Advances in Vascular Medicine
Preface

Understanding the many complex cellular and molecular mechanisms underlying human vascular diseases is essential in improving the treatment of this important and wide-ranging group of diseases that affect a large proportion of the world population. This book is based on lectures presented at an International Vascular Biology Workshop held in London and chaired by Professor Dame Carol Black. The contents are complemented by some invited chapters, all written by world experts in areas of basic science and clinical medicine highly relevant to vascular biology and disease. We are particularly grateful to Professor Arshed Quyyumi, Professor of Medicine and Cardiology at Emory University, who with his research group and clinical colleagues, has provided a substantial contribution to this book. In common with our previous book – Vascular Complications in Human Disease: Mechanisms and Consequences published by Springer in 2008, our aim with this book is to highlight some of the established relationships between basic science and clinical medicine, and to outline new and exciting fields of research and practice in vascular biology and pathobiology.

There are two sections: Basic Science of Vascular Biology and Clinical Aspects of Vascular Biology. In the first section, dealing with basic science, we have included three important growth areas: “Genetics and Gene Therapy” cover approaches to gene therapy and delivery systems, “Animal Models to Study Vascular Disease” with chapters on animal models of scleroderma, animal models of atherosclerosis, and finally on the endothelin system. The final section on basic science titled “Molecules and Mediators and Therapeutic Applications” encompasses the role of endothelin in systemic sclerosis, and other aspects of the genetics and biology of endothelium and vascular function and includes a chapter on Cell Therapy for Cardiovascular Diseases and Cell and Molecular Mechanism(s) Underlying Vascular Remodeling. These basic science topics underpin what may further improve the clinical care of patients with vascular diseases.

The first section on clinical aspects of vascular biology is written by our colleagues from Papworth Hospital, currently the only UK center operating on patients with chronic thromboembolic disease associated pulmonary hypertension; this section also includes a chapter on imaging in acute and chronic thromboembolic disease. Vascular disease in connective tissue diseases includes chapters on pulmonary arterial hypertension in connective tissue disease, registry and epidemiological data
in systemic sclerosis associated pulmonary arterial hypertension, and a review of vascular disease in systemic sclerosis. The final clinical section on Cardiovascular Disease, includes the important topics of coronary heart disease in women, graft performance in coronary artery surgery, predicting cardiovascular risk and the metabolic syndrome.

Although common basic science strands link the chapters, each chapter stands alone as an authoritative, up-to-date and powerful insight into these important topics of vascular biology. The chapters help the basic scientist understand clinical problems as well as explaining to clinicians the scientific foundations of vascular diseases and allude to possible tracks for future research.

Although we are making progress in understanding some of the basic scientific mechanisms of vascular disease, there is much work to be done. The picture is thus far from complete. We hope that the information and insights contained in this book will be a useful contribution to the literature and help other scientists and clinicians make progress in this exciting field of biomedicine.

London, UK

David Abraham
Clive Handler
Michael Dashwood
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Vascular medicine, in the form of vascular surgery, has its origins in attempts to ligate blood vessels to prevent haemorrhage, which is a well-described practice in one of the first extant textbooks of surgery written by the Indian doctor Sushruta over 2,500 years ago. However, reconstructive vascular surgery to repair torn or aneurysmic vessels, or to create anastomoses, only really became routinely feasible in the 19th century when surgery in general benefitted from the introduction of antiseptic and anesthetic procedures. Other areas of vascular medicine are much more recent, and needed for their impetus the realization that blood vessels are far more than structural conduits but have an intrinsic biology and pathology that contribute to a wide range of diseases.

For example, though the atherosclerotic process was accurately described by eminent 19th century pathologists such as Virchow, it is only since after World War II that cardiologists began to take seriously the idea that acute myocardial infarction was a consequence of atherosclerosis in the coronary arteries, leading to the huge upsurge in the last 50 years of novel interventional attempts to remedy the problem – coronary bypass surgery since 1960, angioplasty since the mid 1970s, bare metal stents since the mid 1980s, and drug eluting stents since 2002.

In parallel with developments in intervention, vascular biology has steadily increased our understanding of the cellular and molecular physiology of blood vessels, revealing the characteristic responses of endothelial and smooth muscle cells to insult and injury and defining their active roles in the maintenance of vascular homeostasis. As late as 1960, the endothelium was described as a passive, blood-compatible, semi-permeable membrane – leading to Lord Florey’s rejection of this view of the endothelium as “little more than a sheet of nucleated cellophane” and the beginnings of endothelial cell biology. This was to lead within 20 years to important discoveries, including the key molecular mechanisms by which endothelium controls leukocyte traffic and other aspects of the inflammatory response, and then the Nobel prize-winning discovery of nitric oxide as a novel endogenous endothelium-derived signalling molecule that regulates vascular tone and platelet function. Another hugely significant body of cell and molecular biology has stemmed from Folkman’s careful description of new blood vessel formation (angiogenesis) and his seminal discovery of its importance for tumor growth.
Thus we are currently in an exciting phase, as the molecular discoveries from vascular biology are beginning to be combined with the increasingly sophisticated technical expertise of vascular interventionists and surgeons, leading confidently into an era when a series of novel preventive and regenerative strategies will be applied successfully to vascular medicine. This volume provides a valuable snapshot of several growth points in vascular medicine, both preclinical and clinical, that reflect these strategies and the wide range of diseases where they impact.

King’s College, London

Jeremy D. Pearson
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Section 1
Part 1
Genetics and Gene Therapy
1.1 Plasma Lipoproteins and Dyslipoproteinemia

1.1.1 Delivering Lipid Nutrients

Plasma lipoproteins are large macromolecular particles integral to the transport of lipids between the liver, intestine, and peripheral tissues (Fig. 1.1). Dietary triglycerides and other fat-soluble substances are absorbed from the intestine and packaged into chylomicrons, which are secreted into the bloodstream via the lymphatics. Following lipolysis by endothelium-bound lipoprotein lipase, their energy-rich triglyceride is delivered to peripheral tissues as free fatty-acids, leaving behind cholesterol-enriched chylomicron remnants, which are rapidly cleared by the liver. In contrast, following carbohydrate ingestion or fasting, endogenous triglycerides and also cholesterol are packaged into very-low-density lipoprotein (VLDL) for transport from liver to the periphery. As with chylomicron lipolysis, the delivery of triglycerides decreases VLDL volume and changes its composition, forming initially intermediate-density lipoprotein (IDL) and then low-density lipoprotein (LDL) particles. The half-life of LDL, which carries three-quarters of plasma cholesterol, is much longer than chylomicron remnants, and the particles are catabolized by LDL-receptors in both peripheral tissues and the liver.

1.1.2 Reverse Cholesterol Transport

The remaining major class of lipoproteins, high-density lipoprotein (HDL), is involved in reverse cholesterol transport, the pathway by which excess cellular cholesterol is transferred from the periphery to the liver for excretion (Fig. 1.1).
Newly synthesized or nascent HDL particles are discoidal and secreted by the enterohepatic system. They sequester free cholesterol from peripheral cells, which is esterified by the plasma enzyme lecithin-cholesterol acyltransferase (LCAT) to form cholesteryl esters that move into a hydrophobic core. Loading of nascent HDL with cholesterol produces mature, spherical HDL particles, which can deliver their cholesteryl ester to liver via scavenger receptors. Alternatively, some cholesteryl esters are transferred to VLDL and LDL in exchange for triglyceride by the cholesteryl ester transfer protein (CETP), and so are eventually taken up by hepatic LDL-receptors.

Fig. 1.1 The lipoprotein transport system for plasma lipids. Dietary lipids are absorbed in the intestine and packaged into chylomicrons, which then deliver energy-rich triglycerides to peripheral tissues, particularly muscle and adipose. The resultant remnant particles are cleared by hepatic receptors via ApoE. Endogenous lipids in liver are secreted as very-low-density lipoprotein (VLDL), which undergoes a similar lipolysis to generate cholesterol-rich low-density lipoprotein (LDL) particles that are taken up equally by liver and extrahepatic tissues. Reverse cholesterol transport from periphery to liver occurs when nascent discoidal high-density lipoprotein (HDL) (lipid-poor ApoAI) sequesters cellular cholesterol, which, following esterification to facilitate internal packing and formation of mature spherical HDL, is then transported to liver, for recycling or excretion.
1.1.3 Clinical Relevance

The metabolism and relative concentrations of plasma lipoproteins are of profound clinical importance, as dyslipoproteinemias are common and are major risk factors for cardiovascular disease (CVD), the leading cause of death worldwide. High plasma cholesterol levels in the form of LDL or remnant particles result in deposition of lipids in the arterial wall, ultimately causing atherosclerosis. Moreover, low HDL is an important independent risk factor because of its role in removing excess cholesterol from arterial cells. Controlling risk through lifestyle modification and/or drugs, such as statins to lower LDL, are major public health goals. Nonetheless, about two-thirds of adverse cardiovascular events continue despite these interventions; increasingly, new therapeutic approaches are required.

1.2 Key Protein Targets within Plasma Lipid Transfer Pathways

1.2.1 Apolipoprotein B (ApoB)

1.2.1.1 Structural and Receptor-Binding Functions

Apolipoprotein B (ApoB) is the principal protein component of VLDL, IDL, LDL, and chylomicrons. It is primarily expressed in liver and intestine, but the two translational products differ. In the intestine, ApoB mRNA is subject to post-transcriptional editing, which results in a premature stop codon. Thus, both the full-length protein, ApoB100 (515-kDa), and a carboxy-terminus truncated form, ApoB48 (244-kDa; hence 48% the size of ApoB100), are produced from the same gene. Editing of ApoB mRNA is accomplished by the editosome, a multiprotein complex whose active subunit is APOBEC-1 (ApoB mRNA-editing enzyme catalytic polypeptide 1). Circulating liver-derived VLDL has ApoB100 as its major protein constituent, while ApoB48 is the structural protein of chylomicrons.

This difference determines their catabolism: as ApoB48 lacks the C-terminal moiety, it cannot bind to the LDL-receptor and hence chylomicron remnants are cleared via ApoE interaction with hepatic LDL-receptor-related protein (LRP), a fast catabolic step. In contrast, binding of ApoB100 is a slow process and the half-life of LDL is 100-fold greater than chylomicrons. This exacerbates the susceptibility of LDL to oxidation and ingestion by monocyte-macrophages to form “foam cells,” the lipid streak of early atherosclerotic lesions. As LDL has a central role in atherosclerotic plaque formation, inhibition of ApoB synthesis is considered a therapeutic target to reduce CVD risk. However, genetic evidence suggests that any suppression of ApoB expression must be carefully controlled to prevent adverse consequences.
1.2.1.2 ApoB Deficiency

Familial hypobetalipoproteinemia (FHBL) is an inherited genetic disease, which naturally reduces levels of functional ApoB.\textsuperscript{1,6,10} In most cases of FHBL, the mutation results in production of truncated inactive ApoB, although nontruncating missense mutations with impaired secretion, such as R463W, are also documented.\textsuperscript{11} The more severe form of the disease abetalipoproteinemia (ABL), in which there is complete absence of ApoB-containing lipoproteins, is often caused by inactivating mutations in the microsomal triglyceride transfer protein (MTP), a molecular chaperone essential for the correct folding and lipidation of ApoB in the endoplasmic reticulum.\textsuperscript{12} ABL results in intestinal fat malabsorption, and patients present with severe neurological and other disorders due to lipid-soluble vitamin deficiency.\textsuperscript{12} In FHBL, heterozygous carriers have decreased LDL levels and a high incidence of nonalcoholic fatty liver disease and mild intestinal dysfunction, but are otherwise asymptomatic.\textsuperscript{1,10,13} Homozygous carriers, on the other hand, have a more severe phenotype similar to that of ABL.\textsuperscript{10,12}

1.2.2 Apolipoprotein AI (ApoAI)

1.2.2.1 ApoAI and HDL are Atheroprotective

Apolipoprotein AI is a 243 amino acid (28-kDa) amphipathic protein produced by liver and intestine, and is the main protein component of HDL. It is secreted into the circulation as a longer propeptide, which is proteolytically processed by cleavage of a hexapeptide to mature ApoAI.\textsuperscript{14} ApoAI is essential for formation of HDL and several of its biological functions; thus, impaired ApoAI activity results in diminished reverse cholesterol transport and low HDL levels.\textsuperscript{4,5} Genetic evidence reveals that mutations associated with defective ApoAI correlate strongly with increased risk for premature CVD.\textsuperscript{14} Complete absence of normal ApoAI, a condition known as analphalipoproteinemia, results in undetectable levels of HDL.\textsuperscript{1} Various missense mutations leading to premature termination of the protein, such as codon-2 (Q[-2]X) of the ApoAI propeptide\textsuperscript{14} or codon 136 (E136X),\textsuperscript{15} are associated with markedly decreased plasma HDL and greatly increased risk of premature CVD. Patients homozygous for Q[-2]X show additional pathology such as neuropathy and, due to subretinal lipid deposition, premature cataracts and retinopathy.\textsuperscript{14}

1.2.2.2 ApoAI$_{\text{Milano}}$

In 1980, a novel “gain-of-function” ApoAI variant termed ApoAI$_{\text{Milano}}$ and arising from a point mutation (C$\rightarrow$T; R173C) was identified, which appeared to be atheroprotective.\textsuperscript{16} Heterozygous patients with the ApoAI$_{\text{Milano}}$ mutation have decreased incidence of CVD, despite paradoxical low plasma HDL. Although anti-atherogenic
mechanism(s) of ApoAI\textsubscript{Milano} are poorly understood, the Cysteine-173 substitution allows intramolecular disulfide bond formation, which confers unique structural and functional properties to the HDL particles.\textsuperscript{17,18} Some studies in vitro suggest that ApoAI\textsubscript{Milano} increases cholesterol efflux, while in experimental animals infusion of ApoAI\textsubscript{Milano} stabilizes or even regresses established atherosclerotic plaque.\textsuperscript{19} Indeed, weekly injections of ApoAI\textsubscript{Milano}/phospholipid complexes for 5 weeks caused regression of coronary atheroma in patients with acute coronary syndrome, validating the potential of HDL-based therapeutics to treat CVD.\textsuperscript{20}

1.2.3 Apolipoprotein (ApoE)

Plasma ApoE is a polymorphic glycoprotein of 299 amino acids (34-kDa), primarily secreted by liver (90%) and monocyte-macrophages (10%); it is also synthesized by brain, kidneys, and spleen.\textsuperscript{21} ApoE plays a critical role in clearing remnant chylomicrons by targeting them to liver for receptor-mediated endocytosis.\textsuperscript{21} Two common isoforms arise from wild-type human ApoE3 by single nucleotide polymorphisms (SNPs) and increase risk of coronary heart disease.\textsuperscript{1,21,22} The rarest variant ApoE2 (Arg158Cys; 8% frequency) has defective binding to LDL-receptors and LRP, and in homozygous carriers predisposes to Type III hyperlipoproteinemia.\textsuperscript{22} ApoE4 (Cys112Arg; 15% frequency) produces dominant hyperlipidemia and, additionally, is strongly associated with increased susceptibility to Alzheimer’s disease.\textsuperscript{23}

1.2.4 Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9)

PCSK9 is a serine protease, mainly expressed by liver and intestine, with a key role in the catabolism of the LDL-receptor and other lipoprotein receptors of the same family, such as the VLDL-receptor and ApoE receptor 2 (ApoER2), though not LRP.\textsuperscript{1,24,25} Initially, it was thought that PCSK9 degraded LDL receptors intracellularly. However, later studies showed that PCSK9 was also secreted into the circulation where it acts as a ligand for the LDL-receptor and, following their endocytosis, directs the receptor for degradation rather than recycling to the plasma membrane. Its physiological significance is emphasized by the close positive correlation between levels of plasma PCSK9 and total or LDL-cholesterol. Missense “gain-of-function” mutations in the \textit{PCSK9} gene are associated with autosomal dominant hypercholesterolemia (ADH), a rare form of familial hypercholesterolemia in which both the LDL-receptor and the ligand binding domain of ApoB100 are normal.\textsuperscript{24} Such mutations, including F216L, lead to increased LDL-receptor catabolism and hence higher plasma levels of total cholesterol and LDL.\textsuperscript{24} In contrast, “loss-of-function” mutations increase the amount of LDL-receptor protein, thereby reducing plasma LDL levels\textsuperscript{25,26}; indeed, patients homozygous for the C679X mutation, which produces a 14 amino acid truncated PCSK9, have severe hypobetaliproteinemia.\textsuperscript{26}
1.3 Oligonucleotide Therapeutics

1.3.1 Overview

Oligonucleotides, either chemically synthesized or generated by selective enrichment strategies, are well-established research tools. Moreover, advances in their production and in vivo delivery have seen them emerge as a novel class of nonsmall molecule therapeutics, and several products are in clinical trial. RNA aptamers and short interfering RNA (siRNA) are at the forefront of clinical development, but other technologies also offer promise. Here, we focus on three key types of oligonucleotide therapeutics, which have potential to treat dyslipoproteinemias and atherosclerosis: RNA interference, exon skipping, and oligonucleotide-directed gene editing. The first two, RNA interference and exon skipping, are now established examples of antisense oligonucleotide technology for manipulating gene expression, while gene editing is a radical technique, uniquely suited for introducing small, permanent changes into the genome of the target cells.

1.3.2 RNA Interference

1.3.2.1 Mechanism

Gene silencing or RNA interference is a form of post-translational gene down regulation that is activated in response to double-stranded RNA. Most likely, it evolved as a defense mechanism against parasitic RNA sequences, for example, viruses or transposons, but is now in widespread use as a research tool and as a developing clinical therapeutic. The mechanism of RNA interference has been elucidated (Fig. 1.2). Double-stranded RNA is recognized and processed by Dicer, an enzyme that makes staggered cuts to cleave it into shorter 21 nucleotide double-stranded fragments (short interfering RNA or siRNA). In turn, the siRNA fragments are targets for RNA-induced silencing complex (RISC), a multiprotein cluster, which becomes activated by degrading one of the siRNA strands and incorporating the second. The activated RISC then binds mRNA sequences complementary to the assimilated RNA strand, resulting in mRNA degradation and hence gene downregulation.

This process is commonly exploited experimentally to silence genes in two ways: (1) transfection of synthetic preformed siRNA duplexes; or (2) transfection of a plasmid or viral vector that produces short hairpin RNA (shRNA). Synthetic siRNAs are designed, so their sequence matches only the gene of interest. When transfected into cells, the siRNA is recognized by RISC and used to target and degrade mRNA; this produces a transient and sequence-specific downregulation of gene expression. The siRNA-induced silencing is temporary because the siRNAs and the activated RISCs have finite lifetimes. Vectors producing shRNA are constructed
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Use of unmodified (naked) RNA in vivo is limited by its susceptibility to ubiquitous RNases and its inability to cross cellular plasma membranes. Indeed, naked siRNA molecules administered to animals are eliminated rapidly and fail to reach target tissues. This problem was initially circumvented by using RNA with a modified, nuclease-resistant backbone and by conjugation to lipophilic molecules, such as cholesterol, which facilitate entry into cells. Although this approach is relatively effective, newer liposomal formulations of siRNAs are superior and show much promise as potential future therapeutics.
1.3.2.2 Lipoprotein Targets

There are two reports of reducing ApoB expression in vivo by using siRNA. The first was intravenous injection (liver-directed) of cholesterol-conjugated ApoB-specific siRNA into transgenic mice expressing human ApoB100. Significantly lower levels of plasma ApoB100 were seen, which were accompanied by 40–50% reductions in chylomicrons, LDL, and total cholesterol. A later study in cynomolgus monkeys showed that a single injection of a liposomal formulation of ApoB siRNA reduced levels of ApoB mRNA by >80% for a period of 11 days; concomitant reductions of 62 and 82% in plasma total cholesterol and LDL were also noted. An additional target for cholesterol lowering, well-suited to siRNA treatment and a major focus for drug companies, is PCSK9, as its downregulation would increase LDL-receptor protein and hence reduce circulating LDL. Indeed, a study in vivo in nonhuman primates showed siRNA to reduce PCSK9 expression by 70% and plasma LDL by 60%, while leaving HDL or triglyceride levels unchanged.

1.3.3 RNA Splicing and Exon Skipping

1.3.3.1 Mechanism

An interesting property of eukaryotic genes is their discontinuous nature. The protein coding sequence exists in segments called exons that are separated by stretches of noncoding sequence, known as introns. Gene transcription produces a long pre-mRNA molecule containing both exons and introns, which is then converted to mature mRNA (Fig. 1.3). This process, known as RNA splicing, removes introns and joins the exons into one continuous protein-coding sequence.

The mechanism of RNA splicing has been characterized in some detail (Fig. 1.4). Splicing is carried out by the spliceosome, a large nucleoprotein complex that comprises all necessary enzymes and factors, and assembles directly on the pre-mRNA molecule. There are five types of sequences that are important in defining introns and exons (see Fig. 1.4): the 5’ and 3’ splice sites, the branch point sequence (BPS), the polypyrimidine tract, and the splicing enhancers/silencers. The 5’ and 3’ splice sites are the intron/exon boundaries and are defined through conserved sequence elements and via binding of the splicing machinery components and accessory proteins during spliceosome assembly. As the spliceosome catalyzes intron removal and joining of the two adjacent exons, it follows that if one of the splice sites is incorrectly chosen, or skipped, then this will drastically change the final mRNA sequence, as intron sequence might be included, or exon sequence deleted (Fig. 1.5).

Where a splice site is skipped, leaving the next suitable splice site along the pre-mRNA to be selected instead, one or more exons can be completely omitted. This can occur naturally, in a process termed alternative splicing (Fig. 1.5), which allows more than one protein product to be produced from the same pre-mRNA. In most cases, alternative splice site selection is regulated by the presence of factors that
Fig. 1.3 Structure of eukaryotic genes. Protein coding segments (exons) of eukaryotic genes are interspersed with noncoding regions, termed introns. Gene transcription produces a pre-mRNA that contains both introns and exons. Formation of mature mRNA requires excision of each intron and the merging of adjacent exons.

bind to splicing enhancers/silencers. However, erroneous disruption of the splicing signals by naturally occurring mutations can give rise to aberrant, often nonfunctional, products with pathological consequences. Recently, short antisense RNA oligonucleotides (ASO) were shown to interfere with the splicing machinery by inhibiting the binding of splicing factors and thereby artificially dictating the choice of splice sites. It is feasible, therefore, to use specific ASOs for blocking selection of a splice site and hence force the splicing machinery to use the next available one, causing an exon to be skipped (Fig. 1.5). This outcome, termed exon skipping, can be exploited to remove exons, which through mutations have deleterious effects on the final gene product.

The most successful application of exon skipping is in Duchenne muscular dystrophy (DMD), a muscle wasting disorder caused by dysfunctional forms of the cytoplasmic structural protein, dystrophin. Usually, this occurs through frameshift mutations, or formation of internal stop codons, which completely disrupt protein function. The central part of dystrophin contains multiple repeats of the same sequence and partially functional dystrophin can be produced by small internal deletions, which skip certain repeats but keep the reading frame intact. This type of defect results in a much milder phenotype, termed Becker muscular dystrophy (BMD). Importantly, the tolerance of dystrophin to internal deletions can be utilized
therapeutically: out-of-frame DMD mutations can be “rescued” to partially functional forms, by skipping appropriate exons. This either removes the mutation completely if it preserves the reading frame or, if skipping changes the reading frame, it restores the reading frame in the remaining sequence. This approach was used in a mouse DMD model to remove mutated exon 23 and produced a partially functional dystrophin, rescuing the animals muscle from degeneration. Indeed, phase I/II clinical trials are underway to assess safety and efficacy of locally injected ASOs directed against exon 51.
Exon skipping is also being considered as an alternative to ApoB siRNA for treating hypercholesterolemia. Although RNA interference successfully reduced plasma LDL, it is uncertain whether impaired production of chylomicrons might cause liver toxicity and intestinal dysfunction as is sometimes observed in FHBL patients. The strategy is based on the observation that skipping of ApoB exon 27 generates a product, ApoB87\textsubscript{SKIP27}, which is almost identical to ApoB87\textsubscript{Padova}, a naturally occurring dominant ApoB variant from patients with FHBL. Although ApoB87\textsubscript{Padova} produces functional LDL particles, they have much faster catabolic rates and hence FHBL kindreds have markedly decreased plasma LDL and total cholesterol. At the molecular level ApoB87\textsubscript{Padova}, and a similar variant ApoB89, have frameshift mutations

![Diagram](attachment:image.png)
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(single nucleotide deletions) in exons 28 and 29, respectively, which produce truncated ApoB with novel C-termini that confer increased LDL-receptor affinity. Importantly, both natural variants retain exon 26, within which lies the nucleotide targeted by the editosome to create ApoB48, thereby allowing normal assembly and secretion of chylomicrons. It has been established that suitably selected ASOs can indeed induce skipping of exon 27 to generate ApoB87\textsuperscript{SKIP27} albeit with a low frequency. However, since the increased catabolism conferred by these variants is dominant, it is predicted that expressing a relatively small amount of ApoB87\textsuperscript{SKIP27} will still increase LDL catabolism without affecting chylomicron production. Although not yet tested in vivo, this use of “gain-of-function” exon skipping is an exciting application of oligonucleotide therapeutics.

1.3.4 Gene Editing

1.3.4.1 Early Difficulties Using RNA–DNA Oligonucleotides

Oligonucleotide-mediated targeted gene editing is a novel and potentially very powerful technology for introducing permanent genetic changes into a cell’s genome. In a clinical setting, it represents the ultimate gene therapy protocol for inherited diseases: repair to a defective gene would be permanent and existing enhancers and promoters, and cell-specific control and context to regulate gene expression would be retained. Over a decade ago, chimeric RNA–DNA oligonucleotides (RDOs) were reported to induce 30% editing in an episomal target and 50% in a cell model of sickle cell anemia. Encouraged by such reports, we pioneered the technique in CVD, initially converting the dysfunctional ε2 allele in recombinant CHO cells to wild-type ε3 using 68-mer RDOs. The correction was confirmed at both genomic and protein levels. However, on extending this emerging technology to other targets, we began to question its practicality. Thus, while we demonstrated successful conversions of APOE4 to APOE3 and APOAI to APOAI\textsubscript{Milano} by PCR-based assays, we also noted poor reproducibility and apparently unstable conversions; such concerns were voiced by others. Adverse factors included low-quality RDOs, as these hairpin-capped duplex reagents were difficult to synthesize, which meant higher reagent doses and delivery vehicles were needed to effect gene editing; these amplified cytotoxic and pro-apoptotic actions, or induced cell cycle arrest.

1.3.4.2 Single-Stranded All-DNA Oligonucleotides (ssODN) Give Reproducibility

To resolve such issues, we undertook a back-to-basics approach and targeted cells that stably-express nonfluorescent EGFP due to a point mutation (TAC→TCC; Tyr→Ser). Successful correction produced green cells, which we accurately quantified by flow cytometry. We also switched from problematic RDOs to short (27-mer) single-stranded all-DNA oligonucleotides (ssODNs), as these 2nd generation reagents have high purity and increased reproducibility. Our experiments help clarify certain inconsistent