Revolutionizing gene editing techniques in medicine: siRNAs, saRNAs, miRNAs, CRISPR/Cas system

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0. Abstract

Gene therapy is a new modality of medical treatment that can treat or prevent diseases by directly modifying gene expression. This review focuses on four prominent mechanisms: small interfering RNAs (siRNAs), small activating RNAs (saRNAs), micro RNAs (miRNAs), and the CRISPR/Cas system. siRNA and miRNA silence gene expression by either degrading mRNA or repressing protein translation, respectively, while saRNA promotes gene expression through targeting promoter regions. CRISPR/Cas precisely edits genes through insertion, deletion, or correction of specific sequences. Although these mechanisms show significant therapeutic potential, challenges such as delivery barriers and off-target effects remain as obstacles. This review synthesizes findings from peer-reviewed articles found using Google Scholar, while focusing on research from the past 20 years. While exceptions were made for a few older articles due to their importance to the paper, their credibility was assessed through cross-referencing with reliable medical sources. Preliminary findings highlight the capability of siRNA and miRNA in silencing genes associated with various diseases, the potential of saRNA in activating therapeutic targets, and the precision of CRISPR/Cas in correcting genetic mutations. By providing a comprehensive evaluation of four innovative gene therapy approaches via their mechanisms of action, advantages, challenges, and therapeutic applications, this review highlights the need for continued innovation to overcome current limitations and revolutionize medicine.

Keywords: Gene Therapy, RNA Interference, Gene Editing, Small Interfering RNA (siRNA), Small Activating RNA (saRNA), Micro RNA (miRNA), CRISPR/Cas

1. Introduction

Gene therapy is a groundbreaking scientific advancement in medicine that has the potential to treat and cure genetically impacted disorders by modifying or manipulating the expression of the underlying genetic cause. By increasing, silencing, or correcting the expression of specific genes within a patient's cells, gene therapy aims to cure diseases considered uncurable or have suboptimal cures. These approaches can precisely target the cause of disease in a large variety of disorders, such as cancers, strokes, and genetic disorders. Gene therapy can upregulate the expression of one or multiple genes, allowing the cells to increase the production of the downstream protein(s) deficient in certain diseases[1]. In contrast, gene therapy can also silence genes to block or prevent the production of unwanted proteins[2]. Upregulation and silencing mechanisms are beneficial when altering the expression of specific

genes. By increasing the expression of under-expressed genes or silencing the expression of pathogenic genes, scientists can correct the underlying cause of various diseases. In addition, gene correction techniques that replace faulty genetic sequences within the genome can help treat many genetic disorders. As gene therapy continues to evolve with the rapid increase in the discovery of new gene-editing technologies, it paves the way for revolutionary treatments that can address the underlying causes of diseases rather than just symptom management.

Small interfering RNA (siRNA) is a powerful gene therapy tool that silences specific genes' expressions by targeting and degrading their messenger RNA (mRNA). This tool exploits the natural cellular process of RNA interference (RNAi). In short, double-stranded RNA (dsRNA) molecules that are homologous to the silenced gene guide the degradation of mRNA[3]. These molecules are then cleaved by an enzyme into short fragments of 21-25 nucleotides, and each fragment joins a protein complex that degrades the mRNA[4]. siRNA is an extremely promising method due to its efficiency; however, there are still many challenges in the application of this technique due to potential problems with sequence targeting, off-target silencing, and accidental activation of immune responses[5].

In contrast to siRNAs, small activating RNAs (saRNAs) are short double-stranded molecules that play a role in the upregulation of transcription of specific genes. Unlike siRNAs, which are gene-silencing modalities, saRNAs exploit the cellular process of RNA activation (RNAa) to enhance gene expression[6]. Although the exact mechanism is unknown, the rough process is that saRNAs bind to complementary sequences within gene promoters, recruit the protein Argonaute 2 (Ago2) and its associated proteins CTR9 and RHA, and form the RNA-induced transcriptional activation (RITA) complex that modifies chromatin structure[6]. Like siRNAs, saRNAs have significant benefits as they are highly regulated mechanisms and can be chemically synthesized in high yields. However, they can face similar issues in stability, accidental immune responses, off-target effects, and inaccurate delivery[6].

MicroRNAs (miRNAs) are small, non-coding 21-25 nucleotide single-stranded RNAs produced from hairpin-shaped primary miRNAs (pri-miRNA)[7]. They regulate gene expression post-transcriptionally by binding to their target mRNAs' untranslated region and further repressing protein production[7]. In addition to repressing translation, miRNAs are also able to degrade mRNA. miRNAs have significant functions in the human body, as they participate in many cellular biological processes such as homeostasis, cell growth, cellular differentiation, apoptosis, and stress responses. They also regulate many human diseases, such as neurological diseases, cardiovascular diseases, cancer, and aging[8]. Similar to the previous methods of gene therapy, this method also has some challenges in working efficiently: the degradation and clearing of unmodified miRNAs in blood circulation, limited penetration of miRNAs, unwanted immune system activation, and off-target effects[9].

Lastly, CRISPR/Cas systems are immune systems that exist in most bacteria and archaea and prevent them from being infected by phages, viruses, and other foreign bodies. CRISPR are clustered, regularly interspaced, short, palindromic repeats that can be further transcribed into CRISPR RNA (crRNA), trans-activating CRISPR RNA (tracrRNA), and CRISPR-associated (cas) genes for Cas proteins[10]. These systems are divided into two classes: Class 1 and Class 2, six types: (I-VI), and subtypes (multi-Cas protein and single Cas protein)[11]. CRISPR/Cas systems correct gene expression by targeting and destroying their associated nucleic acids. They precisely cut the DNA and then use the cell's natural body systems to modify the gene. Due to CRISPR/Cas being a relatively new tool for genome editing and expression, some factors and challenges influence its efficacy: off-target effects, efficiency of DNA repair mechanisms, and selection of target site[10].

This review paper solely focuses on mechanisms, challenges, and applications; excluding any ethical, legal, or socioeconomic considerations to the treatments. Peer-reviewed articles from the last 20 years, identified through Google Scholar, were analyzed to ensure credibility and relevance, with older articles cross-referenced for reliability. It provides an overview of various gene therapy mechanisms ***(Fig.1)***, their applications as treatments for various diseases, and their challenges in efficiency and usage in order to bridge gaps in understanding gene therapy and its potential for revolutionizing medicine.

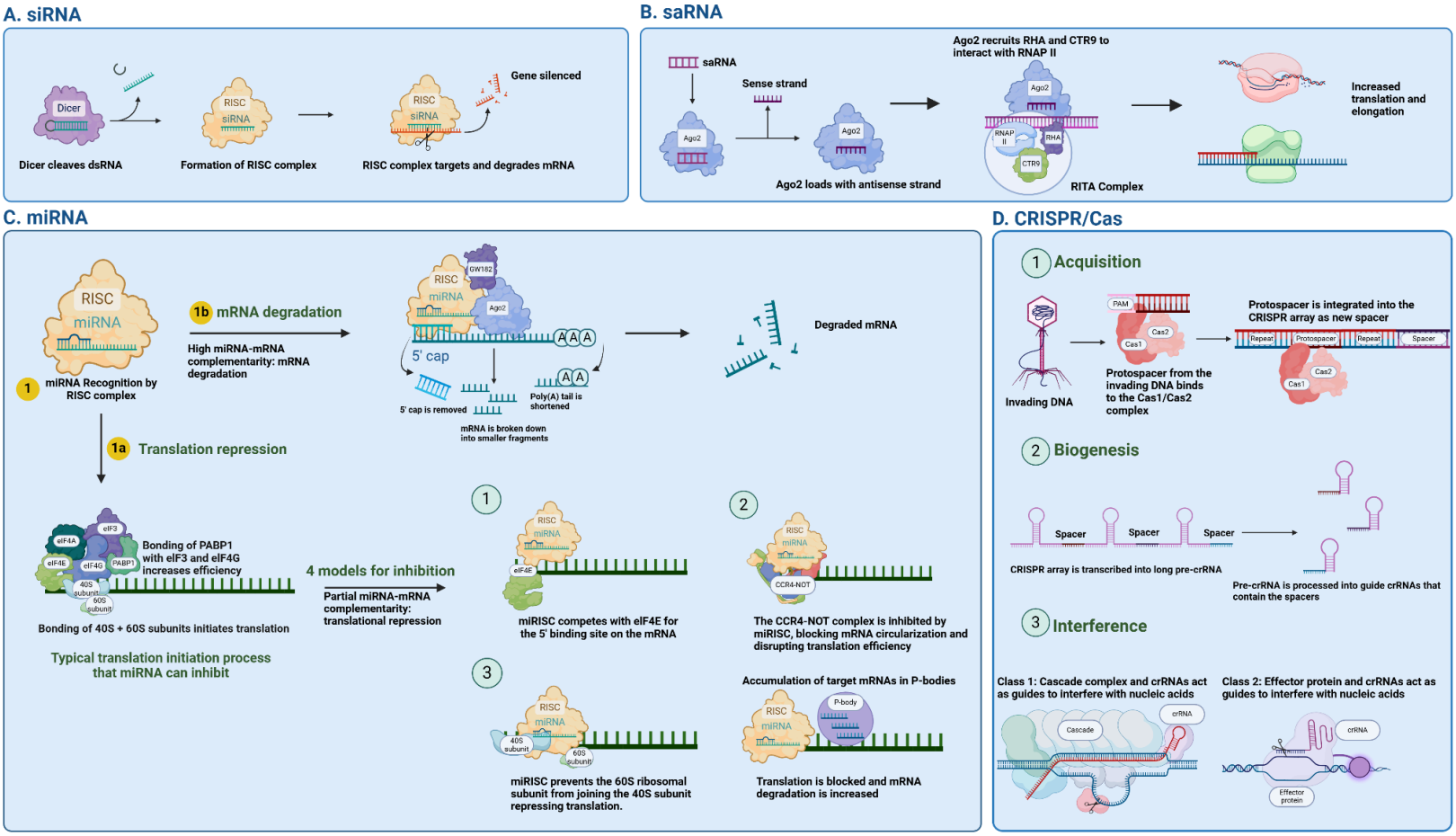

Figure 1. Proposed mechanisms of action of current gene editing techniques: A. siRNA: *The siRNA mechanism begins with the cleavage of double-stranded RNA by the enzyme Dicer. Dicer cleaves the dsRNA into siRNA fragments, which are 21-25 nucleotide long RNA strands. One strand of the siRNA, the guide strand, is incorporated into the RISC (RNA-induced silencing complex). This complex identifies and binds to complementary mRNA sequences within the cell. Once bound, the RISC complex cleaves and degrades the target mRNA, silencing the expression of the gene.* ***B. saRNA:*** *The antisense strand of saRNA is loaded by the Ago2 (Argonaute 2) protein. Subsequently, Ago2 recruits RNA helicase A (RHA) and CTR9 to interact with RNA polymerase II (RNAP II). The interaction forms the RITA (RNA-induced transcriptional activation) complex. This complex promotes transcriptional elongation and translation of the target gene.* ***C. miRNA:*** *miRNA has two separate pathways: mRNA degradation due to high miRNA-mRNA complementarity and translation repression due to partial miRNA-mRNA complementarity. In the first step, the miRISC (microRNA-induced silencing complex) recognizes mRNA strands through guidance by the miRNAs. When there is partial miRNA-mRNA complementarity (1a), the miRNAs are able to inhibit translation. The*

typical method of translation initiation consists of the eIF4F complex (eIF4A, eIF4E, and eIF4G); eIF3, eIF4G, and PABP1 bonding to increase efficiency, while the bonding of the 40S and 60S ribosomal subunits begins translation. There are four models to the inhibition of this process: competition for the 5' binding site on the mRNA between miRISC and eIF4E (model 1), disruption of mRNA circularization by the CCR4-NOT complex (model 2), prevention of the joining of the 60S and 40S ribosomal units (model 3), or the accumulation of mRNA in P-bodies. With high miRNA-mRNA complementarity (1b), the 5' cap of the mRNA is removed, the mRNA is broken down into smaller fragments, and the poly(A) tail is shortened, leading to degradation. ***D. CRISPR/Cas:*** *In the first stage, acquisition, the invading DNA is recognized by the cell, and a segment called a protospacer is extracted from the invading DNA and bound to a complex formed by Cas1/Cas2. The protospacer is integrated into the CRISPR array as a new spacer, guided by the presence of a PAM (protospacer adjacent motif). Repeats separate the spacers in the CRISPR array. In the biogenesis stage, the CRISPR array is transcribed into a long pre-crRNA (precursor CRISPR RNA). The long pre-crRNA is further processed into smaller guide crRNAs with one spacer from the invader DNA. The interference stage is very different based off of the class of the CRISPR/Cas system: Class 1 systems employ Cascade complexes and crRNAs to act as guides to interfere with the invading nucleic acids, while Class 2 systems use a single effector protein and crRNAs to act as guides.*

2. Results

2.1. **Small Interfering RNAs**

2.1.1. **Overview of siRNA**

Small interfering RNAs (siRNAs) are short, 21-25 nucleotide long, double-stranded fragments of RNA. They participate in the biological process that silences genes. siRNAs work by degrading specific messenger RNA (mRNA) sequences, preventing the production of proteins. Due to RNAi being a relatively new discovery, the mechanism of siRNA has become more apparent through extensive research.

The discovery of siRNAs and their function began with studies on post-transcriptional gene silencing (PTGS) in plants[4]. Hamilton and Baucombe discovered an accumulation of 25-nucleotides small RNAs in tomato plants undergoing PTGS[12]. This was the first discovery of the role of small RNAs in the RNAi process. In 1999, when Tuschl et al. tested an in vitro cell-free system obtained from a *Drosophila* syncytial blastoderm embryo, they found that the dsRNA was processed into 21-23 nucleotide siRNAs. However, the single-stranded RNA was not efficiently converted into the same 21-23 nucleotide products, proving the importance of the dsRNA in siRNA processing[13]. The role of siRNAs in RNAi was further confirmed by a study by Elbashir et al., who demonstrated that siRNAs can efficiently guide the degradation of

homologous mRNAs[14]. To further assess the exact role of siRNAs in RNAi, an experiment by Zamore et al. fractionated both the unprocessed and the processed dsRNAs[15]. The fractions with siRNAs induced RNA degradation, proving that siRNAs were the main factors of the RNAi reaction.

To further understand the siRNA-assisted RNAi silencing process, researchers looked for an enzyme responsible for siRNA's binding and cleavage process. Bass discovered the involvement of RNase III-type endonucleases in the degradation of dsRNAs by siRNAs based on the binding and cleavage properties of RNase III enzymes. He found that the RNase III enzyme cuts both strands of the dsRNA and leaves a 3' overhang of 2 nucleotides. These cuts by the RNase III enzyme form the siRNAs[16]. Upon further analysis of the siRNAs generated by the *Drosophila* system, Tuschl et al. found a 5' phosphate, 3' hydroxyl, and a 3' 2 nucleotide overhang in the processed 21- to 23- nucleotide RNAs. One gene, *dicer*, was found to encode an enzyme that cleaves dsRNA into 22 nucleotide fragments. When immunoprecipitated from *Drosophila* extracts, it was found that Dicer produced 22 nucleotide RNAs from dsRNA[13].

2.1.2. **siRNA's components and mechanism of action**

In RNAi, a small amount of dsRNA can lead to prolonged degradation of target mRNA. Although the initial synthesis of dsRNA into siRNAs can lead to degradation of target mRNA, it is insufficient for consistent sustained degradation[4]. This suggests a mechanism beyond simple degradation of target mRNA involved in this phenomenon. Lipardi et al. investigated the dsRNA-dependent degradation of target mRNA in a *Drosophila* embryo system and discovered the generation of dsRNAs from labeled siRNAs. They found that single-stranded RNAs, mimicking the target mRNA, and dsRNAs served as templates for RNA-dependent RNA polymerase (RdRP). This rapidly produced new dsRNAs that were subsequently cleaved into siRNAs[17]. Through an RNAi reaction, Sijen et al. discovered the formation of new secondary siRNAs that were different from the initial dsRNAs but still corresponded to the target mRNA[18]. RdRP is crucial for generating the secondary siRNAs, which sustain the RNAi. Amplification occurs at various stages of the RNAi reaction, enhancing the gene silencing effect[4].

In the last phase of RNAi, the siRNAs bind to the RISC, a component of the RNAi mechanism. RISC is activated using ATP, leading to the siRNA's unwinding and exposing the siRNA's antisense strand[4]. Once the antisense strand is exposed, it guides the RISC to the target mRNA. Once the RISC is activated, its antisense siRNA component pairs with complementary mRNA sequences. The RISC cleaves the target mRNA approximately 11-12 nucleotides down from the 5' end of the guide siRNA[4]. Exoribonucleases likely degrade the resulting mRNA fragments after RISC cleavage. Some of the cleaved mRNA fragments may be further converted into duplex forms by RdRP activity[4]. This forms new siRNA-like molecules that help to contribute to the amplification of the RNAi response. In a different model, the siRNAs

assemble along the target RNA and are combined by RNA ligase to generate cRNA. Dicer then processes the cRNA and target RNA[4].

The RNA-induced silencing complex (RISC) is an essential component of the RNAi mechanism, as it mediates the degradation of target mRNAs through siRNAs[5]. RISC is composed of Argonaute proteins, more specifically, Argonaute2 (AGO2). AGO2 binds the guide siRNA strand, removes the passenger strand, and undergoes several cycles of target mRNA recognition, cleavage, and release. Argonaute selects the guide strand with the less stable 5' end and slices the passenger strand for removal[5]. AGO2 has three functional domains: PIWI, PAZ, and MID. PIWI contains an RNase H fold, which provides the slicing activity essential for mRNA degradation[5]. The PAZ domain recognizes the 3' dinucleotide end and anchors it within its hydrophobic pocket. The terminal nucleotide base stacks with an aromatic ring of one of the aromatic residues on the pocket. The 5' phosphate end of the siRNA interacts with the MID and PIWI domains and binds to a magnesium ion coordinated with the C-terminus of the protein[5].

Dicer is a member of the RNAse III nuclease family, one of the few nuclease families specific for dsRNA cleavage[4]. It breaks the phosphodiester bonds found in the dsRNAs, generating the siRNAs. Dicer can convert dsRNA into uniformly sized siRNAs. The structure contains four domains: an amino-terminal helicase domain, dual RNase III motifs, a dsRNA binding domain, and a PAZ domain, which is present in proteins like Piwi, Argo, and Zwille. These domains are familiar with other families of proteins, such as RDE1, QDE2, and Argonaute, which are all genetically linked to RNAi. The Dicer protein has two forms: Dicer-1 cleaves pre-miRNAs, and Dicer-2 processes dsRNAs. The helicase domain of Dicer proteins recognizes and processes siRNA. It comprises multiple subdomains (HEL1, HEL2i, and HEL2) in various Dicers. Dicer-2 uses its helicase domain to recognize, unwind, and cleave the dsRNAs in organisms with two Dicers. Furthermore, helicases also act as sensors for viral RNA and activate the appropriate RNAi pathway for cleavage[19]. The helicase domain of Dicer helps facilitate substrate recognition, unwinding, and cleavage, contributing to the creation of siRNAs and the RNAi mechanism. The dsRBD (dsRNA binding domain) is a small, conserved protein domain found across eukaryotic, prokaryotic, and viral proteins. dsRBD primarily functions in binding dsRNA and is essential for substrate binding. It helps engage dsRNA substrates, facilitating transfer to the other domains for cleavage[19]. The PAZ (Piwi-Argonaute-Zwille) domain is a crucial component of Dicer proteins. It facilitates the initial recognition and anchoring of RNA substrates with two adjacent pockets. The 3' pocket binds the two nucleotide 3' overhang of RNA, while the 5' pocket binds the phosphate group. This anchors the RNA substrate and facilitates the processing of dsRNA into siRNA. PAZ also mediates interactions between the Dicer and Ago proteins, which assemble the RISC that facilitates gene silencing[19]. Dicer proteins' dual RNase III motifs consist of two RNase III domains (RNase IIIa and RNase IIIb). Each RNase domain independently catalyzes the cleavage of the phosphodiester bonds within one strand of the dsRNA. The RNase IIIa domain cleaves the 3' end of the pre-siRNA with 3'

overhangs, while the RNase IIIb domain cleaves the 5' phosphate arm. This allows Dicer to produce siRNA of exact lengths essential for incorporation into the RISC[19].

In summary, the siRNA mechanism begins with dsRNA processed by the enzyme Dicer, which cleaves it into siRNAs. These siRNAs are then loaded onto the RISC, where the antisense strand guides RISC to complementary target mRNA, leading to cleavage and degradation. Secondary siRNAs form through RdRP-mediated amplification, further sustaining the RNAi effect.

2.1.3. **Applications of siRNA in ischemic stroke treatment**

One notable application of siRNA is its therapeutic capabilities for stroke treatment. Cerebral strokes often leave patients with long-lasting mental, physical, and psychological disabilities. The most common type of cerebral stroke, ischemic stroke, occurs when there is an interruption or reduction of oxygen flow to neurons, leading to hypoxia and cell death[20]. The main goal of therapeutic intervention through siRNA is restoring any lost neurological functions from the strokes. Several attempts in the past couple of years have been to induce neuroprotection and reduce inflammation, delay scar tissue and activate neuronal plasticity, enhance neurogenesis from the SVZ (subventricular zone), and replace lost cells through stem cell insertions. Researchers rely on regulating protein pathways to restore impaired function using siRNAs to do this. siRNAs are used to silence protein pathways activated after stroke, as very few reports suggest that siRNA usage for prevention is a possibility[20].

One of the possible applications of siRNAs in cerebral strokes is the induction of neuroprotection after a stroke to restore neuronal activity and function[20]. A study by Kim et al. tested if RNAi against the protein kinase apoptosis signal-regulating kinase 1 (Ask1) downregulates the expression of Ask1 and prevents apoptotic neuronal cell death after ischemia/reperfusion (I/R) in mice[21]. An I/R injury occurs when blood flow is restored to previously ischemic tissues. Although reperfusion is necessary to save the tissues, it can cause further damage, including cell dysfunction and death[22]. In this treatment, they rescued brain damage after I/R in mice that underwent occlusion of the middle cerebral artery for one hour, followed by more reperfusion. In their results, Kim et al. concluded that Ask1-siRNA reduces the upregulation of Ask1 and found some reduced infarction in the ischemic brain after I/R. However, there were no reports of behavioral outcomes in treated animals[21].

Wang & Yamaguchi found that the transcription factor C/EBP homologous protein that encodes (CHOP, DDIT3, GADD153) promotes apoptosis after stress on the endoplasmic reticulum (ER) in various diseases and delayed adaptation in neurons after hypoxia. Furthermore, CHOP also acts post-transcriptionally through p38 MAPK in response to ER stress and activates the expression of Bim[23]. This expression of the Bim protein leads to

Caspase-3-dependent apoptosis[20]. A study by He et al. proved that intracerebroventricular pre-treatment with CHOP siRNA in a subarachnoid hemorrhaghia (SAH) model significantly upregulated the antiapoptotic protein Bcl2 and downregulated the expression of the problematic protein Caspase-3. Furthermore, any neurological deficits were reduced in any siRNA-treated animals, providing evidence for siRNA potential for apoptotic mechanisms after SAH[24]. Finally, another study by Al-Jamal et al. directly targeted Caspase-3 through a local siRNA delivery by an intraparenchymal injection in an endothelin-induced ischemia rat model. The researchers found that acute local delivery of Caspase-3-siRNA-loaded carbon nanotubes into the primary cortex 24 hours before the stroke reduced neuronal apoptosis and prevented microglia activation after the stroke. Furthermore, forelimb motor function was restored entirely in animals treated with Caspase-3-siRNA, while internalization of the carbon nanotubes by neurons suggested that siRNA delivery was achieved. Also, an improvement was found in motor skills reaching tests, which suggests clinical potential for this method of treating ischemic strokes[25].

Another potential application of siRNA in ischemic stroke treatment is the elucidation of neurodegenerative mediators. A study by Tizon et al. demonstrated how CysC, an inhibitor of cysteine protease activity and regulator of autophagy, can act as a neuroprotective mechanism after cell damage. They found that by blocking autophagy in oxygen-deprived cell culture models and primary neurons using Beclin1-siRNA, they could eliminate the protective effect of CysC. Furthermore, when using a proprietary dendriplex complex (TRANSGEDEN) for siRNA delivery, downregulation and knockdown of Beclin-1 were observed, preventing autophagy[26]. Therefore, Beclin-1 prevention of autophagy can be used as a potential strategy for neuroprotection after stroke damage.

2.1.4. **Challenges in siRNA application**

Generally, applying the siRNA gene therapy technique is promising due to its efficient and specific gene silencing mechanisms. However, some challenges in its therapeutic application still need to be overcome for safe and efficient usage. The first challenge lies in its stability and targeting. siRNAs outside of cells are prone to enzyme degradation in serum and tissues with a half-life of several minutes to an hour[5]. This very short period makes accumulating siRNA to the appropriate target site a significant challenge. To be effective, siRNAs have to not only survive in the serum, but they also have to reach the target cells or tissues. Furthermore, siRNAs face further challenges once at their target site. Their large size and negative charge also prevent diffusion across the plasma membrane, impacting the accumulation of siRNAs in cells. Also, they are even vulnerable to degradation by intracellular RNases and need to be recognized and incorporated by the RISC almost immediately[5]. Aside from challenges in delivery, the siRNA mechanism also has limitations in practice. A microarray analysis by Jackson et al. found that siRNA treatments can accidentally silence off-target genes, leading to harmful mutations and unexpected cellular impacts[27]. Most of these off-target silencing events occur due to homology

with 6-7 nucleotides in the “seed region” of the siRNA. Focusing only on mRNA levels in off-target analyses can also overlook genes suppressed during translation[5]. Furthermore, if the RISC poorly selects the guide strand over the passenger strand, the probability of matching undesired targets increases. A final challenge is the accidental activation of an immune response. siRNA duplexes 23 nucleotides long can activate interferon responses and cause cell death. Also, specific siRNAs can bind and activate the TLR7 receptor, which initiates immune responses, if they have a particular 5’-GUCCUUCAA-3’ sequence or similar GU-rich sequences[5].

2.2. **Small Activating RNAs**

2.2.1. **Mechanism of saRNAs**

Small activating RNAs (saRNAs) are small sequences of dsRNA that participate in the biological process of RNA activation (RNAa). Unlike RNAi, which downregulates or silences the expression of genes, RNAa induces transcription at a faster rate and increases the overall gene expression.

Similar to siRNAs, there is no clear-cut mechanism for saRNAs, so various experiments and studies must find a general mechanism. Previous chromatin immunoprecipitation (ChIP) studies by Portnoy et al. revealed an increase of RNA polymerase II (RNAP II) and different epigenetic marks at saRNA target promoters. These findings suggested that there should be a mechanism in which gene expression is induced through transcription. More recently, another study by Portnoy et al. performed biochemical, proteomic, and functional analysis on the saRNA process using human *CDKN1A* (*p21*) as a gene to explore the exact mechanism of RNAa and how saRNAs can increase the transcription rate[28]. Their first step in the analysis was to confirm that saRNAs increase transcription through a direct, on-target mechanism. The researchers then conducted nuclear run-on assays on two saRNAs- saP21, targeting the *p21* gene, and saEcad, targeting the *E-cadherin* gene.

These experiments showed significant increases in the transcription rates of these genes after saRNA transfection. The sa21 and saEcad caused a 9.3 and 28.3-fold increase in transcription compared to the controls. To confirm that this effect was specifically due to saRNAs and not any off-target effects, the researchers used CRISPR to mutate the saP21 target site in the *p21* promoter in mutated and wild-type PC-3 cells. In mutated cells, mRNA induction by saP21 in *p21* cells was almost completely abolished, while in the wild-type PC-3 cells, the saRNA increased *p21* expression. There was some *p21* protein induction in the mutated cells, but it was most likely due to outside mechanisms unrelated to RNAa. Through this analysis, the researchers confirmed that saRNAs directly increase gene transcription through an on-target mechanism on the transcriptional level.

After confirmation of saRNA activities, Portnoy et al. examined the binding of RNAP II and its phosphorylation at *Ser5* and *Ser2* in response to saP21 treatment to better understand the saRNA-related transcription process[28]. Phosphorylation at *Ser5* indicates transcriptional pausing near the transcription start site (TSS), while phosphorylation at *Ser2* indicates transcription elongation. The differentiation between Ser5 and Ser2 phosphorylation influenced the use of scanning ChIP assays on the *p21* gene to measure the RNAP II and Ago2 binding at the various stages. The results showed significant Ago2 binding around the saP21 target site, suggesting that saP21 guided Ago2 binding to its target. In addition, in saP21-treated cells, there was a substantial increase in RNAP II binding near the *p21* TSS, suggesting that saRNA-Ago2 interactions promote transcription by facilitating RNAP II recruitment to the promoter. Upon further analysis, a localized accumulation of RNAP II Ser5P and Ser2P was found near the TSS in both treated and non-treated cells, while the accumulation of Ser2P was moderate. This suggests that there were potential pauses in transcription without RNAa. After treatment with saP21, the researchers observed a higher accumulation of RNAP II SerP, reflecting transcription elongation. This change from pauses in transcription to transcription elongation highlights how saRNAs can target both the transcription and elongation processes.

To address critical questions about RNAa, such as if saRNAs bind to their intended targets on the promoters, which strand acts as the guiding strand, and which proteins participate in RNAa, Portnoy et al. developed a ChIP assay called chromatin isolation by biotinylated RNA pull-down (ChlbRP). This assay isolates the DNA and protein components associated with saRNAs at target sites to determine if saRNAs are loaded by Ago2. In short, this method biotinylates one strand of an saRNA duplex at the 3’ end, transfects the strand into cells, precipitates the biotinylated saRNA-bound chromatin with magnetic streptavidin beads, and purifies the associated nucleic acids and proteins for analysis. Previous studies have shown that Ago2 loading of duplex RNAs can be impaired by adding chemical groups such as biotin to the 5’ end. The researchers biotinylated the 3’ end of saP21, saEcad, and control saRNAs. These saRNA duplexes maintained total RNAa activity. They successfully induced their respective genes (*p21* and *E-cadherin*). However, when the researchers added a psoralen group to the 5’ end of the antisense strand of saP21, the ability of saP21 to induce *p21* expression was blocked by preventing Ago2 loading. This demonstrated that 3’ biotinylation on either strand doesn’t interfere with RNAa activity, but 5’ end labeling interferes with the activity.

Having confirmed the saRNAs can tolerate 3’ biotinylation, the researchers transfected cells with biotinylated saRNAs and then pulled down the biotinylated strand to detect the Ago2 protein. They observed different patterns of Ago2 loading on the saP21 and saEcad duplexes based on the thermodynamic stability of the 5’ end. Because saP21 has an asymmetric thermodynamic stability, Ago2 was mainly associated with the antisense strand. On the other

hand, saEcad has almost symmetric thermodynamic stability, so the two strands had an equal Ago2 association.

To assess if the Ago2-loaded saRNA strands bind to their intended promoters, qPCR was performed on the DNA pulled down in the ChIbRP assay. The results showed a 24.7-fold enrichment in the antisense strand to the *p21* promoter, but not in the sense strand, indicating that only the antisense strand binds to the *p21* promoter. In contrast, the binding on both strands was similar to their target promoter with a 16.8-fold enrichment in the sense and a 14.8-fold enrichment in the antisense strands. These results suggest that promoter binding by saRNAs is mainly dependent on Ago2. The ChIbRP samples also contained RNAP II, indicating that saRNAs guide Ago2 to target promoters and interact with transcription machinery to enhance transcription.

In the next phase of the study, Portnoy et al. explored the additional proteins recruited by the saRNA-Ago2 complex that facilitate interaction and activation of transcription. By utilizing mass spectrometry analysis and the ChIbRP assay, they compared the proteins of the antisense strand to the proteins on the sense strand. They found 42 proteins associated with the antisense strand and only 15 associated with the sense strand. In addition, more Ago2 peptides were found in the antisense strand, emphasizing that Ago2 preferentially loads the antisense strand and is the guide strand.

Two significant proteins that were associated with the guide strand were CTR9 and RHA. These proteins were chosen for further analysis due to their ability to activate transcription with DNA/RNA unwinding/binding activity and their potential for interacting with RNAP II. To validate the mass spectrometry results and confirm interactions between the chosen proteins (CTR9 and RHA) with Ago2 and RNAP II, the researchers used immunofluorescence staining to reveal the nuclear localization for both CTR9 and RHA. They also used reciprocal co-immunoprecipitation assays to show their interaction with Ago2 and RNAPII in saP21-treated cells. Furthermore, the protein CTR9 was found to be part of the PAF1C, which is involved in histone modification and transcription regulation. This suggests that the saRNA-Ago2 complex recruits PAF1C for transcriptional activation. The saRNA-Ago2 complex recruits CTR9 and RHA, essential components of the RNA-induced transcriptional activation (RITA) complex, which drives saRNA-mediated transcriptional regulation.

Through this study, the mechanism of the pathway can be hypothesized: saRNAs are first loaded by Ago2 and bind to their intended promoter targets, then Ago2 recruits RHA and CTR9 to interact with RNAP II through the RITA complex which then drives transcription and elongation.

2.2.2. **Application of saRNAs in pancreatic cancer treatment**

An important potential application for saRNA therapies includes pancreatic cancer, which is one of the most common causes of cancer-related mortality. The number of people who die each year from pancreatic cancer is rapidly increasing, and less than 5% of patients with pancreatic cancer are still alive 5 years after diagnosis. Even with resection and chemotherapy, most patients face recurrence of the cancer. The current treatment for pancreatic cancer, gemcitabine, is not a highly effective treatment as it only improves the one-year survival rate by three percent[29].

Since the current treatment is suboptimal, this emphasizes the need of new treatment options like saRNA therapy. The current target for saRNA therapy in pancreatic cancer is the transcriptional factor C/EBPα transcription factor, an upregulator of p21. A study by Reebye et al. found that saRNAs can induce antitumor effects by activating C/EBPα and its downstream targets, including p21[30]. In pancreatic cancer specifically, the loss of the *KDM6B* gene that encodes a histone demethylase enhances cancer's aggressiveness by downregulating C/EBPα[31]. Therefore, the upregulation of C/EBPα by using C/EBPα-saRNA is a promising strategy for pancreatic cancer treatment.

To further improve the efficiency of this approach, delivery mechanisms have been developed to target the cancer cells more precisely. RNA aptamers that recognize these cells have been developed to enhance the delivery of saRNA to pancreatic cancer cells. RNA aptamers can target cell-surface motifs through epitope recognition and cell internalization. Even when exposed to harsh conditions, they have a stable three-dimensional structure, offering better stability, lower toxicity, and lower immunogenicity[29]. The specific aptamers for pancreatic cells are found through a method called the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) which is based on their affinity to PANC-1, human pancreatic adenocarcinoma cells. After 14 rounds of SELEX, the researchers selected the aptamers of P19 and P1.

The study further investigated the selected P19 and P1 aptamers with structural similarities and a common motif, GAAUGCCC. The aptamers were evaluated for their ability to target PANC-1 cells selectively through flow cytometry. PANC-1 cells were detached using a nonenzymatic cell dissociation solution incubated with Cy3-labeled aptamers for 30 minutes at 37°C[29]. The results indicated a significant increase in the cell surface binding of the aptamers, highlighting their potential for targeting pancreatic cancer cells.

Further, to confirm the specificity and internalization of these aptamers, Zhao et al. utilized live-cell confocal imaging where a panel of four different pancreatic cancer cell lines was placed in 35-mm dishes and grown for 24 hours before treatment with Cy3-labeled aptamers[29]. Their results indicated an increase of P19 and P1 on all pancreatic cancer cells but not on any

noncancerous pancreatic cells, highlighting P19 and P1's specificity for purely cancerous tissues.

To test the delivery for C/EBPα saRNA, Zhao et al. used 20-fluoropyrimidine RNA (20F-RNA) pancreatic cancer-specific aptamers, P19 and P1[29]. They then created conjugates by synthesizing various RNA components, which then are refolded in a binding buffer and annealed to form scrambled C/EBPα RNAs. The researchers then assessed the internalization of these new saRNA conjugates by incubating PANC-1 cells with Cy3-labeled P19 and P1 conjugates. Imaging after a one-hour incubation found significant uptake of the conjugates in the pancreatic cancer cells, highlighting that using aptamers to deliver saRNA can enhance the activation of C/EBPα expression in pancreatic cancer cells.

Next, the researchers wanted to test gene activation *in vivo* by examining the effects of P19 and P1 conjugated C/EBPα saRNA on PANC-1 cells. The cells were seeded in 24-well plates and treated with 80 nM of either the conjugated C/EBPα-saRNAs or the scrambled RNAs. This was then repeated 24 hours later, and the cells were harvested after 72 hours for RNA extraction[29]. Analyses revealed that cells treated with the conjugated C/EBPα RNAs had majorly higher levels of C/EBPα mRNA and p21 than those treated with only the scrambled saRNAs did. A WST-1 cell proliferation assay measured the impact of the aptamer conjugates on cell proliferation and found an 80% reduction in cell proliferation, highlighting a sharp decreasing effect caused by p21. To analyze protein expression, the PANC-1 cells were treated similarly, and the total protein was extracted for a Western blot analysis. The results found that P19-C/EBPα-saRNA treatment had 3 times higher C/EBPα protein levels than its counterpart. Overall, they found that linking C/EBPα-saRNA to pancreatic cancer-specific aptamers significantly enhances the depression of C/EBPα.

Finally, the researchers evaluated the antitumor effects of C/EBPα-saRNA *in vivo* by establishing traceable tumor animal models using firefly luciferase-expressing cells. PANC-1 and AsPC-1 cells were transfected and selected for stable clones using G418, so the resulting cell lines expressed luciferase, allowing for monitoring of tumor growth through bioluminescence. The researchers injected a suspension of luciferase-expressing PANC-1 or AsPC-1 cells mixed with a growth factor-reduced Matrigel matrix into the dorsal skin of 5 week-old SCID mice[29]. Once the tumors reached approximately 1 cm, the mice were divided into groups for treatment, receiving injections of various amounts of the aptamer-saRNA conjugates. The results showed that groups treated with either the 100 or 250 pmol dose of P19-C/EBPα-saRNA and P1-C/EBPα-saRNA significantly reduced tumor growth. P19-C/EBPα-saRNA exhibited a 30% more efficient antitumor response than the current treatment gemcitabine.

Furthermore, the study reported no signs of blood toxicity from the P19-C/EBPα-saRNA treatment with normal hemoglobin, white blood cell, and platelet counts. In a gemcitabine-resistant pancreatic cancer xenograft model, P19-C/EBPα-saRNA induced a 40% decrease in tumor growth without toxic effects[29]. Overall, this study suggests that targeted delivery of C/EBPα through pancreatic cancer-specific aptamers can serve as a promising therapeutic approach for pancreatic cancer.

2.2.3. **Challenges in saRNA application**

The therapeutic potential of saRNA is very promising due to its precise gene activation mechanism and small size. Due to its small size, it is much easier for saRNAs to be chemically synthesized in large amounts. However, several challenges still have to be addressed before the wide usage of saRNA in medicine, including risks of immune response activation, off-target effects, and poor stability. Small dsRNAs are often prone to instability in serum, with very little half-life due to nuclease degradation. Therefore, there have been efforts to modify and increase the nuclease resistance for efficient saRNA usage[6]. Place et al. attempted to incorporate 2'-fluoro into both sense and antisense strands to saRNA, which improved saRNA stability. However, it did decrease the gene activation mechanism when applied to both strands[32]. The researchers found that restricting modifications to only the guide strand does not affect the efficiency of gene activation[6]. Additionally, locked nucleic acid modifications were applied to saRNAs, which usually decreased the activity. They also found the gene activation efficiency can be restored to similar levels when applied to both ends of the sense strand.

Another possible modification is adding a 5' inverted abasic modification to the sense strand, enhancing saRNA activity with better antisense strand loading[6]. Furthermore, because saRNA is a dsRNA, it also has the potential to be immunogenic. Efforts have been made to mute this issue, such as 2' fluoro and 2'-O-methyl modifications. Place et al. have shown that 2'-fluoro modifications on cytidines and uridines in the guide strand can significantly inhibit the immune activation of saRNA[32]. Voutila et al. have also shown that incorporating 2'-O- methyl modifications on the passenger strand and overhangs on the guide strand can abolish the immune activation effect of saRNA[33].

Additionally, saRNA can induce off-target effects like siRNA[6]. To prevent this, various strategies have been developed to ensure the specificity of saRNA for its intended target site. Voutila et al. analyzed the seed sequence of the saRNA to predict complementary binding sites through bioinformatics. Experiments can then determine whether the saRNA causes activation at those sites[33]. Finally, while saRNA has the potential to be an effective treatment option, development of methods to deliver the saRNA is still crucial for any therapeutic application. Naked saRNA has significant challenges *in vivo* due to its susceptibility to degradation by

nucleases. Its nucleobases face the inside, exposing the negatively charged phosphate backbone on the outside, hindering interactions between saRNA and the cell membrane[6].

2.3. **MicroRNAs**

2.3.1. **Mechanism of MicroRNAs**

MicroRNAs (miRNAs) are small, endogenous RNAs 21-25 nucleotides long that can target specific mRNAs for either degradation or translation repression. Scientific advancements have revealed the regulatory mechanism of miRNAs in animals, including *C. elegans* and *D. melanogaster*[7].

miRNAs guide the microRNA-induced silencing complex (miRISC) to recognize mRNA and downregulate gene expression through translational repression and mRNA cleavage[7]. The degree of miRNA-mRNA complementarity is crucial in determining the regulatory mechanism process of miRNAs. Complementarity refers to the extent to which the nucleotide sequence of the miRNA matches with the sequence of the target mRNA. High complementarity usually leads to mRNA cleavage and degradation through Ago proteins, while central complementarity prevents cleavage and promotes translational repression of target mRNA. Once miRISC binds to target mRNAs, the degree of miRNA-mRNA complementarity either facilitates Ago-catalyzed degradation or translation repression of target mRNA sequences[7].

One mechanism by which miRNA decreases gene expression is through translational repression; however, it is unknown whether this decrease in expression occurs during translational initiation or translational levels. A popular model points to the eIF4F complex as the initiating factor in translation. This complex has four subunits, eIF4A, eIF4E, and eIF4G, that can recognize the 5’ cap of mRNA, triggering translation initiation. Another initiation factor, eIF3, binds with eIF4G, assembling the 40S ribosomal subunit at the 5’ end of the mRNA. This binding forms a pre-initiation complex that joins with the 60S ribosomal subunit at the mRNA’s start codon to initiate translation. Further, the polyA-binding protein PABP1 interacts with eIF4G and eIF3, increasing translation efficiency[7]. A study by Petersen et al. found that miRISC can repress translation by repressing this elongation process. Their analysis showed that blocking initiation with hippuristanol, a potent inhibitor of eIF4A, in the presence of miRNAs led to ribosome dissociation[34]. This implies that miRISC can prompt ribosome detachment, therefore repressing any elongation.

There are three main proposed models for the mechanism of miRISC-mediated inhibition of transcription initiation[7]. The first model hypothesizes that miRISC competes with eIF4e for the 5’ cap-binding site on mRNA, slowing translation initiation. In this model, the protein GW182 or another downstream factor is proposed as a candidate for the eIF4e competitor. The second

model suggests that miRISC prevents mRNA circularization, thus inhibiting efficient translation. A proposed complex is the CCR4-NOT complex, which is hypothesized to contribute to translation inhibition by miRISC. Finally, the third model suggests that miRISC can prevent the 60S ribosomal subunit from joining the 40S preinitiation complex. The 40S ribosomes are attached to the target mRNA, but the 602 subunits cannot join, repressing translation.

Another pathway for miRNA-mediated repression involves the accumulation of target mRNAs in processing bodies (P-bodies), which lack machinery for translation. When miRNA guides mRNA to the P-bodies, the accumulation without ribosomes can lead to translation repression[7]. miRNAs may increase the amount of ribosome-free mRNA, blocking translation and promoting mRNA degradation in P-bodies.

miRNAs with high sequence complementarity can facilitate target mRNA degradation through Ago protein activity. mRNA levels decrease with an increase of miRNAs, so miRNAs likely have a direct role in mRNA degradation[7]. Many mechanisms are involved in the Ago-catalyzed mRNA degradation process, including deadenylation, decapping, and exonucleolytic digestion of mRNA[7]. Deadenylation shortens mRNA's poly(A)-tail in eukaryotic cells. It plays a significant role in mRNA translation inhibition and degradation[35]. mRNA degradation also requires Ago2, GW182, and other cellular organelles in the previous processes. However, the exact method by which mRNAs select targets for degradation has yet to be fully understood or determined. It is known that specific factors influence the process in the miRNA/RNA duplex, including the number, type, and position of the mismatches. The variation in complementarity is essential for determining if the miRNA will degrade or inhibit the mRNA's translation[7].

miRNAs regulate gene expression by guiding the miRISC to target mRNAs, leading to either translational repression or mRNA degradation. This outcome is determined by the degree of miRNA-mRNA complementarity: where high complementarity triggers mRNA cleavage, while partial complementarity causes translational repression. In cases of high complementarity, Ago proteins initiate mRNA degradation through processes like deadenylation and decapping.

2.3.2. **Applications of miRNAs in thoracic cancer treatment**

miRNAs have gained significant attention for causing gene expression changes in cancer as they function as oncogenes (oncomiRs) or tumor suppressors (oncosuppressor miRs). Their dysregulation is closely linked with cancer initiation, progression, and metastasis. In cancer, normal cells progressively develop into malignant cells by undergoing tumorigenesis, becoming malignant, and initiating cancer[36]. Therefore, correcting miRNA expression is a potential treatment pathway for cancer patients. Currently, most therapeutic approaches focus on miRNA replacement using miRNA mimics designed to replenish the oncosuppressor miRs. These

dsRNA molecules have previously restored tumor suppressor function in various cancer models[37].

Lung cancer was one of the first diseases that miRNA mimic-based therapy was explored in. Most previous studies concentrated on specific miRNA families: let-7, miR-34, and miR-15/16[37]. The let-7 family is known to regulate the RAS oncogene and lung cancer cell growth. Synthetic let-7b was tested for tumor control in vivo and showed potential in tumor growth inhibition through cell cycle inhibition. miR-34a, popularly known as a tumor suppressor, reduced tumor growth after intratumoral and intravenous administration. miR-34a acts as a tumor suppressor through repression of proteins involved in regulating the cell cycle and apoptosis[37]. Further, the delivery of let-7 and miR-34a mimics led to significant inhibition of lung tumors after eight systemic injections[37]. These promising results with let-7 and miR-34a in lung cancer models have provided the backbone for researchers to explore similar miRNA-based therapies in other cancers, such as malignant pleural mesothelioma (MPM).

MPM is a rare form of cancer that grows in the membrane of the walls of the chest and lungs, and is mainly caused by asbestos exposure. Asbestos refers to tiny, airborne particles that settle in the lungs when inhaled. Because these particles are too harsh to be broken down, they can cause scarring, inflammation, and cell changes in the lungs over time[38]. Reid et al. examined miRNA expression in MPM and found the downregulation of tumor-suppressive miRNAs[37]. One particular miRNA that was significantly reduced in MPM patient samples was miR-16. When the expression of miR-16 in MPM cells was restored through the downregulation of BCL2 and CCND1, apoptosis was induced. In addition, the *in vivo* activity of miR-193a-3p mimics can cause the downregulation of MCL1, an apoptosis regulator[37]. In MPM tumors and cells, Reid et al. found consistent downregulation of miR-16 and the miR-15/107 group. Based on this observation of the downregulation of miR-16 and the miR-15/107 family in MPM and the efficacy of miRNA mimics in previous studies, further advancements, such as developing the MesomiR-1 clinical trial, have been pursued.

MesomiR-1 is a Phase I trial that tested the efficacy of TargomiRs in treating patients with MPM. The trial began in December 2014 and is currently nearing completion as of 2024. TargomiRs use patented miRNA mimics with the miR-15/107 family's sequence packaged in EDV nanocells (EDVs). These are nonviable minicells produced by de-repressing sites of cell division in bacteria. EDVs are coated with bispecific antibodies for delivery, with one arm designated for binding to a receptor on the surface of cancer cells. They then bind to overexpressed target receptors on tumor cells and participate in endocytosis[37]. The trial started by testing $5x10^9$TargomiRs through a 20-minute intravenous infusion once a week. Currently, 18 patients with MPM have been tested with this clinical trial and have received different doses. TargomiRs were generally well received, as most patients experienced mild inflammatory responses post-infusion.

Current data show that disease control was achieved in five out of six patients after they underwent 8 weeks of the treatment[37]. One specific patient even experienced significantly reduced tumor size following the treatment. This patient had previously undergone pleurodesis and chemotherapy but progressed further after treatment. His therapy began with a lower dose due to elevated baseline IL-6 levels and eventually escalated to a total $5x10^9$ dose over eight weeks. After eight weeks, the tumor size decreased, so the patient continued the therapy for over 40 more weeks, emphasizing the potential for this therapy to control tumor growth and treatment for cancers with significant miRNA dysregulation[37].

2.3.3. **Challenges in miRNA application**

Delivering miRNA effectively is challenging due to some specific properties of RNA oligonucleotides that impact their stability, cell entry, and target specificity[9]. First, unmodified miRNAs face rapid degradation in the bloodstream due to their exposed 2'-OH group in the ribose, making them highly susceptible to nucleases like RNase A. The kidneys also quickly excrete them, limiting their half-life and therapeutic potential. One attempt to counteract this issue is the modification of the phosphodiester backbone and the position of 2' ribose. These modifications improve the stability of the RNA oligonucleotides, increase binding affinity to the target, and load into the miRISC[9]. miRNA also has the challenge of poor penetration into target tissues. Because the veins in tumors are usually not as stable, there is inadequate blood flow and limited delivery of unmodified miRNAs.

There have been some solutions to this issue of inadequate delivery of unmodified miRNAs, including developing several delivery systems. Nanocarriers like liposomes and polymeric nanoparticles utilize enhanced permeability and retention to improve targeting, conjugating miRNAs with molecules like sugars or peptides to increase tissue specificity, and using exosomes and adenoviral vectors for in vivo delivery as some examples of the modified delivery systems. These methods help address miRNA's poor stability and negative charge, which usually limits tissue penetration[9]. Finally, once miRNAs are delivered into the cytoplasm, there is potential for off-target effects. Because miRNAs bind imperfectly to the 3' UTRs of multiple genes, they can unintentionally silence non-target genes, leading to off-target effects and reduced therapeutic efficiency. Also, individual miRNAs can target various mRNAs, which can increase the risk of unexpected side effects. Even when miRNAs successfully target a specific gene, there is still a chance for unintended on-target impacts to occur. Some mitigation methods for these risks are combination therapies that contain low doses of complementary miRNAs[9].

2.4. **CRISPR/Cas System**

2.4.1. **Introduction to CRISPR/Cas**

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) and their associated proteins (called Cas proteins) are a prokaryotic immune system that integrates short sequences of invading genomes (called spacers) into the CRISPR genomic locus. Their associated proteins can target and destroy the associated nucleic acids, providing immunity against viruses, plasmids, and other genetic elements[39]. There are thousands of CRISPR systems across all genomes, but most consist of an AT-rich leading sequence followed by identical short repeats interspaced with spacers complementary to mobile genetic elements (MGEs)[40]. This complementarity produces CRISPR RNAs (crRNAs), which target and disable the MGEs. The crRNAs are first transcribed as longer pre-crRNAs but then processed into mature guides for targeting and disabling foreign genetic material with high specificity. The CRISPR loci are bordered by Cas genes that encode proteins in the CRISPR-Cas mechanism.

The Cas proteins fall into two main categories: those for adaptation and those for effector modules. Cas1 and Cas2 are universal across all CRISPR systems, which is crucial in acquiring spacers. There are two classes in effector modules: Class I and Class II[41]. Class I consists of types I, III, and IV and uses multiprotein complexes for interference, while class II systems consist of types II, V, and VI and use a single effector protein for targeting and neutralizing foreign genetic elements[40].

2.4.2. **Mechanism of CRISPR/Cas system**

The first phase of the mechanism of the CRISPR/Cas system is the integration of a sequence from an invading genetic element (called protospacers) into the CRISPR array, forming a new spacer[40]. This helps the host organism to remember the intruder's DNA. Cas1 and Cas2 usually do this process across CRISPR-Cas types. The mechanism of spacer acquisition is not currently fully understood because of the selection of protospacers and their processing before integration, as this process varies and remains unclear across the different CRISPR Cas/types[40]. However, some studies have shown that Cas1 and Cas2 of the type I system of *E.coli* form a complex that promotes the integration of new spacers through a process similar to viral enzymes. A new spacer is usually placed at the leader-repeat boundary of the CRISPR array, while the first repeat is duplicated.

Specific CRISPR/Cas types have unique spacer acquisition requirements in addition to Cas1 and Cas2. For example, type I-B requires the presence of Cas4 for adaptation, and type II-A requires Csn2, Cas9, and tracrRNA (trans-activating CRISPR RNA)[40]. Further, the selection of target sequences integrated into the CRISPR locus is highly specific and guided by the presence of the protospacer adjacent motif (PAM), which is crucial for efficient spacer acquisition in types I, II, and V systems. In type II-A, the PAM helps guide Cas1, Cas2, and

Csn2 to integrate new spacers[40]. In type I-E systems, Cas1 and Cas2 work independently, but the PAM improves spacer integration. Additionally, type I systems use a process called "priming", where crRNA-guided binding to a protospacer boosts the acquisition of new spacers, making the process more efficient[40].

In the next stage, biogenesis, the CRISPR array is first transcribed into a long precursor crRNA (pre-crRNA) that is then processed into guide crRNAs that contain sequences from previous invaders[40]. For type I and III systems, Cas6 proteins process pre-crRNA and create intermediate crRNAs with a short 5' tag. The 3' end is trimmed to produce the mature crRNAs with a complete spacer on the 5' end and a repeat on the 3' end[40]. However, this process is different in class 2 CRISPR/Cas systems. Type II CRISPR/Cas systems rely on tracrRNA to form an RNA duplex with each of the repeats of the pre-crRNA. The host RNase III then recognizes and processes the duplex, creating an intermediate crRNA that undergoes further maturation[40]. On the other hand, in type II-C CRISPR/Cas systems, transcription initiation occurs within repeats without interference of RNase III[40]. In addition, in type V-A CRISPR/Cas systems, Cpf1 (a type V CRISPR effector protein) processes pre-crRNAs and then uses the processed crRNAs to cleave target DNA[40].

In the final stage of CRISPR, mature crRNAs act as guides to specifically interfere with invading nucleic acids[40]. The exact mechanism of this process differs across the different CRISPR/Cas systems and classes. Class 1 systems utilize Cascade (CRISPR-associated complex for antiviral defense) complexes to achieve degradation, while class 2 systems use a single effector protein for interference[40]. Type I, II, and V CRISPR/Cas systems rely on the PAM sequence to prevent self-targeting and degradation, while type III systems use a 5' tag on the mature crRNA. Cascade locates the target DNA in type I systems and recruits Cas3 to degrade it by inducing a nick on the foreign DNA. The tracrRNA + crRNA duplex in type II systems guides Cas9 in introducing a double-strand break in the target DNA. In type III systems, Cas10-Csm and Cas10-Cmr complexes target DNA and RNA with transcription-dependent targeting. Cas10 cleaves the DNA, while Csm3 and Cmr4 cleave transcribed mRNA. In type V systems, an RNA duplex of tracrRNA and crRNA are required for target interference and degradation[40].

The CRISPR/Cas system enables precise gene editing by defending against genetic intruders in three stages. First, Cas1 and Cas2 integrate sequences from invaders into the CRISPR array and form spacers that help the host remember the intruder's DNA. In the next phase, these spacers are transcribed into pre-crRNAs and are processed into mature crRNAs that guide interference. In the interference stage, crRNAs direct the Cascade complexes to degrade invading genetic material.

2.4.3. **CRISPR/Cas9**

CRISPR was first identified in the DNA of *Escherichia coli* in 1987 by scientists at Osaka University[42]. The significance of these sequences was initially unclear, but further research in bacterial genotyping revealed CRISPR loci's potential for differentiating bacterial strains. In 1995, Francisco Mojica discovered similar DNA patterns in the genomes of archaea, causing him to hypothesize that CRISPR functioned as an immune system by incorporating viral DNA to help bacteria and archaea defend against viruses[42]. However, the key turning point that caused CRISPR to be a leading gene-editing tool was the identification of the Cas9 protein[42]. Researchers identified that when guided by crRNA, Cas9 was able to target and cut specific DNA sequences. tracrRNA was then found to be essential for crRNA processing and Cas9 function[42]. Through these discoveries, scientists developed CRISPR/Cas systems as a tool for precise genome editing. The mechanism of cutting specific DNA sequences varies from CRISPR's naturally occurring mechanism. The Cas9 protein complex has several functional domains that enable it to target and cut specific DNA sequences with high precision[43]. The REC I domain binds to the gRNA, while a bridge helix triggers cleavage when targeting the DNA. The PAM-interacting domain of Cas9 recognizes the sequence (5'-NGG-3') near the target site. When gRNA binds to Cas9, it activates the protein, causing the protein to search for a DNA sequence that matches the PAM. Cas9 then uses the HNH and RuVC nuclease domains to create a double-stranded break in the DNA[43].

2.4.4. **Applications of CRISPR/Cas9 in sickle cell anemia treatment**

Sickle cell disease (SCD) is an autosomal recessive disorder caused by mutations in the HBB gene, which encodes the β-globin subunit of adult hemoglobin[44]. The most common mutation, a substitution of p.Glu6Val, results in sickle hemoglobin (HbS) which polymerizes under hypoxic conditions. When the HbS polymerizes, it causes red blood cells (RBCs) to become sickle-shaped and fragile, leading to chronic anemia, recurrent pain, multiorgan damage, and increased risk of early fatality[44]. Current treatments for SCD, such as hydroxyurea blood transfusions, and newer drugs, can provide only partial symptom relief[44]. OTQ923 is an autologous, CRISPR-Cas9-edited CD34+ hematopoietic stem cells (HSC) product that disrupts the HBG1/HGB2 promoters by utilizing a ribonucleoprotein complex of Cas9 and gRNA-68. gRNA-68 suppresses HBG1 and HBG2 transcription by targeting a site 246 base pairs upstream of the TSS for each gene[44]. Some preclinical testing showed that by introducing a gRNA-68-Cas9 ribonucleoprotein complex into CD34+ HSCs, there was a successful production of fetal hemoglobin in RBCs.

In a clinical trial by researchers Sharma et al., three participants with severe SCD received a single infusion of OTQ923, resulting in increases in total hemoglobin, fetal hemoglobin, and F-cell levels as well as no detectable off-target effects[44]. In preparation for the study, the participants received monthly red-cell exchange transfusions for at least two months

prior to CD34+ cell collection. CD34+ HSCs were mobilized, collected, cryopreserved, and shipped to a manufacturing facility. After thawing, these cells were electroporated with the CRISPR-Cas9-gRNA-68 ribonucleoprotein complex to create OTQ923. Further, before OTQ923 infusion, participants went through myeloablative conditioning with busulfan[44].

Participants did experience some vaso-occlusive episodes post-infusion, however; these events were minimal and occur throughout various studies showing fetal hemoglobin induction[44]. Participants 1 and 2 displayed stable or improved cardiac, pulmonary, and renal function 12 months post-infusion, which suggests protection against organ damage. However, they also displayed worsened osteonecrosis, potentially linked to exposure to busulfan or ongoing SC damage[44]. Furthermore, despite improvements in total hemoglobin and RBC counts and reduced symptoms, all participants displayed mild hemolysis. This indicates that fetal hemoglobin levels were insufficient to fully prevent sickle hemoglobin polymerization[44]. Bone marrow assessments displayed balanced trilineage hematopoiesis without dysplasia, as well as a rise in total hemoglobin, highlighting improved hematopoiesis after OTQ923 infusion. This total hemoglobin increase has led to clinical improvement for all three participants; however, the observed fetal hemoglobin induction did not fully resolve the disease[44].

The findings of this clinical trial indicate that Cas9-mediated disruption of the HBG1 and HBG2 promoters in HSCs from individuals with SCD led to an increase in RBC fetal hemoglobin and a partial correction of the disease[44].

2.4.5. **Challenges in CRISPR/Cas application**

In order for the CRISPR/Cas mechanism to efficiently edit the genome, there are some challenges that need to be considered and overcome such as the selection of the target sites and mitigation of off-target effects and the efficiency of homology-directed repair[10]. Although CRISPR-Cas systems utilize the PAM sequences and guide cRNA, they have a high off-target cleavage rate of more than 50%. These off-target effects can compromise the stability and function of normal genes. One of the major causes of these unintended effects is the sgRNA (single guide RNA) with involvement from PAM sequences. Selecting sgRNA with optimal DNA targets is crucial to minimize off-target activity. Some efficient computational tools are able to evaluate sequence composition, GC content, nucleotide position, genetic and epigenetic features, and properties to choose efficient sgRNA[10]. In addition, there have been several methods developed to detect off-targets such as the T7E1 assay, deep sequencing, in silico prediction, ChIP-seq, and GUIDE-seq. Other approaches such as sgRNA truncation, use of high-fidelity Cas9 variants, use of high-quality reference genomes, and reduction of nuclease expression can also lower off-target activity. Another issue in CRISPR/Cas application is the efficiency of HDR (homology-directed repair)[10]. Non-homologous end joining (NHEJ) often causes errors in CRISPR/Cas application. For example, in mice, HDR efficiency is very low

(0.5-20%), while NHEJ repair is more frequent (20-60%). Some methods of increasing HDR efficiency or reducing NHEJ occurrences include inhibiting key NHEJ enzymes like DNA ligase IV, or enhancing HDR using siRNA[10].

3. Discussion

Each of the previously-covered gene alteration techniques- siRNAs, saRNAs, miRNAs, and CRISPR/Cas systems present unique challenges and advantages, making them suitable for various treatment goals. siRNAs, by targeting and degrading mRNA, are desirable in diseases that are impacted when there is a reduction of overactive or harmful proteins. siRNAs are also ideal in disorders where gene expression regulation is beneficial without the need for permanent alteration in the genome, such as in ischemic strokes. In the case of ischemic strokes, siRNAs are able to target and silence specific mRNA to reduce the production of proteins involved in cell death and inflammation following a stroke. By silencing these genes, siRNAs can reduce the damage that worsens stroke outcomes. With the precise targeting of siRNAs, it also mitigates any adverse effects on healthy cells and concentrates its effects to the affected brain region.
In contrast, saRNAs are able to upregulate gene expression, which is suitable for diseases where increasing the expression of a beneficial gene can restore normal function or provide therapeutic relief. In the case of pancreatic cancer, they can target genes associated with tumor suppression and upregulate genes that combat tumor growth. This mechanism allows saRNAs to enhance the body's defenses against cancerous cells. Unlike other treatments that attack both healthy and cancerous cells, saRNAs are more targeted to a specific site and have a lower risk of damaging non-tumor cells. miRNAs are able to target multiple genes within a pathway, which is advantageous in diseases where multiple genes contribute to the development of the disorder, such as thoracic cancer. miRNAs can treat thoracic cancer by modulating gene expression linked to cancer progression. They can downregulate oncogenes or enhance tumor suppressor genes, disrupting pathways that support tumor survival and spread. By disrupting these pathways, miRNAs can limit disease progression and restore healthier cellular functions. In addition, because miRNAs are able to target multiple genes within cancer-driving pathways simultaneously, the treatment approach has potential to be more effective than current chemotherapy treatments. Finally, the CRISPR/Cas system is very desirable for achieving permanent gene correction, which is beneficial for genetic disorders caused by singular mutations. CRISPR's ability to edit DNA directly provides an approach that is long-lasting and leads to irreversible modifications of the genome. CRISPR technology holds large potential in treating sickle cell anemia by directly editing the HBB gene, which codes for the abnormal hemoglobin in sickle cell anemia. CRISPR is able to restore normal hemoglobin production and reduce the formation of sickle-shaped cells, allowing CRISPR to address the disease's main cause, not just manage symptoms. Furthermore, by correcting the mutation in HSCs, healthy red blood cells are produced.

Each of the gene therapy techniques have therapeutic promise but have some room for improvement to optimize their potential. For siRNAs, the main challenge is their stability and cellular delivery. Some possible improvements include advancements in delivery methods such as nanoparticle-based delivery systems to ensure that the RNA reaches its target cells without degradation, as well as enhancing its stability and reducing off-target effects. For saRNA, enhancing the specificity and stability of gene activation is a possible area for improvement. A possible solution for this can be the development of more efficient delivery strategies to prevent degradation by nucleases, modifications to the saRNAs to maintain stability, and further understanding of the intended target site for saRNAs to offset off-target effects. miRNAs also share similar issues of stability, cell entry, and target specificity. Some improvements that have been made are modifications to the miRNAs in certain areas to improve the stability, increase binding and loading affinity, and the development of several delivery systems to improve targeting and tissue specificity. Finally, the main challenges for CRISPR/Cas systems include the selection of target sites, mitigation of off-target effects, and maintaining efficiency of repair. Some possible solutions are to use tools to evaluate the sequences to choose the efficient guide RNA, as well as using methods to detect off-target effects.

Beyond delivery and off-target effects, the diversity of disease mechanisms and patient responses poses a significant limitation to the generalizability and usage of gene therapy techniques. For instance, CRISPR is limited to disorders caused by a singular mutation, such as sickle cell anemia, where a specific genetic defect can be targeted for correction. However, its application in polygenic diseases is more challenging due to the involvement of complex gene interactions. Additionally, genetic diversity among patients is also a limitation of treatment efficacy. A therapy effective for one population may not yield the same results in a different group due to differences in genetic backgrounds. These differences can influence how patients respond to treatments.

siRNAs, saRNAs, miRNAs, and the CRISPR/Cas system represent powerful tools in gene therapy, each offering unique mechanisms and therapeutic potentials for addressing diseases. siRNAs and miRNAs enable for specific silencing of gene expression, providing targeted approaches for managing conditions such as strokes and cancers which stem from an increased expression of harmful genes. saRNAs increase the activation of genes, giving them promise in the treatment of diseases like pancreatic cancer where the expression of a beneficial gene can help return the body to a normal function. CRISPR/Cas is able to use its precise gene-editing capabilities to treat diseases that stem from a mutation in a gene, such as sickle cell disease. Despite their promise, each technique faces significant challenges, such as barriers in delivery, instability, and off-target effects. These challenges can be addressed through refinement of the delivery processes, enhanced specificity, and modification of the mechanisms themselves. With continued advancements, these gene therapy techniques can

redefine the future of medicine by offering precise and potentially curative treatments for some of the most challenging diseases.

4. Methods

To gather information for my analysis, I utilized Google Scholar as the primary search engine, with key terms including “gene therapy”, “siRNA”, “saRNA”, “miRNA”, “CRISPR/Cas”, as well as specific searches for their mechanisms, challenges, and therapeutic applications. Search results were filtered to include only peer-reviewed articles from the past 20 years, with exceptions made for older studies that were reviewed for credibility and relevance by cross-referencing with more recent findings. Additionally, reputable medical websites, such as from the National Institute of Health (NIH), PubMed, and Cleveland Clinic were reviewed to ensure accurate information and corroborate my understanding, particularly in sections relating to therapeutic applications. Data extraction focused on identifying key findings, such as the mechanisms of each therapy, challenges, and clinical applications. Details including publication dates and study outcomes were analyzed for each relevant article. Studies cited within the primary articles were also reviewed and incorporated into the analysis if they contributed relevant information for the review. The information gathered was synthesized through a form of narrative synthesis by organizing findings into key sections related to mechanisms, therapeutic challenges, and applications of specific methods of gene therapy. This approach allows for a more comprehensive understanding of the current knowledge in the field of gene therapy.

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