A Review: possible optimization of Cas9-sgRNA nuclease delivery via ingested lipid-nanoparticles bioencapsulated within plant cell-based enfolding

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Abstract

The possibility of gene editing to correct disorders is one of the most impactful therapeutic agents, currently. CRISPR Cas9-sgRNA nucleases can be used to cleave and to delete harmful or pathogenic DNA sequences, which cause genetic disorders. Cas9 nuclease includes palindromic repeats that cut and delete a single point mutation or multiple DNA target site sequences. The Cas9, attached to a sgRNA or a guiding RNA, finds and then cleaves the target DNA sequence. The Cas9-sgRNA method of cleavage has corrected DNA mutations that cause cataracts in the eyes, cystic fibrosis, and chronic granulomatous disease. However, there are issues with an effective delivery of Cas9-sgRA to target DNA sequences. Delivering Cas-9 nucleases are negatively affected by off-target DNA sites, sgRNA design, off-target cleavage, Cas9 activation, and the method of delivery. This review focuses on oral and ingested delivery methods to effectively guide the transport of Cas9-sgRNA nucleases in vivo. This review presents possible alternatives for nuclease delivery within optimized lipid-nanoparticles, plant, algae, and bacterial-based orally ingested edibles. This review attempts to provide evidence in support of the higher effectiveness of ingesting therapeutic bioencapsulated edibles because the edibles can directly contact immune cells within the gastrointestinal tract for blood or lymph circulation.

Introduction

The sgRNA of the CRISPR CAS-9 system attached Cas9 to a specific genomic area that is complementary to a 20 bases of the sgRNA, consisting of a 5'-NGG-3' protospacer-adjacent motif or a PAM (Yin et al., 2014). Cas9 breaks the double stranded DNA structure at the specific gene loci and then are repaired through nonhomologous end-joining or homology-directed repair, HDR. Dominquez et al. (2016) define a CRISPR–Cas as a prokaryotic RNA a part of the adaptive immune system, protecting its cells from foreign DNA sequences. Dominquez et al. (2016) described the structure of CRISPR-Cas as a large protein with multiple domains but with only two active sites for nuclease activity. In fact, after researchers studied the Cas9 of *S. thermophilus* it was found to block the entrance of viruses (Doudna et al., 2014). The CRISPR Cas-9 system has corrected gene mutations that cause disease as cataracts and cystic fibrosis, however, there has not been a direct injection into mammalian organs to genetically edit gene mutations. Genome editing was initially applied to *Drosophila melanogaster*, and rapidly extends to a broad range of organisms (Wu et al., 2015).

Benefiting from the simplicity and adaptability of CRISPR/Cas9, it opens the door for revealing gene function in biology and correcting gene mutations in diseases. Genome editing can modify DNA sequences and treat genetic disorders through CRISPR. CRISPR-Cas9 consists of palindromic repeats that can change a single to multiple genes (Wang, 2017). CRISPR corrected mutations in NOX2. NOX2 is responsible for an immunodefiency disorder called chronic granulomatous disease (De Ravin, 2017). The modified stem cells were transplanted into mice, and the NOX2 protein was functional for 5 months. Therefore, CRISPR/Cas9 has the potential to become an effective and a more facile genome editing tool, after addressing multiple issues.

Further studies are necessary to explore the characteristics and improve the performance of CRISPR/Cas9, especially its specificity for off-target mutations and the delivery methods of the CRISPR/Cas9. For instance, Zhang et al. (2014) discussed an issue of off-target mutations versus ZFNs and TALENs, which CRISPR/Cas9 offered a higher propensity for developing off-target mutations. The human cell has a large amount of DNA material, which frequently consists similar homologous DNA target sequences. As a result, choosing target sites and more specifically designing sgRNAs will improve with the total sequencing of bacterial genomes.

However, oral ingestion of Cas9 nuclease may be a possible effective route of delivery through the GI tract. Many immune cells are located near large aggregations of commensal bacteria in skin and in the GI tract. The microbiota provides a barrier to assist with the immune response. The host cells have a close interaction with microbiota, which provide a buffer system between microbes and epithelial cells. Called the "Mucosal firewall" it consists of epithelial cells, mucous, immunoglobin A, antimicrobial peptides, and immune cells (Belkaid and Hand, 2015). This review will present and discuss alternatives for optimizing Cas9 nuclease delivery mainly through the gastrointestinal tract, GIT, after oral ingestion. The delivery issues for Cas9-sgRNA, alternative methods, and possible oral ingestion delivery of Cas9 will be discussed in this review.

Survey Methodology

Research Questions: What are the current challenges of the Cas-9 nuclease? How can the delivery of Cas9-sgRNA be improved? Can lipid-nanoparticles improve delivery of Cas9? Can oral ingestion of lipid-based nanoparticles with Cas9 become a more effective approach for its delivery? Why may the GI tract be a more successful route for Cas9 delivery?

Search Terms: CRISPR Cas9 description, Structure of the immune system within the gut, the structure of the Peyer's patches, lipid-nanoparticles and Cas9 delivery, liposomes versus lysosomes, bioencapsulation and Cas9 current issues and challenges.

Search Engines: google scholar, google images, Science Direct, and ResearchGate

Total Research Articles Viewed: 20

Chosen Research Articles: 16

Eliminated Research Articles: 4

The four articles were eliminated based on their title and abstract, which did not align with the search terms. The first article was eliminated because it reviewed the coagulation or blood clotting gene mutations corrected by CRISPR gene editing, the 2nd article's title included the term Rheumatoid Arthritis, the 3rd article studied muscular dystrophy, the 4th article only discussed the history or the development of CRISPR nucleases.

Issues and Methods for CRISPR CAS9 Delivery

Some challenges for targeting genes include sgRNA design. For example, challenges with Cas9 occur when sgRNA-2 and sgRNA-5 detect and change the wildtype and the mutant genes for the mCrygc embryonic stem cells or ESCs. The sgRNA-1, sgRNA-3, and sgRNA-4 only target the gene mutation of mCrygc. The sgRNA-4 was selected due to it inducing deletions, insertions, and it closely targets the mutant allele. To correct the mutation that causes cataracts, a mouse model was used. Zygotes were injected with Cas9 mRNA and sgRNA-4 and were the offspring of females that procreated with homozygous males, expressing the phenotypic cataracts. Approximately 91 percent of the zygotes matured into embryos and 22 mice were born (Wu et al., 2013). Of all the 22 mice none of the wildtype alleles were affected or changed, showing the accuracy of sgRNA-4 to only target the mutant alleles.

Other challenges may occur with direct injection or through unguided Cas9. The Cas9 mRNA, sgRNA-4, and Oligo-1 were injected into the cytoplasm to test rather adding a single-stranded DNA oligo called Oligo-1 can make HDR-mediated precise genome more efficient. The coinjection was projected into the cytoplasm of heterozygous cataract mutation-bearing zygotes. The results included: the DNA sequencing 14 of the 29 mice displayed gene changes in the mutant allele (Wu et al., 2013). Nine of the fourteen mice did not form cataracts. To genetically edit the Fah gene three sgRNAs: FAH1, FAH2, and FAH3, were cloned into the plasmid pX330. The vector pX330 expresses sgRNA and Cas9 complementary to the target gene mutation. Mice were then injected with unguided Cas9 or with ssDNA oligos with pX330, dispatching Cas9 and

one of the sgRNAs of FAH1 to FAH3. The unguided Cas9 injected mice needed to be euthanized due to their 20 percent loss of body weight (Yin et al., 2014). The FAH-2 injected mice after 30 days exhibited less liver damage and disease. After the histological analysis of the liver of the mice, applying serum biomarkers as aspartate aminotransferase, AST, alanine aminotransferase (ALT), and bilirubin to the liver, the Fah gene increased with less liver damage. Next, the structure and function of the immune system and the Peyer's patches within the gut will be discussed.

The Immune System within the Gastrointestinal Tract

Mucous in the intestines provide a barrier between microbiota and epithelial tissue. The goblet cells produce the mucous. The epithelial cells form antimicrobial peptides to reduce a physical interaction with commensal microflora. The antimicrobial peptides act as enzymes to degrade bacterial cell walls and its inner membranes. The peptides help to create and maintain a physical barrier between the host intestines and the microbiota. The immune response in the intestines includes FOXp3 for the regulatory Treg cells, which maintain balance in the mucous (Belkaid and Hand, 2015). The degradation of Treg cells causes effector responses in the gut. Tissue co-factors as Vitamin A, MUC2, a mucous glycoprotein formed from intestinal goblet cells differentiate dendritic cells for regulation. Treg cells in the colon also have a high affinity for antigens processed by commensal bacteria.

Peyer's Patch and Drug Delivery

The immunoglobulin type A is derived from commensal bacteria and produced by dendritic cells. The microflora interacts with the epithelial cells, T cells, and B cells in the Peyer's Patch. The microbiota-immune relationship yields IgA, which bind specific antigens only processed by commensal bacteria. Microflora bacteria bound to dendritic cells move through the lymph node but cannot cross the IgA barrier. Then, the B cells carrying IgA traverse to the lamina propria of the intestines to release the IgA antibody. The transfer of IgA from the B cell monitors the host to microflora-bacterial gene expression. The host-commensal bacteria immunity blocks bacteria from attaching to the epithelial lining of the GI tract.

The general mechanism involves a drug compacted within a liquid and solid lipid. The drug travels through the small intestines, and then binds to M cells that line the lumen of the intestines. The drug transports through the M cells to be engulfed and phagocytized by NCLs (Fig.1). The drug is carried by dendritic cells to the Peyer's patch and released into the lymph nodes for lymphatic circulation. In the lymph nodes the dendritic cells release the drugs to enter blood circulation. Therefore, the route of drug ingestion and delivery through the intestines may be a high-yielding method for a direct interaction between the immune cells, the circulatory system, and Cas-9-sgRNA nucleases. However, Cas9 nucleases will require packaging into lipid-based nanoparticles to successfully cross the lipid bilayer of the M cells.

Lipid nanoparticles and Liposome Ingestion of CRISPR CAS9 Nucleases.

Lipid nanoparticles have much potential for improving CRISPR-CAS9 delivery. Because DNA is negatively charged, DNA can be encased within cationic or positively charged lipid

nanoparticles. The lipid encased CRISPR Cas9 nucleases can cross the lipid cell membrane as a result of its positive charge. The liposome reduces an immune response, rendering it a safer delivery method. The Cas9 and sgRNA are intensely anionic, therefore, can be auspiously packed within lipid nanoparticles. However, there are issues present with administering the lipidnanopartles.

The issues include: 1) the Cas 9 nuclease liposomes, entering the cell membrane, are immediately engulfed and degraded by lysosomes and 2) delivering the liposome rarely reaches the cell nucleus. Crossing the nucleus presents an arduous obstacle with a lower delivery efficiency. Lipofectamine can be a possible method and solution. It is positively charged, can bind DNA targets, diffuse through the cell membrane, and avoid endocytosis. The Cas9 and sgRNA plasmid DNA was transfected into human pluripotent stem cells. The Cas9 and sgRNA have successfully been delivered through Lipofectamine to correct Facial anomalies syndrome (ICF) syndrome with a high rate of transfection efficacy at 63% (Lino et al., 2018). Also, other methods to optimize lipid nanoparticle delivery will be reviewed.

The pH-sensitive liposome can avoid lysosome degradation. The pH-sensitive unit can be added to the liposome through blending pH-sensitive lipids with polymers. The liposome will remain stable in a neutral pH and intact until it reaches a lower pH environment, which is more acidic, pH of 5, in the cytoplasm (Liu & Huang, 2013). After entering the cell through an endosome, a controlled and gradual release of the contents inside the pH-sensitive liposome occurs, bypassing the lysosome degradation. Tumors are highly acidic with a pH of 5; however, Monteiro et al., (2018) currently crafted a pH-sensitive liposome consisting of paclitaxel or PTX. The pH-sensitive-PTX liposomes can extend its reach to tumors, and then release the encapsulated drugs as it contacts the acidic tissue masses. Hasset et al. (2019) enhanced lipid nanoparticles with ionizable lipids, called MC3, for delivering a nucleic acid-based vaccine for influenza. Their vaccine trials with 100 micrograms of the ionizable lipid-based mRNA vaccine yielded a 100% seroconversion or a total detection of the antibodies produced.

The size and dosage of oral ingested particles absorbed within the GIT can be a factor for its efficacy as well. For example, Brocks and Davies (2018) modeled the pharmacokinetics and the absorption of drugs or other particles across the enterocytes of the gut. They confirmed a competition between the lymphatic flow of drugs versus the blood flow, which is 500 times faster. The expected positive result for an oral ingested drug entering the lymphatic flow depends upon the drug size, a molecular weight less than 500g/mol, and the dosage. Larger drugs enter the blood flow instead of the lymphatic system to become cleared from the blood circulation by the liver. A smaller dosage of a drug enters the lymphatic flow, delivering it more efficiently to the lymph and nodes. However, the Cas9 nuclease will require efficient packaging to successfully navigate through digestive enzymes of the stomach after ingestion.

Possible Ingested Delivery Methods:

The bioencapsulation of liposomes carrying Cas-9-sgRNA nucleases with plant, algae, and bacterial-based oral delivery could be considered as well. For example, consider the most

proven and studied vaccine delivery to induce an enormous adaptive and humoral immune response include edible vaccines. Vaccines ingested signal an immune response through the gut-associated lymphoid tissue or known as GALT. The GALT consists of the lymph nodes, the Peyer's Patch, and lymphoid tissues in the GI tract. The Peyer's patch are 75% B cells, and include 20% of T cells (Criscuolo et al., 2019). When oral vaccines are ingested, the antigens are transferred through the mucosal layer via the M cells into the Peyer's patches and presented to the T-Cells.

However, oral vaccines need to surpass the mucosal tolerance to overcome and attract more effector cells than regulatory cell types. Oral vaccines need to be revised to increase their absorption by M cells. Three possible ways to enhance oral vaccines are through plant, algae, and bacteria-based bioencapsulation. Plant-based encapsulation allow for ample folding of proteins as antigens and are more affordable. Plant-based delivered vaccines have been in use since the 1990s. Also, because of plants thick and sturdy cell walls, the plant cells can tightly protect antigen proteins from degrading or denaturing. With the delivery of oral vaccine components, the cross-contamination with animalistic disease-causing antigens can be avoided. Also, plant cell-based oral delivery can prevent bacterial corruption.

Algae-Based oral vaccines, encapsulated in green microalgae called *Chlamydomonas reinhardtii*, readily acquired and amplified the yield of antigens. The growth rate of algae is extremely rapid. The FDA has identified and approved green algae as safe and it can be stored at 4 degrees Celsius for 20 months (Criscuolo et al., 2019). The cell walls in algae increase the stability for the bioencapsulation form, blocking antigen denaturing by the enzymes in the GI tract. Gram-positive bacteria as Lactic acid bacteria do not cause disease and have been utilized for many years, decades. The Lactic acid bacteria have been used to preserve food and used to compose antibodies against HIV infection. Because the bacteria-base delivery allows for a more facile acceptance in the GI tract among other commensal bacteria, the antigen compressed inside will not degrade.

Bacteroidetes and Firmicutes are the only bacteria in the gut microbiome that use enzymes called cellulosomes that bind to the cellulose of plant cell walls. Activating the cellulosome, bound to cellulose, the glyosidic bonds are broken. This was proven through an in-situ hybridization study (Kwon & Daniell, 2016). In this study there was an enormous measure of Bacteroidetes in the mucous layer and its substrates were predominantly mucopolysaccharides. In the mucous layer, bacteria in the gut grasp and attach to a plant cell with their pilli consisting of cellulosomes. The plant cell is ruptured, releasing the Green Fluorescent Protein. The GFP was not visible in the stomach, but in the upper GI tract near the ileum and the villi absorbed in the epithelial cells. The release of visible GFP signaled a successful oral delivery of a plant-based encased protein.

Conclusion

This review discussed possible methods for optimizing the delivery of Cas9 nucleases. Delivering Cas-9 nucleases are negatively affected by off-target DNA sites, sgRNA design, off-target cleavage, Cas9 activation, and the method of delivery. This review focuses on delivery

methods to effectively guide the transport of Cas9-sgRNA nucleases through the GI tract in vivo. Delivery through the digestive and GI tract may provide a valuable alternative method. The GI tract consist of 70% of the total immune system. The lining of the GIT contains a layer of microflora bacteria, a mucosal layer, and epithelial cells, and immune cells. The Peyer's Patch is located within the GIT, containing immune B cells and T cells. The Peyer's patches within the gut represent a fastidious transport of prescription drugs or other lipid nanoparticles. The ingestion of lipid-based nanoparticles travels through the epithelial lining of GIT via M cells, attach to dendritic cells transferring them to the Peyer's patches where they are presented to B-Cells and T-Cells. The immune cells deliver the lipid nanoparticles to the lymph nodes or into blood circulation.

Our review attempted to present alternatives for delivering CRISPR Cas9-sgRNA nucleases through the gastrointestinal tract. We reviewed the internal processes for delivery through the ingestion of nanoparticles as the lipid nanoparticles. Evidence for a more successful Cas9 delivery through the GIT was explained by revisiting the structure and function of the gut immune system. For example, ingested nanoparticles as drugs can immediately travel through the epithelial lining of the lumen of the GIT and interact directly with immune cells in the Peyer's patches. Lipid nanoparticles are a possible method that can immensely impact the delivery of Cas9 nucleases through the GIT to the Peyer's patches to the lymphatic and circulatory systems. Because the Cas9 nucleases are negatively charged, they can be tightly packed into positively charged lipid nanoparticles.

Sheathing Cas9 nucleases into lipids can allow prompt diffusion through the lipid bilayer of cell membranes lining the lumen of the GIT. However, delivering the Cas9-liposomes are engulfed quickly by lysosomes, rarely entering the cell nucleus. Because liposomes lack viral subunits, it lessens an immune response, but liposomes could be re-designed to more precisely target cells and tissues. Plant, algae, and bacteria-based bioencapsulation can be a possible option for re-designing Cas9-liposomes. Plant cells have strong cell walls, which enclose antigens, averting its degradation. Using plant cells for encapsulation can prevent contamination with mammalian and bacterial material. Algae-base liposomes can protect the contents inside from GI tract enzymes. Algae-based encapsulation also have strong and durable cells walls to protect Cas9 nuclease cargo. Bacterial-based liposomes can have an even safer travel through the GIT because Lactic acid bacterial encapsulation will be accompanied by more commensals within the GIT microflora.

However, more emphasis is given to bioencapsulation with plant cells. Because the enzymes in the human stomach can not digest or degrade the cellulose in the plant cell walls, the Cas9-sgRNA contents within plant-based casing should not degrade. When the plant-based Cas9-sgRNA liposome receptacle enters the GI tract, commensal bacteria begin to metabolize the plant cell walls, and then the contents are released to transport across the GIT lumen via the M cells. The contents permeate into the circulatory or the immune system. However, more research is needed to more effectively elucidate and apply methods that optimize delivery of CRISPR Cas9 nucleases. Specifically, continued improvement to optimize lipid nanoparticle delivery of Cas9 cargo components should be a future goal for further research. Further study

needed could include confirming the efficacy of combined plant-based bioencapsulation with optimized lipid nanoparticles as an outer and mid covering of the Cas9 contents. For example, for oral digestion, the plant cells, as an outer covering, can protect the mid pH-sensitive liposome and the inner Cas9 nuclease cargo from stomach enzymes after it is orally ingested. In addition, when designing a plant-based bioencapsulated lipid-nanoparticle for Cas9 delivery, its size, molecular weight, and dosage need to be considered to assure its absorption by the lymphatic system.

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