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Review Article

Exploring the Advantages and Limitations of CRISPR-Cas in Breast Cancer

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Abstract

Breast cancer (BC) is the type of cancer with the highest incidence and mortality rates in women in the world. In the treatment of this neoplasia, several therapies are applied, including radiotherapy, hormonal therapy, chemotherapy, and biological therapy. Although most patients respond to these types of therapy, some patients over time, develop resistance or eventually relapse. Considering the above, future therapeutic concepts in BC are being directed at individualization of therapy and escalation of treatment based on tumor biology through the use of gene therapy. In this regard, a new genomic engineering technology, called the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein-9 (Cas9), has acquired great importance in recent years, as a potential gene editing tool, extensively applied in human cancer research and cancer treatment. The aim of this review was to describe the advantages, limitations, and applications of CRISPR gene editing technology in BC treatment. Our review emphasizes the innovative facets and profound importance of CRISPR gene editing technology within the BC treatment landscape. Additionally, it provides valuable information to consider when evaluating the risks associated with the implementation of CRISPR-Cas9 technology in BC therapy.

Keywords: Breast cancer; Gene therapy; Resistance to therapy; Therapeutic targets.

Abbreviations: ABC, ATP binding cassette; *ACKR3*, Atypical Chemokine Receptor 3; AKT, protein kinase B; AKT, protein kinase B; *ATP8B3*, ATPase Phospholipid Transporting 8B3; BC, breast cancer; BCL2, B-cell lymphoma 2; *BRCA1*, Breast cancer type 1; Cas9, CRISPR associated protein-9; C-MYC, MYC Proto-Oncogene; CRISPR, clustered regularly interspaced short palindromic repeat; *CXCR4*, C-X-C Motif Chemokine Receptor 4; dCas9, deactivated Cas9; DSBs, DNA double-strand breaks; *DSTYK*, Dual Serine/Threonine And Tyrosine Protein Kinase; EGFR, Epidermal Growth Factor Receptor; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FOXO1, Fork head Box O1; *FOXR2*, Fork head Box R2; *FRG2*, FSHD Region Gene 2; GLUT4, Glucose transporter type 4; GSK3A, Glycogen Synthase Kinase 3 alpha; GSK3B, Glycogen Synthase Kinase 3 beta; HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; HER4, human epidermal growth factor receptor 4; HR, homologous recombination; *MAP3K11*, Mitogen-Activated Protein Kinase Kinase Kinase 11; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; *MDR1*, multidrug resistance protein 1; MEK, mitogen-activated protein kinase kinase; MN, micronuclei; mTOR, Mechanistic Target Of Rapamycin Kinase; mTOR2, Mechanistic Target Of Rapamycin Kinase; *MYC*, MYC Proto-Oncogene; NHEJ, non-homologous DNA end joining; *OPN*, osteopontin gene; *PARP1*, Poly [ADP-ribose] polymerase 1; PI3K, phosphatidylinositol 3-kinase; PIP2, Phosphatidylinositol 4,5-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; PR, progesterone receptor; PTEN, Phosphatase and Tensin homolog; PTEN, Phosphatase and Tensin homolog; RAF, Raf Proto-Oncogene, Serine/Threonine Kinase; RAS, Rat sarcoma virus protein; TNBC, triple negative breast cancer; TNBC, triple negative breast cancer; *TP53*, Tumor Protein P53; TSC1/2, TSC Complex Subunit ½; TSGs, tumor suppressor genes; VEGF, Vascular Endothelial Growth Factor; VPR, transactivator VP64-p65-Rta.

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Introduction

Breast cancer (BC) is the type of cancer with the highest incidence and mortality rates in women in the world, being therefore, a worldwide concern considering the high rates of incidence (47.8%) and mortality (13.6%) recently reported. Specifically, in 2020, 2,300,000 new cases (11.7%) and 684,996 deaths from this neoplasia were reported. Therefore, it is the type of cancer with the highest incidence and mortality rates in women.[1] At the molecular level, BC is a very heterogeneous disease, being classified by molecular subtypes based mainly on the presence of hormone receptors (estrogen-ER, and progesterone receptors-PR), human epidermal growth factor receptor 2 (HER2), and/or *BRCA* mutations. Depending on the tumor subtypes, treatment strategies have been developed that generally include: endocrine therapy (for ER-positive BC patients), poly(ADP-ribose) polymerase (for *BRCA* mutation carriers), anti-HER2 therapy (for HER2 positive BC patients), chemotherapy (for triple negative breast cancer—TNBC), and immunotherapy, among others. However, despite the development of several new technologies and the emergence of new classes of anticancer drugs, current clinicopathological, immunohistochemical and molecular markers, leave a significant number of patients at risk of side effects, over-treatment or eventually the development of resistance. Considering the above, future therapeutic concepts in BC are being directed at individualization of therapy and escala-

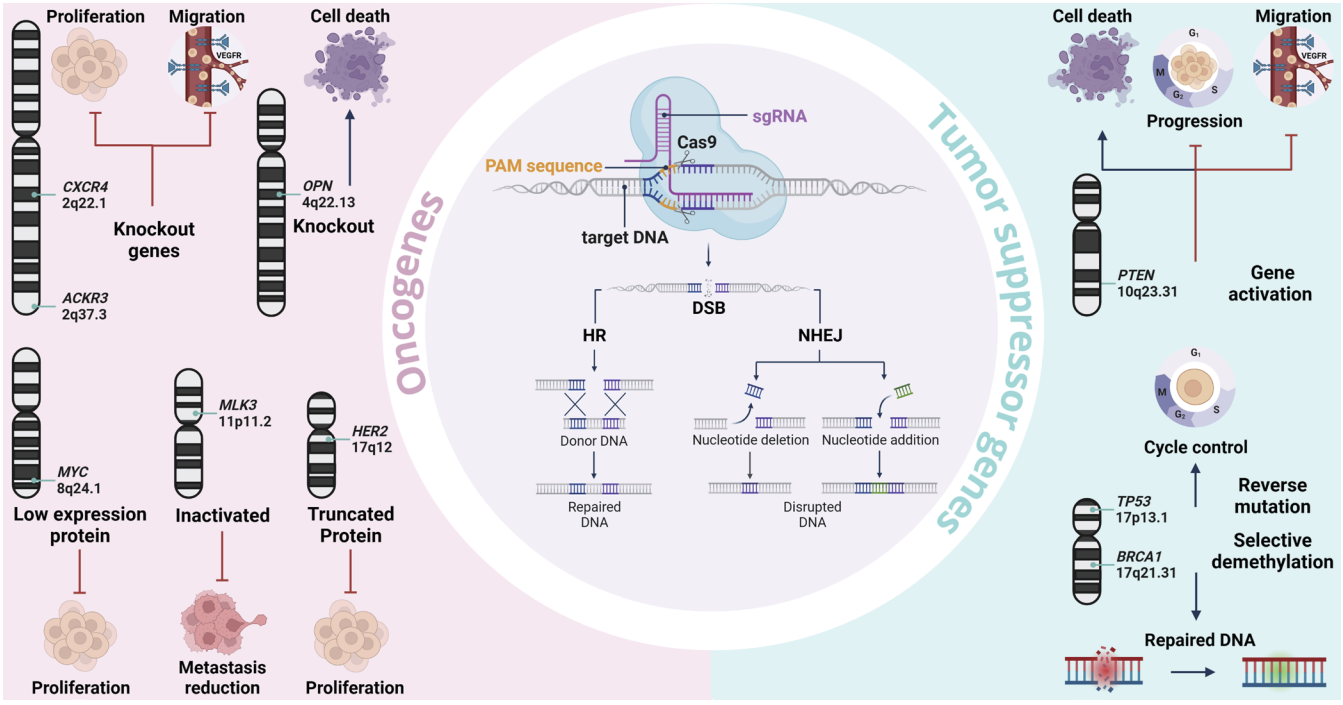

Fig. 1. CRISPR mechanism of action and potentials target genes. CRISPR "spacer" sequences are transcribed into short RNA sequences (single guide RNA- sgRNA) capable of guiding the system toward matching or complementary DNA sequences. When the target DNA is found, Cas9 binds to the DNA and cuts it, generating DNA double-strand breaks (DSBs). Then, the DSB repair pathway, including homologous recombination (HR) and non-homologous DNA end joining (NHEJ), is activated to repair DSBs. The error-prone NHEJ pathway can lead to random indel mutations in the binding site. Indel mutations that occur within the coding region of a gene, can lead to gene knockout. CRISPR-Cas9 has been used in breast cancer research to edit oncogenes and tumor suppressors genes, leading to their inactivation or activation, respectively. *ACKR3*, Atypical Chemokine Receptor 3; *BRCA1*, Breast cancer type 1; Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeat; *CXCR4*, C-X-C Motif Chemokine Receptor 4; DSBs, DNA double-strand breaks; HR, homologous recombination; *HER2*, human epidermal growth factor receptor 2; MYC, MYC Proto-Oncogene; NHEJ, non-homologous DNA end joining; *OPN*, osteopontin gene; PAM, Protospacer Adjacent Motif; *PTEN*, Phosphatase and Tensin homolog; sgRNA, single guide RNA; *TP53*, Tumor Protein P53.

tion of treatment based on tumor biology through the use of gene therapy. In fact, gene therapy has become a potential tool to correct defective genes and treat various types of cancer.

In recent years, a novel targeted genome editing technology, known as clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein-9 (Cas9), is being extensively applied in human cancer research and cancer treatment. In this review, we describe the advantages, limitations, and applications of CRISPR gene editing technology in BC treatment. By exploring the intricacies of CRISPR-Cas9 technology and its potential implications in BC therapeutics, this review provides valuable insights that are crucial for understanding its scientific significance and clinical relevance. Moreover, it offers a comprehensive analysis of the risks associated with the implementation of CRISPR-Cas9 technology in BC treatment.

CRISPR-Cas9 technology

CRISPR-Cas technology has developed rapidly and is now an efficient alternative for cancer treatment. CRISPR is naturally occurring genome editing systems found in bacteria. The system serves as a genetic memory that helps the cell detect and destroy invading viruses.[2] CRISPR-Cas is composed of two components: CRISPR repeat-spacer arrays and a set of CRISPR-associated (Cas) genes, which encode Cas proteins with endonuclease activity. According to the involvement of the different Cas proteins within the CRISPR framework, CRISPR-Cas technology has been classified into 2 classes (Class 1 and Class 2), 6 types (I to VI) and 33 subtypes.[3,4] In general, the system works as follows: CRISPR "spacer" sequences, are transcribed into short RNA sequences (single guide RNA- sgRNA) capable of guiding the system towards matching or complementary DNA sequences. When the target DNA is found, Cas9 binds to the DNA and cuts it, generating DNA double-strand breaks (DSBs). Then, DNA double-strand break repair pathway, including homologous recombination (HR) and non-homologous DNA end joining (NHEJ), is activated to repair DSBs.[5–7] (Fig. 1). Considering that activation mainly of NHEJ repair pathways is error-prone, chromosomal rearrangements and large deletions, as consequences of target activity, have been reported.[8,9]

Although the apparent advantages of the use of CRISPR-Cas9 have been elucidated, its limitations have also been indicated, including: cancer risk, immunological reactions and ethical concerns, among others.

CRISPR-Cas9 technology in the editing of genes involved in BC

BC is characterized by being a very heterogeneous neoplasm, which is governed by genes that control proliferation, apoptosis, genomic instability, replicative immortality, cell metabolism, invasion and metastasis, among others.[10] Despite the development of several new technologies and the emergence of new classes of an-

Table 1. Application of CRISPR-Cas9 in the treatment of Breast Cancer by targeting different genes.

Target gene	CRISPR-Cas9 approach	Effects	References
HER2	Induction of a frameshift mutation in exons 5, 10 and 12	Cell growth inhibition in HER2 positive cell lines	11
MYC	Epigenetic modifications of MYC regulatory elements. Elimination of the MYC enhancer docking site	Cell proliferation reduction and MYC decreased expression levels	12
	Alteration of MYC binding sites (E-boxes)	Alterations in MYC binding to target genes, in target gene expression, in tumor growth *in vivo* and in cell proliferation *in vitro*	13
CXCR4	Gene knockout	Cell proliferation and cell invasion reduction	14
ACKR3	Gene knockout	Cell proliferation and cell invasion reduction	14
MAP3K11	Gene depletion	Metastasis reduction	15
OPN	Gene knockout	*OPN* gene expression reduction. Apoptosis and cell viability decrease	16
TP53	Reversion of a missense mutation	Base substitution in the *TP53* gene	18
PTEN	Activation of gene expression	Increased PTEN expression, and repression of the AKT, mTOR and MAPK signaling pathways.	19
BRCA1	Decreased DNA methylation	Transcriptional up-regulation of *BRCA1* gene	17
MDR1	Gene disruption	Increased response to doxorubicin and cell death	20
PARP1	Gene depletion	Sensitization of TNBC cells to chemotherapeutic drugs: doxorubicin, gemcitabine and docetaxel,	21
DSTYK	Gene deletion	Activation of apoptosis in chemoresistant cells in *in vitro* and *in vivo* models.	22
ATP8B3	Gene knockout	Increased resistance to paclitaxel in TNBC	23
FOXR2	Gene knockout	Increased resistance to paclitaxel in TNBC	23
FRG2	Gene knockout	Increased resistance to paclitaxel in TNBC	23

ACKR3, Atypical Chemokine Receptor 3; AKT, protein kinase B; *ATP8B3*, ATPase Phospholipid Transporting 8B3; *BRCA1*, Breast cancer type 1; Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeat; *CXCR4*, C-X-C Motif Chemokine Receptor 4; *DSTYK*, Dual Serine/Threonine And Tyrosine Protein Kinase; *FOXR2*, Fork head Box R2; *FRG2*, FSHD Region Gene 2; *HER2*, human epidermal growth factor receptor 2; MAPK, mitogen-activated protein kinase; *MAP3K11*, Mitogen-Activated Protein Kinase Kinase Kinase 11; *MDR1*, multidrug resistance protein 1; *MYC*, MYC Proto-Oncogene; mTOR, Mechanistic Target Of Rapamycin Kinase; *OPN*, osteopontin gene; *PARP1*, Poly [ADP-ribose] polymerase 1; PTEN, Phosphatase and Tensin homolog; TNBC, triple negative breast cancer; *TP53*, Tumor Protein P53.

ticancer drugs, resistance to therapy, side effects, overtreatment or, eventually, the development of resistance, continue to be the biggest concerns for the correct management of BC patients. Therefore, in recent years, a large amount of research has been directed at understanding the biological and molecular mechanisms that lead to resistance, as well as the design of personalized therapies that allow such resistance to be overcome. In fact, the investigations carried out to date, have made it possible to identify the role played by oncogenes and tumor suppressor genes (TSGs), in the resistance to therapy in BC. Taking the above into account, both, oncogenes and TSGs, constitute potential therapeutic targets for the treatment of BC, using CRISPR (Table 1 and Fig. 1).[11–23]

Targeting oncogenes

Proto-oncogenes are normal genes involved in cell proliferation and differentiation. Among the mechanisms associated with the conversion of proto-oncogenes into oncogenes are amplifications, translocations and mutations. These types of alterations, lead to the permanent activation of oncogenes, and therefore to the alteration of the cellular functions in which they participate,[24] promoting tumorigenesis.[25] In BC, oncogenes that have often been found to be deregulated include: *HER2*, *MYC, CXCR4*, *ACKR3, MAP3K11 (MLK3)* and *OPN*, among others. Therefore, CRISPR-Cas9 can be used to target directly these oncogenes, inhibiting cell proliferation and tumorigenicity.

HER2 gene

The *HER2* gene is amplified in approximately 15% of primary BC.[26] *HER2* amplification/overexpression, has been identified as oncogenic driver and potential therapeutic target in BC patients.[27] In fact, *HER2* gene amplification is used for both, the prognosis and guide treatment with trastuzumab in BC patients.[28,29] However, although anti-HER2 therapies have dramatically improved the prognosis of cancers that overexpress *HER2*, some BC patients relapse or develop resistance over time. Therefore, it is necessary to use treatments that allow such resistance to be overcome. In this regard, some studies have been directed to the use of CRISPR-Cas9 technology to target the *HER2* gene in HER2 amplified BC cell lines. The results of one of such studies, show that CRISPR-Cas9-mediated HER2 targeting, inhibited cell growth in HER2 positive cell lines. Additional analyzes showed that the inhibition of cell growth of CRISPR-Cas9 on the cell lines, was related to the induction of a frameshift mutation in exon 12 of HER2, which

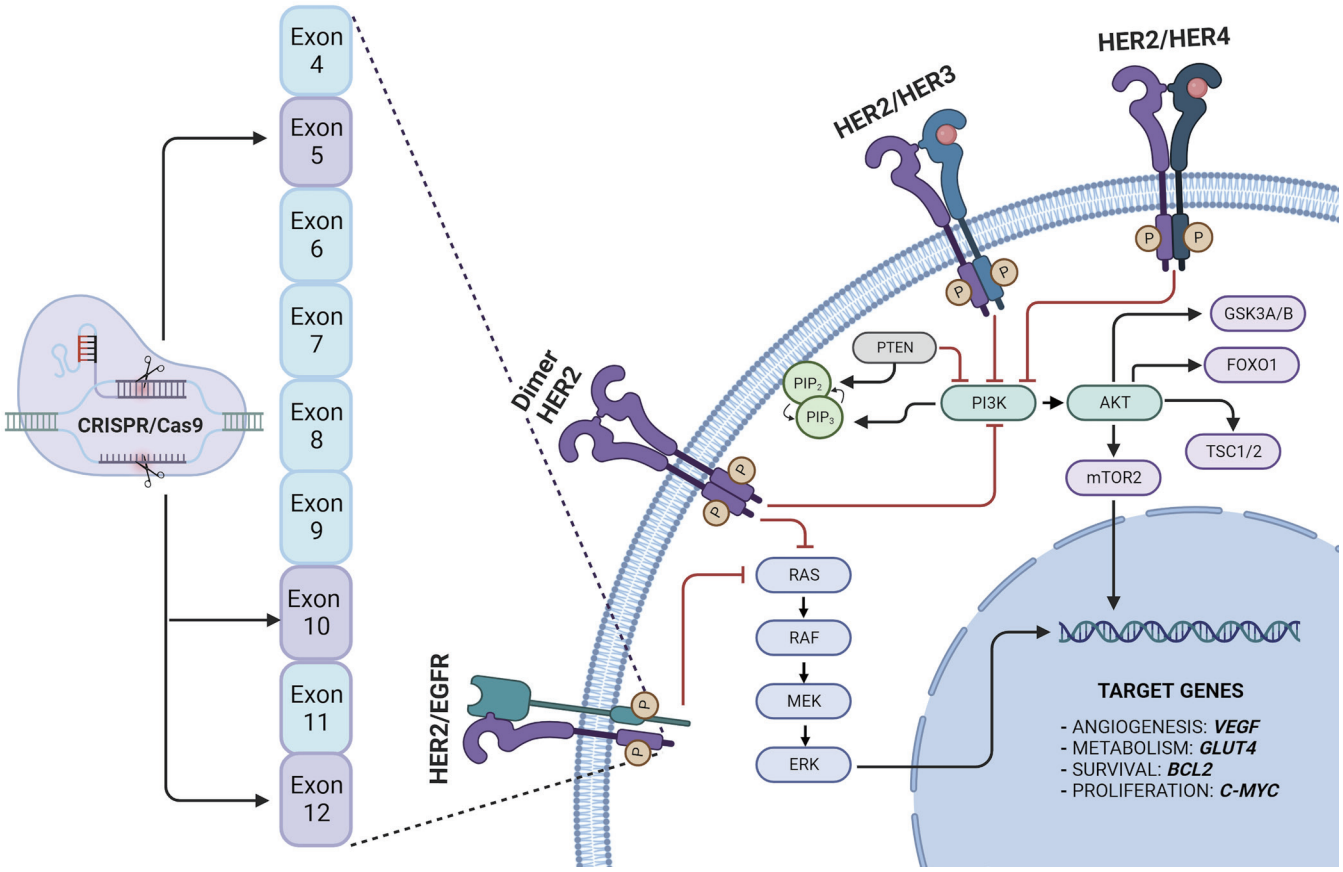

Fig. 2. CRISPR/Cas9 as a gene editing tool in HER2+ Breast Cancer. CRISPR/cas9 induces a frameshift mutation in exons 5, 10, and 12 of HER2, generating a truncated HER2mut protein. The mutated HER2 protein leads to growth inhibition and negative regulation of the endogenous MAPK-ERK and PI3K signaling pathways. These signaling pathways are associated with the activation of target genes related to some hallmarks of cancer. AKT, protein kinase B; *BCL2*, B-cell lymphoma 2; Cas9, CRISPR associated protein 9; *C-MYC*, MYC Proto-Oncogene; ERK, extracellular signal-regulated kinase; *FOXO1*, Fork head Box O1; *GLUT4*, Glucose transporter type 4; CRISPR, clustered regularly interspaced short palindromic repeat; EGFR, Epidermal Growth Factor Receptor; GSK3A, Glycogen Synthase Kinase 3 alpha; GSK3B, Glycogen Synthase Kinase 3 beta; HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; HER4, human epidermal growth factor receptor 4; MEK, mitogen-activated protein kinase kinase; mTOR2, Mechanistic Target Of Rapamycin Kinase; PIP2, Phosphatidylinositol 4,5-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphatidylinositol 3-kinase; PTEN, Phosphatase and Tensin homolog; RAF, Raf Proto-Oncogene, Serine/Threonine Kinase; RAS, Rat sarcoma virus protein; TSC1/2, TSC Complex Subunit ½; *VEGF*, Vascular Endothelial Growth Factor.

led to the production of a truncated HER2mut protein (Fig. 1). Additionally, it was indicated that the inhibition of cell proliferation was accompanied by the suppression of the MAPK-ERK signaling pathway (Fig. 2).[30] This study showed that, targeting exons 5, 10 and 12 of *HER2* using CRISPR-Cas9, led to the inhibition of cell growth of two HER2+ BC cell lines (BT474 and SKBR3), but not in HER2- BC cells (MCF7).[30] Exons 5, 10, and 12, encode parts of the extracellular domain of the HER2 protein.[11] Specifically, in this study, Cas9 together with three gRNAs, were introduced into HER2+ (BT474 and SKBR3) and HER2- BC (MCF7) cell lines, observing suppression of cell growth in the HER2+ cell lines but not in the HER2- cell line. These results indicate that the use of CRISPR-Cas9 to target HER2, results in a decrease in cell growth in HER2+ BC cell lines.[30]

MYC gene

MYC is an oncogene frequently amplified in BC and associated with apoptosis inhibition and activation of cell proliferation. The application of CRISPR-Cas9 technology in c*MYC* gene editing, has been directed at the transcriptional deregulation of *MYC*, either through epigenetic modifications of *MYC* regulatory elements or through mediated elimination of the *MYC* enhancer docking site. This leads to reduced cell proliferation, associated with inhibition of transcription factor binding and thus decreased expression levels of the MYC protein (Fig. 1).[12] Considering that *MYC* binds to specific E-box sequences in the genome to regulate gene expression, a recent study showed the potential application of CRISPR-Cas9 in altering MYC binding sites (E-boxes) in the MCF7 BC cell line. The results of this study showed that E-box disruption, in genes essential for tumor cell growth, affected MYC binding, target gene expression, tumor growth in *in vivo* studies, and cell proliferation in *in vitro* studies. The authors conclude that this approach could constitute a useful tool for the genome-wide identification of E-boxes that are important for MYC-dependent networks in cancer cells.[13]

CXCR4 and ACKR3 genes

CXCR4 and *ACKR3* genes, encodes a G-protein-coupled seven transmembrane receptor, and are highly expressed and dysregulated in BC. Both, *CXCR4* and *ACKR3* genes, play important roles in

the progression, metastasis and prognosis of BC.[31] In fact, higher expression of *CXCR4* and *CKR3* has been associated with both, poor prognosis and metastasis in TNBC. Further, upregulation of cytoplasmic expression of *CXCR4* was also suggested as one of the molecular mechanisms that could facilitate lymph node metastasis in invasive micropapillary breast carcinoma (IMPC).[32,33] IMPC is a relatively rare subtype of invasive ductal breast carcinoma, accounting for less than 5% of all BC cases, associated with lymphovascular invasion.[34] Considering the high implications of CXCR4 and CKR3 genes in BC, CRISPR-Cas9 technology was used in the TNBC cell line (MDA-MB231), in order to create *CXCR4* or *CKR3* knockout or co-knockout (Fig. 1). The results of these assays showed a significant reduction in both, cell proliferation and cell invasion.[14]

MAP3K11 (MLK3) gene

Mitogen-Activated Protein Kinase Kinase Kinase 11 (*MAP3K11*), encodes for a member of the serine/threonine kinase family. This kinase activates MAPK8/JNK kinase, and functions as a positive regulator of the c-Jun N-terminal kinase (JNK) signaling pathway, being implicated in the metastasis process in TNBC.[35] Given its implication in TNBC, previous studies demonstrated that editing of *MLK3* gene, using CRISPR-Cas9, led to a significant reduction of TNBC metastasis (Fig. 1), thus revealing the crucial role that *MAP3K11* gene plays in this BC tumoral subtype.[15]

OPN gene

OPN gene, encodes for a member of Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING).[36] *OPN* is overexpressed in several diseases,[37,38] being associated with poor prognosis, survival, growth and radio resistance in BC.[39,40] Given the implications of *OPN* in BC, this gene has been proposed as a potential prognostic biomarker and also as therapeutic target.[41] For instance, a recent study showed that the combined use of the CRISPR-Cas9 system with radiation, in the MDA-MB231 BC cell line, led to a significant reduction in the *OPN* gene expression, as well as an increase in the rate of apoptosis (Fig. 1) and a greater decrease in cell viability.[16] These results suggest that the combination of conventional radiotherapy with *OPN* gene knockout, could become an effective treatment for the treatment of BC.

Targeting TSGs

TSGs are genes that regulate several cellular functions, such us: Cell cycle regulation, apoptosis induction, DNA repair mechanisms and surveillance of genomic integrity, among others. Inactivation or loss of function of TSGs has been correlated with a high risk of cell growth deregulation, a well-known mechanism for the development and progression of many types of cancers.[17] Among the mechanisms associated with the inactivation of TSGs are: chromosomal deletions, mutations and loss of expression due to transcriptional silencing mediated by hypermethylation at the promoter site. Considering the important roles that TSGs play in cancer control, CRISPR-Cas9 technology has acquired great importance in recent years, as a promising tool aimed at activating suppressed TSGs. Among the TSGs that may be potential targets for BC treatment using CRISPR, are: *TP53, PTEN* and *BRCA1*, among others.

TP53 gene

The *TP53* gene, encodes a tumor suppressor protein with important functions in maintaining cellular integrity. The p53 protein, responds to various cellular stresses to regulate the expression of target genes, activating cell cycle arrest, DNA repair, apoptosis, senescence or alterations in metabolism.[42] Mutations in this gene have been associated with a variety of human cancers, with the most frequent mutations occurring within the DNA-binding domain.[43] An increase in *TP53* mutation burden (predominantly missense mutations) in BC, has been correlated with advanced disease, higher genetic instability and metastatic risk,[44] worse overall survival and poor clinical outcome.[45,46] Given the high implications of *TP53* gene mutations in cancer prognosis, this gene constitutes an attractive therapeutic target for cancer therapy. In fact, several studies have been directed at reversing mutations in *TP53* (Fig. 1). For instance, a recent study used CRISPR-Cas9 technology to reverse a *TP53* missense mutation (L194F), in the T47D luminal A BC cell line.[18] The results of this study showed success in the desired base substitution in the *TP53* gene, although the editing efficiencies were lower than expected by the authors. Despite the above, the importance of improving the efficiency of the main edition is highlighted, proposing ways forward that could be beneficial for research in BC.

PTEN gene

Phosphatase and tensin homolog (PTEN), is a TSG that encodes for a negative regulator of the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway.[47] Therefore, this gene is involved in the inhibition of cell cycle progression, survival and migration. Deletions, mutations, transcriptional repression and epigenetic silencing due to promoter methylation, are the mechanisms mainly associated with *PTEN* inactivation.[19] Despite the above, deletions together with promoter methylation, are the main causes of the loss of expression of the PTEN protein observed in many BCs.[48,49] In addition, loss of *PTEN* activity has been associated with resistance to therapy, poor outcomes in BC,[48,50] and also with more aggressive phenotypes.[51] Taking into account both, that the inactivation of *PTEN* has been associated with the severity of BC,[52,53] and that the expression of *PTEN* can be regulated transcriptionally and epigenetically in the absence of mutations in *PTEN*,[54,55] several studies have focused on transcriptional reactivation of *PTEN* expression. The goal of these types of studies has been to achieve inhibition of progression and increase drug sensitivity in aggressive *PTEN*-deficient cancers in which *PTEN* is not mutated. In this regard, one of these studies activated PTEN expression in TNBC cells with low levels of PTEN expression (Fig. 1),[19] by using the dCas9-VPR system. The dCas9-VPR system, consists of a deactived (d) Cas9 fused to the transactivator VP64-p65-Rta (VPR). The results of this study showed that the dCas9-VPR system, increased the *PTEN* expression in TNBC cells, also observing repression of the AKT, mTOR and MAPK oncogenic signaling pathways. The results of this study constitute the basis for the design of potential therapies for the treatment of triple-negative breast tumors, for which there is currently no specific target therapy available.

BRCA1 gene

BRCA1 is another relevant gene in BC, associated with the DNA double-strand break repair and DNA stability. Among the mechanisms associated with genetic silencing of *BRCA1*, in non-familial BC, including TNBC, is promoter methylation, leading to genetic silencing and conferring poor prognosis.[56–59] With the aim of decreasing DNA methylation, a recent study reactivated gene expression and restoring the functional activity of *BRCA1* in BC (Fig. 1), by using the CRISPR/deactivated Cas9 (dCas9)-Ten-Eleven Translocation dioxygenase1 catalytic domain (TET1cd) demethyl-

ation system.[17] The CRISPR/dCas9-TET1cd system has the ability to bind to the target site without cutting the DNA strands. The results of this study showed that CRISPR/dCas9-TET1cd, lead to the transcriptional up-regulation of *BRCA1* gene. The results of this study open the possibility of using the CRISPR/dCas9-TET1cd system, as a gene editing tool, for the targeted demethylation of epigenetically silenced TSGs in human cancers.

CRISPR-Cas9 system and treatment resistance in BC

Treatment resistance has been reported as the main cause of high mortality in BC, where more than 90% of failed treatments are due to multidrug resistance (MDR) and acquired resistance.[60,61] In fact, it has been indicated that exposure to chemotherapeutic agents can lead to an MDR phenotype, and may also involve various cellular and molecular changes.[62]

For instance, overexpression of ATP binding cassette (ABC) transporters has been associated with MDR. This is due to the involvement of such transporters in the elimination of drugs from breast tumor cells, before they accumulate in therapeutically active concentrations.[63] Given their role in resistance to therapy, the induction of alterations in these membrane transport proteins, could facilitate re-sensitization to existing therapies and reduce the possibility of applying new therapies.[20] Established strategies to improve drug therapy include increasing drug efflux by altering the membrane transporter protein, thereby enhancing DNA repair, and reversing MDR.[64] Specifically, blocking resistance factors using CRISPR-Cas9, is an attractive strategy to overcome MDR and thus continue using existing anticancer agents. In fact, CRISPR-Cas9 has also been suggested as a potential therapeutic tool to overcome chemoresistance in BC.

Another drug efflux pumps commonly overexpressed in BC and contributing to drug resistance are: P-gp and breast cancer resistance protein (BCRP).[65] In addition, Glutathione S-Transferase Pi 1 (*GSTP1*) gene, has also been associated with chemoresistance in BC.[66]

P-glycoprotein (P-gp) is encoded by the multidrug resistance gene 1 (*MDR1*).[67] P-gp is a membrane glycoprotein transporter that belongs to the largest superfamily of ATP-binding cassette (ABC) proteins.[68] Recently, the CRISPR-Cas9 technology was used to edit the *MDR1* gene, and thus overcome doxorubicin resistance in a MDR BC cell model (MCF7/ADR cells). Disruption of *MDR1* by CRISPR-Cas9 in MCF7/ADR (adriamycin-resistant cell line) cells, showed an increase in doxorubicin potency in treated cells, which also led to an increase in cell death compared to cells not edited.[20] The results of this study suggest that, Cas9-mediated disruption of *MDR1* gene, could be considered as a potential tool to overcome MDR in BC cells.

BCRP is encoded by the ATP-binding cassette subfamily G member 2 (*ABCG2*) gene.[65] BCRP is an ABC transporter, associated with MDR in various cancer cells. ABCG2 acts as energy-dependent efflux pumps capable of effluxing out of the cell a wide range of xenobiotics, such us: chemotherapeutics (doxorubicin) and anticancer drugs based on natural products.[65,69] Additionally, it has been indicated that the expression of BCRP/ABCG2 in cancer cells, in addition to being associated with drug resistance mechanisms, could be associated with invasiveness, self-renewal and with poor prognosis For example,[69] BCRP/ABCG2 has been associated with an MDR phenotype in the MCF7 cell line.[70] Taking the above into account, blocking active efflux mediated by BCRP/ABCG2 could constitute a promising therapy to overcome resistance in cancer.

Additionally, the glutathione S-transferase P1 (*GSTP1*) gene has also been associated with chemoresistance in BC. *GSTP*1 is a gene that encodes a protein involved in many cellular processes, including: phase II detoxification of xenobiotics, in the metabolism of a variety of carcinogenic compounds and in the protection of cells against DNA damage, among others. Indeed, overexpression of *GSTP1*, has been associated with cisplatin resistance in BC.[71]

Taking into account that one of the main causes of therapeutic failure in BC is chemoresistance, especially in TNBC, the CRISPR-Cas9 system is being applied in *in vitro* and *in vivo* studies with the aim of sensitizing tumor cells to the chemotherapy. For instance, in this tumoral subtype, mutations in the *BRCA1* gene (*BRCA1*m) have been frequently associated with chemoresistance. In this regard, a recent study, aimed at sensitizing *BRCA1*m cancer cells to chemotherapy, used CRISPR-Cas9 to generate Poly(ADP-Ribose) Polymerase 1 (PARP1) deficient TNBC cell lines (MDA-MB231 and MDA-MB436). The results of this study showed that CRISPR-Cas9-mediated PARP1 deficiency, sensitized TNBC cells with *BRCA1*m (MDA-MB436) to chemotherapeutic drugs: doxorubicin, gemcitabine and docetaxel, compared to the wild-type cell line (MDA-MB231).[21]

In addition, another gene that has recently been associated with promoting chemoresistance in TNBC, is the Dual Serine/Threonine and Tyrosine Protein Kinase (*DSTYK*). *DSTYK* gene encodes a dual serine/threonine and tyrosine protein kinase which is thought to function as a regulator of cell death. *DSTYK* has recently been established as a potential therapeutic target, given its involvement in resistance to chemotherapeutic treatment in TNBC cells. In fact, a recent study demonstrated that deletion of *DSTYK* by using CRISPR-Cas9, led to apoptosis of chemoresistant cells after drug treatment, both in *in vitro* and *in vivo* models. These findings suggest that *DSTYK* exerts an important and previously unknown role in promoting chemoresistance.[22]

Additional studies have shown the potential use of CRISPR in identifying cancer vulnerabilities and developing new therapeutic strategies. This is the case in a recent study that identified potential paclitaxel-sensitizing/resistant genes, using a combined *in vitro/in vivo* genome-wide CRISPR synthetic lethality screening approach in a TNBC cell line.[23] The results of this study showed that silencing of the *ATP8B3, FOXR2* and *FRG2* genes led to increased resistance to paclitaxel in TNBC. Altogether, these results suggest the potential therapeutic value of the *ATP8B3, FOXR2* and *FRG2* genes for chemotherapy treatments in TNBC.

Overall, the results of all of the above studies suggest the potential use of CRISPR-Cas 9 to restore drug sensitivity and overcome chemotherapy resistance in BC.

CRISPR-Cas9 limitations—induction of chromosomal alterations

As indicated above, CRISPR genome editing has emerged in recent years as a potential tool for the treatment of cancer and other diseases. However, since CRISPR technology is primarily based on creating specific DNA DSBs in almost any part of the genome, has been indicated that gene editing with CRISPR-Cas9, in addition to inducing DSBs, can lead to the induction of a broad spectrum of genomic rearrangements, chromosomal variations and structural chromosomal alterations.[72–74] In fact, it has been reported that CRISPR induces structural chromosomal alterations, such as: dicentric chromosomes, chromosomal translocations, micronuclei and chromothripsis.[72] Dicentric chromosomes (dic) can be generated because DNA breaks, produced by Cas9, can lead to ligation of the central fragments of sister chromatids cleaved by Cas9.[75,76]

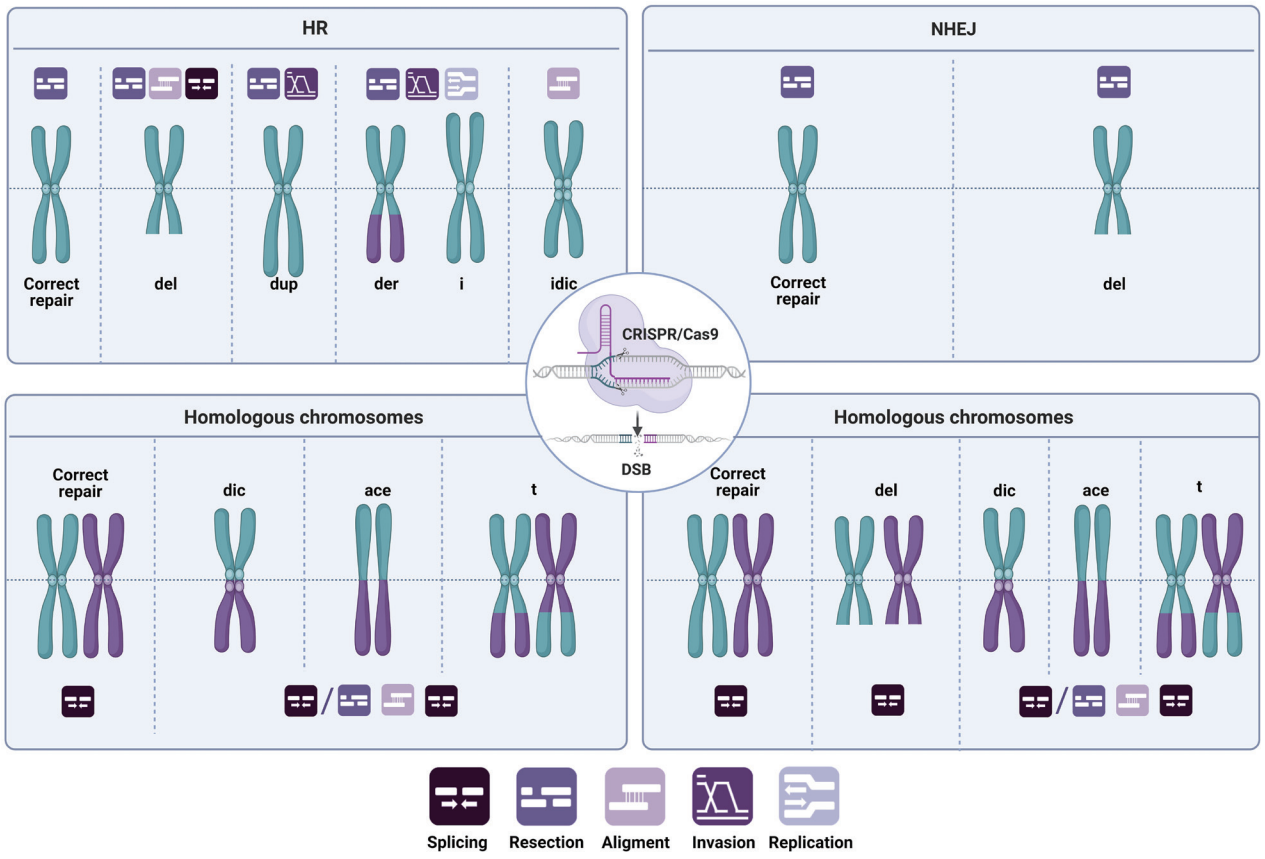

Fig. 3. Induction of chromosomal alterations by CRISPR/Cas9. CRISPR technology is primarily based on creating specific Double-strand breaks in DNA (DSBs) in almost any part of the genome. These DSBs are subsequently corrected, through the activation of DSBs repair mechanisms, including homologous recombination (HR) and non-homologous end joining repair (NHEJ). In HR repair, the pair of homologous chromosomes are brought together and the region of the undamaged homolog or chromatid is taken as a template to reconstruct the DSB of the affected chromosome. While, Non-homologous end joining (NHEJ) repair, allows the joining of broken ends without requiring a homologous or complementary sequence to guide the repair. These mechanisms are error prone and can leave the ends of the affected chain free, which after erroneous processes of splicing, resection, alignment, invasion and/or replication, lead to structural chromosomal alterations as: chromosomal deletions (del), chromosomal duplications (dup), derivatives chromosomes (der), isochromosomes (i), isodicentric chromosomes (idic), dicentric chromosomes (dic), acentric fragments chromosomes (ace), and chromosomal translocations (t). Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeat; del, chromosomal deletions; dup, chromosomal duplications; der, derivatives chromosomes; i, isochromosomes; idic, isodicentric chromosomes; dic, dicentric chromosomes; ace, acentric fragments chromosomes; t, chromosomal translocations.

In regard to chromosomal translocations (t), has been indicated that gene editing protocols that induce more than one on-target DSB, could lead to incorrect joining of DNA ends and, therefore, the induction of chromosomal translocations (Fig. 3) that could persist in treated patients over time.[77]

Furthermore, recent findings reveal that CRISPR-Cas9 genome editing can induce the formation of micronuclei (MN) and nucleoplasmic bridges in dividing cells, leading to both numerical and structural chromosomal alterations, including chromothripsis (Fig. 4).[72] Chromothripsis is a mutational process, in which up to thousands of massive chromosomal rearrangements occur in a single event and in genomic regions confined to one or a few chromosomes. This phenomenon is involved in cancer and congenital diseases. In fact, it has been indicated that in cancer, chromothripsis leads both, to the amplification of oncogenes, mainly through the formation of double minute chromosomes (dmin), and to the loss of TSGs.[78–80]

For instance, it has been suggested that the mechanisms by which chromothripsis can emerge include, the fragmentation and subsequent reassembly of a single chromatid into small nuclear structures surrounded by membranes called MN and,[81,82] the breakage of dicentric chromosomes during telomere crises (Fig. 4).[83,84] The above constitutes a limitation for the application of therapeutic genome editing strategies that require the induction of DSBs, since these DSBs, have been associated with the induction of structural chromosomal alterations including chromothripsis. Furthermore, chromothripsis can lead to the acquisition of multiple additional alterations that can promote tumorigenesis in many tissues, including those with cells with intact p53.[78,79,85] However, it is important to highlight that to date, the rates of MN formation, nucleoplasmic bridges and chromosomal alterations, including chromothripsis, associated with genome editing therapies in humans are unknown.

Regarding p53, it was recently indicated that in addition to inducing chromosomal alterations, CRISPR-Cas9 technology can also, cause changes in the p53 signaling pathway in many cell lines, leading to an increase in mutations that inactivate p53 and therefore promoting the development of cancer.[86,87] Further, recent studies indicated that numerical (chromosomal losses) and structural (chromosomal translocations) chromosomal alterations caused by gene editing, apparently do not disappear over time and, on the contrary, increase in frequency, thus showing a clear ran-

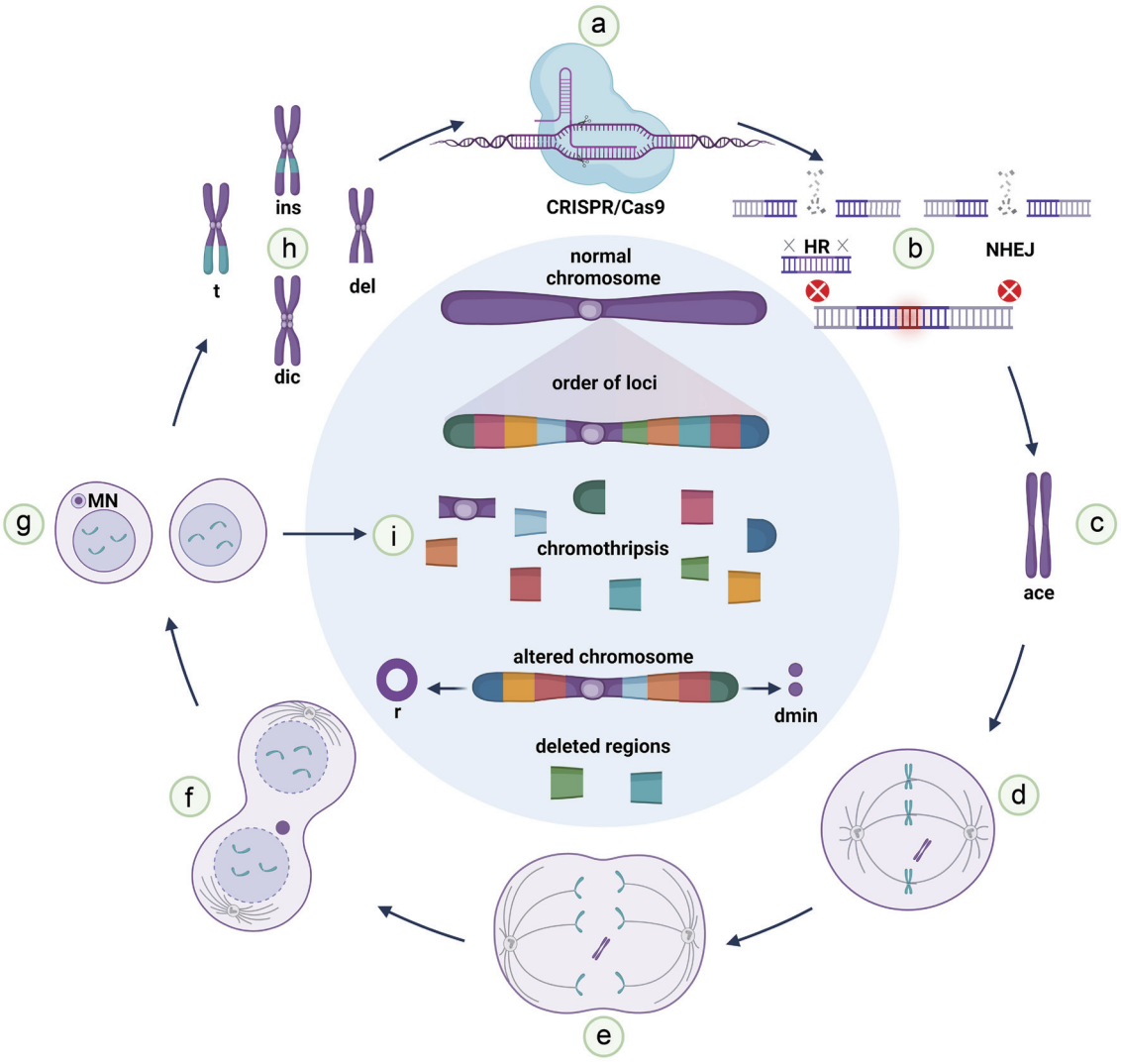

Fig. 4. Micronuclei formation and chromothripsis induced by CRISPR/Cas9. The main limitation of CRISPR/Cas9 as a therapeutic alternative is the induction of chromosomal alterations. (a) DNA double-strand breaks (DSBs) may not be corrected by (b) DNA DSB repair mechanisms, which may favor the formation of (c) acentric (ace) chromosome fragments. Acentric chromosome fragments that persist until mitosis, fail to align in metaphase (d), or even anchor to the mitotic spindle in (e) anaphase. The above leads to the fact that, while the chromosomes go to the opposite poles of the mitotic spindle, the acentric chromosome fragments remain lagging. In telophase (f), the formation of a new nuclear membrane leads to the emergence of daughter nuclei, each with a copy of the complete genetic material, except for the lagging chromosome fragments, which are (g) surrounded by their own membrane, leading to the formation of micronuclei (MN). MN formation, can favors the acquisition of additional chromosomal alterations (h), including, chromosomal translocations (t), chromosomal insertions (ins), chromosomal deletions (del), dicentric chromosomes (dic), and chromothripsis (i). Chromothripsis is an event of genetic chaos in which one or more chromosomes are fragmented into many segments and then randomly rearranged, losing some regions and promoting the formation of double chromosomes (dmin) and ring chromosomes (r). ace, acentric chromosome fragments; Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeat; del, chromosomal deletions; dic, dicentric chromosomes; dmin, double chromosomes; DSBs, DNA double-strand breaks; ins, chromosomal insertions; MN, micronuclei; r, ring chromosomes; t, chromosomal translocations.

dom clonal expansion.[73] The above could be explained because during the active period of gene editing, changes occur in chromosome segregation and nuclear division, evidencing a probable mechanism for the induction of chromosomal alterations following gene editing.[88] Overall, the research carried out to date shows a limitation for the application of DSB-inducing CRISPR therapy in the clinic, so it is important to consider the possibility that extensive chromosomal rearrangements may be induced as a result of the application of this gene editing technology.

Our study presents limitations related to the reduced literature available about the use of CRISPR-Cas9 in *in vivo* models and in BC patients. This is possibly due to the recent application of this gene editing system in cancer research. Despite the above, CRISPR-Cas9 constitutes a potential tool in the treatment of cancer.

Conclusions

Although, CRISPR-Cas9 genome editing technology has therapeutic potential both, to direct personalized therapy in BC and to overcome drug resistance, it is necessary to consider and monitor

the possibility of induction of genomic rearrangements, chromosomal variations and structural chromosomal alterations. This is due to the fact that such genomic rearrangements could have an implication in the prognosis of the disease and in the response to therapy. The studies carried out to date provide important data to take into account when determining the risks associated with the use of CRISPR-Cas9 technology in the clinic. In fact, more studies are necessary to delve into both, the mechanisms related to the safety of CRISPR and the potential risks involved with CRISPR-Cas9 system.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by NR, VC, GC, MF-C and MR-L. The first draft of the manuscript was written by MR-L and NR. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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