CRISPR/Cas in Atherosclerosis

Subjects: Biology

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Atherosclerosis represents one of the major causes of death globally. The high mortality rates and limitations of current therapeutic modalities have urged researchers to explore potential alternative therapies. The clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) system is commonly deployed for investigating the genetic aspects of Atherosclerosis. Besides, advances in CRISPR/Cas system has led to the extensive options for researchers to study the pathogenesis of this disease. The recent discovery of Cas9 variants, such as dCas9, Cas9n, and xCas9 have been established for various applications, including single base editing, regulation of gene expression, live-cell imaging, epigenetic modification, and genome landscaping. Meanwhile, other Cas proteins, such as Cas12 and Cas13, are gaining popularity for their applications in nucleic acid detection and single-base DNA/RNA modifications. To date, many studies have utilized the CRISPR/Cas9 system to generate disease models of atherosclerosis and identify potential molecular targets that are associated with atherosclerosis.

Keywords: CRISPR/Cas9 ; atherosclerosis ; gene editing ; gene therapy

1. Introduction

Cardiovascular diseases (CVDs) refer to a group of disorders that affect the heart and blood vessels, including hypertension, coronary heart disease, heart failure, rheumatic heart disease, congenital heart disease and cardiomyopathies, cerebrovascular disease, and peripheral vascular disease [1]. Atherosclerosis represents one of the main underlying causes of CVD, characterized by the presence of fibro-fatty lesions in the artery wall due to lifelong exposure to elevated low-density lipoprotein (LDL) cholesterol [2]. Lipid-lowering drugs are the primary therapeutic strategy for managing atherosclerosis. A drug such as statin helps in lowering LDL cholesterol and can be prescribed as a primary [3] and secondary prevention drug [4] for atherosclerosis treatment. Statins inhibit 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), a key enzyme which is involved in the synthesis of cholesterol. Non-statin lipid-lowering drugs, such as bile-acid sequestrants (e.g., Ezetimibe), are alternative options which inhibit the absorption of cholesterol into enterocytes of small intestine and reducing the LDL cholesterol levels. In combination therapies, statin can be combined with ezetimibe, and the treatment was shown to provide additional 15–20% reduction in LDL cholesterol levels [5]. On the other hand, there is an increasing focus on the proprotein convertase subtilisin/kexin type 9 (PCSK9) enzyme, which plays a key role in plasma cholesterol metabolism [6]. PCSK9 inhibitors such as evolocumab [7][8][9], alirocumab [10] that target PCSK9 enzyme have been tested in clinical trials and showed efficacy in lowering LDL cholesterol levels.

Considering that atherosclerosis constitutes a long pre-clinical phase, early detection of atherosclerosis may allow identification of individuals at risk for developing atherosclerotic clinical events and provides an opportunity for prevention. Both subclinical and clinical atherosclerosis has known genetic components, and novel causal therapeutic targets are being identified in several genetic studies. For instance, two prominent loci, *SERPINA1* and *AQP9*, were identified as potential candidate genes of atherosclerosis in a multi-phenotype genome-wide association study (GWAS) [11]. *SERPINA1* gene encodes for alpha 1-antitrypsin (A1AT), a protease inhibitor that enhances the degradation of LDL [12]. On the other hand, the *AQP9* gene coordinates the transport of glycerol in liver, and it is associated with reducing lipid accumulation in hepatocytes [13]. Besides, a genome-wide interaction study (GWIS) between genetic and environmental exposures uncovered several novel genetic variants in *FCAMR* (Fc fragment of IgA and IgM receptor)-*PIGR* (polymeric immunoglobulin receptor) locus that are associated with coronary atherosclerosis in individuals who are chronically exposed to traffic air pollution [14]. Another GWIS on gene-smoking interactions identified two novel genetic variants (e.g., rs1192824 and rs77461169) in the regulatory region of TBC1 domain family member 8 (*TBC1D8*) gene that affect carotid intima-media thickness and thus, increased consequent risk for atherosclerosis [15].

In line with these genetics and genomics studies, nucleic acid-based cardiovascular therapies are developing rapidly and have shown significant progress in the safety and efficacy for atherosclerosis treatment. Some prominent clinical studies of RNA-targeted nucleic acid-based therapeutics utilize small interfering RNAs (siRNAs) and antisense oligonucleotides

(ASOs) to inhibit the production of proteins that are involved in lipid homeostasis such as apolipoprotein B (*APOB*), *PCSK9*, angiopoietin such as 3 (*ANGPTL3*), and apolipoprotein C3 (*APOC3*). The double-stranded siRNA has the capability to target and induce cleavage of mRNAs [16]. Inclisiran is the first-in-class cholesterol lowering siRNA conjugated to triantennary N-acetylgalactosamine carbohydrates (GalNAc) which inhibits the translation of PCSK9 and reduces levels of LDL cholesterol [17]. It was approved by EU in Dec 2020 for treatment of primary hypercholesterolaemia (heterozygous familial and non-familial) or mixed dyslipidaemia. ASO, on the other hand, is a short, single-stranded oligo that prevents protein translation by binding to mRNA target [18]. Mipomersen is an FDA approved ASO drug that binds to *APOB*-encoding mRNA, which prevents the translation of APOB and reduces LDL cholesterol level [19][20][21].

The discovery of CRISPR/Cas has emerged as an effective genome editing tool due to its ease of customization, feasibility to target almost any genome regions, and high editing efficiency with multiplexing capability. Numerous experimental studies have shown that correction of single gene defect can be achieved by the use of CRISPR/Cas technology in atherosclerosis models. This genome editing tool provides compelling alternatives to current treatment options (statins and ezetimibe), which require multiple dosages during the course of the disease. It has immense potential in facilitating development of atherosclerosis disease models and nucleic acid-based cardiovascular therapy. Despite its potential, there are two major limitations associated with this technology for its clinical translation. First, low delivery efficacy of therapeutic CRISPR tools results in non-specific targeting. Second, there are possible off-target mutations which may cause unwarranted side effects. Further research in this field is essential before it can be expanded for clinical treatment and prevention of atherosclerosis.

2. CRISPR/Cas System: Experimental Considerations in Atherosclerosis Models

CRISPR/Cas system was first discovered in the genome of prokaryotes in 1987 [22], but its role in adaptive immunity was not known until 2007 [23]. The basic mechanism of CRISPR/Cas genome editing has been extensively discussed elsewhere [23][24][25]. Briefly, genome editing takes advantage of the CRISPR/Cas-mediated double-strand break (DSB) at desired genome sites. DSB activates either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathway. In NHEJ, the repair pathway mediates direct re-ligation of the excised DNA, which often results in the introduction of insertions and/or deletions (indels). The introduced indels result in either frameshift mutations or in-frame insertions/deletions, generally resulting in gene “knockout”. Conversely, HDR repair requires a donor DNA template (i.e., single or double stranded DNA) with flanking homology arms for precise replacement or repair at the cleavage site [26][27][28][29]. In general, NHEJ is the dominant repair pathway, while HDR tends to occur at a lower frequency and is usually limited to proliferating cells (i.e., S phase or G2 phase cell proliferation) [30][31][32].

Among all identified types of CRISPR/Cas systems, the one derived from *Streptococcus pyogenes* (Type II) is the most commonly used and well-characterized [33][34][35], which consists of Cas9 protein and a single guide RNA (sgRNA). Meanwhile, *Streptococcus pyogenes* Cas9 (spCas9) derivatives, such as spCas9-NG and xCas9, are also being used for genome editing. Both spCas9-NG and xCas9 variants have better PAM flexibility, and the former recognizes any target site with NG without any preference for the third nucleotide, while the latter has broader PAM compatibility that allows recognition of NG, GAT, and GAA [36][37]. Some studies utilize catalytically impaired Cas9 protein, also known as dCas9 (dead Cas9). A dCas9-gRNA ribonucleoprotein (RNP) complex can bind the promoter or regulatory regions of a target gene to induce transcriptional activation or inhibition [38][39][40][41][42][43]. Both CRISPR-activation (CRISPRa) and CRISPR-interference (CRISPRi) approaches can regulate multiple gene expressions simultaneously [44][45][46].

2.1. Types of Cells Used in CRISPR/Cas9 Applications

CRISPR/Cas9 can be performed on various cellular sources including somatic cells, zygotes/embryos, and pluripotent stem cells [47][48]. However, the choice of cells for genome editing application may present different technical and ethical issues. Depending on the cell type and cell state, the efficiency of DNA repair mechanisms, either NHEJ or HDR, varies substantially [49]. NHEJ can happen in most cell types including actively dividing and post-mitotic cells, whereas HDR perform better in proliferating cells [50].

Specific cell types, such as liver Kupffer cells (KCs) or liver resident macrophages, were used in the investigation of iron metabolism and atherosclerosis development. It was shown that KCs play a central role in transferring LDL-derived cholesterol to hepatocytes via ATP binding cassette subfamily A member 1 (*ABCA1*) in the presence of iron [51]. Human liver cell line Huh7, on the other hand, was used to investigate coronary artery disease (CAD) risk and atherosclerosis associated with increased milk fat globule EGF and factor V/VIII domain containing (*MFGE8*) expression [52]. Other immortal leukemic cell lines, such as K562 and Meg-01, were used to investigate the relationship

between *CD36* expression and the risk of thrombo-embolism [53]. Meg-01 cell line displays phenotypic properties that resemble megakaryocytes and produces functional platelets, which is suitable for studying platelet functions [54], whereas K562 cell line possesses myelogenous origin which allowed high transfection efficiency and comparable expression profile with megakaryocytes [53].

Primary cells, such as mesenchymal stem cells (MSCs) are capable of self-renewal and differentiating into various cell types, and they are recognized as a promising tool with high therapeutic utility and disease modeling [55][56]. For instance, MSCs from individuals with both atherosclerosis and T2DM have been used for the evaluation on the role of NF-κB in immuno-potency, and it was shown that constitutively active nuclear factor kappa B kinase subunit beta (IKKB) reduces the immuno-potency by changing their secretome composition [57]. Recent studies have also used MSC as a model to evaluate cardioprotective effects of *LEF1* from oxidative stress conditions [58]. Besides, the human aortic endothelial cells (HAECs) are also widely used to study endothelial pathophysiology, due to its essential role in pro- and anti-thrombotic activities as well as modulating inflammatory processes [59]. HAEC was used to investigate the effects of a noncoding polymorphism involved in endothelial mechanosensing mechanisms [60].

Human pluripotent stem cells (hPSCs) which encompass both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), is an attractive option for in vitro atherosclerosis model system [61]. Both cell lines can be reprogrammed and differentiated into specific cells for functional analysis. However, the use of hESC for research purposes remain controversial due to the use of early embryos [62]. However, the scientific community argues that hESCs should not be regarded as equivalent to embryos since the isolated hESC is unable to develop into a human being [62][63]. Meanwhile, iPSCs is genetically identical to the donor which allows reaffirmation of the patient phenotype with the in vitro cellular phenotype [61]. For instance, iPSC is used to generate vascular smooth muscle cells (VSMCs) to model the protective effects of arylacetamide deacetylase (AADAC) over-expression against atherosclerosis [64].

Nevertheless, the drawback of iPSC is that the comparison of iPSC lines between different individuals can be confusing due to the difference in epigenetics modifications and its capacities to differentiate into desired specialized cells [65]. Besides, iPSC is also prone to undesired genetic modifications during reprogramming [66]. Furthermore, genome editing in germ cells has been controversial and sparked considerable debate, where the argument revolves around permanent DNA modifications that can be passed on to future generations [67]. Hence, somatic cell genome editing appears to be more widely accepted for disease treatment of affected individuals without influencing the genetic makeup of future descendants [49].

2.2. Off-Target Effects of CRISPR/Cas

Off-target effects have been observed in many CRISPR/Cas9-mediated atherosclerosis model systems. For instance, a hypercholesterolemia and atherosclerosis mouse model developed by the AAV-based delivery of CRISPR/Cas9 (AAV-CRISPR) approach have detected 5% mutations in one of the predicted off-target sites in an intron of the syntaxin 8 (*STX8*) gene [68][69]. Undesired off-target effects can be reduced by using a sgRNA design tool, which helps in predicting the off-target sites across the genome [70][71] as well as using truncated sgRNA which composed of shorter number of nucleotides (<20 nt) to increase the specificity of CRISPR/Cas9 system [72]. The use of engineered Cas9 nucleases, such as enhanced *S. pyogenes* Cas9 (eSpCas9) [73] and Cas9-HF-1 [74], could provide higher on-target fidelity without affecting the cleavage efficiency. Recent studies have used two CRISPR/Cas9 nickases to flank the target DNA and generate DSB with increased specificity [75][76]. Meanwhile, other researchers have used an inactive fusion protein complex that comprises of two FokI-dCas9 fusion proteins that are recruited to adjacent target sites to facilitate efficient genome editing [77]. Modifying the binding sites of Cas9 nuclease also reduces the chance of off-target binding [78].

Meanwhile, it is suggested that different screening methods such as exome- and whole-genome sequencing (ES and WGS) are used to detect off-target events on a genome-wide scale. Specifically, the **G**enome-wide, **U**nbiased **I**dentification of **D**SBs Enabled by **seq**uencing (GUIDE-seq) and in vitro Cas9-**Di**gested whole-**genome seq**uencing (Digenome-seq) can detect specific DSBs in the genome. Guide-seq relies on the detection of double-stranded oligodeoxynucleotides in DSBs [79], while Digenome-seq involves in vitro digestion and profiling of all DSBs [80]. Another strategy to evaluate off-target assessment in vivo, also known as ‘verification of in vivo off-targets’ (VIVO), was developed and involves the identification of off-target sites using the **CIR**cularization for in vitro reporting of **CLE**avage effects by **seq**uencing (CIRCLE-seq) method [81], followed by confirmation through the targeted amplicon sequencing approach [82]. This strategy was shown to be robust and sensitive in detecting off-target mutations with minimal frequencies (0.13%) [82].

2.3. Types of Mutations

Genome editing can be performed in many ways to achieve the desired mutational outcome. For example, disruption of a particular gene of interest can be achieved by the formation of indels, which often cause frameshift mutations [83]. NHEJ pathway is the main mechanism involved for the gene deletion approach and often utilizes two different guide RNAs to create two DSBs flanking the target sequence [84][85]. The method can also be used to create exon skipping by inducing DSBs at two different intron regions flanking a targeted exon. Recently, it was demonstrated that similar results can be obtained by using single guide RNA only [85]. In another example, Madan et al. [53] has successfully deleted a 573 base pair fragment in vitro using two guide RNAs which flank a targeted genomic locus containing the CVD-associated genetic variants (rs2366739 and rs1194196). On the other hand, base editors and HDR approaches are both applicable for point mutation correction. Base editors are capable of precise nucleotide substitution without the need of donor template [86][87][88], whereas the HDR method requires donor template such as a copy of the wild-type gene that serves as a corrective template [32][89]. Base editors (e.g., cytosine deaminase) that fused to CRISPR/Cas9 has the ability to convert cytosine bases into uracil, and have been successfully used to introduce nonsense mutations in *PCSK9* gene [90].

2.4. Delivery of Genome Editing Components

Delivery of the CRISPR system into cellular or animal model systems can be challenging [91], and efficient delivery is necessary to minimize off-target effects [92]. CRISPR/Cas9 systems contain two main components—the Cas9 endonuclease and guide RNA. The two components can be delivered into the cells in different forms such as plasmids, mRNAs, and RNP. Plasmid-based method utilize plasmids containing expression cassettes for Cas9 and guide RNA, and the expression of the two components are controlled by the endogenous U6 promoter [93]. Besides, mRNA for Cas9 and guide RNA can be delivered into target cells simultaneously to achieve genome editing [94]. Next, the plasmid-free method emphasized on the formation of RNP complexes before being introduced into the cells for genome editing [93]. The RNP approach was found to exhibit higher editing efficiency with lower off-target effects in hard-to-transfect cells [95]. This method allows transient genome editing effect in transfected cells where the CRISPR/Cas9 components gradually cleared from cells over time [49].

Generally, ex vivo and in vitro genome editing can be performed using non-viral and viral delivery systems. Non-viral approach involves physical and chemical delivery strategies such as electroporation, transfection agents, nanoparticles, and cell-penetrating peptides, whereas viral delivery systems involve viral transduction using adeno-associated viruses (AAVs) or lentiviruses [91]. Electroporation method uses electrical currents to increase permeability of the cell membranes which allows the delivery of genome editing components into the cells. Electroporation method may be a better option against difficult-to-transfect cells. However, it is more laborious and expensive [96]. On the other hand, chemical methods involving the use of positively charged lipid-based nanoparticles encapsulate negatively charged nucleic acids and facilitate the delivery across the cell membranes of the targeted cells [97]. Similarly, non-lipid polymeric reagents (e.g., polyethylenimine and poly-L-lysine) share the same principle by mediating the encapsulation of CRISPR/Cas9 and allows the positively charged complexes to enter the cells via endocytosis [98].

Alternatively, viral systems offer higher genome editing efficiency in vitro/in vivo and provide the advantage of natural tropism for specific cell types, along with long-term transgene expression [49][91]. To date, the AAV viral delivery systems are frequently used for gene transduction due to its non-pathogenicity, mild immunogenicity, serotype specificity, and ability to infect proliferating and non-proliferating cells indiscriminately [99]. AAV-packaging plasmids such as adenoviral helper plasmid pAdDeltaF6 (PL-F-PVADF6) and AAV8 packaging vector pAAV2/8 (PL-T-PV0007) were co-transfected with the AAV-CRISPR plasmid into HEK293T to produce high viral titer, before intraperitoneally injected into mice. Besides, lentivirus vector is also widely used. For instance, sgRNA-Cas9-expressing lentiviruses were produced from co-transfection of gRNA-integrated Cas9-producing lentiviral plasmid (e.g., pLentiCRISPR v2) and lentiviral packaging plasmid (e.g., pMDLg/pRRE, pRSV-REV, and pVSV-G), where the former is capable of expressing CRISPR/Cas9 components upon expression, and the latter is involved in the packaging of lentivirus [57]. In addition, lentivirus has high infection efficiency, which can be a better option to transfect hard-to-transfect or non-dividing cells [100].

3. Therapeutic Potential of CRISPR/Cas9 System for Atherosclerosis Treatment

Development of nucleic acid-based approaches has shown promising results, the CRISPR/Cas9 gene editing technique, on the other hand, have been explored as a novel therapeutic approach for atherosclerosis. Inactivation of gene targets such as *PCSK9* [101], *APOC3* [102], and *ANGPTL3* [103] have shown to be athero-protective. For instance, CRISPR-mediated inhibition of *PCSK9* showed reduced serum PCSK9 levels and lowered plasma cholesterol by 30–40% in mice [104]. In 2016, Wang et al. [105] used the same approach on the chimeric liver-humanized mice bearing human hepatocytes and demonstrated reduced human PCSK9 levels. *PCSK9* gene modification through adenoviral delivery of CRISPR/Cas9

showed high on-target mutagenesis (close to 50%) and relatively low off-target effects [105]. Besides, robust editing of *PCSK9* (more than 40%) in murine can be achieved using a *Staphylococcus aureus* Cas9 (SaCas9) nuclease with more restrictive PAM that can reduce the probability of off-target mutagenesis [106]. Furthermore, Thakore et al. [107] demonstrated gene silencing of *PCSK9* though systemic administration of SaCas9-based repressor (dSaCas9^{KRAB}) that is compatible with AAV delivery. Specific base editing was also successfully achieved by using a base editor 3 (BE3), which comprises of a CRISPR/Cas9 that is fused to a cytosine deaminase domain, and the resulting gene-edited mice showed more than 50% reduction of plasma PCSK9 protein levels and approximately 30% reduction of cholesterol levels without detectable off-target mutagenesis [108]. Moreover, following the subsequent success of in utero gene editing of *PCSK9* with positive results in murine models, gene editing before birth was made possible [109]. Hence, the idea of 'one shot' treatment from the elimination of liver *PCSK9* [110] in humans is appealing and seemingly possible to treat dyslipidemias. Meanwhile, *ANGPTL3*, is another new promising candidate that influences human lipoprotein metabolism by inhibiting lipoprotein lipase (LPL) [111] and endothelial lipase [112]. *ANGPTL3* gene silencing in the mouse model successfully lowered plasma cholesterols (e.g., triglyceride, HDL and LDL cholesterols) [113]. Lower triglyceride and cholesterol levels were also obtained by using CRISPR/Cas9 to mediate base editing of *ANGPTL3*, which introduced loss-of-function mutations of *ANGLTL3* in the transgenic mice model, highlighting the immense potential and feasibility of CRISPR/Cas9 technologies in gene therapy [90].

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