CRISPR/Cas-Based Gene Editing

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There is a growing need for a molecular vehicle that can successfully load and deliver CRISPR/Cas ribonucleoprotein complexes (and other gene editing systems) into target tissues. Synthetic delivery vehicles are being developed but so far have been only moderately successful. Extracellular vesicles are ideal candidates for a universal biological platform to produce ready-to-use, programmable, and highly biocompatible CRISPR therapeutics. Using extracellular vesicles in the CRISPR/Cas research and, ultimately, in the clinic, demands novel, advanced techniques for protein/RNA loading, surface engineering, and manufacturing. Safety of CRISPR/Cas systems and EVs also need to be tested extensively for every particular application.

Keywords: gene editing ; biodistribution ; pharmacokinetics ; nanomedicines ; nanovesicles ; exosomes ; nanoparticles ; nanoblades ; stem cells ; mesenchymal stem cells

1. Introduction

CRISPR/Cas-based gene editing is a prominent, recently developed molecular technique that has already revolutionized biology and could dramatically transform clinical management of genetically defined conditions, including cancer, infectious, and genetic diseases[1]. CRISPR/Cas systems function by recruiting the Cas protein to a specific locus on a DNA or RNA molecule using a short RNA called single guide RNA (sgRNA)[2]. The Cas protein then introduces a break into the targeted nucleic acid[3][4]. Alternatively, nuclease-null Cas9 (or dead Cas9; dCas) proteins may serve as carriers to bring enzymes or functionally active factors to certain locations in the genome[5][6]. CRISPR/Cas systems provide the powerful means to directly modify genetic, epigenetic, and protein-based pathogenic mechanisms, projecting their application for treating numerous diseases[7][8].

2. Challenges

So far, one of the major challenges is the lack of an optimized tissue-specific CRISPR/Cas delivery tool[9]. Many nanotechnological vehicles have been devised in recent years to deliver CRISPR/Cas systems into target cells (reviewed in[10][11][12][13]). Current strategies have numerous limitations, including: (1) high molecular mass and positive charge of Cas proteins that make them difficult to package using common drug delivery tools[14]; (2) the lack of robust tissue-specific delivery vehicles suitable for cell-specific gene editing applications[15]; (3) immunogenicity[16][17][18][19][20] and other safety issues (molecular, cellular and tissue toxicity)[21] to which the majority of novel synthetic delivery vehicles are prone; and, finally, (4) the lack of a universal CRISPR/Cas delivery platform that can be utilized for a wide array of CRISPR/Cas systems. Such a platform must allow use of CRISPR/Cas systems that are highly variable in size and molecular features[22]; systems isolated from various species (e.g., *Neisseria meningitides*[23], *Streptococcus thermophiles*[24], *Streptococcus pyogenes*[3], and others such as the recently described small CasX from Deltaproteobacteria[25]); and engineered CRISPR/Cas[26], such as CRISPRa/i tools[27][28][29], CRISPR base editors[30][31], and the PrimeEditing system[32]. The lack of robust and safe CRISPR/Cas delivery tools, especially with tissue-targeting modalities, delays translation of CRISPR/Cas-based therapeutics into the clinic. In particular, CRISPR/Cas systems have been shown to be highly potent antivirals eliminating or dramatically reducing viral loads in such infections as hepatitis B virus[33][34][35], hepatitis C virus[36], human immunodeficiency virus (HIV)[37][38][39], human papillomavirus[40], and even the recently emerged coronaviral SARS-CoV-2 infection[41]. Notably, CRISPR/Cas systems have been successfully leveraged to genetically modify the human genome for making primary $CD4^+$ T cells resistant to HIV[42]; several ongoing clinical trials are underway using CRISPR/Cas for correcting mutations associated with genetic disorders and treating cancer.

Three principal methods are available to deliver Cas and their guiding RNAs (gRNAs) into target cells: (1) coding DNA sequences; (2) coding RNA/mRNA; and (3) ribonucleoprotein complexes (RNPs), i.e., readily available Cas protein complexes with *in vitro*-transcribed or synthetically generated gRNAs. Delivery of coding DNA sequences can be performed by both viral (including adeno-associated virus and adenovirus) and non-viral methods; packaging and delivery

of mRNA/RNA and RNPs are usually performed by non-viral methods[15]. Nanotechnological methods mostly rely on the use of liposomes and cationic lipids[43][44][45], amphiphilic peptides[46], DNA nanoclews[47][48], gold nanoparticles[49][50][51], and graphene-based nanosheets[52].

Delivering CRISPR/Cas as DNA coding sequences is fraught with poorly controllable intracellular synthesis of CRISPR/Cas components with an ensuing increase in off-target activity[53][54][55] and potential integration of DNA into the genome[56]. Although plenty of novel approaches have been proposed to hone the specificity of CRISPR/Cas systems (e.g., self-inactivating delivery systems[57][58], on/off-inducible systems[59][60]) and build additional levels of tunability (e.g., anti-CRISPR proteins[61][62]), these approaches add complexity and safety issues. Delivering large amounts of DNA is also associated with toxicity, may induce activation of the host factors involved in foreign DNA recognition, and may even cause cell death[63][64][65][66]. Additionally, the large molecular size of traditional CRISPR/Cas nucleases and, especially, dCas-based molecular tools exceeds the packaging capacity of commonly used AAV viral vectors and thus hampers their use. This is particularly true for hybrid CRISPR/Cas systems fused to additional functional moieties (epigenome modifiers[67], transposases[68][69], reverse transcriptases[32], etc.), that add molecular weight to Cas proteins.

Delivery of CRISPR/Cas as mRNA/RNAs is associated with instability and fragility of the long Cas mRNAs and may be substantially compromised by reduced efficacy of on-target editing[70][71][72]. The most straightforward approach is direct delivery of CRISPR/Cas RNPs into the cells[73]. Successful gene editing for treating a disease, whether a genetic disorder or an infectious illness, usually requires very transient expression of CRISPR/Cas, which may permanently correct the malfunctioning gene or rapidly destroy the viral genomes. Many recent studies demonstrated that the delivery of CRISPR/Cas RNPs is characterized by the highest efficacy and specificity of gene editing[74][75][76].

Proteins or RNPs cannot be delivered systemically as naked molecules. Human serum contains proteases that can rapidly destroy unprotected proteins. Protein and RNA components of CRISPR/Cas are therefore vulnerable to rapid degradation upon systemic injection and must be protected by nanoparticles for in vivo applications.

Moreover, pre-existing antibodies against Cas proteins[16] and immune response to Cas and sgRNAs[18] can limit efficacy of CRISPR/Cas approaches. Reducing and evading immune recognition can be achieved by rationally designing Cas proteins (e.g., epitope masking or limiting presentation of Cas epitopes to the immune system)[77] [78], using CRISPR/Cas systems from non-pathogenic organisms, inducing immune tolerance[78], or shielding Cas proteins in systemic circulation. Short-lived CRISPR/Cas complexes are sufficient for most clinical applications, especially in immune-privileged organs, and are less likely to induce a meaningful immune response. Nevertheless, in order to increase efficacy and preserve a second-use opportunity, it is desirable to shield Cas and RNPs from immune recognition.

Cas proteins are not naturally able to cross biological barriers without specially designed delivery vehicles and do not tend to accumulate in specific organs or tissues. High positive charge and molecular mass (>160 kDa for *S. pyogenes* Cas9) make CRISPR/Cas RNPs unsuitable for traditional methods of nanotechnological packaging and protein delivery. Thus, engineering advanced molecular vehicles encapsulating the CRISPR/Cas RNPs with penetrating and targeting ability is needed for tissue-specific delivery of gene editing complexes and clinical implementation of devised molecular techniques.

The ideal non-viral method for targeted in vivo drug delivery should fit the following criteria: (a) effectively package CRISPR/Cas RNPs of any type and species and with any modifications; (b) shield RNPs from an aggressive environment and the immune system; (c) effectively deliver RNPs into target organs; (d) escape endolysosomal pathways; and (e) be simple and scalable. To date, no such methods exist.

To date, the most successful approach for local targeted delivery of gene editing systems in the form of gold-linked nanoparticles combined with penetrating peptides demonstrated ~30% efficacy[49]. However, this method does not shield Cas proteins and suffers from other disadvantages, such as immunogenicity, toxicity, and rapid clearance upon systemic administration. On the other hand, EVs have emerged as a promising delivery system for proteins and RNAs, substantially outperforming synthetic nanocarriers in terms of safety and pharmacokinetics[79]. Evs are natural nanoparticles secreted by numerous cell types that exhibit very high biocompatibility and extraordinary ability to cross biological barriers[80]. Because Evs can transfer RNA, protein, and lipid cargo, display preferential tropism for certain tissues, and are amenable to engineering, they have been extensively utilized as potential drug delivery systems. Genetic engineering of EV-producing cells and modification of purified Evs enables direct loading of therapeutic macromolecules into the vesicles and targeted drug delivery.

The advantages of Evs have been increasingly utilized for CRISPR/Cas delivery, but translating EV-CRISPR/Cas therapies to the clinic requires the invention of new, more efficient techniques for EV cargo loading and surface engineering. Overall, there is great demand for developing effective, programmable, versatile, and safe delivery platforms

that ideally can be used for any type of CRISPR/Cas system.

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