eriksson M, Falk G, Graff C, Presnell SC, Read MS, Nichols TC, Blomback M and Anvret M (1998) Failure to achieve gene conversion with chimeric circular oligonucleotides: potentially misleading PCR artifacts observed. **Antisense Nucleic Acid Drug Dev** 8, 531-536.

Zhu T, Mettenburg K, Peterson DJ, Tagliani L and Baszczyński CL (2000) Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. **Nat Biotechnol** 18, 555-558.



James S. Owen