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REVIEW

Off-Target activity as a Translational Barrier in Programmable Gene-Editing Strategies for Nontuberculous Mycobacteria: Narrative Review

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ABSTRACT

Objectives: *To review the clinical and translational implications of off-target activity associated with clustered regularly interspaced short palindromic repeats (CRISPR)-based approaches in nontuberculous mycobacteria (NTM) and discuss current strategies aimed at specificity and safety.*

Materials and methods: *The relevant published literature on the application of CRISPR-Cas systems, including Cas9, Cas12a and CRISPR interference (CRISPRi), in NTM research was reviewed. Particular attention was given to off-target mechanisms, mycobacteria-specific genomic challenges, computational predictions, experimental detection methods, high-fidelity nucleases and delivery optimisation approaches.*

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Results: *Nontuberculous mycobacteria infections often require prolonged treatment and are frequently associated with relapse and rising antimicrobial resistance, particularly in* Mycobacterium abscessus *infections. CRISPR-based technologies provide advantages in precision diagnostics, functional genomics and therapeutic development; however, high guanine-cytosine (GC) content, repetitive PE/PPE gene families, mismatch tolerance and unique DNA repair mechanisms contribute considerably to off-target effects. Emerging high-fidelity nucleases, guide RNA optimisation, artificial intelligence (AI)-assisted prediction platforms and alternative editing systems demonstrate considerable potential for improving editing specificity and translational safety.*

Conclusions: *Advances in nuclease engineering, computational modelling, delivery systems, and genome-wide validation approaches may improve therapeutic precision and diagnostic reliability. Addressing these challenges through interdisciplinary innovation will be essential for the future clinical integration of CRISPR-based antimycobacterial strategies.*

Keywords: nontuberculous mycobacteria, CRISPR-Cas9, CRISPR-Cas12, CRISPRi, off-target effects.

INTRODUCTION

The clinical challenge of NTM

Nontuberculous mycobacteria (NTM) represent a diverse assembly of over 200 species found in the environment. These bacteria are ubiquitous in soil, natural water bodies, manufactured water systems and within biofilms. Nontuberculous mycobacteria infections have increased globally, particularly within industrialised nations characterised by ageing populations and better diagnostic tools (1, 2). Patients with chronic lung disease or immunosuppression are among the vulnerable categories. Pediatric cases are increasingly recognised, often presenting as cervical lymphadenitis or pulmonary problems (3). *Mycobacterium abscessus*, *Mycobacterium neoaurum* and the *Mycobacterium avium* complex (MAC) are linked to persistent lung disease, soft-tissue infections and systemic illness (2, 4). Nontuberculous mycobacteria exhibit marked intrinsic and acquired antimicrobial resistance. This is caused by a lipid-dense hydrophobic cell wall that contains efflux pumps, drug-modifying enzymes, mycolic acids and inducible resistance genes such as (*erm*) (4, 5). Biofilm formation further increases treatment resistance. These infections are difficult to manage, often requiring prolonged multidrug therapy (2). Treatment outcomes remain poor, particularly for *M. abscessus* infections (5). The growing burden of NTM infections, high recurrence and treatment-related toxicity highlights the urgent need for better diagnostic and therapeutic strategies.

Clustered regularly interspaced short palindromic repeats (CRISPR) as a strategic tool

CRISPR-Cas systems, initially recognised as bacterial defence mechanisms, are now being explored for studying and treating NTM infections. Nucleases like Cas9 and Cas12a enable targeted gene modification, facilitating both the development of new bactericidal agents and the identification of novel drug targets (6, 7). In addition, platforms like SHERLOCK allow for rapid detection of drug resistance markers (8). However, off-target effects remain a major translational barrier, as unintended modifications can lead to cellular toxicity. This duality-therapeutic promise *versus* specificity risk forms the central theme of this review.

NTM pathogenesis and biological characteristics

Architecture of the mycobacterial cell wall – Bacteria are shielded from drugs and host immunological responses. Their cell wall is made up of layers of arabinogalactan and peptidoglycan connected by a thick outer "mycomembrane" made of long-chain mycolic acid (9, 10). This hydrophobic layer contributes to bacteria's intrinsic resistance by severely limiting the penetration of antimicrobial compounds (9, 11). Mycolic acid production involves fatty acid synthase (FAS-I and FAS-II) pathways in the cytoplasm, with trehalose monomycolate (TMM) serving as a transport intermediate across the membrane (10, 12). Pks13 is the enzyme responsible for the final condensation

step in this process (13). Because Pks13 lacks a human homolog, it is considered a high-priority target for drug development.

Strategies for immune evasion – Intracellular survival within macrophages contributes to NTM virulence. These bacteria inhibit phagosome-lysosome fusion and can persist within the environment of the host cell (14). Studies involving *M. bovis* BCG and *M. neoaurum* have demonstrated that these pathogens activate the NLRP3 and AIM2 inflammasomes, triggering caspase-1 activation and elevated cytokine expression, including IL-1β (15, 16). Host autophagy induction may improve clearance; 2,4-di-tert-butylphenol can help macrophages clear intracellular mycobacteria (17). Persistence is supported by biofilm formation and increased resistance to therapy, particularly in *M. abscessus* (4).

Genomic hurdles in NTM research – The guanine-cytosine (GC) content of NTM species is remarkably high, often exceeding 65%. This genomic feature influences codon use, DNA stability and the distribution of protospacer adjacent motifs (PAMs), thereby complicating CRISPR applications. Furthermore, high GC levels may promote the formation of secondary structures in guide RNAs (gRNAs), which may increase off-target cleavage (6). The PE/PPE gene families account for nearly 10% of the genome. These genes are highly repetitive and homologous, contributing to immune evasion. Their similarity complicates the design of target-specific guide RNAs (18). Strong DNA repair mechanisms, including homologous recombination (HR) and non-homologous end joining (NHEJ), influence repair outcomes following programmable gene-editing (19). Figure 1 illustrates the pathogenesis and biological characteristics of NTM.

CRISPR-Cas frameworks in NTM studies

The role of CRISPR-Cas9 – The Cas9 nuclease from Streptococcus pyogenes (SpCas9) is a widely used tool for genome editing. It uses a single guide RNA (sgRNA) to locate a 20-nucleotide target adjacent to a 5′-NGG-3′ PAM sequence. Once bound, Cas9 creates a double-strand break (DSB) that the cell's internal repair machinery then attempts to fix (6, 20). This technique has been applied to NTM research for functional studies and diagnostics, including the identification of drug-resistant mutations (21). However, a major obstacle to therapeutic usage is still SpCas9's propensity to cut at unwanted locations with identical sequences (22).

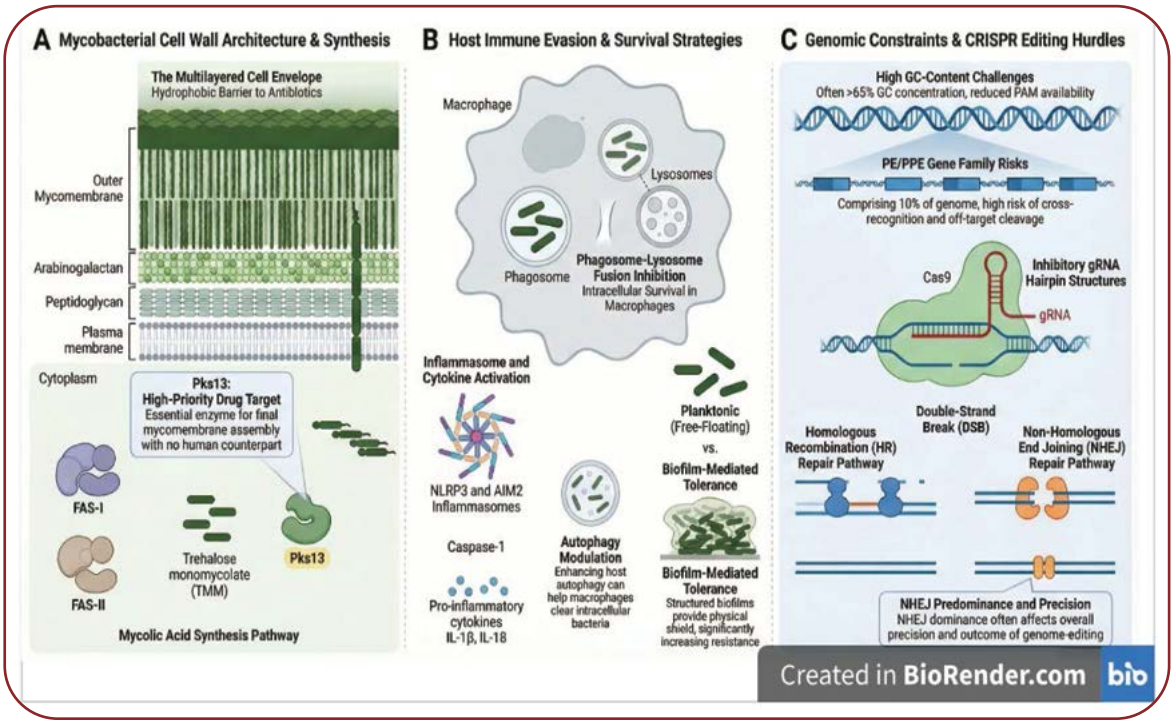

FIGURE 1. Biological and genomic determinants of non-tuberculous mycobacteria (NTM) pathogenesis and clustered regularly interspaced short palindromic repeats (CRISPR) targeting

Advancements with CRISPR-Cas12 – Cas12a (Cpf1) offers unique advantages over Cas9. It recognises T-rich PAMs (5′-TTTV-3′), which help target specific genomic regions (23). Cas12a produces staggered DNA cuts (5′ overhangs) that may improve editing precision. It also processes its own crRNA, enabling multiplex targeting. Cas12a exhibits "collateral" cleavage activity, in which it cuts nearby single-stranded DNA after finding its primary target, supporting sensitive diagnostic tests (24). Combining Cas12a with amplification methods such as recombinase polymerase amplification (RPA) or reverse transcription-loop-mediated isothermal amplification (RT-LAMP) has led to the development of rapid sensitive tools for detecting mycobacterial infections, even in low-resource settings (25, 26).

CRISPR interference (CRISPRi) – The gene-silencing technique known as CRISPR interference (CRISPRi) makes use of dCas9, a nuclease-deficient form of Cas9. Despite its inability to cleave DNA, dCas9 retains sequence-specific binding when directed by a single guide RNA (sgRNA). dCas9 blocks transcription by interfering with RNA polymerase activity, targeting promoter or coding sequences, thereby suppressing gene expression without DNA cleavage (27). This reversible system is particularly useful for studying mycobacteria and essential genes. CRISPRi has advanced mycobacterial functional genomics. For instance, a multiplexed screening approach in *M. abscessus* has been used to suppress multiple genes for therapeutic target identification simultaneously (28). Studies have demonstrated that inhibition of the PrrAB regulatory system reduces bacterial survival under stress (29). Gene silencing also alters morphology and antibiotic susceptibility when the cell division-related gene *mab_1999* (an FtsL homolog) is silenced (30). Another article shows that using CRISPRi to target *inhA* can reduce transcription by more than 90%, substantially affecting bacterial survival (31). Advances such as the pJL fluorescence series enable tunable repression and real-time monitoring of gene expression (32), highlighting the importance of the CRISPRi tool.

Mechanism of off-target activity

Tolerance for DNA-RNA mismatches – The extent to which the gRNA complements the genomic DNA, especially in the "seed region", the 8-12 nucleotides next to the PAM, dictates Cas9 specificity. Although stable binding and enzyme activation require strong complementarity, distal PAM

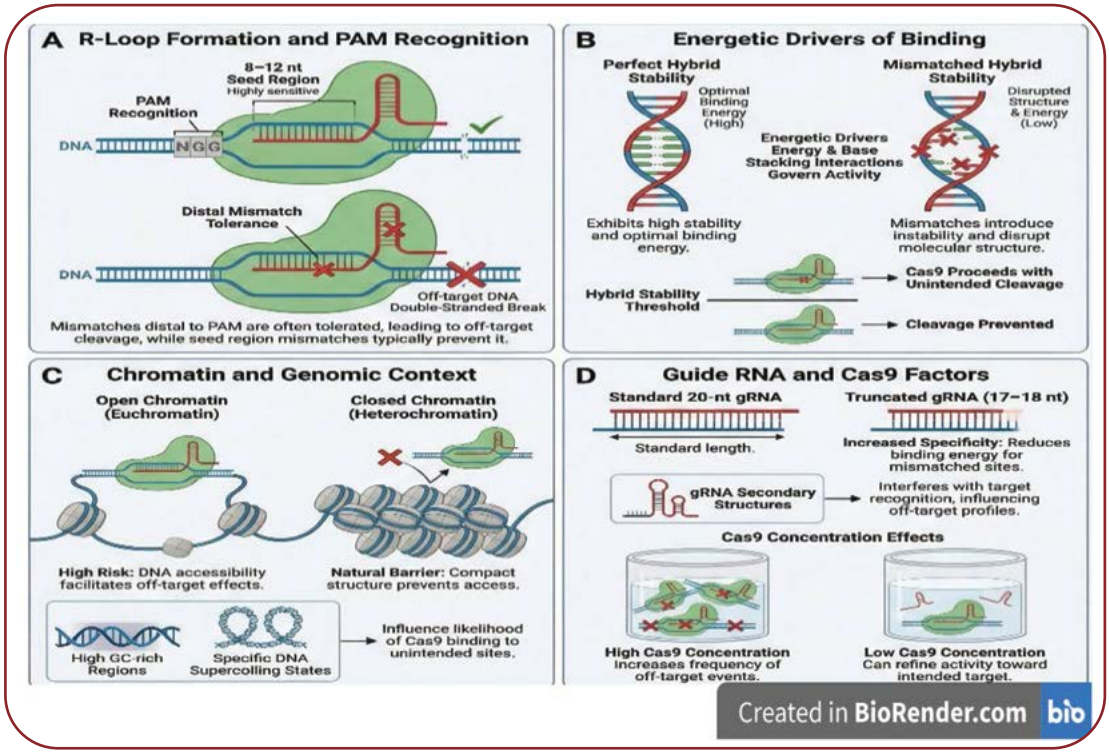

FIGURE 2. Mechanism of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas off-target effects

mismatches are often tolerated, resulting in unintended cleavage at similar genomic sites (20, 33). Even single or double mismatches can cause detectable off-target cleavage, particularly in genomically accessible regions (33). Biophysical parameters also influence interaction stability; simulations of binding energy and conformational stability improve the accuracy of off-target prediction (34).

Influence of chromatin and genomic context – The structural organisation of DNA influences CRISPR efficiency. In eukaryotes, Cas9 preferentially binds accessible euchromatin (35). Although mycobacteria lack nucleosomes, DNA accessibility is influenced by supercoiling, nucleoid-associated proteins and a high GC content. These factors influence R-loop formation and DNA melting, suggesting that local topological and biochemical environments influence cleavage efficiency.

Impact of nuclease levels and duration – Cas9 concentration and the duration of its activity influence editing specificity. High enzyme levels increase transient off-target binding. Reducing Cas9 mRNA levels in porcine embryos improves specificity by reducing off-target mutations (36). Inducible promoters or the delivery of ribonucleoproteins (RNPs) reduce cumulative genomic damage by limiting nuclease exposure (33, 37).

Guide RNA architecture – The accuracy of the gRNA is influenced by its content and structure. Stable internal gRNA complexes that obstruct target binding can form due to high GC content. Optimising gRNA thermodynamic stability is essential. Using truncated gRNAs (17-18 nucleotides) improves specificity by making the RNA-DNA duplex less tolerant of mismatches (37). Figure 2 displays the diagrammatic explanation of this section. Systematic studies on off-target determinants in mycobacteria remain limited. Cas nuclease fidelity remains poorly characterised in relation to high GC content, DNA supercoiling and species-specific nucleoid organisation. The lack of experimentally validated genome-wide off-target maps in NTM species limits the development of predictive models for mycobacterial genomes, a significant unmet challenge.

Genomic obstacles unique to mycobacteria

GC concentration increases the possibility that the gRNA will form inhibitory secondary structures and reduce the number of optimal target locations (6). These GC-rich areas may further increase the thermodynamic stability of partial gRNA-DNA duplex formation, thereby enhancing mismatch tolerance (22). The 10% PE/PPE gene families

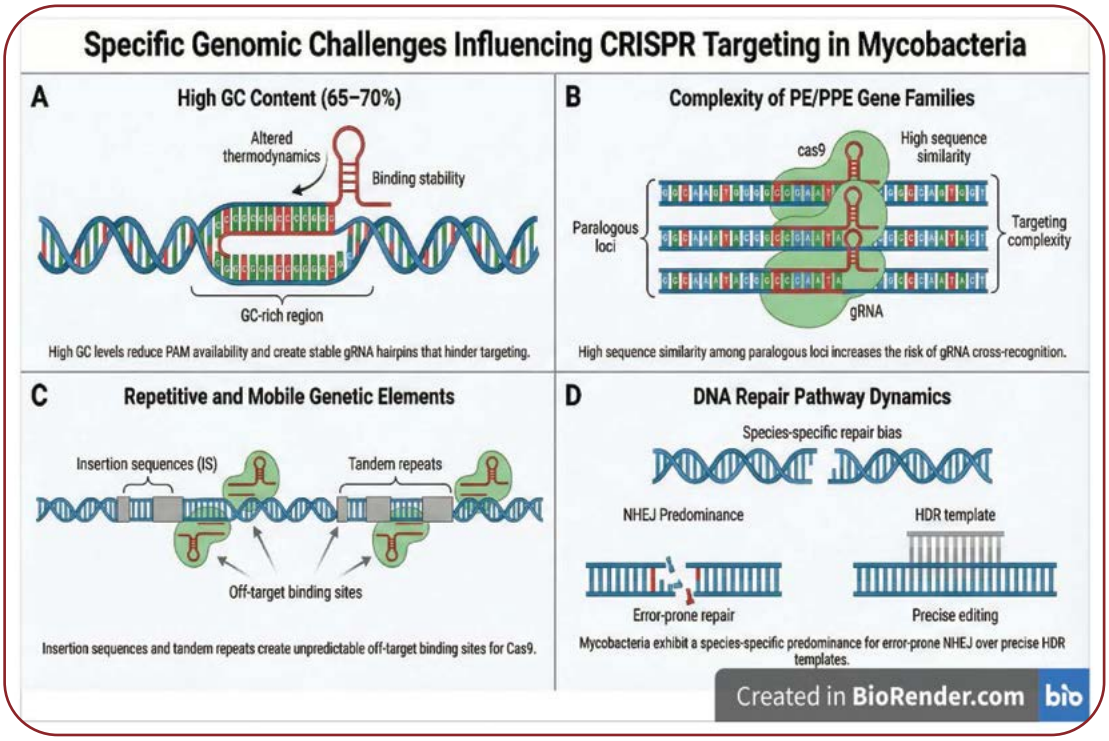

FIGURE 3. Specific genomic challenges influencing clustered regularly interspaced short palindromic repeats (CRISPR) targeting in mycobacteria

make it difficult to target a specific gene without affecting its paralogs. Effective CRISPR strategies must therefore focus on unique sequences flanking these genes (18). The next obstacle is that multiple identical or nearly identical binding sites are provided by prophages, insertion sequences and duplicated segments (22). Dynamics of DNA repair indicate that both homologous recombination (HDR) and non-homologous end joining (NHEJ) are used by mycobacteria. Error-prone repairs are usually the outcome of CRISPR-induced breaks since NHEJ is frequently the prevailing route. Achieving precise genome modifications *via* HDR is more challenging because it requires external repair templates (38). All four obstacles mentioned in this section were demonstrated in Figure 3. However, there is still a lack of quantitative information outlining the proportional contributions of NHEJ and HDR across clinically significant NTM species. The majority of what is now known comes from model organisms like *M. smegmatis*, which might not fairly represent the kinetics of repair in pathogenic NTM. To rationally optimise CRISPR techniques for therapeutic development, future research must find species-specific editing efficiency.

Methodologies for detecting off-target effects

Computational prediction – Digital tools provide initial off-target screening; Cas-OFFinder performs genome-wide mismatch searches (39) and its variant-aware version accounts for genetic variation (40). While machine-learning tools like DeepCRISPR and CRISOT use molecular dynamics and epigenetic data to improve accuracy (34, 41), CHOPCHOP optimises gRNA design (42). However, ensemble frameworks such as iGWOS integrate multiple prediction methods to improve off-target identification (43).

Experimental validation – While cell-based techniques like GUIDE-seq and DISCOVER-seq detect double-strand breaks or repair protein recruitment *in vivo* (44, 45), *in vitro* techniques like CIRCLE-seq and SITE-seq map cleavage sites using pure DNA (46). Amplicon-based next-generation sequencing (NGS) remains the reference standard due to its high sensitivity, even though whole-genome sequencing (WGS) is comprehensive (47).

Strategic recommendations – Experts recommend a three-step strategy, including *in silico* screening, experimental detection and finally, amplicon NGS validation of candidate sites. There is currently no standardised framework for off-target evaluation in mycobacterial systems. Since most validation platforms were created for eukaryotic genomes, they may not accurately represent GC-rich bacterial genomes. A crucial improvement in clinical translation is the development of mycobacteria-specific benchmarking datasets and validation workflows.

Strategy to improve CRISPR specificity

Cas9 variants with high fidelity – High-fidelity Cas9 variants with fewer non-specific DNA contacts have been produced through engineering efforts. By weakening non-target strand contacts, eSpCas9 (enhanced specificity Cas9) reduces mismatch tolerance (48). Porcine embryos used in *in vivo* experiments showed excellent on-target effectiveness and no observable off-target events (36). SpCas9-HF1, with reduced non-specific DNA interactions (49); HypaCas9, which is optimised by changes in the HNH domain to enhance proofreading (50); HiFi Cas9 and LZ3 Cas9 are further designed variations. According to comparative studies, eSpCas9 frequently offers the best possible compromise between catalytic efficiency and specificity in vivo.

Guide RNA (gRNA) optimisation – The design of guide RNA is critical for specificity. Secondary structure prediction, avoiding repeating motifs, and maintaining an ideal GC content (40-60%) all improve fidelity (6). By reducing mismatch tolerance, truncated gRNAs (17-18 nucleotides instead of 20) decrease off-target binding while preserving adequate on-target cleavage (37). By increasing binding accuracy and resistance to degradation, chemical changes such as terminal modifications, phosphorothioate linkages and 2′-O-methyl substitutions increase nuclease stability and decrease unwanted interactions (23).

Alternative CRISPR system – Alternative CRISPR systems may further improve specificity. In some situations, Cas12a may improve specificity by recognizing T-rich PAM sequences and displaying unique cleavage kinetics (23). Cas13 avoids permanent genomic alterations by targeting RNA rather than DNA. Base editors reduce indel-associated effects by enabling accurate nucleotide conversions without creating double-stranded breaks (DSBs) (51). Prime editing enables targeted insertions, deletions and base substitutions with fewer unintended changes (52). Furthermore,

CRISPRi/CRISPRa technologies provide reversible and less dangerous options by modifying transcription without cleaving DNA.

Dosage and delivery optimisation – Cas9 is transiently delivered as ribonucleoprotein (RNP) complexes, thereby limiting nuclease exposure and off-target activity. Lower Cas9 concentrations reduce off-target events. Dose optimisation improves specificity at the expense of editing efficiency (22). Adeno-associated virus (AAV), lentiviral vectors, lipid nanoparticles, polymeric nanoparticles and electroporation influence editing kinetics and specificity (53).

Machine learning and computational design – AI-guided tools integrate large experimental datasets to predict off-target risk and on-target efficiency. RNA-DNA interaction fingerprints are produced using the CRISOT framework for thermodynamically informed specificity assessment (34). Optimising guide selection across several CRISPR platforms, such as base and prime editors, requires the use of integrative computational techniques.

Clinical and therapeutic implications

Given safety considerations, the clinical translation of CRISPR-based NTM treatments requires a thorough safety assessment. To avoid inadvertent genetic changes that might impair cellular function or encourage neoplastic transformation, off-target mutations must be reduced (22). A serum CRISPR assay targeting cell-free DNA from *Mycobacterium avium* complex (MAC) achieved 97.6% sensitivity and 97.6% specificity, while declining cfDNA levels during therapy suggest potential utility for treatment monitoring (54). In addition, CRISPR-Cas12a-based platforms such as CADEM and MyTRACK enable rapid species-level identification of major NTM pathogens within approximately two hours, with detection limits of 1-100 copies per reaction, sensitivities of 92.6-100% and 100% specificity in clinical samples (55, 56). These developments demonstrate the potential of CRISPR not only as a research tool but also as a clinically relevant platform for rapid diagnosis, species differentiation, detection of antimicrobial resistance and longitudinal monitoring of NTM disease. In order to identify uncommon or delayed adverse effects, long-term safety monitoring and thorough genomic profiling, including patient-specific genome sequencing, may be necessary. Risk-benefit analysis is especially crucial in cases of severe or refractory NTM infections, as restricted genome-editing operations may be warranted due to therapeutic need (6). The effective transport of CRISPR components to intracellular NTM in macrophages is a significant translational obstacle. Nuclease access is restricted by the mycobacterial cell membrane, biofilm formation, and phagosomal localisation; additionally, there are currently no clinically proven, bacterial-specific delivery methods available. For treatment to be successful, overcoming delivery is important for specificity. For functional genomics and drug discovery, CRISPRi techniques using nuclease-deficient Cas9 facilitate comprehensive gene essentiality screening (4). These methods facilitate the identification of new therapeutic targets, validation of potential pathways and identification of synthetic lethal interactions, such as enhanced antibiotic resistance following targeted gene suppression (7, 27).

Emerging technologies and future directions

The goal of the upcoming generation of CRISPR-based technologies is to improve therapeutic variety, safety and accuracy. Prime editors and base editors are examples of advanced editing platforms that are being improved to increase targeting scope, increase editing efficiency and decrease unwanted bystander mutations. While epigenome editors made from catalytically inactive Cas9 linked to transcriptional regulators allow reversible gene activation or repression, RNA-targeting devices based on Cas13 allow transitory transcriptome control without lasting genomic modification. New perspectives for CRISPR applications in NTM include efficient Cas9 and Cas12a-mediated genome editing that exploits endogenous DNA repair pathways for rapid gene knockout and large genomic deletions, advanced CRISPRi platforms enabling multiplex gene silencing, fluorescent tracking, functional genomics and drug-target discovery, as well as highly sensitive Cas12a-based diagnostic systems for rapid species-level identification of clinically relevant NTM. Furthermore, emerging proof-of-concept CRISPR antimicrobials have demonstrated the ability to selectively eliminate or re-sensitise mycobacterial cells to antimicrobial agents. Collectively, these advances are transforming NTM from genetically challenging organisms into tractable systems for mechanistic research, precision diagnostics and future therapeutic innovation.

CONCLUSION AND RECOMMENDATIONS

High-fidelity Cas variants, especially eSpCas9, offer promising approaches to improve specificity. A comprehensive off-target assessment should combine multiple *in silico* prediction tools with amplicon-based next-generation sequencing validation. CRISPRi systems using nuclease-deficient Cas9 provide safer alternatives for functional genomics, especially when targeting essential genes. Mycobacteria-specific genetic databases may further improve predictive accuracy. Cas12a-based platforms are promising for multiplexed diagnostics with enhanced specificity. Reliable clinical translation will require diverse clinical isolate validation, long-term safety monitoring, off-target profiling and strict regulatory oversight. Combination approaches integrating CRISPR with antibiotics or immunomodulatory therapy may further improve therapeutic efficacy while limiting resistance. ❑

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