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Designing CRISPR-Cas9 for the Treatment of Breast Cancer: Molecular Strategies, Delivery Systems, and Translational Advances

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ABSTRACT

The most prevalent female cancer worldwide is breast cancer because of its multiple genetic subtypes and complex cancer-promoting mechanisms. The gene-editing technology CRISPR-Cas9 is a revolutionary therapeutic approach that enables targeted attacks on cancer-causing genes, tumour suppressor genes, and treatment-resistant mechanisms. The article provides an overview of the CRISPR-Cas9 system for breast cancer treatment, highlighting its fundamental concepts and current research progress. The review examines guide RNA (gRNA) selection methods, Cas9 enzyme variants, base and prime editing systems, epigenome-editing methods, and immunomodulation strategies via combinatorial approaches. The review evaluates new delivery methods, including lipid nanoparticles, viral vectors, exosomes, and tumour-specific biomaterials. The review presents preclinical research findings and explains therapeutic targets, including HER2, PIK3CA, TP53, BRCA1/2, and PD-L1, and discusses safety aspects and translation barriers. The review demonstrates how CRISPR-based therapies will transform precision oncology through multiplex editing, AI-guided gRNA design, and integration with single-cell genomics.

Introduction

The worldwide occurrence of breast cancer as the leading female cancer results in 2.3 million new cases each year, which contributes to substantial cancer-related deaths [1,2]. The different breast cancer subtypes, including luminal A and B and Human epidermal growth factor receptor 2 (HER2) positive and triple-negative breast cancer (TNBC), present treatment challenges because of their diverse biological and clinical characteristics [3,4]. The different breast cancer subtypes show unique genetic patterns and epigenetic changes, metabolic patterns, and immune system responses, which affect how patients respond to treatment and how their disease advances [5–7].

The development of new treatments, including chemotherapy, endocrine therapy, immune checkpoint inhibitors, and HER2-targeted monoclonal antibodies, has not eliminated the problem of treatment failure and cancer relapse [8,9]. The evolution of tumours, their internal genetic diversity, and adaptive signalling networks make conventional treatments less effective, underscoring the need for more specific molecular therapies [10]. The development of new therapeutic approaches becomes essential for TNBC patients because this aggressive breast cancer subtype lacks sufficient targeted treatment options [11].

The clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9 (CRISPR–Cas9) system is a programmable genome-editing technology derived from the adaptive immune system of bacteria and archaea, in which a guide RNA directs the Cas9 endonuclease to a specific DNA sequence adjacent to a protospacer-adjacent motif (PAM), enabling precise genetic modification through cellular DNA repair pathways [12]. The cancer research field has received a revolutionary boost from CRISPR-Cas9 technology, which enables scientists to edit genomes with high precision and programmability [12]. The CRISPR system has proven helpful for studying breast cancer mutations, understanding cancer pathways, building accurate disease models, and identifying new treatment targets [13]. Genetic changes in breast cancer, including Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), Tumour Protein p53 (TP53), Estrogen Receptor 1 (ESR1), and Breast Cancer gene 1 (BRCA1) and Breast Cancer gene 2 (BRCA2) mutations, provide a solid basis for CRISPR-based gene editing and targeted gene repair [2] (Table 1). The growing understanding of breast cancer genomics, together with advances in CRISPR technology, makes it essential to evaluate all available CRISPR-Cas9 design methods [14]. The review combines current developments in guide RNA optimisation, target gene selection, and editing methods (including knockout, knock-in, base editing, and prime editing), delivery systems, preclinical therapeutic results and safety evaluations, and future translational applications. The developed technologies demonstrate how CRISPR-Cas9 will transform precision oncology, enabling advanced treatments for breast cancer in the future.

Fundamentals of CRISPR–Cas9 genome-editing technology

Biological function of CRISPR–Cas systems

CRISPR–Cas systems originated as adaptive immune mechanisms in bacteria and archaea, enabling these organisms to recognise and eliminate invading genetic elements such as bacteriophages and plasmids. Upon initial exposure to foreign DNA, short fragments of the invader genome are incorporated into the host CRISPR locus as spacer sequences [12]. These spacers serve as a molecular memory, allowing sequence-specific recognition during subsequent infections. Transcription of the CRISPR locus produces CRISPR RNAs (crRNAs), which guide Cas effector proteins to complementary nucleic acid targets, resulting in precise cleavage and neutralisation of the invader [13]. The repurposing of this naturally occurring defence system for genome editing relies on the programmability of RNA-guided Cas nucleases, which can be redirected to virtually any genomic locus of interest. Among the diverse CRISPR–Cas systems, Cas9 has emerged as the most widely used effector protein due to its robust DNA cleavage activity, relative simplicity, and adaptability to mammalian cells [15] (**Figure 1**).

Classification and subtypes of CRISPR–Cas systems

CRISPR–Cas systems are broadly classified into two classes, six types, and multiple subtypes based on their effector architecture and mode of action. Class 1 systems (Types I, III, and IV) utilise multi-protein effector complexes for target recognition and cleavage. Class 2 systems (Types II, V, and VI) rely on a single multidomain Cas protein, making them more suitable for genome engineering applications [16]. The Type II CRISPR–Cas9 system, derived primarily from Streptococcus pyogenes and Staphylococcus aureus, is the foundation of most therapeutic genome-editing strategies. Other Class 2 systems, such as Cas12 (Type V) and Cas13 (Type VI), expand the functional repertoire of CRISPR technologies by enabling staggered DNA cleavage or RNA targeting, respectively. While Cas9 remains the dominant platform for DNA editing in cancer research, these alternative Cas proteins provide complementary capabilities for multiplex editing and reversible gene regulation.

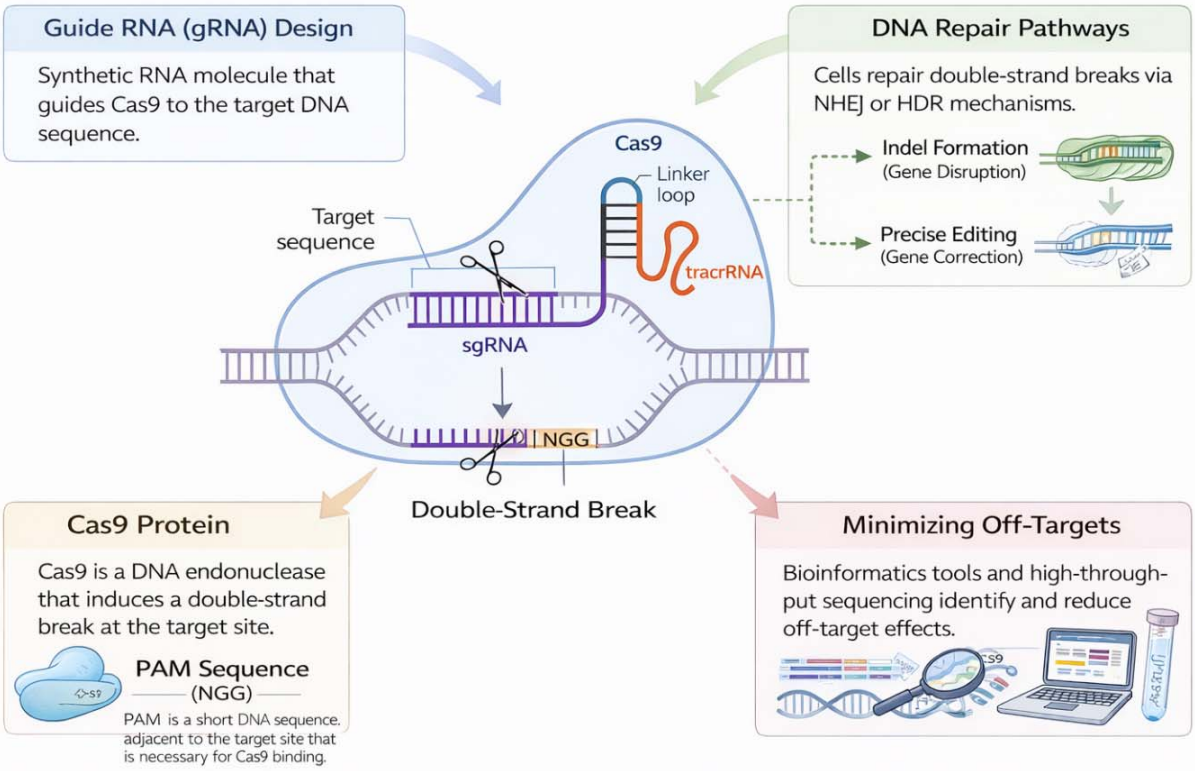

Figure 1. Fundamentals of CRISPR–Cas9 genome-editing technology. Schematic overview of the core molecular components and workflow of CRISPR–Cas9–mediated genome editing. A synthetic single-guide RNA (sgRNA), composed of CRISPR RNA (crRNA) fused to trans-activating crRNA (tracrRNA), directs the Cas9 endonuclease to a complementary genomic target sequence adjacent to a protospacer-adjacent motif (PAM; NGG). Upon target recognition, Cas9 induces a site-specific double-strand DNA break (DSB). Cellular DNA repair pathways subsequently resolve DSBs through either non-homologous end joining (NHEJ), resulting in insertion/deletion mutations and gene disruption, or homology-directed repair (HDR), which enables precise sequence correction in the presence of a repair template. The figure also highlights strategies to minimise off-target editing, including optimised guide RNA design, bioinformatics-based prediction tools, and high-throughput sequencing-based validation approaches.

Structure and mechanism of Cas9 endonuclease

Cas9 is a multidomain RNA-guided endonuclease composed of two principal catalytic lobes: the recognition (REC) lobe, responsible for guide RNA–DNA heteroduplex formation, and the nuclease (NUC) lobe, which contains the histidine–asparagine–histidine nuclease (HNH) and resolvase-like (RuvC) catalytic domains. Upon binding to a guide RNA, Cas9 undergoes a conformational rearrangement that enables scanning of genomic DNA for target sites adjacent to a compatible protospacer-adjacent motif (PAM) [17]. Once a correct PAM is recognised and base pairing between the guide RNA and target DNA is established, the HNH domain cleaves the target strand, while the RuvC domain cleaves the non-target strand, generating a site-specific double-strand break. This break is subsequently repaired by endogenous cellular DNA repair pathways, primarily non-homologous end-joining (NHEJ) or homology-directed repair (HDR), thereby forming the mechanistic basis for gene disruption or precise sequence modification (**Figure 1**).

Protospacer-adjacent motifs (PAMs): role and importance

The requirement for a protospacer-adjacent motif (PAM) is a fundamental determinant of CRISPR–Cas9 specificity and safety. PAMs are short DNA sequences immediately flanking the target site that are essential for Cas protein binding and activation [18,19]. For example, Streptococcus pyogenes Cas9 (SpCas9) recognises the NGG PAM, whereas Staphylococcus aureus Cas9 (SaCas9) recognises the longer NNGRRT motif. PAM dependence serves as a biological safeguard, preventing Cas nucleases from targeting the host's own CRISPR locus. In therapeutic genome editing, PAM constraints limit targetable

genomic regions and influence guide RNA design, editing efficiency, and off-target risk. Recent engineering of PAM-flexible Cas9 variants has expanded the editable genome, but often at the cost of increased off-target activity, highlighting a trade-off between targeting scope and specificity that is particularly relevant for clinical applications in breast cancer (**Figure 1**).

Relevance of CRISPR–Cas fundamentals to breast cancer therapy

A mechanistic understanding of Cas protein biology, system classification, and PAM constraints is essential for rational design of CRISPR-based breast cancer therapies. Differences in PAM availability, chromatin accessibility, and DNA repair pathway activity across tumour subtypes directly affect editing outcomes [20]. Consequently, therapeutic success depends not only on target gene selection but also on the informed choice of Cas variants and editing strategies tailored to the molecular context of breast cancer.

Molecular targets for CRISPR-Cas9 therapy in breast cancer

Oncogenic drivers

The CRISPR-Cas9 genome-editing system is an advanced tool that enables scientists to study and treat the genetic factors that drive breast cancer development and treatment resistance [15]. Multiple breast cancer-related genes have been successfully edited using CRISPR systems, revealing disease mechanisms and creating new targets for targeted treatments [4]. HER2 (ERBB2): The HER2 gene is overexpressed in breast cancer cells, leading to aggressive disease progression. The use of CRISPR to reduce ERBB2 gene expression results in decreased tumour cell growth, increased cell death, and improved response to HER2-targeted therapies such as trastuzumab. The research shows that CRISPR technology can improve or defeat existing HER2-targeted treatments for breast cancer patients [2].

PIK3CA mutations: The most common breast cancer mutations in PIK3CA are H1047R and E545K, which activate the PI3K/AKT/mTOR signalling pathway [16]. Scientists have used precise CRISPR gene editing to correct these mutations, thereby restoring normal signalling pathways and blocking cancer-promoting effects [17]. The method demonstrates that allele-specific gene editing can be performed in tumours harbouring targetable mutations [18]. KRAS and MYC: The occurrence of KRAS and MYC mutations in breast cancer is less common than in other cancer types, yet they lead to uncontrolled cell growth, metabolic changes, and tumour progression [19]. The use of CRISPR technology allows scientists to study how these genes cause cancer and discover new treatment options that target their synthetic lethal interactions [20]. ESR1 mutations: ESR1 mutations, including Y537S and D538G, and others, lead to endocrine therapy resistance in patients [21]. The CRISPR-Cas9 system enables researchers to study ESR1 mutations that activate receptors in the absence of estrogen binding and to create models for studying therapy-resistant breast cancer [21]. The therapeutic potential of ESR1 mutation correction or disruption is to overcome endocrine resistance and restore cancer cells' responsiveness to treatment (**Figure 2** and **Table 1**).

Tumour suppressor genes

The CRISPR-Cas9 system enables scientists to study and modify tumour suppressor genes commonly harboured by breast cancer patients [22]. The CRISPR system allows precise gene editing, helping scientists study tumour-suppressing pathways and their associated vulnerabilities in breast cancer cells [23] (**Figure 2** and **Table 1**).

TP53: The Tumour protein p53 (TP53) gene harbours mutations in 30–40% of breast cancer cases, with TNBC showing the highest incidence. Mutations in TP53 disrupt p53-dependent apoptosis, cell-cycle arrest, and the DNA damage response, leading to tumour progression and genomic instability. Scientists have used CRISPR to edit TP53 mutations, restoring normal p53 function, restoring apoptotic responses, and decreasing malignant cell growth in laboratory tests. The research demonstrates that genome-editing technology can reverse the harmful effects of dysfunctional tumour suppressor genes [24, 25]. BRCA1/2: The occurrence of BRCA1 and BRCA2 mutations in cells leads to homologous recombination repair failure, which results in genomic instability and increases cancer development risk. Scientists use CRISPR-Cas9 to edit BRCA1/2 genes in the laboratory to study breast cancer

Table 1. CRISPR–Cas9 Therapeutic Landscape for Breast Cancer.

Category	Subcategory / Component	Details	CRISPR Strategy / Role	Therapeutic Impact
1. Molecular Targets	Oncogenes	HER2, PIK3CA (H1047R/E545K), MYC, KRAS	Gene knockout, base/prime editing	Inhibits oncogenic signaling, reduces proliferation
	Tumor suppressors	TP53, PTEN, BRCA1/2	HDR-mediated repair, gene restoration	Restores apoptosis, DNA repair, and reduces aggressiveness
	Immune evasion genes	PD-L1, PD-1, CTLA-4	Knockout or CRISPR-enhanced CAR-T	Boosts anti-tumor immunity
	Drug resistance genes	ABCB1, PARP1, ESR1 mutants	Gene disruption or correction	Improves chemotherapy or PARP inhibitor sensitivity
	Metastasis regulators	MMP9, MMP2, EMT genes (Snail, Twist)	Knockout	Reduces invasion and metastasis
2. CRISPR Editing Tools	Cas9 Nucleases	SpCas9, SaCas9	Cuts DNA to induce DSB NHEJ/HDR	Gene knockout or precise repair
	High-Fidelity Cas9	SpCas9-HF1, eSpCas9	Reduced off-target cleavage	Safer genome editing
	Cas12a (Cpf1)	AsCas12a, LbCas12a	Staggered cuts, multiplex editing	Efficient editing with T-rich PAM
	Base Editors	BE3, ABE8e	CT or AG conversions	Single-nucleotide correction without DSBs
	Prime Editors	PE2, PE3	Insertions, deletions, any base change	Highest precision therapeutic editing
	CRISPRi/CRISPRa	dCas9-KRAB, dCas9-p300	Epigenetic suppression/activation	Transcriptional modulation without DNA cuts
3. Delivery Technologies	Lipid nanoparticles (LNPs)	mRNA + gRNA encapsulation	Non-viral, tumor-targetable	Transient, safe delivery; clinical-grade
	AAV vectors	SaCas9-compatible	Long-term expression	Preclinical *in vivo* editing
	Lentiviral vectors	Integrating vectors	Stable long-term gene editing	*Ex vivo* cell engineering
	Exosomes	Natural vesicles	Immune evasion, tissue targeting	CRISPR RNP delivery into tumors
	Polymeric nanoparticles	PLGA, PEG-based	Controlled release	Local tumor delivery
	Physical methods	Electroporation, microbubble ultrasound	Direct RNP entry	Ideal for *ex vivo* editing (e.g., CAR-T)
	Biomaterial scaffolds	Injectable hydrogels	Sustained, localized release	Enhances tumor-specific CRISPR action
4. Preclinical Findings	Cell line models	MCF-7, MDA-MB-231, BT-474	CRISPR knockout or HDR repair	Reduced growth, restored tumor suppression
	Mouse xenografts	HER2+, TNBC models	*In vivo* genome editing	Reduced tumor volume; altered signaling
	Immune models	PD-L1 knockout, CAR-T CRISPR editing	Enhanced T-cell killing	Improved immunotherapy outcomes
5. Translational Workflow	Target identification	WES, WGS, RNA-seq, CRISPR screens	Identify actionable mutations	Defines therapeutic targets
	gRNA design & optimization	CHOPCHOP, Benchling, Deep CRISPR	High specificity, low off-target	Therapeutically viable gRNAs
	Cas9/editor selection	Nuclease, base editor, prime editor	Match the tool to the mutation type	Precision therapeutic editing
	Vector construction & formulation	AAV, LNP, plasmid, RNP	Delivery packaging	Preclinical-grade CRISPR
	Safety & preclinical validation	Off-target WGS, cytotoxicity, xenografts	Ensure therapeutic safety	Regulatory compliance
	Delivery assessment	Viral, non-viral, exosomes	Determine the best *in vivo* route	Efficient tumor-specific editing
	Clinical translation	GMP manufacturing, IND preparation	First-in-human trials	Move therapy to the clinic

*Footnote Abbreviations: AAV, adeno-associated virus; ABE, adenine base editor; ABCB1, ATP-binding cassette subfamily B member 1; AKT, protein kinase B; AsCas12a, Acidaminococcus sp. Cas12a; BE3, third-generation cytosine base editor; BRCA, breast cancer susceptibility gene; CAR-T, chimeric antigen receptor T-cell; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRi, CRISPR interference; CRISPRa, CRISPR activation; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; Deep CRISPR, deep learning–based CRISPR guide design tool; DSB, double-strand break; EMT, epithelial–mesenchymal transition; eSpCas9, enhanced specificity Cas9; ESR1, estrogen receptor 1; gRNA, guide RNA; GMP, good manufacturing practice; HDR, homology-directed repair; HER2, human epidermal growth factor receptor 2; IND, investigational new drug; KRAS, Kirsten rat sarcoma viral oncogene homolog; LbCas12a, Lachnospiraceae bacterium Cas12a; LNP, lipid nanoparticle; MMP, matrix metalloproteinase; NHEJ, non-homologous end joining; PARP1, poly(ADP-ribose) polymerase 1; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PEG, polyethylene glycol; PE2/PE3, prime editor 2/3; PI3K, phosphoinositide 3-kinase; PLGA, poly(lactic-co-glycolic acid); PTEN, phosphatase and tensin homolog; RNP, ribonucleoprotein complex; SaCas9, Staphylococcus aureus Cas9; Snail, SNAI1 transcription factor; SpCas9, Streptococcus pyogenes Cas9; TNBC, triple-negative breast cancer; TP53, tumor protein p53; Twist, TWIST1 transcription factor; WES, whole-exome sequencing; WGS, whole-genome sequencing.

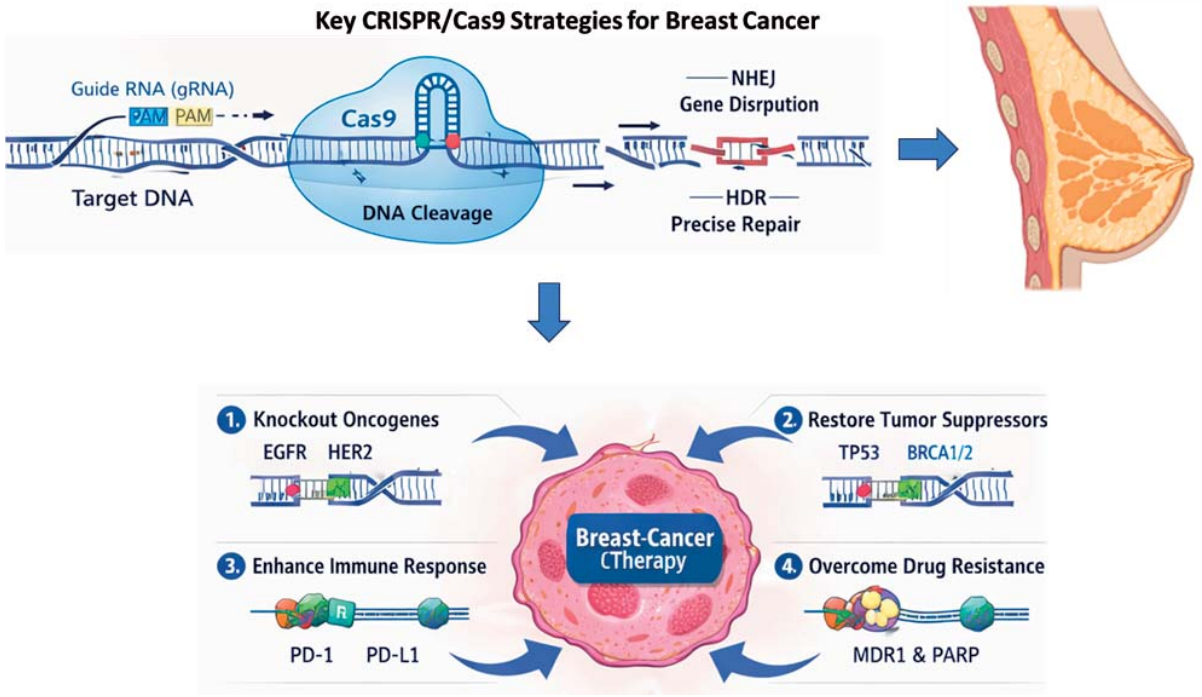

Figure 2. CRISPR-based therapeutic strategies for breast cancer. This figure summarises four major CRISPR–Cas9 therapeutic approaches currently under investigation for the management of breast cancer. CRISPR can (1) target and disrupt oncogenes driving tumour growth, (2) restore the function of tumour suppressor genes lost or mutated during cancer progression, (3) enhance anti-tumour immune responses by modifying immune-related pathways or immune cells, and (4) overcome drug resistance by editing genes responsible for chemoresistance and treatment failure. Together, these strategies illustrate the versatile applications of genome editing in the development of precision therapies for breast cancer.

development, synthetic lethal interactions, and PARP inhibitor responses [26]. The editing process of loss-of-function genes produces HR-deficient cell phenotypes, but targeted gene correction enables DNA repair function and affects treatment responses [27]. The research demonstrates that CRISPR technology serves two purposes: helping scientists understand breast cancer mechanisms and developing new treatments for BRCA1/2 mutations (**Figure 2**).

Immune-modulating targets

The CRISPR-Cas9 system enables scientists to edit essential immune checkpoint proteins and regulatory pathways, which determine breast cancer immune responses [28]. The CRISPR-based approaches show promise for enhancing immunotherapy effectiveness in breast cancer patients who have "cold" tumours, including triple-negative breast cancer (TNBC) [29]. PD-1/PD-L1 Axis: The programmed death pathway is a primary immune evasion strategy that breast tumours use to evade immune detection [30]. The removal of PD-1 from T cells and PD-L1 from tumour cells via CRISPR-Cas9 editing enhances T-cell killing and increases interferon-γ production, resulting in greater tumour cell destruction in laboratory tests. Disrupting this immune checkpoint axis via editing techniques enhances the effectiveness of PD-1/PD-L1 antibody therapies, creating a new approach to boost the immune system's anti-tumour function (**Figure 2**).

CTLA-4, TIGIT, and LAG-3: The immune checkpoint receptors CTLA-4, TIGIT, and LAG-3 work together to exhaust T cells, creating a significant obstacle to treating TNBC and other aggressive breast cancer types [31]. The removal of immune checkpoints using CRISPR technology enables T cells to regain function, leading to improved antigen recognition, enhanced proliferation, and increased effector cell activity [32]. The combination of multiple immune checkpoint gene-editing approaches enhances immune system activation, which scientists can use to create personalised

T-cell therapies for breast cancer treatment and drug Resistance Mechanisms [33]. The application of CRISPR-Cas9 technology has enabled scientists to identify drug resistance genes in breast cancer, thereby validating their functions and discovering new therapeutic approaches [34]. The CRISPR system allows scientists to edit specific genes that control drug resistance, EMT, and DNA repair, helping them reverse drug resistance and improve treatment outcomes [35] (**Figure 2**).

ABC Transporters (ABCG2): The ATP-binding cassette (ABC) transporters, including ABCG2, function as major drug resistance factors by actively effluxing chemotherapeutic agents, tyrosine kinase inhibitors, and anthracyclines from cells [36]. Deletion of ABCG2 using CRISPR-Cas9 results in decreased drug efflux and increased drug accumulation within cells, leading to greater cancer cell death in drug-resistant breast cancer lines [37]. The study demonstrates that ABCG2 functions as a primary chemoresistance factor that scientists can target for developing combination therapies. EMT Drivers (Snail, Twist): The epithelial–mesenchymal transition promotes cancer spread and confers tumour resistance to chemotherapy and targeted therapies [38]. The transcription factors Snail and Twist direct EMT programs that promote stem cell development and enhance cell movement and survival during treatment [39]. Disruption of Snail or Twist genes using CRISPR technology leads to reversal of EMT, decreased migration and invasion, and enhanced responses to chemotherapy and endocrine therapy [40]. The research demonstrates that gene-editing techniques offer practical methods for combating aggressive tumour states that exhibit plasticity (**Figure 2**).

DNA Repair Genes and PARP Inhibitor Resistance: The development of PARP inhibitor resistance occurs through three main mechanisms: restoration of homologous recombination, return of BRCA1/2 mutations, and enhancement of DNA repair mechanisms [41,42]. The CRISPR-Cas9 system enables scientists to create BRCA-reversion mutations, delete essential DNA repair proteins, and modify replication-fork stability regulators [43]. Targeted editing of resistance-associated genes using CRISPR technology opens new possibilities for restoring PARP inhibitor sensitivity in HR-deficient breast cancer treatment (**Figure 2**).

Designing CRISPR-Cas9 tools for breast cancer

Guide RNA (gRNA) design

Guide RNA (gRNA) design is a critical determinant of editing efficiency and specificity. Widely used platforms such as CHOPCHOP, CRISPOR, and Benchling integrate sequence features, PAM compatibility, and predicted off-target sites to assist in selecting optimal gRNAs. However, computational predictions alone are insufficient for therapeutic applications, and experimental validation remains essential, particularly in genetically heterogeneous breast cancer models [44]. The success of CRISPR-Cas9 editing depends heavily on guide RNA (gRNA) design because it determines both the precision of the edit and the safety of the treatment [44]. The selection of optimal gRNAs for breast cancer treatment requires precise targeting of oncogenic drivers, tumour suppressors, and resistance-associated genes to achieve effective on-target results with minimal genomic alterations [45,46]. The development of new computational prediction tools, high-fidelity Cas variants, and expanded PAM-recognising enzymes has improved the accuracy of gRNA engineering for cancer treatment (**Table 1**).

Off-target detection and genome-wide safety assessment

To experimentally evaluate off-target activity, several genome-wide detection methods have been developed. GUIDE-seq (Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing) identifies double-strand breaks in living cells by incorporating short oligonucleotide tags at cleavage sites. CIRCLE-seq (Circularisation for In vitro Reporting of Cleavage Effects by Sequencing) detects Cas9 cleavage sites in vitro using purified genomic DNA, offering high sensitivity [47]. SITE-seq (Selective Integration of Targeted Ends by Sequencing) enables quantitative mapping of cleavage sites by sequencing adapter-ligated DNA ends. These complementary approaches are increasingly regarded as essential components of preclinical safety assessment for CRISPR-based cancer therapies [48].

Cas nuclease selection and engineering

Among available nucleases, SpCas9 remains the most widely used due to its high activity

and well-characterised behaviour, while SaCas9 offers advantages for adeno-associated virus delivery owing to its smaller size. High-fidelity variants such as SpCas9-HF1 and eSpCas9 are increasingly adopted to reduce off-target cleavage in therapeutic contexts [48].

Advanced editing platforms

Base editors such as BE3 and ABE8e enable single-nucleotide substitutions without inducing double-strand breaks, making them particularly attractive for correcting point mutations in genes such as PIK3CA. Prime editing systems, including PE2, offer broader editing capabilities but currently exhibit lower in vivo efficiency, highlighting a trade-off between precision and practicality [49].

gRNA Selection Criteria

- **GC Content (40–60%):** The stability of gRNA and Cas9 binding efficiency reaches its peak when GC content reaches 40–60% [49]. The binding affinity of gRNAs decreases when GC content is too low, but becomes less specific when GC content exceeds 60%.
- **Off-Target Motifs Must Be Avoided:** The design of specific gRNAs requires researchers to remove all sequences that closely match other genomic regions, especially in the seed region near the PAM. The software tools CRISPOR, CHOPCHOP, and Benchling enable users to evaluate off-target sites and select guides with high precision [50].
- **Exonic and Conserved Regions Should Be Targeted:** Selecting gRNAs targeting essential coding sequences and functional domains yields better results for oncogenic driver disruption and more consistent knockout/repair outcomes [51]. The preferred therapeutic editing approach targets mutation hotspots and essential coding sequences rather than non-coding regions.
- **PAM Compatibility and Cas Variants:** The SpCas9 enzyme requires an NGG protospacer adjacent motif (PAM) to function, which restricts its ability to target specific genomic locations [52]. The xCas9, SpG, and SpRY variants of Cas9 enzymes recognise different PAM sequences, including NG, GAA, and GAT, providing researchers with more target options [53]. The selection of Cas variants depends on the specific genomic region and therapeutic goals to achieve maximum accessibility.

AI-Assisted Tools

Modern CRISPR design pipelines benefit from artificial intelligence (AI) and machine learning (ML), which improve gRNA prediction accuracy, on-target activity prediction, and off-target risk evaluation [54]. AI platforms analyse genomic data, chromatin accessibility, sequence characteristics, and experimental results to generate precise guide selection for breast cancer therapy (**Table 1**).

CHOPCHOP and CRISPR-DO

The CRISPR design tool CHOPCHOP provides a simple interface for selecting gRNAs and displays scores that assess their efficiency, potential for off-target effects, and compatibility with the genome [55]. The CRISPR-DO platform helps users identify optimal gRNA positions for knockout, knock-in, and promoter editing using alternative splicing data and exon structure information, which are crucial for breast cancer genes such as ESR1 and BRCA1.

Deep CRISPR uses deep learning models that were trained on extensive CRISPR screening data to predict both the efficiency of on-target editing and the patterns of off-target cleavage [56]. The system uses convolutional neural networks to analyse sequence motifs, genomic features, and epigenomic signals, achieving better predictive accuracy than traditional rule-based approaches [57]. The design of gRNAs targeting BRCA1/2 and ESR1 resistance mutations requires high precision, making Deep CRISPR an essential tool (**Figure 3**).

Elevation

The Elevation platform uses machine learning to generate comprehensive predictions of off-target sites by analysing DNA shape, chromatin structure, and genomic sequence elements. The system generates a probability-based list of potential off-target sites, along with their estimated occurrence rates, which helps users prepare CRISPR constructs for therapeutic *in vivo* delivery [58].

Target prediction algorithms

The next generation of algorithms uses deep learning to analyse ATAC-seq and DNase-seq

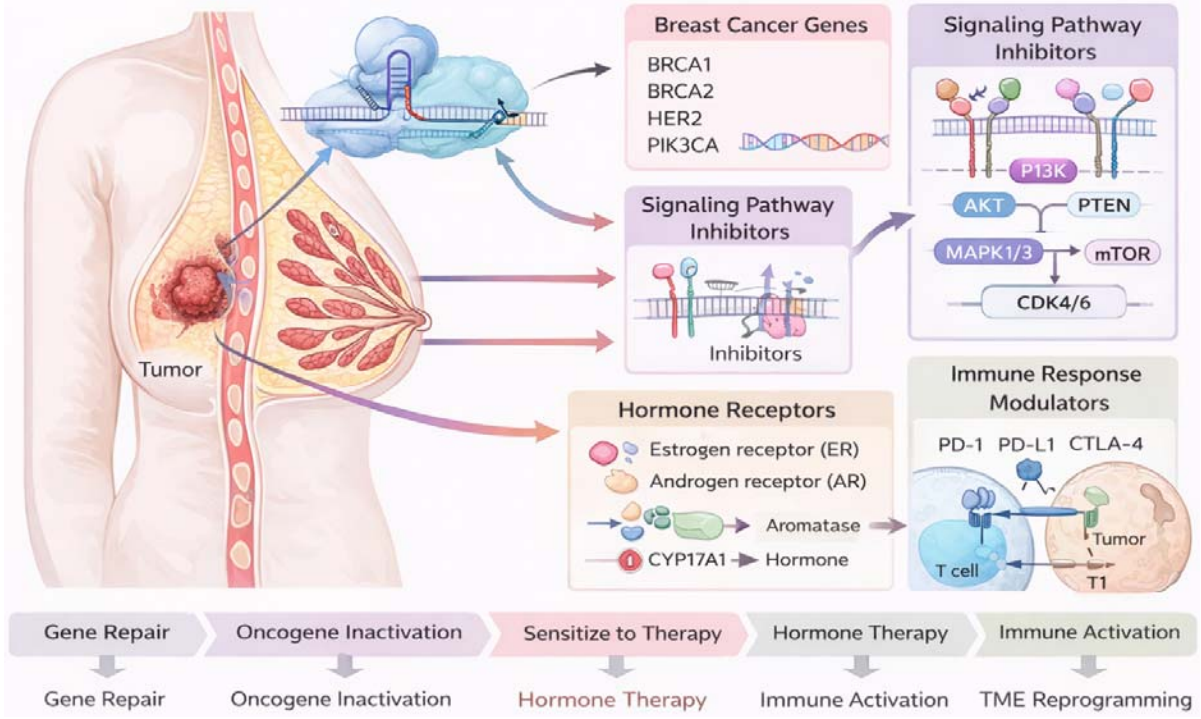

Figure 3. CRISPR–Cas9 therapeutic targets and pathway interventions in breast cancer. This figure summarises key molecular pathways in breast cancer that can be modulated using CRISPR–Cas9 editing strategies. Left panel: Oncogene targeting. Hyperactive oncogenic signalling pathways, such as those involving HER2, PI3K, AKT, and PIK3CA mutations, can be disrupted by CRISPR-mediated gene knockout, thereby attenuating downstream signalling that drives proliferation. Loss-of-function mutations in PTEN or TP53, which are common in aggressive breast cancers, represent additional targets for CRISPR to silence oncogenic signalling or restore proper regulatory control. Right panel: Tumour suppressor restoration and immune enhancement. CRISPR-based correction of tumour-suppressor genes (e.g., TP53, PTEN) may reverse drug resistance and restore cellular function. Furthermore, genome editing can enhance anti-tumour immunity by modifying immune-related genes or pathways, such as introducing targeted double-strand breaks or precise base substitutions to optimise immune activation. Collectively, these editing strategies illustrate the broad therapeutic potential of CRISPR systems in reprogramming oncogenic circuits, reversing resistance mechanisms, and strengthening immune responses in breast cancer.

data, along with nucleosome positions and transcription factor binding sites, to predict actual CRISPR cleavage risks in living cells. The AI-assisted tools enhance the reliability of gRNA design for complex tumour genomes by analysing chromatin states that determine Cas9 accessibility [59].

Cas9 engineering

The development of Cas9 engineering techniques has expanded the applications of CRISPR for breast cancer treatment by improving precision and therapeutic effectiveness. The new generation of editing platforms, together with modified Cas nucleases, offers improved delivery methods, reduced off-target effects, base-conversion capabilities, and sequence-rewriting options, all of which are essential for breast cancer model research [60].

SpCas9

The CRISPR nuclease from Streptococcus pyogenes, known as SpCas9, serves as the primary CRISPR system because it provides high cutting efficiency and works well with NGG PAM sites. The intense activity of SpCas9 has established it as the standard tool for knockout research, therapeutic gene editing, and high-throughput CRISPR screening in breast cancer studies [61].

SaCas9

The Staphylococcus aureus Cas9 enzyme (SaCas9) is smaller than SpCas9, enabling its packaging into adeno-associated virus (AAV) vectors for efficient delivery of gene therapies. The smaller size of SaCas9 does not affect its strong DNA-cleavage ability, while it recognises more extended PAM sequences (NNGRRT), which provides additional flexibility for therapeutic genome editing [62].

High-Fidelity Cas9 variants (SpCas9-HF1, eSpCas9)

Scientists developed high-fidelity Cas9 variants by mutating specific DNA-binding sites, thereby reducing their tendency to cause off-target effects. The SpCas9-HF1 and eSpCas9 variants exhibit reduced off-target effects while maintaining precise DNA cutting, making them suitable for medical applications that require high precision [63].

Cas12a/Cpf1

The CRISPR nuclease Cas12a (Cpf1) produces staggered 5' overhangs during DNA cleavage, and it recognises T-rich PAM sequences (TTTV), which differ from Cas9's G-rich PAM preference. The system requires only one CRISPR RNA (crRNA) to function and produces insertions that are more predictable due to its unique cutting mechanism. The specific cutting pattern of Cas12a, together with its low off-target activity, makes it an ideal tool for performing multiple gene edits and disruptions in breast cancer research [64].

Base Editors (BE)

Base editors use a disabled Cas nuclease together with cytosine or adenine deaminases to perform single-nucleotide changes in DNA without generating double-strand breaks [65]. The CBE system converts C•G base pairs into T•A, while the ABE system converts A•T into G•C. The systems can help treat point mutations in the PIK3CA and ESR1 genes via single-base edits, thereby reducing genomic damage.

Prime Editors (PE)

Prime editing technology allows users to perform all types of base substitutions, insertions, and deletions through a process that does not require double-strand breaks or donor templates [66]. The Prime editing system combines a Cas9 nickase with reverse-transcriptase activity and a programmable pegRNA guide for precise genome editing [67]. The system provides exceptional precision for repairing BRCA1/2 mutations and ESR1 resistance mutations and for making delicate changes to breast cancer genome regulation.

Epigenome-editing CRISPR tools

Scientists have developed CRISPR-based technologies that enable precise epigenetic modifications via DNA-sequence-independent methods. The dCas9 tool with effector domains serves as an epigenome-editing platform to control chromatin structure, DNA methylation, and transcriptional activity. The tools show exceptional value for breast cancer research because they enable modification of epigenetic marks that drive oncogene expression, tumour suppressor silencing, drug resistance, and metastatic spread [68].

dCas9-KRAB: Transcriptional Repression via Chromatin Condensation**.** The dCas9-KRAB system combines nuclease-dead Cas9 with the Krüppel-associated box (KRAB) repressor domain to achieve site-specific gene silencing through heterochromatin formation [68]. The dCas9-KRAB system brings histone deacetylases and methyltransferases to target sites, leading to H3K9me3 marks and transcriptional shutdown. The method enables researchers to disable cancer-promoting genes and block expression of EMT factors, as well as genes linked to drug resistance and stem cell maintenance, in breast cancer models [69].

dCas9-p300: Transcriptional Activation via Histone Acetylation The dCas9-p300 system links Cas9 to the catalytic core of p300, a histone acetyltransferase, for precise H3K27ac histone acetylation at enhancer and promoter regions [70]. The platform enables robust activation of tumour suppressor genes, immune-related pathways, and apoptosis regulators. The dCas9-p300 system allows researchers to study enhancer-controlled oncogenic networks by reactivating silenced breast cancer genes (**Figure 4**).

dCas9-TET1: DNA demethylation for epigenetic reprogramming

The dCas9-TET1 fusion protein performs targeted DNA demethylation by linking TET1 catalytic activity to specific DNA locations. The method activates genes silenced by promoter hypermethylation by targeting tumour suppressors, DNA repair enzymes, and hormone receptor regulators. The dCas9-TET1 system demonstrates potential for treating breast cancer by reversing cancer-promoting epigenetic changes and making tumours more responsive to hormone therapy and targeted treatments [70] (**Figure 4**).

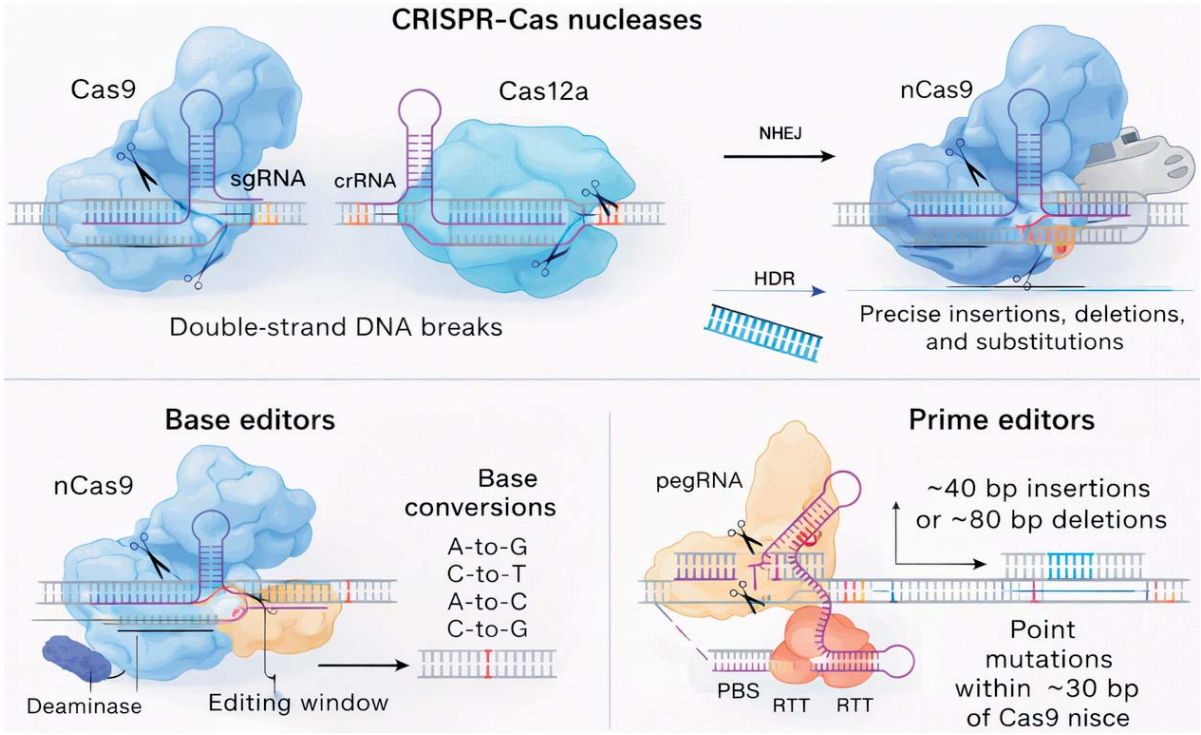

Figure 4. Comparison of Cas9 variants and next-generation genome-editing tools. This figure illustrates the major Cas9-based editing platforms used in therapeutic genome engineering. SaCas9 and SpCas9 function as endonucleases guided by crRNA–tracrRNA complexes to generate targeted DNA double-strand breaks. Cas9 nickase, containing a single catalytic mutation, introduces a strand-specific nick that reduces off-target activity and forms the basis for more precise editing systems. Base editors couple a catalytically impaired Cas9 to a deaminase enzyme, enabling single-nucleotide conversions without double-strand breaks. Prime editors integrate a Cas9 nickase with a reverse transcriptase, enabling precise programmable insertions, deletions, and substitutions. Together, these tools represent the expanding CRISPR toolkit for precise therapeutic gene modification in breast cancer.

Delivery strategies for CRISPR-Cas9 in breast cancer

The success of CRISPR-Cas9 therapy depends heavily on achieving efficient and safe delivery of its components. Researchers have tested different delivery methods for breast cancer treatment by using viral and non-viral systems to deliver Cas nucleases and guide RNAs.

Viral delivery

AAV Vectors: The use of adeno-associated virus (AAV) vectors for gene delivery has become popular because they offer high transduction efficiency and are safe. The delivery system uses AAV vectors to transport Cas9 mRNA and Cas9 nickase constructs into cells [71]. The small size of AAV vectors limits their capacity to carry substantial Cas9 variants, so researchers use SaCas9 or split-Cas9 systems for genome editing [72]. Lentiviral Vectors: The lentiviral vector system provides stable genomic integration, which results in prolonged expression of CRISPR components. The system provides excellent conditions for studying gene function and cell-based genome editing applications [72]. The permanent nature of lentiviral integration, along with the risks of insertional mutagenesis, makes them unsuitable for cancer therapy applications (**Table 1**).

Non-viral delivery

Lipid nanoparticles (LNPs): The success of lipid nanoparticles (LNPs) in the COVID-19 vaccine has proven their effectiveness as clinical mRNA delivery systems [73]. The delivery of Cas9 mRNA and gRNAs via tumour-specific LNP formulations results in efficient genome editing with short-term effects, without causing permanent genomic damage [74]. The addition of HER2-targeted ligands to surface modifications enhances the specificity of breast cancer cells for LNP delivery. Polymeric nanocarriers: The delivery of therapeutic agents is more effective with biodegradable polymeric systems, such as PEG-PLGA

nanoparticles and tumour-responsive hydrogels [75]. The combination of HER2- and EGFR-targeting aptamers with nanoparticles enables specific entry into breast cancer cells and simultaneous delivery of Cas9 and therapeutic compounds. Exosome-Based Delivery: Exosomes are natural biological vesicles that exploit their immune-evading properties to enter cells efficiently [76]. The combination of tumour-homing peptides (RGD4C, iRGD) with engineered exosomes carrying Cas9 RNPs or gRNAs shows promising results for targeted CRISPR delivery with minimal immune response (**Table 1**).

Physical delivery methods

Electroporation is the primary method for *ex vivo* genome editing of T cells and NK cells because it delivers high-efficiency Cas9 RNP complexes for adoptive cell therapy [87]. The combination of microbubbles with ultrasound technology enables targeted CRISPR delivery to breast tissue and tumour sites while reducing systemic exposure (**Table 1**).

Preclinical studies and therapeutic outcomes

Knocking out HER2 using CRISPR-Cas9 reduces cell proliferation in breast cancer cells that express HER2 [78]. Research using HER2-positive breast cancer models shows that HER2 knockout through CRISPR-Cas9 results in smaller tumours and better response to HER2-targeted therapies, including trastuzumab and lapatinib [78]. Base editing and prime editing systems have proven successful in correcting PIK3CA mutations that cause disease by repairing the H1047R mutation, thereby decreasing AKT phosphorylation, blocking PI3K signalling, and restoring normal cellular function [79]. The application of CRISPR editing technology yields CAR-T cells that demonstrate enhanced efficacy in treating TNBC [80]. Combining PD-1 knockout with CAR-T cells results in greater tumour cell killing, improved tissue penetration, and prolonged cell survival [81]. Disrupting metastasis-related genes with CRISPR technology reduces migration and invasion and prevents metastatic spread [82]. The dCas9-TET1

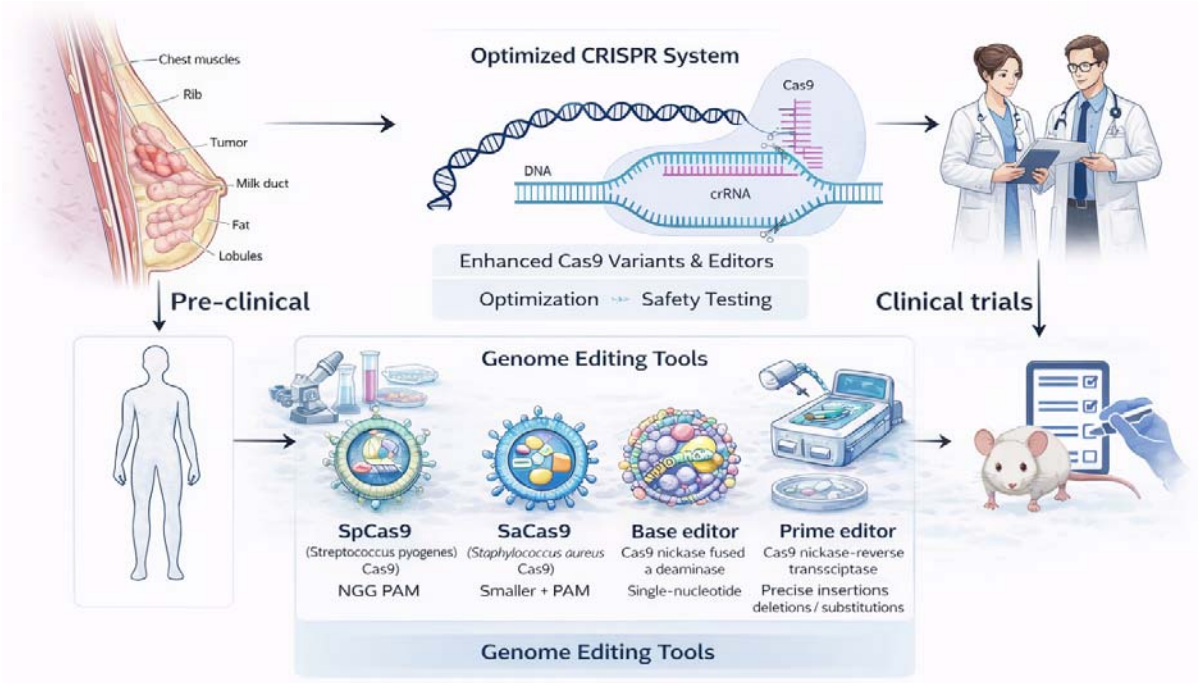

Figure 5. Translational workflow for developing CRISPR-based breast cancer therapies. This figure outlines the sequential stages involved in translating CRISPR gene-editing strategies from concept to clinical application. The process begins with target identification, in which actionable oncogenes or tumour suppressor mutations are defined. The CRISPR system is then optimised, including guide RNA design and verification of efficient, specific DNA editing. Preclinical validation follows, using engineered breast cancer cell lines and mouse models to evaluate therapeutic efficacy, off-target effects, and safety. Various delivery strategies, such as nanoparticles, viral vectors, or exosomes, are then assessed to determine the most effective means of delivering CRISPR components in vivo. Successful candidate therapies advance to clinical application, where they may be tested in human patients for safety and therapeutic benefit. This workflow demonstrates the multi-step pathway for developing clinically viable CRISPR-based breast cancer interventions.

and dCas9-p300 epigenetic tools use CRISPR to edit microRNAs that control EMT processes and metastatic signalling pathways [83]. The system enables researchers to study enhancer-controlled oncogenic networks by reactivating silenced breast cancer genes (**Figure 5** and **Table 1**).

Safety, ethical considerations, and challenges

The process of genome cleavage at incorrect sites remains a significant problem. The combination of SpCas9-HF1 and eSpCas9 with nickase systems and base/prime editors provides enhanced protection against unwanted genome modifications [84]. The development of GUIDE-seq, CIRCLE-seq, and SITE-seq requires complete off-target screening for all translational applications [84]. The process of double-strand break induction leads to chromothripsis, large deletions, and translocations in genomic DNA [85]. The evaluation of structural genome stability after editing requires both Oxford Nanopore and PacBio HiFi long-read sequencing, as well as whole-genome sequencing, for clinical-grade applications [85].

The body recognises Cas9 proteins through the immune system, triggering inflammatory responses and leading to their elimination [85]. The use of RNPs and nanoparticle formulations for transient delivery helps minimise exposure time and decreases the risk of immune responses [99]. The presence of different cancer cell types within tumours makes it difficult to identify appropriate targets for treatment [86]. The development of precision CRISPR therapeutics requires single-cell CRISPR screens and multiomic profiling to identify state-specific dependencies and vulnerabilities [86]. The implementation of clinical gene therapy requires strict guidelines that ban germline editing, protect patients, and require extended monitoring for genetic changes and late-onset complications.

Future directions and conclusions

The therapeutic options for breast cancer continue to expand due to recent developments in CRISPR technology, delivery systems, and computational biology. The following emerging fields will determine the direction of CRISPR-based oncology research over the next 10 years. Artificial intelligence serves as an essential tool for developing CRISPR systems, ensuring safety and achieving therapeutic goals [51]. Deep learning models that analyse extensive CRISPR screening and sequencing databases enable users to Select gRNAs based on their predicted ability to cut DNA efficiently and their stability when bound to chromatin. Artificial intelligence-driven platforms are increasingly being integrated into CRISPR therapeutic development to enhance precision and safety. These approaches enable accurate prediction of guide RNA efficiency, editing outcomes, and potential off-target effects by integrating sequence features, chromatin accessibility, and large-scale CRISPR screening data. In addition, optimisation of prime-editing components, including pegRNA design and editing windows, enables more precise and flexible genome modification. Together, these advances are expected to accelerate the translation of CRISPR-based strategies from experimental models to clinically relevant applications in breast cancer.

The different subpopulations within breast tumours create therapy resistance, immune evasion, and metastasis through their distinct characteristics. The combination of single-cell technologies enables researchers to achieve better CRISPR targeting results through three primary methods. scRNA-seq technology enables the detection of distinct cancer cell states, stem-like populations, and resistant clones through transcriptional analysis. scATAC-seq technology enables researchers to identify which chromatin-accessible regulatory elements can be targeted using CRISPRi/CRISPRa. Spatial transcriptomics enables researchers to study how tumours interact with their surrounding tissue and identify specific areas where CRISPR interventions will be most effective. Researchers can create personalised CRISPR treatment plans by combining different data types that reveal specific weaknesses in TNBC cells, micrometastatic lesions, and minimal residual disease (MRD) cells.

Multiple independent oncogenic programs operate in most breast cancer cases. The new generation of CRISPR systems, including Cas12a, Cas13, and multiple gRNA variants, enables researchers to simultaneously edit numerous targets, including oncogenes and immune

checkpoints such as PIK3CA and PD-1, as well as metastasis-promoting genes such as MMP9, VEGFA, and Snail. The system enables researchers to disable cancer resistance mechanisms while activating genes that enhance immune system function. The combination of multiple gene-editing approaches enhances therapeutic outcomes by allowing the tumours to adapt less effectively and reducing their ability to develop new resistance mechanisms. The CRISPR-Cas13 system targets RNA molecules rather than DNA, enabling reversible gene regulation without permanently altering the genome. The system provides two primary benefits to users. The system allows researchers to temporarily block oncogenic mRNA expression from the MYC and HER2 genes. The system prevents genomic instability by not creating double-strand breaks during operation. The system operates safely in critical situations by allowing temporary changes in gene expression during chemotherapy and radiation treatment. The system enables researchers to correct abnormal RNA splicing patterns, which are frequently observed in breast cancer. The RNA-editing capabilities of Cas13 systems provide researchers with a flexible and secure means to enhance their DNA-targeting CRISPR approaches.

The main obstacle to clinical CRISPR therapeutics reaching patients is the development of safe, tumour-specific delivery methods. Multiple advanced delivery systems, including tumour-specific lipid nanoparticles, exosome-mimetic vesicles, biomaterial scaffolds, and hydrogels, are being developed for clinical use. The delivery systems use tumour-specific surface ligands to direct Cas9 mRNA or Cas9 RNP complexes to breast cancer cells. The delivery system uses exosome-mimetic vesicles that exhibit exosome-like characteristics to transport CRISPR cargo through tissues and deliver it with targeted precision. The delivery system comprises biomaterial scaffolds and hydrogels that serve as implantable depots that release CRISPR tools at controlled rates for post-surgical tumour-bed treatment. The delivery systems use microbubble ultrasound and injectable formulations to enhance tumour exposure while minimising systemic side effects. The new delivery platforms work to meet safety standards and reproducibility requirements, as well as targeted biodistribution and scalability needs, bringing CRISPR therapies closer to human clinical testing. The therapeutic potential of CRISPR in oncology remains limited due to multiple obstacles. The main challenges to CRISPR therapy development include off-target gene modifications, immune responses, changes in DNA structure, and the heterogeneity of breast tumours. The path to clinical adoption of CRISPR-based therapies requires extensive safety testing, ethical oversight, and the development of efficient delivery systems. The CRISPR-Cas9 system, along with its growing collection of editing tools, has ushered in a new era in breast cancer research and treatment. The full realisation of the benefits of genome editing for breast cancer patients depends on ongoing research, collaborative work, and proper implementation of these technologies.

Declarations

Authors contribution
Shafee Ur Rehman collected the data and wrote the manuscript.

Ethics approval and consent to participate
Not applicable.

Availability of data and materials
All data are derived from published studies and available in the manuscript.

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