REVIEW

Review of the scientific evolution of gene therapy for the treatment of homozygous familial hypercholesterolaemia: past, present and future perspectives

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ABSTRACT

Familial hypercholesterolaemia (FH) is a devastating genetic disease that leads to extremely high cholesterol levels and severe cardiovascular disease, mainly caused by mutations in any of the main genes involved in low-density lipoprotein cholesterol (LDL-C) uptake. Among these genes, mutations in the LDL receptor (*LDLR*) are responsible for 80%–90% of the FH cases. The severe homozygous variety (HoFH) is not successfully treated with standard cholesterol-lowering therapies, and more aggressive strategies must be considered to mitigate the effects of this disease, such as weekly/biweekly LDL apheresis. However, development of new therapeutic approaches is needed to cure HoFH. Because HoFH is mainly due to mutations in the *LDLR*, this disease has been proposed as an ideal candidate for gene therapy. Several preclinical studies have proposed that the transference of functional copies of the *LDLR* gene reduces circulating LDL-C levels in several models of HoFH, which has led to the first clinical trials in humans. Additionally, the recent development of clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 technology for genome editing has opened the door to therapies aimed at directly correcting the specific mutation in the endogenous *LDLR* gene. In this article, we review the genetic basis of the FH disease, paying special attention to the severe HoFH as well as the challenges in its diagnosis and clinical management. Additionally, we discuss the current therapies for this disease and the new emerging advances in gene therapy to target a definitive cure for this disease.

INTRODUCTION

Familial hypercholesterolaemia (FH) is usually a monogenic autosomal-dominant disease characterised by abnormally elevated serum levels of low-density lipoprotein cholesterol (LDL-C) and an extremely high risk of atherosclerosis and cardiovascular disease (CVD)[1] figure 1. Whereas the heterozygous variety (HeFH) shows two to four times higher LDL-C levels,[2] patients with the severe homozygous FH (HoFH) form can present plasma levels of LDL-C >13 mmol/L.[3] Thus, patients with HoFH are at increased risk of developing severe CVD during the first decade of life,[1] including aortic valve stenosis and coronary heart disease, requiring invasive therapies, ultimately leading to heart transplant. Additionally, they show both subcutaneous and tendinose cholesterol deposits (xanthomas) and corneal arcus before 10 years of age. These patients develop symptomatic atherosclerosis before 20 years of age and could die before they reach 30 years of age.[4] The historical frequencies of HeFH and HoFH were estimated to be 1:500 and 1:1 000 000, respectively.[4] However, given the founder effect of the genetic population, high frequencies have been described in specific populations.[5] Thus, recent studies estimate that HeFH affects 1 in 200 people,[2] and the prevalence of HoFH is approximately 1 in 160 000–300 000 people, including both true homozygous and composed heterozygous (both referred to as homozygous).[3 6 7]

Elevated levels of serum LDL-C are the result of the inability of FH patients to clear circulating cholesterol caused by mutations in any of the main genes involved in LDL uptake, including the LDL receptor (*LDLR*; OMIM #606945),[4] apolipoprotein B (*APOB*; OMIM #107730),[8] proprotein convertase subtilisin/kexin type 9 (*PCSK9*; OMIM #607786)[9 10] and low-density lipoprotein receptor adaptor protein 1 (*LDLRAP1*; OMIM #695747).[11] Among these genes, mutations in the *LDLR* are responsible for 80%–90% of the cases,[12 13] encompassing >2000 mutations[12] with a range of dysfunction of pathogenic variants between <2% and 50% activity. Accordingly, it is possible to classify the LDLR into two categories: (1) receptor-negative or null-receptor mutations, with <2% of activity and (2) receptor-defective, with ≥2% of residual activity.[14] The severity of the disease depends on the mutation, and *LDLR* null mutations produce the most severe phenotype.[3 6 15–18]

DIAGNOSIS AND CLINICAL MANAGEMENT OF THE DISEASE

Given the severity of FH, particularly the HoFH form that affects children during the first decade of life, early detection is critical for mitigating the genetically induced hypercholesterolaemia effects in these patients. Thus, optimising the diagnosis of children affected by this pathology is of utmost importance.

At the clinical level, HoFH must be suspected with untreated plasma LDL-C levels >13 mmol/L in non-treated patients or >8 mmol/L in individuals treated with standard cholesterol-lowering therapies, according to European Atherosclerosis Society guidelines.[3] However, lower plasma LDL-C in children or treated patients does not exclude HoFH.

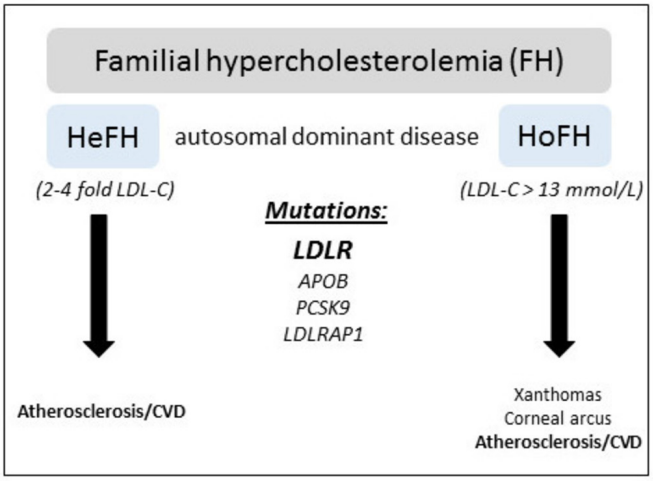

Figure 1 Schematic representation of FH disease. FH is an autosomal dominant disease characterised by elevated serum LDL-C levels as a result of mutations in the genes involved in cholesterol clearance, such as *APOB*, *PCSK9*, *LDLRAP1* and *LDLR*. Mutations in the *LDLR* explain 80%–90% of the cases. The HoFH shows LDL-C levels >13 mmol/L. Therefore, HoFH patients present a higher risk of developing severe CVD than HeFH patients. CVD, cardiovascular disease; FH, familial hypercholesterolaemia; HeFH, heterozygous familial hypercholesterolaemia; ; HoFH, homozygous f amilial hypercholesterolaemia; LDL-C, low-density lipoprotein cholesterol; *APOB*, apolipoprotein B; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *LDLRAP1*, low-density lipoprotein receptor adaptor protein 1; *LDLR*, low-density lipoprotein receptor.

The presence of xanthomas in children <10 years old and/or high LDL-C levels without treatment in both parents is suggestive of the disease. However, these features do not always allow for the differentiation between homozygous and heterozygous individuals due to different grade in the severity of the disease, which is critical given the severity and the poor response to traditional lipid-lowering therapies in HoFH.

Genetic testing is mandatory to confirm a definite HoFH diagnosis. HoFH has been confirmed by mutations in both copies of *LDLR*,[4] *APOB*[8] and *PCSK9*.[9 10] Additionally, recessive autosomal FH has been described in patients with mutations on both *LDLRAP1* alleles.[11] Nevertheless, genetic confirmation is not always possible, potentially due to additional genes involved in FH development.[2] Other factors that may be at play when a mutation is not found include (1) mutation type that is not detectable with the laboratory method (eg, copy number variation) and (2) polygenic hypercholesterolaemia, which is seen in 20%–30% of cases of suspected HeFH. Moreover, genetic classification of the disease may be challenging given the possibility of two functional mutations in the same gene, which can be in the same allele (compound heterozygous in *cis*), usually associated with the HeFH phenotype, or in two different alleles (compound heterozygous in *trans*), leading to the HoFH phenotype. Additionally, mutations in two different *LDLR*-associated genes are found in double heterozygous patients. In patients with a clinical diagnosis of FH in whom a specific mutation cannot be found, it is likely that the disease presents a polygenic aetiology.[19]

CURRENT THERAPIES

Whereas HeFH patients are successfully treated with standard cholesterol-lowering therapies, these therapies are not usually effective for HoFH individuals.[20 21] Although statins can be considered the foundation of treatment for HoFH,[22–26] even the highest doses of the most potent statins (ie, atorvastatin and rosuvastatin) cause only a modest reduction in LDL-C plasma levels (10%–25% reduction) due to the lack of functional receptors that can be upregulated in HoFH. A combined therapy of statins with the cholesterol absorption inhibitor ezetimibe could achieve reductions of 30%–35% in circulating LDL-C levels. Although this may seem like a considerable reduction, taking into account that HoFH patients can present with untreated plasma levels of LDL-C >13 mmol/L, this reduction results in an achieved LDL-C that still markedly exceeds ideal target levels, that is, LDL-C <2.5 mmol/L in adults, <3.5 mmol/L in children and <1.8 mmol/L in patients with clinical atherosclerotic CVD.[2] The addition of other lipid-lowering drugs, such as fibrates or bile acid-binding resins, showed no further reduction in LDL-C levels. The recently approved monoclonal PCSK9 inhibitors have a very limited effect on LDL-C levels in receptor-negative HoFH patients,[27] although they are able to reduce LDL-C by 25% in patients who have at least one receptor-defective allele. Therefore, other strategies must be considered to prevent atherosclerotic CVD in HoFH patients, including newly approved pharmacologic modalities, such as lomitapide and mipomersen. These drugs are focused on inhibiting the microsomal triglyceride transfer protein[28] or reducing apoB synthesis,[29] respectively, therefore, interfering with the production of apoB-carrying lipoprotein rather than increasing its blood elimination. Although these drugs have shown promising results in reducing plasma LDL-C levels in HoFH patients, their degree of efficiency correlates with clinically relevant side effects, such as an increase in liver fat content,[30 31] gastrointestinal distress and malabsorption or, in the case of mipomersen, skin reactions. Additionally, both human genetic analyses and preclinical studies have suggested that ANGPTL3 inhibition reduces LDL-C levels independently of LDLR function.[32] Interestingly, the administration of Evinacumab, an ANGPTL3-blocking antibody, reduced the LDL-C levels in nine HoFH patients by a mean of 49% after 4 weeks of treatment,[33] although patients with the most severe forms of disease did not reach therapeutic objectives.

Apart from the drug-based approaches, liver transplantation in HoFH has shown reductions in LDL-C plasma levels of up to 80%.[34–36] However, this therapeutic option is practically in disuse given its associated issues, such as surgical complications, post-transplant mortality, shortage of donors and the need for lifelong treatment with immunosuppressants.[37]

Thus, pharmacologic-treated HoFH patients are commonly subjected to weekly or biweekly lipid apheresis.[38–42] Using this treatment, it is possible to reduce LDL-C plasma levels almost to the levels found in healthy individuals. Increasing clinical evidence suggests that prolonged LDL apheresis may contribute to plaque reduction and/or stabilisation and improves its prognosis.[43] Although LDL apheresis is the best current therapeutic approach for severe HoFH patients, it requires specialised hospital infrastructure with a substantial budget and has a great personal impact, confining patients to a lifelong extracorporeal LDL clearance. Moreover, the availability of this treatment is limited in many countries. Therefore, the development of alternative approaches is eagerly awaited to mitigate the lethality of this disease.

GENE THERAPY FOR HOFH

Given that HoFH is mainly due to mutations in the *LDLR*, restoring LDLR function using gene therapy may be a useful strategy to mitigate the effects of the disease. Increasing evidence supports the idea that introducing functional copies of the *LDLR* gene reduces LDL-C plasma levels and reduces the negative cardiovascular effects

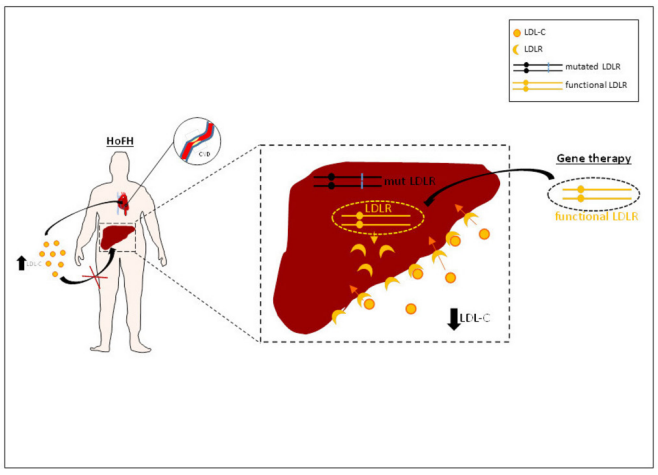

Figure 2 Gene therapy for HoFH. In HoFH patients, LDL-C cannot be uptaken by the liver, thus increasing plasma LDL-C levels and risk for CVD. Insertion of functional copies of the *LDLR* gene in the liver increases hepatic LDL-C clearance and reduces plasma LDL-C. CVD, cardiovascular disease; HoFH, homozygous familial hypercholesterolaemia; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor.

of HoFH (figure 2). Nevertheless, not all forms of gene therapy tried in the past were efficacious. Next, we will review the scientific evolution of gene therapy for HoFH from the initial approaches using ex vivo transduction with retroviruses in the 1990s[44] and the first clinical trial based in this technology in 1995[45] to the in vivo adenoviral[46–50] and helper-dependent adenoviral (HD-Ad) transduction[51–53] and the development of the adeno-associated virus (AAV) vectors[54–60] that have allowed the current clinical trial in development since 2016 (figure 3). The limitations of these strategies and their replacement by advanced approaches will also be discussed. Additionally, we will briefly discuss future perspectives based on the clustered regularly interspaced short palindromic repeat/CRISPR-associated 9 (CRISPR/Cas9) technology for genome editing.[61]

Origins and evolution of gene therapy for HoFH

Retrovirus-mediated gene transfer

Autologous hepatocytes that were genetically modified by recombinant retroviruses containing a functional human *LDLR* gene were transplanted into livers from Watanabe heritable hyperlipidaemic (WHHL) rabbits via portal vein infusion.[44] Transgene expression decreased total serum cholesterol levels from the second to the sixth day after transplantation, obtaining a maximum reduction of 70% on the third day. After this peak, total serum cholesterol levels progressively increased, reaching the pretreatment levels from the seventh day after the hepatocyte transplant.[44] Using this approach, Grossman *et al* performed the first human pilot gene therapy clinical trial to transfer human *LDLR*-expressing retrovirus-transduced hepatocytes to the liver of five HoFH patients.[45] Transgene expression was detected in a limited number of hepatocytes in liver biopsies 4 months after treatment. A modest and variable reduction in the LDL-C levels (6%–25%) was found in three of the five patients enrolled, potentially due to the low efficiency of the transgene transference [45]. Thus, these data ruled out the clinical application of retroviral-based vectors in gene therapy without consistent and sustained gene transfer.

Adenovirus-mediated gene transfer

To avoid problems related to ex vivo approaches, such as failure of cell engraftment, a direct in vivo approach using recombinant adenovirus encoding the human *LDLR* was used in *Ldlr*$^{-/-}$ mice.[46] The adenovirus-mediated *LDLR* overexpression increased very low-density lipoprotein (VLDL) clearance and reduced the elevated intermediate-density lipoprotein (IDL)/LDL plasma levels in the *Ldlr*$^{-/-}$ mice.[46] Similar results showing reduced serum cholesterol levels were found in WHHL rabbits treated with adenovirus overexpressing the *LDLR* gene.[47 48] In addition to *LDLR*, the overexpression of the VLDL receptor (*VLDLR*) by adenovirus in the liver of *Ldlr*$^{-/-}$ mice also reduced total cholesterol levels between twofold and fourfold in these animals.[49 50] Nevertheless, similar to the retrovirus vectors, the adenoviral vectors led to a non-permanent transgene expression due to the immunogenicity of these viruses.

HD-Ad-mediated gene transfer

To increase adenovirus-mediated transgene persistence, Oka *et al* overexpressed *VLDLR* in the livers of *Ldlr*$^{-/-}$ mice using HD-Ad vectors.[51] Transgene expression persisted after 6 months of virus infusion, which was in line with the reduction in plasma cholesterol levels and an almost complete prevention of atherosclerotic aorta lesions in these animals.[51] However, since *VLDLR* mediates the uptake of

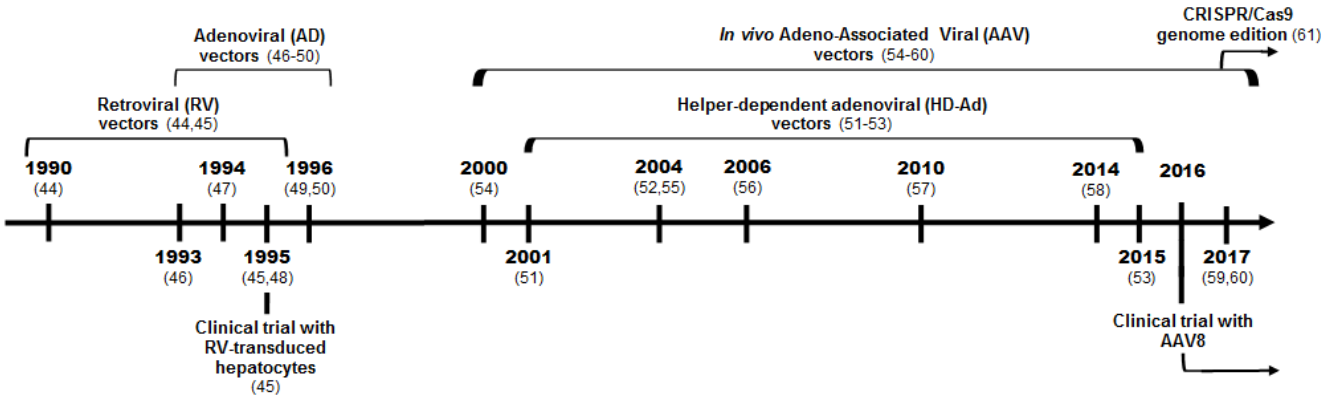

Figure 3 Timeline showing the historic overview of gene therapy research for the treatment of HoFH. The time period during which each approach was used before it was replaced by more advanced technologies is indicated. References for the publications are indicated under each year. The clinical trials with retroviruses (1995) and AAV (2016) are indicated below. AD, adenoviral; RV, retroviral; AAV, adeno-associated viral; HD-Ad, helper-dependent adenoviral; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat/CRISPR-associated 9; .

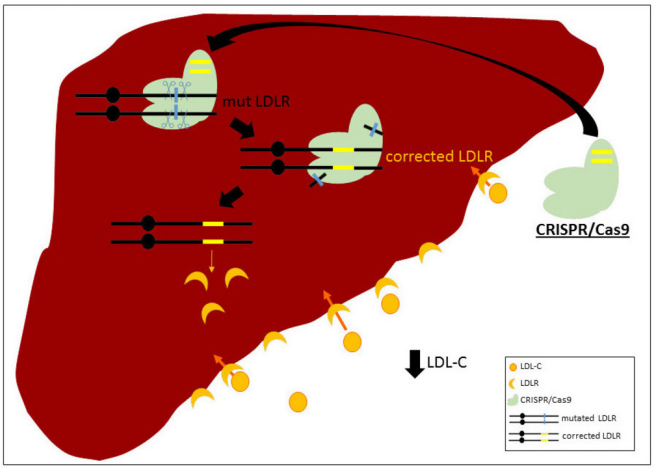

Figure 4 CRISPR/Cas9 technology for HoFH genetic correction. Using CRISPR/Cas9 technology for gene edition is potentially feasible to repair specific mutations inside endogenous *LDLR* in HoFH patients. CRISPR/Cas9, clustered regularly interspaced short palindromic repeat/CRISPR-associated 9; HoFH, homozygous familial hypercholesterolaemia; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density llipoprotein cholesterol receptor.

only IDL, not LDL, *VLDLR* overexpression is not considered for clinical application. Additionally, the effect of *LDLR* overexpression by HD-Ad in lowering the plasma cholesterol levels was greater than that achieved by *VLDLR* overexpression.[52] However, although *LDLR* overexpression by HD-Ad in rhesus macaques heterozygous for a nonsense mutation in the *LDLR* gene (*LDLR*$^{+/-}$) effectively reversed hypercholesterolaemia, the heterogeneous and unsustained long-term response precludes its future application.[53]

AAV precursors of the current gene therapy

Since 2000, recombinant AAV vectors have been available for long-term persistent expression.[54 62 63] However, not all AAV serotypes are equally efficient. *LDLR* overexpression using serotype 2 (AAV2) showed a low transduction efficiency and the loss of liver-associated vector DNA in fat-fed *Ldlr*$^{-/-}$ mice.[54] Nevertheless, the identification of novel capsid AAVs[64] has overcome some of the deficiencies related to AAV2. Specifically, serotype 8 (AAV8) has shown high transduction efficiency in the livers of mouse and dog models, with reduced pre-existing humoral immunity and T cell response to the capsid.[56 65 66] Additionally, the AAV8 vectors were well tolerated in *LDLR*$^{+/-}$ macaques, showing only sporadic mild histopathology, low level and transient transaminase elevations, and an adaptive immune response restricted to early time points.[60] Using these new AAV vectors, a long-term correction of the metabolic defect was achieved in several HoHF animal models. Compared with AAV2, chimeric AAV2/7 and AAV2/8 vectors encoding the common human apolipoprotein E (*APOE*) E3 isoform reduced cholesterol levels and prevented atherosclerosis in chow-fed *ApoE*$^{-/-}$ mice.[56] Similarly, whereas AAV7 and AAV8 vectors encoding human *LDLR* achieved nearly complete normalisation of serum lipids and prevented severe atherosclerosis in *Ldlr*$^{-/-}$ mice fed a high-fat diet, the AAV2 vector constructs showed only partial lipid correction and a modest atherosclerosis improvement.[55] The AAV8 vectors were further explored in a humanised model of HoFH, the *Ldlr*$^{-/-}$*ApoBec1*$^{-/-}$mice, which develop atherogenesis with a chow diet.[57] Delivering the mouse *Ldlr* gene by AAV8 to these animals reduced plasma cholesterol and non-HDL cholesterol levels and achieved an 87% plaque regression compared with the animals treated with the AAV8-null vector.[57] To test the AAV8 vectors in a model more closely resembling human HoFH, the *Ldlr*$^{-/-}$*ApoBec1*$^{-/-}$*hApoB* mice were generated to simulate the in vivo interactions between human LDLR and human ApoB100.[67] AAV8 encoding the human *LDLR* significantly corrected hypercholesterolaemia in the *Ldlr*$^{-/-}$*ApoBec1*$^{-/-}$*hApoB*[67] mice. The effect of the AAV8-*LDLR* vectors on cholesterol reduction was greater when transducing *LDLR* variants that expressed LDLR proteins resistant to PCSK9 and/or inducible degrader of LDLR (IDOL) in both in vitro and in vivo models overexpressing these proteins.[58]

AAV-mediated gene transfer limitations

Although AAV has emerged as a powerful tool for long-term persistent transgene expression, it is worth noting that none of the gene therapy platforms used previously in the clinic based on AAV vectors have achieved a complete reversal of the disease phenotype. Additionally, human hepatocytes are less efficiently transfected by AAV vectors than murine hepatocytes.[68 69] More importantly, constitutive expression of the *LDLR* in experimental models results in cytotoxicity due to excessive lipid internalisation.[47 70 71] While hepatocyte-specific promoters do not appear to show lipotoxic effects,[67 72] given the complexity of LDL metabolism and LDLR physiological regulation, the lack of transgene expression regulation could lead to unexpected long-term consequences, such as the pathological accumulation of lipids and cholesterol in hepatocytes.[70]

Present advancements of gene therapy for HoFH: the AAV8.TBG.hLDLR clinical trial

As mentioned above, most of the gene therapy approaches for HoFH show several limitations that have impeded its clinical application. Ex vivo approaches have shown problems related to cell engraftment. Transgene transference based on both retrovirus and adenovirus vectors have failed to achieve permanent expression due to the immunogenicity of these particles. Although AAV leads to long-term persistent expression, not all serotypes are equally efficient for human hepatocyte transduction. Therefore, most of these approaches have been ruled out for their clinical inadequacies.

However, most of these concerns have been overcome with AAV8 vectors. The safety of these vectors for *LDLR* gene therapy was assessed in non-clinical pharmacological/toxicological studies in both *Ldlr*$^{-/-}$*ApoBec1*$^{-/-}$ mice[59] and *LDLR*$^{+/-}$*Rhesus Macaques*[60] prior to initiation of a phase I clinical trial. In these studies, *LDLR* expression was driven by a liver-specific thyroxine-binding globulin (TBG) promoter (AAV8.TBG.mLDLR) to drive sustained *LDLR* expression in the liver. Based on these advances, after more than two decades from the first HoFH gene therapy clinical trial,[45] phase I/IIa of a new human clinical trial was started in March 2016 to test the AAV8.TBG.hLDLR (NCT02651675), which is currently in development. This clinical trial enrolled 12 HoFH individuals with follow-up up to 5 years after receiving a recombinant *LDLR*-expressing AAV8 vector. The primary outcome of this study involved the assessment of vector-related adverse effects by 52 weeks following administration. The secondary outcomes include the fractional catabolic rate of LDL apoB, the percentage of change in LDL-C and lipid parameters at 12 weeks compared

with baseline, and the percentage change in lipid parameters at 260 weeks compared with baseline. Data from this clinical trial are eagerly awaited by the scientific community and HoFH patients.

Future perspectives in gene therapy for HoFH

Transplantation of autologous genetically modified hepatocyte-like cells derived from induced pluripotent stem cell

An alternative option to hepatocyte transplantation may be the autologous transplantation of hepatocyte-like genetically corrected cells derived from induced pluripotent stem cells (iPSCs). Transfecting the iPSCs of an HoFH patient with a plasmid containing the *LDLR* allows for the formation of hepatocyte-like cells that are able to uptake the extracellular LDL.[73] Interestingly, this response was regulated by lovastatin and sterol, thereby showing a reestablishment in the LDL physiological regulation.[74] However, although promising, the hepatocyte-like derived iPSCs transplant technology that was used is unlikely to be used in a clinical setting given the potential risk of tumourigenesis.[75]

Gene editing with CRISPR/Cas9: expectations and limitations

Experimental approaches using transgene-containing *LDLR* genomic regulatory elements in combination with statins[76] or small interfering RNA oligonucleotides and miRNA against 3-hydroxy-3-methylglutaryl-CoA reductase[77] enhanced transgene expression and activity. Therefore, combining gene therapy with strategies aimed at obtaining physiological/pharmacological transgene regulation would minimise the risk of unwanted gene therapy effects. Furthermore, the recent development of technology based on CRISPR/Cas9 for genome editing has opened the door to directly repair specific mutations in the endogenous *LDLR* gene, thereby giving rise to a functional gene copy subjected to physiological regulation (figure 4). Using this technology, Omer *et al* permanently corrected a 3-base pair homozygous deletion in *LDLR* exon 4 with <5% receptor activity[78] in iPSCs derived from skin fibroblasts of an HoFH patient.[61] The hepatocyte-like cells derived from the corrected iPSCs showed physiologic control of *LDLR* expression and restored receptor-mediated LDL endocytosis and cholesterol metabolism.[61] Nevertheless, several technical challenges must be refined before seriously considering CRISPR/Cas9 gene editing as a realistic therapeutic option for the treatment of HoFH. Although the use of modified Cas9 nickase with paired single-guide RNAs enhances the likelihood for homology-directed repair (HDR)[79 80] and reduces off-target mutations,[79 81] indels are still found within the corrected gene.[61] Additionally, other off-target modifications may occur in non-predicted sites. Furthermore, after the selection of the suitable corrected clone, there are still the aforementioned problems in cell engraftment and/or the potential risk of tumourigenesis.[75] A direct CRISPR/Cas9 in vivo approach is potentially feasible. In fact, this technology has already been used for hepatic gene editing in adult mice to knock out a gene.[82] However, gene disruption was achieved by random indels within the targeted gene. Specific repair of point mutations by HDR seems difficult in differentiated somatic cells. Thus, further research is warranted to develop CRISPR/Cas9-based strategies to specifically edit single mutations in differentiated cells of adult individuals and to minimise any off-target unwanted effects.

CONCLUSIONS

In conclusion, effective therapies to definitively resolve the most severe forms of HoFH are currently needed. HoFH is an autosomal dominant disease commonly caused by mutations in the *LDLR* gene and can lead to severe CVD in the first decade of life due to the inability to clear circulating LDL-C levels. Although LDL-apheresis in patients using a traditional combination lipid-lowering therapy is able to reduce LDL-C levels to near those found in healthy individuals, this approach does not cure the disease, causes patient discomfort and requires specialised personnel and facilities. Therefore, additional therapies aimed at curing the most severe forms of this disease are eagerly awaited. Given the genetic nature of HoFH, research has focused on gene therapy to restore LDLR function. Numerous experimental approaches have shown promising results in delivering functional copies of the *LDLR* gene to several animal models of the disease, and these findings have encouraged the onset of the first clinical trials. Additionally, the recent development of CRISPR/Cas9 technology for gene editing has opened the door to correct the specific mutations causing the disease in the endogenous *LDLR* gene, thereby restoring the *LDLR* function under physiological regulation. Nevertheless, there is still a long way to go before using this approach in clinic, and several issues have to be refined before seriously considering CRISPR/Cas9 technology as a realistic therapeutic option for HoFH treatment.

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