

# Therapeutic applications of CRISPR/Cas9 in breast cancer and delivery potential of gold nanomaterials

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Jananee Padayachee and Moganavelli Singh

## Abstract

Globally, approximately 1 in 4 cancers in women are diagnosed as breast cancer (BC). Despite significant advances in the diagnosis and therapy BCs, many patients develop metastases or relapses. Hence, novel therapeutic strategies are required, that can selectively and efficiently kill malignant cells. Direct targeting of the genetic and epigenetic aberrations that occur in BC development is a promising strategy to overcome the limitations of current therapies, which target the tumour phenotype. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system, composed of only an easily modifiable single guide RNA (sgRNA) sequence bound to a Cas9 nuclease, has revolutionised genome editing due to its simplicity and efficiency compared to earlier systems. CRISPR/Cas9 and its associated catalytically inactivated dCas9 variants facilitate the knockout of overexpressed genes, correction of mutations in inactivated genes, and reprogramming of the epigenetic landscape to impair BC growth. To achieve efficient genome editing *in vivo*, a vector is required to deliver the components to target cells. Gold nanomaterials, including gold nanoparticles and nanoclusters, display many advantageous characteristics that have facilitated their widespread use in theranostics, as delivery vehicles, and imaging and photothermal agents. This review highlights the therapeutic applications of CRISPR/Cas9 in treating BCs, and briefly describes gold nanomaterials and their potential in CRISPR/Cas9 delivery.

## Keywords

Breast cancer, genome editing, CRISPR/Cas9, gold nanoparticles, gold nanoclusters

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## Introduction

Advances in the treatment of breast cancers (BCs) have led to significant improvements in the overall survival of patients. Local therapies, including surgery and radiotherapy, in conjunction with adjuvant targeted therapies and chemotherapy are mainstays of BC treatment.<sup>1</sup> Based on immunohistochemical staining of hormone receptors (HR), human epidermal growth factor receptor-2 (HER2), and ki67, a marker of cell proliferation, BCs can be divided into four subtypes which respond to different therapies. The majority of BCs are HR+ luminal tumours, which can be further subdivided into luminal A (HR+, HER2-, low ki67) and B (HR+, HER2+/high ki67) subtypes.<sup>2</sup> These cancers generally respond well to endocrine therapies targeting the

estrogen receptor (ER) and display relatively good prognoses.<sup>3</sup> HER2+ BCs, which are HR- and HER2+, are treated using anti-HER2 drugs. Triple negative breast cancers (TNBC) are HR- and HER2-, with chemotherapy and

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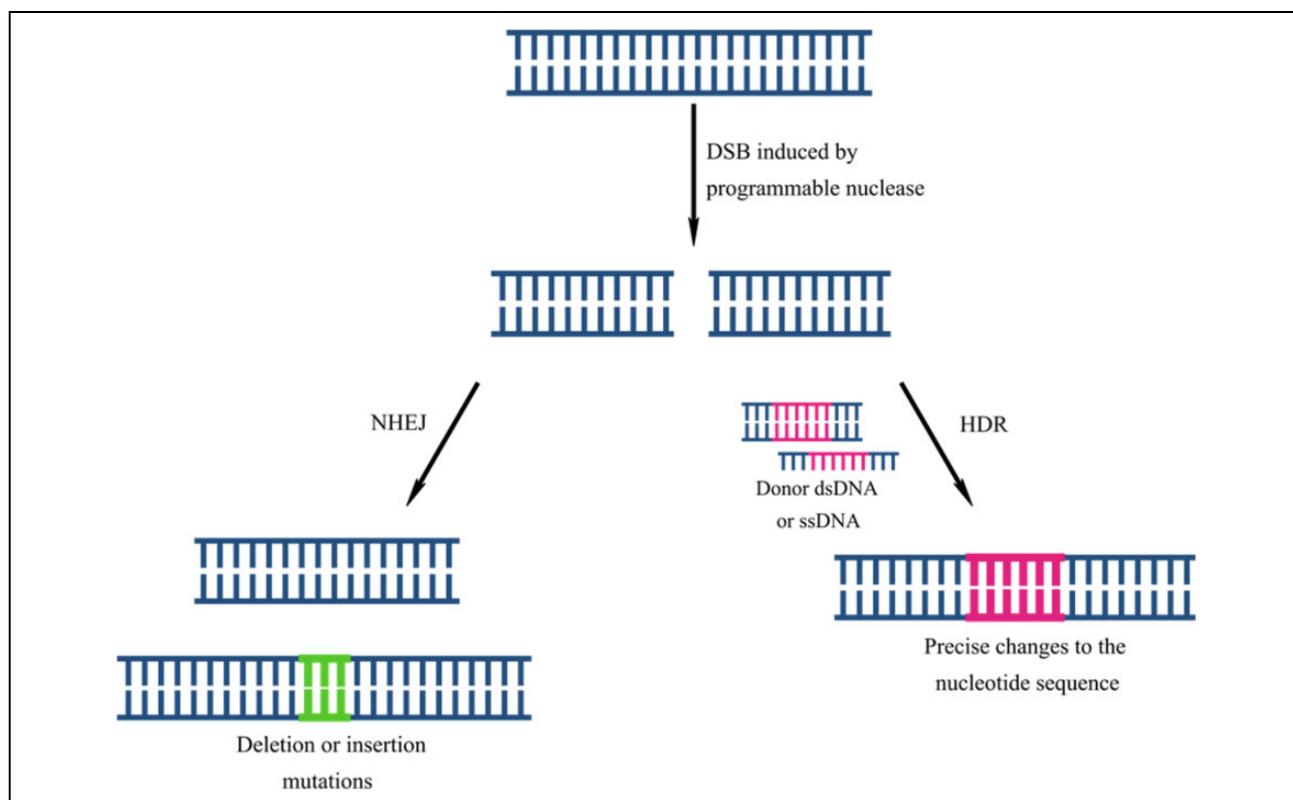
Nano-Gene and Drug Delivery Laboratory, Discipline of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Durban, South Africa

### Corresponding author:

Moganavelli Singh, Nano-Gene and Drug Delivery Laboratory, Discipline of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal (Westville Campus), Private Bag X54001, Durban 4000, South Africa.  
Email: [singhm1@ukzn.ac.za](mailto:singhm1@ukzn.ac.za)



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**Figure 1.** The process of genome editing by exploiting the natural repair mechanism of DSB repair, leading to random insertions or deletions via NHEJ or precise corrections via HDR.

radiotherapy as the options for treatment. The aggressive nature of TNBC coupled with the lack of targeted therapies has led to poor prognosis and high risk of relapse and metastasis compared to other subtypes.<sup>3-5</sup>

Current treatments such as chemo- and radiotherapy are associated with adverse physical and cognitive side effects, as they are non-specific and also affect healthy cells.<sup>6</sup> Many patients show intrinsic or acquired resistance to targeted therapies, chemotherapy, or radiotherapy, causing treatments to fail and leading to metastasis.<sup>7,8</sup> Even if tumours display a pathologic complete response, the risk of relapse remains for decades after therapy.<sup>9</sup> A major issue with current therapies is their dependence on the tumour phenotype. BCs show great molecular heterogeneity, with mutations in a variety of genes controlling cell growth, epigenetic modification, and transcription ultimately resulting in the malignant phenotype. Thus, genome editing techniques, which can directly target these genetic changes, show great promise in cancer therapy.

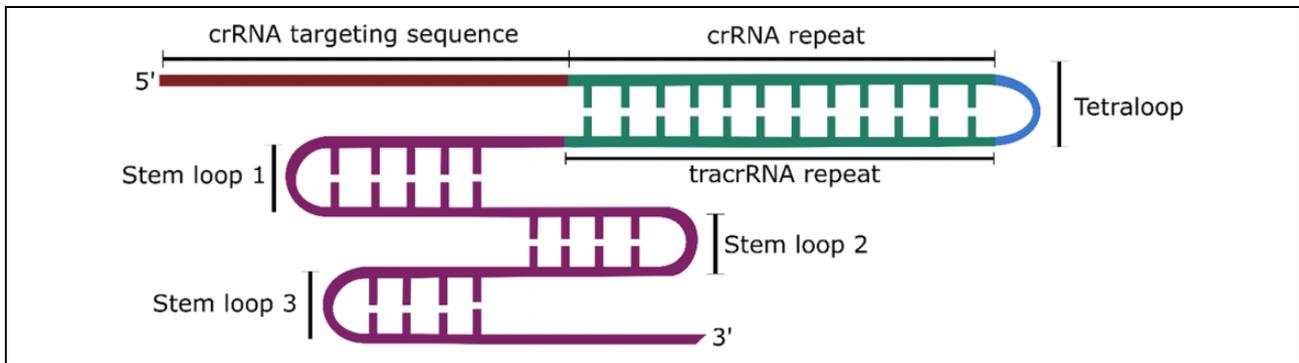
Genome editing involves modification of DNA, through the insertion, removal, or replacement of sequences. Current methods exploit endonucleases that introduce double-stranded breaks (DSB) into the DNA. Following cleavage, the DNA may be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Figure 1). HDR uses a donor DNA molecule as a template for

repair.<sup>10</sup> In this way, precise changes that correct gene function can be introduced into the genome. In contrast, NHEJ is an error-prone process in which the two ends of the DSB are ligated together, often leading to insertion or deletion (indel) mutations that may knock the gene out.<sup>10</sup> Since NHEJ targets the gene directly, it allows for more effective silencing than RNA interference (RNAi), which indirectly silences gene expression by targeting mRNA.

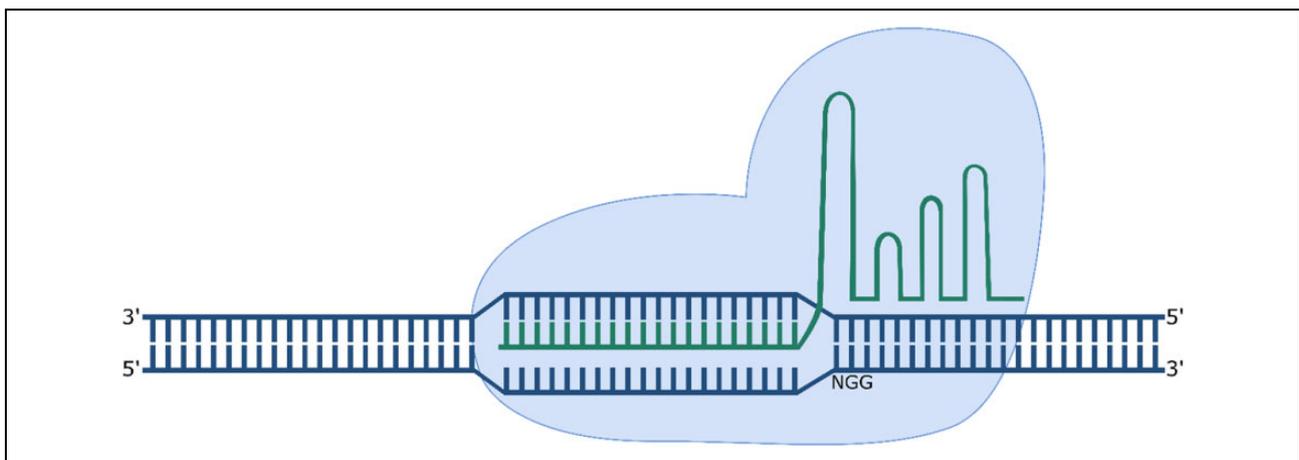
Four genome editing systems with different programmable nucleases have been developed: meganucleases, which have not seen extensive use for genome editing, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system. Despite being the youngest genome editing system, CRISPR/Cas9 has shown the most potential in cancer therapy, and will be discussed in this review.

### CRISPR/Cas9

In contrast to earlier genome editing systems, which mediate sequence recognition through protein-DNA interactions, the CRISPR/Cas system uses an RNA molecule to mediate binding. It is derived from a prokaryotic adaptive immune system protecting against invading viruses and plasmids, and is composed of CRISPR loci, comprised of



**Figure 2.** Structure of the chimeric sgRNA, containing the targeting sequence of the crRNA and the hairpin loops of the tracrRNA. The repeat sequences of the crRNA and tracrRNA sequences are linked via a tetraloop to form one structure.



**Figure 3.** CRISPR/Cas9 mediated cleavage of DNA. The sgRNA mediates binding to the 20 nt directly upstream of the 5'-NGG-3' PAM sequence on the target strand.

alternating repeat-spacer units, and CRISPR-associated (Cas) proteins.<sup>11</sup> Immunisation occurs in three stages: (i) adaptation, in which invading nucleic acids are cleaved by a complex of Cas endonucleases and the resulting fragments, called protospacers, are integrated into CRISPR loci between identical repeats; (ii) expression, in which the locus is transcribed into pre-CRISPR RNA (pre-crRNA) and processed into individual CRISPR RNA (crRNA) molecules; and (iii) interference, where the crRNA directs a single Cas endonuclease or a protein complex to cleave the foreign nucleic acids.<sup>12,13</sup>

CRISPR/Cas systems are broadly classified into two classes, further divided into six types and numerous subtypes, based on the mechanism by which recognition and cleavage occur.<sup>14</sup> Class 1 systems use protein complexes to effect cleavage, while Class 2 systems only utilise one protein, making them more applicable for genome editing.<sup>15</sup> All Class 2 systems (types II, V, and VI) have certain targeting constraints. Type VI systems, which utilise Cas13 to cleave RNA, recognise a protospacer flanking sequence (PFS).<sup>16</sup> Type II and V systems recognise a conserved 2–5 bp sequence called the protospacer adjacent motif

(PAM).<sup>17</sup> Type V Cas proteins recognise a PAM directly upstream of the protospacer, such as 5'-TTTN-3' recognised by the Cas12a, or Cpf1, protein.<sup>13,18</sup> In contrast, the Cas9 endonuclease of type II systems recognise a PAM downstream of the protospacer.<sup>19</sup>

The type II CRISPR/Cas9 system is the best characterised and most commonly used CRISPR system. For cleavage, Cas9 requires an additional RNA molecule called the trans-activating crRNA (tracrRNA), which facilitates crRNA binding and maturation. However, for use in genome editing, the tracrRNA and crRNA can be connected via a linker into one molecule termed the single guide RNA (sgRNA) (Figure 2).

Cas9 undergoes a conformational change following binding of the gRNA, allowing it to search for the PAM sequence and cleave the target strand 3 bp upstream of the PAM (Figure 3).<sup>20</sup> Cleavage is dependent not only on the presence of the PAM, but also on the complementarity of the “seed” sequence, the 10–12 nt of the target sequence adjacent to the PAM.<sup>21</sup> The *Streptococcus pyogenes* Cas9 (spCas9) is the most popular Cas9 used for genome editing. It is a large 1368 amino acid protein; however, it recognises

a short 5'-NGG-3' PAM sequence compared to orthologues from other bacterial species.<sup>19</sup>

The simplicity of CRISPR/Cas9 has made it the most used genome editing system. Compared to earlier systems, it is cheaper, easier to design, and can be retargeted without the need for protein engineering.<sup>22</sup> The PAM requirement limits the choice of target sequence; however this may be overcome by using different Cas proteins, or spCas9 variants that have been modified to recognise different PAMs.<sup>23,24</sup> The CRISPR system is also highly versatile and catalytically inactivated Cas9 s (dCas9), with mutated nuclease domains, have been conjugated to a variety of proteins and enzymes to perform different functions. CRISPR interference (CRISPRi) and activation (CRISPRa) techniques fuse dCas9 s with transcription repressors, such as the Krüppel associated box (KRAB) domain, or activators, to inhibit or promote transcription, respectively.<sup>25,26</sup> Fusions with DNA methyltransferases (DNMT) and DNA deaminases can be used to modify methylation patterns and specific bases. These variants greatly broaden the scope of CRISPR applications in treating diseases such as cancer, which result from a variety of mutations.

## Therapeutic potential of CRISPR in BC

CRISPR/Cas9 is a powerful tool for the study and treatment of various cancers, including BCs. Genes involved in cancer development are often categorised into two broad groups: proto-oncogenes, which promote cell growth and proliferation and, when mutated or activated drive tumour development; and tumour suppressor genes (TSGs), which are involved in DNA repair and control of cell growth, which when mutated or inactivated, lead to genomic instability and uncontrolled proliferation. Many different types of mutations, at the nucleotide, transcriptional, and epigenetic levels, may lead to their aberrant or inhibited expression. CRISPR/Cas9 and its variants can target mutations at each level and thus have great potential in treating BCs.

### Targeting oncogenes and TSGs

Genes encoding growth factors and their receptors, transcription factors (TFs), signalling transducers, and chromatin remodelling proteins have oncogenic potential.<sup>27</sup> CRISPR/Cas9 can be used to target these oncogenes directly, knocking them out and inhibiting cancer growth through various mechanisms. The technique has been successfully applied to knocking out both cellular and viral oncogenes in diverse cancer models, including leukaemia,<sup>28</sup> cervical cancer,<sup>29</sup> endometrial cancer,<sup>30</sup> and prostate cancer.<sup>31</sup> The *PI3KCA*, *HER2/ErbB2*, and *MYC* oncogenes have been implicated in BC.<sup>32</sup> Knockout of *HER2* has been observed to reduce the viability of *HER2*+ BT-474 and SKBR-3 cells.<sup>33</sup> Notably, targeting of *HER2* exon 12 produced a truncated protein with a dominant negative

function rather than knocking out protein expression, suggesting that knockout of all copies of an oncogene in cancer cells may not always be necessary to exert a therapeutic effect. *In vitro* CRISPR/Cas9-mediated knockout of the Lipocalin 2 (*Lcn2*) oncogene, implicated in BC growth and metastasis in TNBC cells, did not show reduced cell proliferation, but instead inhibited cell migration by suppressing epithelial to mesenchymal transition, while *in vivo* treatment led to significantly reduced tumour growth.<sup>34</sup>

Alternatively, oncogenes can be targeted indirectly, by inhibiting aberrant transcriptional programmes that lead to overexpression. The upregulation of the *MYC* oncogene, overexpressed in 30–50% of high grade BCs, is often mediated by super enhancers, regions surrounding the gene bound by enhancer elements that bind TFs.<sup>35–37</sup> TF binding can be impaired through CRISPR/Cas9-mediated mutagenesis or dCas9-DNMT-mediated methylation of the binding site, both of which have been observed to reduce *MYC* expression and cell proliferation *in vitro*.<sup>36</sup> CRISPRi strategies have also shown potential in suppressing oncogene expression in squamous cell carcinoma cells and may be applied to BC therapy.<sup>38</sup>

Mutations in multiple TSGs such as *PTEN*, *BRCA1* and *BRCA2* have been identified in BCs. These TSGs play central roles in maintaining genome integrity by directing repair of DSBs through HR and NHEJ, and by ensuring progression of replication forks and restarting stalled forks.<sup>39–41</sup> Restoring TSG function is more difficult than knocking out an oncogene.<sup>42</sup> TSG expression, which may be repressed by dysregulated TFs or hypermethylated promoters, can be promoted using CRISPR variants. Expression of the *PTEN* TSG, whose loss is associated with more aggressive BC, has been activated in TNBC SUM159 cells using a CRISPRa approach, fusing dCas9 with the VPR domain consisting of the transcriptional activators VP64, p65, and Rta.<sup>43,44</sup> Hypermethylation of TSGs, including *PTEN* and *BRCA1*, represses their expression.<sup>45–47</sup> Removal of methylation can be facilitated by fusion with ten-eleven translocation (TET) dioxygenases. These enzymes convert 5'-mC to 5-hydroxymethylcytosine (5-hmc), which is corrected to C during DNA replication, removing methylation.<sup>48,49</sup> dCas9-TET fusions have been used to demethylate the *BRCA1* gene *in vitro* in MCF-7 and HeLa cells, upregulating *BRCA1* expression and enhancing the cytotoxic effect of the chemotherapeutic Mitomycin-C.<sup>49</sup> Fusion of dCas9 with an R2-stemloop, a short RNA sequence that recruits the DNMT1 enzyme and inhibits its activity, has also shown potential as a demethylation strategy.<sup>50</sup>

CRISPR/Cas9-mediated HDR can be used to correct small mutations, such as single nucleotide polymorphisms (SNPs) or indels, that knock out TSGs. The *TP53* gene is estimated to be mutated in 30–35% of BCs and 80% of TNBCs.<sup>51</sup> While it is not a “pure” TSG, as it may undergo gain-of-function mutations that drive oncogenesis, *TP53* remains a prominent target for therapy.<sup>52</sup> Correction of the

*TP53* 414delC null mutation in PC-3 prostate cancer cells, leading to increased protein expression and apoptosis, has been facilitated using CRISPR, highlighting its potential for correcting mutations in BC.<sup>53</sup> Base editing techniques provide a more precise method of correcting SNPs than HDR, as they facilitate corrections without inducing a DSB, reducing the chance of NHEJ-mediated repair. These techniques conjugate dCas9 to cytidine or adenosine deaminases. Correction of the *TP53* Tyr163Cys mutation in HCC1954 BC cells has been achieved by fusing a Cas9 nickase with cytidine deaminase and uracil DNA glycosylase inhibitor (UGI) proteins, facilitating conversion of the mutant C-G pair to T-A.<sup>54</sup> Adenosine deaminases convert A to inosine, which bonds C, ultimately leading to replacement of an A-T bp with C-G.<sup>55</sup>

### Targeting DNA repair pathways

In addition to TSGs such as *BRCA1/2* and *PTEN*, genome integrity is also maintained by poly-(ADP-ribose) polymerase (PARP) enzymes, involved in single-stranded break (SSB) repair and restarting stalled replication forks.<sup>56,57</sup> Their inhibition impedes repair and replication, and has synthetic lethality with HR deficiency in *BRCA1/2*-deficient cancers. However, PARP knockout in these cancers may not be as effective as treatment with inhibitors, as the cytotoxic effect results from PARP trapping at the SSBs rather than from PARP downregulation.<sup>58–60</sup> Instead, PARP gene knockout may be used in conjunction with platinum-based chemotherapeutics that induce DNA damage. CRISPR targeting of the *PARP1* gene has been shown to enhance the cytotoxicity of cisplatin in ovarian cancer cells.<sup>61</sup> The gene also presents a therapeutic target in BC patients receiving concurrent chemotherapy, as co-treatment with PARP inhibitors and the platinum-based drugs has shown improved survival in metastatic TNBC patients.<sup>62</sup>

### Targeting the kinome

The kinome refers to kinase proteins involved in the phosphorylation of proteins and lipids.<sup>63</sup> Their dysregulation is a common feature of many cancers, including BC. The tyrosine kinase family, which phosphorylate tyrosine residues, includes transmembrane receptor and cytoplasmic kinases.<sup>64</sup> Well-studied oncogenes such as *HER2*, *PI3KCA*, and *FGFR* that are susceptible to knockout via CRISPR/Cas9, are members of this family.<sup>65</sup>

Cyclin-dependent kinases (CDKs) are serine/threonine kinases. They bind cyclins and regulate cell cycle progression through phosphorylation of the retinoblastoma (Rb) TSG, and transcription and RNA splicing through phosphorylation of RNA polymerase II and TFs.<sup>66</sup> CDK4/6 inhibitors have been approved for combination therapy for HR+ BCs and THZ inhibitors have been developed for transcriptional CDKs (tCDKs); however, the

development of drug resistance remains a potential issue.<sup>67,68</sup> CRISPR/Cas9 knockout of the cell cycle CDK2 has been observed to induce cell cycle arrest *in vitro* in cutaneous melanoma cells.<sup>69</sup> This CDK is a potential target for TNBC treatment, as it has been observed to promote tumorigenesis *in vivo*, with inhibition inducing ER expression in TNBC MDA-MB-231 cells, sensitising them to ER-targeted therapies.<sup>70</sup> Overactivity of tCDKs can lead to transcriptional addiction, where cancers become “addicted” to transcription of genes that drove the initial stages of tumour formation, but remain necessary for cancer cell survival after tumorigenesis.<sup>71</sup> Their knockout may thus induce tumour death. Knockout of tCDKs has identified CDK7 and CDK9 to be required for TNBC growth.<sup>72</sup> CDK7 dependence was observed to be TNBC-specific, with knockout leading to reduced proliferation *in vitro* and reduced tumour growth *in vivo*.

### Altering the epigenome

Epigenetic modifications are heritable changes to the DNA that do not involve changes to the nucleotide sequence. These modifications work together to regulate gene expression, and their dysregulation is thus a feature of tumorigenesis. The major mechanisms are DNA methylation, histone modification, and non-coding RNAs (ncRNA).

DNA methylation commonly occurs on the C of CpG repeats in promoter regions, called CpG islands, preventing TF binding and thus inhibiting gene expression.<sup>73</sup> The process is carried out by DNMT1, DNMT3A, and DNMT3B enzymes, which convert C to 5'-methylcytosine (5'-mC) and ensure methylation is maintained during cell division.<sup>74</sup> In addition to TSG hypermethylation, hypomethylation at the promoter level, facilitating expression of genes promoting tumour growth; and at the genome level, potentially destabilising chromosomes, have been observed in BCs.<sup>75–77</sup> These abnormal methylation patterns can be altered using dCas9 variants, as described above, or by inhibiting mutated or overexpressed DNMT and TET enzymes.<sup>78–80</sup> Current DNMT inhibitors are associated with adverse side effects, and their non-specific mechanism of action can induce global hypomethylation or demethylation of oncogenes, potentially promoting cancer growth.<sup>81,82</sup> RNAi-mediated knockdown of *DNMT1* has been observed to inhibit transformation without inducing excessive demethylation.<sup>81</sup> *DNMT1* knockout by CRISPR/Cas9 has shown significant anti-tumour activity in *in vivo* ovarian cancer models, and can thus potentially facilitate knockout of specific *DNMT* genes in BCs, inhibiting cancer growth with fewer side effects than DNMT inhibitors.<sup>83</sup> *TET1* has also been observed to be overexpressed in TNBC cells, inducing hypomethylation, with CRISPR knockout reducing cell migration and proliferation.<sup>84</sup>

Histone post-translational modifications in transcription start sites similarly influence DNA accessibility to TFs, by controlling whether the chromosomes are tightly bound,

forming heterochromatin with repressed TF binding; or loosely bound, forming euchromatin open for transcription.<sup>85</sup> Histone acetylation is associated with weakened DNA binding, promoting transcription, while methylation may increase or inhibit transcription.<sup>86</sup> Various aberrant modifications have been identified in BC that alter the chromatin state and gene expression patterns, resulting from dysregulation of histone-modifying enzymes, such as lysine-specific demethylase 1 (LSD1).<sup>87–92</sup> RNAi knockout of LSD1 has been observed to inhibit cellular proliferation in BC cell lines.<sup>90</sup> Histone-modifying enzymes also modify non-histone proteins, altering their activity and potentially promoting tumour growth.<sup>93–95</sup> Thus, overexpressed enzymes present a therapeutic target for CRISPR/Cas9 knockout. Alternatively, fusions of dCas9 with acetyltransferases,<sup>96</sup> deacetyltransferases,<sup>97</sup> methyltransferases,<sup>98,99</sup> and demethylases can potentially be used to correct abnormal modifications, repressing oncogenes and activating TSGs.

Most non-coding DNA is transcribed as ncRNA, some of which conduct essential housekeeping and regulatory functions. The most widely studied ncRNA are microRNA (miRNA) and long non-coding RNA (lncRNA). miRNA (~22 nt) regulate gene expression by binding complementary mRNA, leading to its degradation or repressed translation<sup>100</sup>; while lncRNA (>200 nt) alter chromatin states and TF binding, and influence mRNA and miRNA activity.<sup>101</sup> Their dysregulated activity has been associated with chemoresistance and metastasis in BCs.<sup>102–106</sup> However, their knockdown by RNAi is inhibited by the short length of miRNAs, and the nuclear localisation of some lncRNAs.<sup>107,108</sup> CRISPR/Cas9 knockout of lncRNA and miRNA has been observed to impair growth and invasion in bladder, ovarian, and hepatocellular carcinoma models, and can potentially be applied to knockout in BC.<sup>107,109,110</sup> Moreover, CRISPR has been observed to induce long-term knockouts of miRNA *in vitro* in colon cancer cells.<sup>108</sup> However, NHEJ may not efficiently knockout ncRNAs, as non-coding sequences may tolerate indels.<sup>111</sup> This may be overcome by using multiple sgRNA to delete the gene, a strategy which has shown potential for lncRNA knockout.<sup>112,113</sup> CRISPRi has also shown potential for inhibition of ncRNA transcription in a study which identified the *PVT1* promoter for the *PVT1* lncRNA to act as a tumour suppressor, and can thus potentially be used to inhibit oncogenic ncRNA.<sup>114</sup> Type VI Cas13 systems may also be exploited to target and cleave ncRNA.

### Reversing drug resistance

BCs may develop resistance to chemotherapeutics through a variety of mechanisms. Resistance in HR+ and HER+ BCs may result from the loss of ER $\alpha$  and HER2 expression, often resulting from epigenetic changes that silence these genes.<sup>115,116</sup> Multiple SNPs associated with resistance, such as ER $\alpha$  Tyr537Ser and Asp538Gly, which drive

constitutive expression by promoting interactions with coactivators, and HER2 mutations Lys753Glu and Leu755-Ser, have also been associated with resistance to lapatinib and trastuzumab.<sup>117–120</sup> Resistance to PARP inhibitors may result from *BRCA* reactivation or *PARP1* point mutations.<sup>121,122</sup> These mutations can be altered using CRISPR/Cas9 HDR, or the previously described Cas9 variants, conjugated to epigenetic modulators or base editors, to re-sensitise tumours to therapy.

Multidrug resistance is often mediated by overexpression of the ATP-binding cassette (ABC) transporters that remove drugs from cancer cells before therapeutically active concentrations can accumulate. The P-glycoprotein (P-gp), Multidrug Resistance-Associated Protein 1 (MRP1), and Breast Cancer Resistance Protein (BCRP) transporters have been recognised as playing a major role in resistance.<sup>123</sup> Knockout of these efflux pumps facilitates re-sensitisation to existing drugs and avoids the need to develop new therapies.<sup>124</sup> CRISPR-mediated knockout of P-gp has been observed to increase chemosensitivity of A2780/ADR ovarian cancer cells, and increase the intracellular doxorubicin concentrations in resistant MCF-7/ADR cells, leading to increased cell death following treatment compared to unedited cells.<sup>124,125</sup>

### Immunotherapy

Immunotherapy involves boosting the immune response to tumour cells. Cancers may avoid immune responses through overexpression of immune checkpoint proteins, normally responsible for preventing autoimmune responses.<sup>126</sup> These proteins bind receptors on the surface of immune cells, averting immune attack. The expression of various checkpoint proteins has been observed in BCs and particularly in TNBC, including CD155 and programmed cell death-ligand 1 (PD-L1), which recognises the programmed cell death 1 (PD-1) receptor on immune cells.<sup>127–130</sup> CRISPR/Cas9 knockout of either PD-L1 or its receptor may trigger an immune response against the tumour.<sup>131,132</sup> CRISPR knockout of CDK5 has also been shown to downregulate PD-L1 expression, inhibiting tumour growth *in vitro* and *in vivo*.<sup>133,134</sup> shRNA-mediated knockdown of CD155 has also been observed to inhibit the growth of *in vitro* and *in vivo* BC models, highlighting its potential in BC therapy.<sup>135</sup>

Knockdown of checkpoint proteins may be used to enhance the efficacy of chimeric antigen receptor (CAR) T-cells, which express CARs recognising tumour-associated antigens (TAAs).<sup>136</sup> Multiple TAAs, such as HER2, mucin1, and TEM8 have shown potential as targets for BC CAR T-cell therapy.<sup>137–139</sup> CRISPR/Cas9 knockout of PD-1 has been shown to enhance the anti-cancer activity of CAR T-cells targeting mesothelin, overexpressed in TNBC BT-459 cells.<sup>140</sup> However, T-cells must be isolated from the patient and edited *ex vivo* in a laborious and lengthy process. Universal T-cells eliminate the need for

isolation; however, class I human leucocyte antigens (HLA) and T-cell receptors (TCR) present on the surface of donor T-cells must be removed to prevent graft-vs-host disease.<sup>141</sup> CRISPR/Cas9-mediated HDR allows for simultaneous knockout of TCRs and/or HLAs and knockin of CAR-encoding genes. Introduction of the anti-CD19 CAR into the TCR locus has been shown using CRISPR, leading to effective CAR expression while avoiding T-cell exhaustion.<sup>142</sup> Multiplex strategies, allowing simultaneous knockout of TCRs, beta-2 microglobulin (B2 M), a subunit of HLA-I, and other proteins including PD-1 and CTLA-4, have also been used to generate allogeneic CAR T-cells with enhanced anti-cancer activity.<sup>141,143,144</sup>

### Targeting cellular adhesion

Integrins are transmembrane proteins that function in cell adhesion to the extracellular matrix (ECM).<sup>145</sup> When dysregulated, they play important roles in both tumour development and metastasis, by promoting cell motility, growth and survival, modifying the ECM to promote growth, and facilitating survival of circulating cancer cells and metastatic colonisation.<sup>145</sup> CRISPR knockout of these integrins may thus reduce the tumour-forming and metastatic potential of BC cells, as shown by knockout of integrin  $\alpha 5$  (*ITGA5*), an integrin that promotes tumour cell migration and metastasis to the lymph nodes and lungs.<sup>146,147</sup>

### Delivery of CRISPR/Cas9

Therapeutic efficacy of CRISPR/Cas9 is dependent on its ability to reach target cells with minimal biodegradation. The Cas9 and sgRNA may be delivered in the form of the ribonucleoprotein (RNP), a plasmid, or as a combination of Cas9 mRNA and sgRNA. Plasmids are stable and easily synthesised, and can simultaneously encode and deliver all required components. However, their large size, with the  $\sim 4.2$  kbp spCas9 gene, and strong negative charge hinder delivery.<sup>148</sup> Moreover, plasmids must first undergo transcription and translation, with expression continuing for prolonged periods after transfection, leading to delayed editing and increased off-targets compared to RNP delivery.<sup>149</sup> In contrast, delivery of the RNP produces rapid therapeutic effects, and its relatively short expression time before protease degradation reduces the chance of off-targets. However, the large protein size and the net negative charge of the complex may interfere with cellular uptake.<sup>150</sup> Delivery of the mRNA and sgRNA similarly avoids the need for nuclear localisation and leads to transient Cas9 expression with reduced off-target effects.<sup>151</sup> However, the instability of RNA and the long Cas9 mRNA length of  $\sim 4500$  nt complicates delivery.<sup>152</sup>

Delivery methods can be broadly categorised as physical, viral or non-viral. Physical methods include electroporation, hydrodynamic injection, and microinjection. However, these techniques are often difficult to apply

*in vivo* and may damage cells.<sup>153</sup> Viral vectors have seen widespread use due to their high transfection efficiencies; however, issues such as their limited packaging size, difficult synthesis, and immunogenic and carcinogenic risks have resulted in a shift to non-viral vectors, and nanoparticles (NP) in particular.<sup>154,155</sup> NPs, ranging from 1–100 nm in size, have shown great potential as gene and drug delivery vehicles due to their lower immunogenicity, tunable synthesis, and large loading capacity. Their large surface area-to-volume ratio allows for coating with polymers that facilitate CRISPR/Cas9 binding and protection, and functionalisation with compounds that promote targeting and increased circulation times.<sup>156</sup> Inorganic NPs, composed of metals, magnetic compounds, selenium and silica, have been widely investigated as delivery vehicles for cancer therapy. Gold nanomaterials are among the most popular and have the potential as carriers for CRISPR/Cas9-mediated genome editing.

### Gold nanomaterials

Gold nanomaterials display many unique optical and physiochemical properties that facilitate their use as imaging agents, biosensors, and vectors. Among their attractive properties are their small size, and facile synthesis and functionalisation. Their highly tunable synthesis allows for modification of NP size and shape to optimise characteristics for therapy. Moreover, the ability of gold nanomaterials to convert absorbed light energy into heat following irradiation with near infrared light, leading to thermal ablation of surrounding tumour tissue, permits their use as photothermal therapy agents.<sup>157</sup>

Spherical gold nanoparticles (AuNPs) are among the most popular NPs for gene and drug delivery. Their unique optical properties include their localised surface plasmon resonance (LSPR), the phenomenon where free electrons on the NP surface oscillate in response to light exposure. The electrons absorb and scatter light energy, facilitating the use of AuNP as imaging agents.<sup>158</sup> AuNP are most commonly synthesised using the citrate reduction method, in which chloroauric acid ( $\text{HAuCl}_4$ ) is reduced by trisodium citrate, to produce citrate-capped AuNPs 10–20 nm in diameter and with a net negative charge.<sup>159,160</sup> This method can be easily modified by varying the ratios of reagents to produce AuNPs of 15–150 nm in diameter.<sup>161</sup>

Gold nanoclusters (AuNCs) have more recently been investigated as carriers, as their advantageous qualities facilitate their use as simultaneous delivery and imaging agents. These ultrasmall ( $< 2$  nm) NPs consist of only a few to tens of Au atoms, imparting them with characteristics unique from conventional AuNPs, such as the absence of SPR and size-dependent fluorescence.<sup>162</sup> They display a large Stokes shift, good photostability, resistance to photobleaching, and low toxicity compared to conventional fluorophores such as dyes and quantum dots.<sup>163,164</sup> These optical properties have led to AuNCs being widely studied

as sensors and cellular imaging agents.<sup>165–168</sup> AuNCs are commonly synthesised through reduction of HAuCl<sub>4</sub> with biomolecules such as peptides,<sup>169,170</sup> proteins,<sup>168,171</sup> and polymers.<sup>164,172,173</sup> Glutathione (GSH) is often used as a capping agent in a simple and eco-friendly synthesis process requiring no additional reducing agents, to produce biocompatible AuNCs with a relatively high quantum yield.<sup>174,175</sup>

For use as delivery agents, inorganic NPs are often coated with polymers and conjugated to various ligands to enhance stability, biocompatibility, and transfection. As-synthesised capped AuNPs and AuNCs can easily bond with cationic polymers, such as chitosan and polyethyleneimine (PEI), facilitating interactions with anionic cell membrane components and anionic groups of other ligands. Functionalisation can alternatively exploit the semi-covalent interactions between sulphur and gold to bind thiol groups, allowing conjugation with peptides, proteins, and thiol-modified nucleic acids. The hydrophilic polymer polyethylene glycol (PEG) is widely used to inhibit interactions with plasma proteins, thus preventing uptake by the reticuloendothelial system and increasing circulation times and bioavailability. Specific tumour accumulation may be achieved through functionalisation with targeting ligands that bind receptors on the surface of the target cells, facilitating uptake by receptor-mediated endocytosis. In addition to HRs and HER2, BCs have been shown to overexpress folate and transferrin receptors, and their ligands are commonly attached to NPs for targeting.<sup>176–178</sup> The difficulties associated with nuclear localisation can be overcome through conjugation with nuclear localisation signals (NLS), peptides that promote interactions with the nuclear pores.<sup>179</sup> The cationic HIV-1 trans-activator of transcription (TAT) peptide is the most popular NLS, and also acts as a cell-penetrating peptide (CPP) promoting transport across the cell membrane.<sup>180</sup>

### Gold nanomaterials for CRISPR delivery

AuNPs and AuNCs are relatively novel vehicles for CRISPR delivery and have not been exploited, compared to viral vectors and organic lipid and polymeric NPs. While they have not been used to mediate genome editing of BC, they have shown their potential in studies delivering CRISPR components to various malignant cells *in vivo* and *in vitro*, achieving editing efficiencies comparable to other delivery systems.

Arginine-coated AuNPs have been shown capable of delivering RNPs, and facilitating *AASV1* and *PTEN* knock-down *in vitro*.<sup>181,182</sup> AuNPs were able to enter cells via a membrane fusion process, avoiding potential lysosomal degradation, leading to editing efficiencies of ~20–30%. RNP binding has been achieved by binding a thiol-modified crRNA to the AuNP, and reacting the resulting AuNP-crRNA with Cas9 to form the RNP.<sup>183</sup> Delivery of the large CRISPR/Cas9 plasmid targeting the *Plk-1* gene

has been described using lipid-encapsulated TAT-coated AuNPs in *in vitro* and *in vivo* melanoma tumours.<sup>184</sup> Controlled irradiation with 514 nm light following transfection was used to promote plasmid release without inducing cell death, producing a synergistic effect in tumour ablation.

The delivery of an HDR template can be achieved in multiple ways using AuNPs, and may be performed simultaneously with the Cas9 and sgRNA due to AuNPs' large loading capacity. A layer-by-layer approach has been used, in which the ssDNA template was complexed with a PEI layer coating the AuNP-RNP.<sup>183</sup> Another study utilised AuNPs complexed with thiol-linked ssDNA molecules that bound the donor DNA and RNP, and coated with the polymer PAsp(DET).<sup>185</sup> These AuNPs achieved an *in vitro* HDR frequency of 3–4%, significantly higher than lipofectamine transfection, and facilitated *in vivo* correction of the dystrophin gene.

The potential of AuNCs for CRISPR delivery has been highlighted in several studies. Lipid-encapsulated TAT-coated AuNCs carrying both the Cas9 protein and *Plk1* sgRNA encoded as a plasmid produced editing efficiencies of 26.2% *in vitro*, and significantly inhibited tumour growth *in vivo* in melanoma models.<sup>186</sup> A proof-of-concept study showed that GSH-AuNCs can self-assemble with Cas9 proteins at physiological pH, producing spCas9-AuNC complexes which dissociate under acidic pH.<sup>187</sup> Transfection with these complexes and sgRNA targeting the viral E6 oncogene in HeLa cells produced editing efficiencies of 34% and significantly reduced protein expression. These Cas9-AuNCs could facilitate genome editing in systems where the components (Cas9, sgRNA, template for HDR) are delivered separately.

While AuNCs have not been exploited to deliver CRISPR nucleic acids, they have shown potential as gene delivery agents. PEI-coated AuNCs have facilitated improved delivery of the EGFP gene compared to PEI in a proof-of-concept study.<sup>172</sup> AuNCs capped with the positive tetrapeptide K4 have also been shown capable of assembling with DNA and RNA.<sup>188</sup>

### Conclusion

The use of CRISPR/Cas9 has exploded since it was first adapted for use in genome editing in 2013.<sup>189,190</sup> Its simplicity, ease of use, and versatility have led to its widespread use in all aspects of cancer research. CRISPR/Cas9 has the ability to target any oncogenic mutation through simple redesign of an RNA sequence, and provides a means of exploiting the molecular heterogeneity of BCs to tailor therapies to specific individuals, thus avoiding treatment with ineffective or cytotoxic drugs. Personalised treatments for BC, targeting the hormone and HER2 receptors, have significantly improved the outcomes of many patients. However, CRISPR now allows for extension of these tailored treatments to individuals suffering from TNBC.

However, before the progression of CRISPR therapies into clinical settings, their safety must be thoroughly evaluated to avoid adverse side effects. Off-target cleavage is a concern, as mismatches outside the PAM and seed sequences in the sgRNA are tolerated.<sup>191</sup> The use of bioinformatics tools that evaluate sgRNA and identify possible off-targets and spCas9 variants with improved fidelity reduces non-specific activity.<sup>192–194</sup> Studies have also observed innate and adaptive immunity against the spCas9 protein since it originates from pathogenic bacteria.<sup>195,196</sup> Cas9-induced immune responses may also lead to responses being raised against the edited cells. Further studies are required to assess the likelihood of adverse immune responses as, thus far, the majority of CRISPR clinical trials (as listed on U.S. National Library of Medicine's site, ClinicalTrials.gov) focus on *ex vivo* editing of T-cells. However, an ongoing trial (NCT03872479) is assessing the safety of adenoviral-packaged CRISPR/Cas9 correcting the *CEP290* gene, in the first trial to attempt editing in humans.<sup>197,198</sup>

Efficient and selective delivery of therapeutics is a universal issue faced in gene and drug therapy. However, the development of CRISPR delivery systems will benefit from the extensive research that has already been conducted. AuNPs have proven themselves as delivery agents for gene therapy, and show great promise for the delivery of CRISPR/Cas9 systems. Their capacity for multifunctionalisation allows for efficient delivery of all CRISPR/Cas9 formats. Before their clinical translation, the biodistribution and fate of various sizes and shapes of AuNPs and AuNCs must be clearly determined, as gold is non-biodegradable.

Overall, the CRISPR/Cas9 system can be applied to the treatment of BCs to develop highly effective precision medicines. With further optimisation, these systems may produce treatments that overcome the limitations faced by current therapies and significantly improve the survival of patients suffering from BC.

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### ORCID iD

Moganavelli Singh  <https://orcid.org/0000-0002-9985-6567>

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