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CRISPR/Cas9-mediated activation of *CDH1* suppresses metastasis of breast cancer in rats

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ABSTRACT

Background: Cancer is a life-threatening disease that affects approximately 18 million individuals worldwide. Breast cancer is the most common female neoplasm globally with more than 276,480 new cases of invasive breast cancer expected to be diagnosed in women in the U.S. alone in 2020. Genetic and epigenetic factors play role in the carcinogenesis and progression of this disease. In this study, MCF-7 adenocarcinoma cells were transfected with CRISPR/Cas9 plasmid to either knock out CDK11 or to activate CDH1. Treated cells were allografted into the mammary glands of female rats (150–190 g, 6–8 weeks) to evaluate the capability of these cells to control cancer progression and metastasis.
Results: qPCR data revealed a significant downregulation of CDK11 and upregulation of CDH1. Cell cycle analysis and apoptosis assays indicated the knockout of CDK11 and simultaneous activation of CDH1 resulted in cell cycle arrest at G2/M phase and accumulation of cells at G2. Meanwhile, the percentage of cells that underwent late apoptosis increased in both genome editing hits. Histopathological sectioning data indicated that untransfected MCF-7 cells were capable of developing tumors in the mammary gland and initiation g angiogenesis. Transfected cells significantly restricted cancer cell infiltration/invasion by minimally localizing tumors and inhibiting angiogenesis.
Conclusions: Although further investigation is needed, the present data indicate the potentiality of using CRISPR/Cas9-based therapy as a promising approach to treat breast cancer. Impact: these data indicate targeting cancer-related genes via any genome editing tool might represent a novel approach to combat cancer.

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1. Introduction

Cancer is a leading cause of global mortality worldwide and the second leading cause of mortality in the United States; with about 1,806,590 new cancer cases and approximately 606,520 cancer related deaths are expected to occur by the end of 2020 [1]. Cancer is a complex disease that manifests itself in several forms; all characterized by uncontrollable cell proliferation [2,3].

Breast Cancer (BC) is the most common female neoplasm, affecting one in nine women worldwide [3]. It is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide [4]. The development of BC involves a progression; starting with atypical hyperplasia, followed by intermediate stages, then invasive carcinoma, and finally the metastatic disease.

Modern techniques have been proposed to diagnose the disease at an earlier stage and in some cases, to achieve a cure for the

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disease [5]. Epigenetics play a crucial role in the development and progression of BC via methylation-based silencing of tumor suppressor genes or abnormal activation of oncogenes [6,7,8]. Restoring the normal functions of these mutated genes is considered a promising approach to treat cancer [9,10]. Clustered regularly interspaced short palindromic repeats-associated Cas9 (CRISPR-Cas9) is one of the recently developed multipurpose genome-editing tool for editing eukaryotic genomes. Using viral or non-viral delivery, CRISPR/Cas9 can activate, knock out, correct, and disrupt specific genes [11,12]. Thus, the genome editing technology had once again returned to the central stage of disease treatment [13]. Moreover, a promising application of CRISPR/Cas9 has been proposed for tissue engineering, bioelectronics, and diagnostics [14].

CDH1 is one of genes that strongly contributes to sporadic breast cancer (BC) progression. It encodes for epithelial cadherin (E-cadherin), the membrane adhesion protein that controls different cellular processes such as regulation, cell growth, cell proliferation, and apoptosis [15,16]. Loss of *CDH1* function leads to epithelial to mesenchymal transition (EMT), which increases the metastatic potential of malignant cells [17] and causes dysregulation of cell–cell adhesion and anoikis resistance [15,18]. Defective *CDH1* is frequently found in breast cancer and are particularly prevalent in lobular breast cancers [19].

Cyclin-dependent kinase 11 (CDK11) is one of the components of the mediator co-activator complex, which is a multi-protein complex required for transcriptional activation. *CDK11* is implicated in cell cycle progression, tumorigenesis, apoptotic signaling [20,21], and RNA splicing [22]. Recent reports have shown that *CDK11 is* upregulated in most human malignancies [21], thus, downregulating this gene via CRISPR/Cas9, for example, has become a promising therapeutic approach.

In this study, we propose that targeting *CDH1* (by activation) and *CDK11* (by suppression) in breast cancer cells MCF-7 could restrict the ability of these cells to form tumors in the mammary glands of allografted rat model. This might represent a promising approach to dually targeting cancer cells using genome editing technologies such as CRISPR/Cas9.

2. Materials and methods

2.1. Cell line maintenance

Breast adenocarcinoma cells (MCF-7) were purchased from the Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt. Cells were maintained under the normal laboratory conditions in 5% CO_2 and 95% humidity at 37°C. Cells were cultured on EMEM (EBSS) supplemented with 2 mM Glutamine and 1% Non-Essential Amino Acids NEAA and 10% Fetal Bovine Serum FBS.

2.2. CRISPR/Cas9 transfections

CRISPR/Cas9 kits and reagents were purchased from Santa Cruz Biotechnology, Germany. E-cadherin (*CDH1*) activation plasmid (h) (Cat#: sc-400031-ACT), CDK11knock out plasmid (h) (Cat#: sc-405330), Transfection Reagent (Cat#: sc-395739), and Plasmid Transfection Medium (Cat#: sc-108062) were used in the present study. The transfection procedures were performed according to the manufacturer's protocol.

2.3. Animals

Female Sprague–Dawley rats were obtained from the Animal House of the Faculty of Veterinary Medicine, Cairo University, Egypt. The rats weighted approximately 150–190 g and aged at the time of the experiment 6–8 weeks. The animals were reared under controlled conditions at room temperature and 50–60% humidity with a 12-h light–dark cycle. Animals were fed ad libitum with free access to normal diet and water.

Animals were dosed intraperitoneally Cyclophosphamide at 120 mg kg^{-1} daily for 4 d to compromise immunity as a preparatory treatment for animals not to reject the subcutaneous injection of foreign human cells [23].

Animals were divided into five experimental groups (5 animals in each). Group I: served as control animals and injected with saline, group II: received subcutaneous injections with cells challenged with empty CRIPSR/Cas9 transection media and reagents, group III: received subcutaneous injections with breast cancer cells in which *CDH1* was activated using CRISPR/Cas9, group IV: received subcutaneous injections breast cancer cells in which *CDK11* was knocked out using CRISPR/Cas9, and group V: received subcutaneous injections of breast cancer cells with dual treatment (activation of *CDH1* and knockout of *CDK11*).

After 3 weeks of treatment, animals from each group were sacrificed, and mammary tissues were extracted along with blood samples (drained from dissection). This study was approved by the ethics committee for animal experimentation of the Faculty of Veterinary Medicine, Cairo University, Egypt (VetCU051 92019036).

2.4. Apoptosis detection

Treated and untreated breast cancer cells MCF-7 were harvested to assess the apoptotic cells using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. The harvested cells were resuspended in 100 μL Annexin V binding buffer and 5 μL Annexin V Alexa Fluor 488. The mixture was incubated for 15 min. PI (4 μL) diluted in 1× Annexin V binding buffer (1:10) was added and the mixture was incubated again for 15 min in a dark place. Annexin V binding buffer (500 μL) was added to wash the Annexin/PI-stained cells. Annexin/PI staining was visualized on flow cytometer (BD FACSCalibur™, BD Biosciences-China). Annexin V-FITC binding was analyzed at Ex = 488 nm; Em = 530 nm using FITC signal detector (FL1) and PI staining by the phycoerythrin emission signal detector (FL2).

2.5. Cell cycle analysis

To determine the changes in cell cycle progression after treating cells with CRISPR/Ca9 to activate *CDH1* and to knock out *CDK11*, cells (5×10^5) were collected, centrifuged at 600 rpm for 5 min and resuspended in 500 μL PBS. Cells were centrifuged again at 600 rpm for 5 min and left at 4°C for 2 h in cold ethanol. Cells were centrifuged for the third time, pelleted at low speed and ethanol was decanted. Cells were resuspended in PBS and pelleted again. Cells were then resuspended in 50 μg mL^{-1} PI, 0.1% Triton X-100 and 50 μg mL^{-1} RNAse and incubated for 30 min at room temperature. The PI fluorescence was read on a FACScan flow cytometer (BD FACSCalibur™, BD Biosciences-China). Pulse width/pulse area signal was used to discriminate between G2/M cells and the cell doublets. DNA content histogram deconvolution software was used.

2.6. Gene expression analysis

Total RNA was isolated from untreated and CRISPR/Cas9-treated MCF-7 cells using RNA Isolation System (Qiagen, GmbH, Germany). cDNA was synthesized by mixing 5 μg of the extracted RNA with 1 μg random 6-mer primers, 1 μM deoxyribonucleotides (dNTP), and 10 units of M-MLV SuperScript II Reverse Transcriptase (Invitrogen). The mix was incubated for 1 h at 42°C.

Quantitative real time PCR was performed to analyze the expression profiles of *CDK11* and *CDH1* in cells transfected with either transfection and media and reagent, CDH1-targeting CRISPR/Cas9 activation plasmid, *CDK11*-targeting CRISPR/Cas9 KO plasmid, and dual transfection. Primer sequences are listed in Table 1. About 100 ng (2 μL) of cDNA was mixed with 12.5 μL CYBR Green master mix, 10 pM (1.5 μL) of each of the primers and the final volume was brought to 25 μL with molecular biology grade water. The thermal profile was as follows: pre-PCR heating for 5 min at 95°C, then 35 cycles of 94°C for 40 sec, 56°C and 58°C (for *CDH1* and *CDK11*, respectively) for 45 s, 72°C for 50 s, followed by a final extension step of 72°C for 10 min. All reactions were performed in triplicates on StepOne Plus thermal cycler (ABS, UK).

2.7. Histopathological sections of mammary gland

Histopathological sections were prepared from treated and untreated rats' mammary gland using ultra-microtome sectioning machine. The slide preparation procedures were carried out using the standard procedures of pathological sectioning, in which tissues were fixed with formalin and embedded on paraffin, and then cut into a thickness of 3–5 μm sections and staining with H&E.

2.8. Immunohistochemistry

The sections of the mammary gland from different groups were stained against estrogen receptors as follows. First, antigen retrieval was applied by citrate buffer [10 mM trisodium citrate dihydrate, 0.05% (v/v) Tween-20, pH 6.0] for 10 min before the staining process. The samples were then incubated with primary antibodies against anti–estrogen receptor (ER; clone 1D5, 1:300; Dako, Glostrup, Denmark) overnight at 4°C, followed by staining of the sections using diaminobenzidine and counterstaining was performed with hematoxylin.

2.9. Statistical analysis

Results were presented as mean ± standard error (SE) in triplicate experiments. All comparisons between treated and untreated cells were carried out by analysis of variance (ANOVA) tests, where $p < 0.05$ was considered significant. Analysis of gene expression was performed using student's *t*-test to compare control with treatment.

3. Results

3.1. Disruption of cell cycle

In the present study, MCF-7 breast adenocarcinoma cells were transfected with CRISPR/Cas9 plasmids to activate *CDH1* and knock down *CDK11* genes. After the incubation period, cells were harvested using trypsinization and analyzed for changes in the cell cycle stages using flow cytometry. Results (Fig. 1A, 1C) indicated that transfection media had no significant effect on the distribution of cell cycle phases in all treatments ($p = 0.48$, 0.40, 0.32, and 0.39 for TM, *CDH1*, *CDK11*, and dual treatments against the control cells, respectively).

However, an increase in the G2/M phase was observed in the *CDH1*, *CDK11*, and dual treatment, which might indicate that these treatments caused the cells to arrest at G2/M phase compared with C and TM treatments.

Table 1
Primer sequences used Designed in NCBI (https://www.ncbi.nlm.nih.gov/).

Primer name	Sequence (5′ to 3′)	Tm	Product size (bp)
CDH1-F	TGCCCTCAGCACTGTAGAGA	60.25	190
CDH1-R	ATGGTGCTTAATGGGGCCTG	60.4	
CDK11-F	TGCTGTGTCCTGATGTAGGC	59.75	299
CDK11-R	GATTTTGGGCTCACCTGCGA	60.96	

3.2. Apoptosis detection

To further evaluate the effect of challenging MCF-7 breast cancer cells with CRISPR/Cas9 targeting *CDH1* and *CDK11*, apoptosis levels were assessed in treated and untreated cells. Results showed a significant increase in the percentages of late apoptosis in the *CDH1*, *CDK11*, and dual transfection (Fig. 1B, 1D). The percentages of late apoptosis were 0.25% and 0.22% in control and TM treatment, respectively, which indicate non-significant differences.

3.3. Activation of CDH1 and KO of CDK11

In the present study, breast cancer cells were treated with CRISPR/Cas9 plasmid to activate *CDH1* and to knock out *CDK11*genes. To further investigate the efficiency of the transfection, the expression of both *CDH1* and *CDK11* genes was profiled. Data (Fig. 2) indicated a significant upregulation of the CDH gene in the cells challenged with activating plasmid compared with control and cells treated with TM. *CDK11* also showed a significant downregulation in the breast cancer cells transfected with CRISPR/Cas9 compared to the control and cells treated with only TM. However, dual transfection of cells with both *CDH1*-targeting CRISPR/Cas9 activation plasmid and *CDK11*-targeting CRISPR/Cas9 KO plasmid showed non-significant changes in *CDK11* while *CDH1* was significantly upregulated.

3.4. Tumor growth and histopathological analysis

The presence of malignant tumor could not be confirmed macroscopically, therefore it was confirmed by histopathology. The sections of the mammary glands of the TM group revealed colonization of tumor cells with invasion of the adjacent stromal tissue and presence of tumor cells in the newly formed blood vessels, suggesting the induction of angiogenesis and formation of invasive carcinoma together with focal necrosis (Fig. 3-I -C-F). The control group displayed normal architecture of rodent mammary tissue; with stromal connective tissue surrounding the ducts and embedded in adipose tissue (Fig. 3-I-A). On the other hand, rodents that received cells transfected with CRISPR/Cas9 showed minimal colonization of the tumor cells with tubular hyperplasia without any sign of angiogenesis (Fig. 3-I-B).

3.5. Immunohistochemistry for detection of estrogen receptors

Immunostaining revealed that rats of TM group strongly expressed high levels of estrogen receptors as evidenced by strong positive staining (Fig. 3-II-D), while the control group showed negative immunostaining of estrogen receptors (Fig. 3-II-A). The rats that received cells transfected with CRISPR/Cas9 showed mild estrogen receptors immunostaining (Fig. 3-II-B and C).

The examined sections showed a marked increase in the number of positive estrogen receptors cells in mammary tissues of TM group (83.4 ± 2.61704). A significant ($p \leq 0.05$) reduction in number of positive estrogen receptors cells was observed in both the control group (3.2 ± 0.55377) and rats that received cells transfected with CRISPR/Cas9 (13.6 ± 0.76303) (Fig. 3-III).

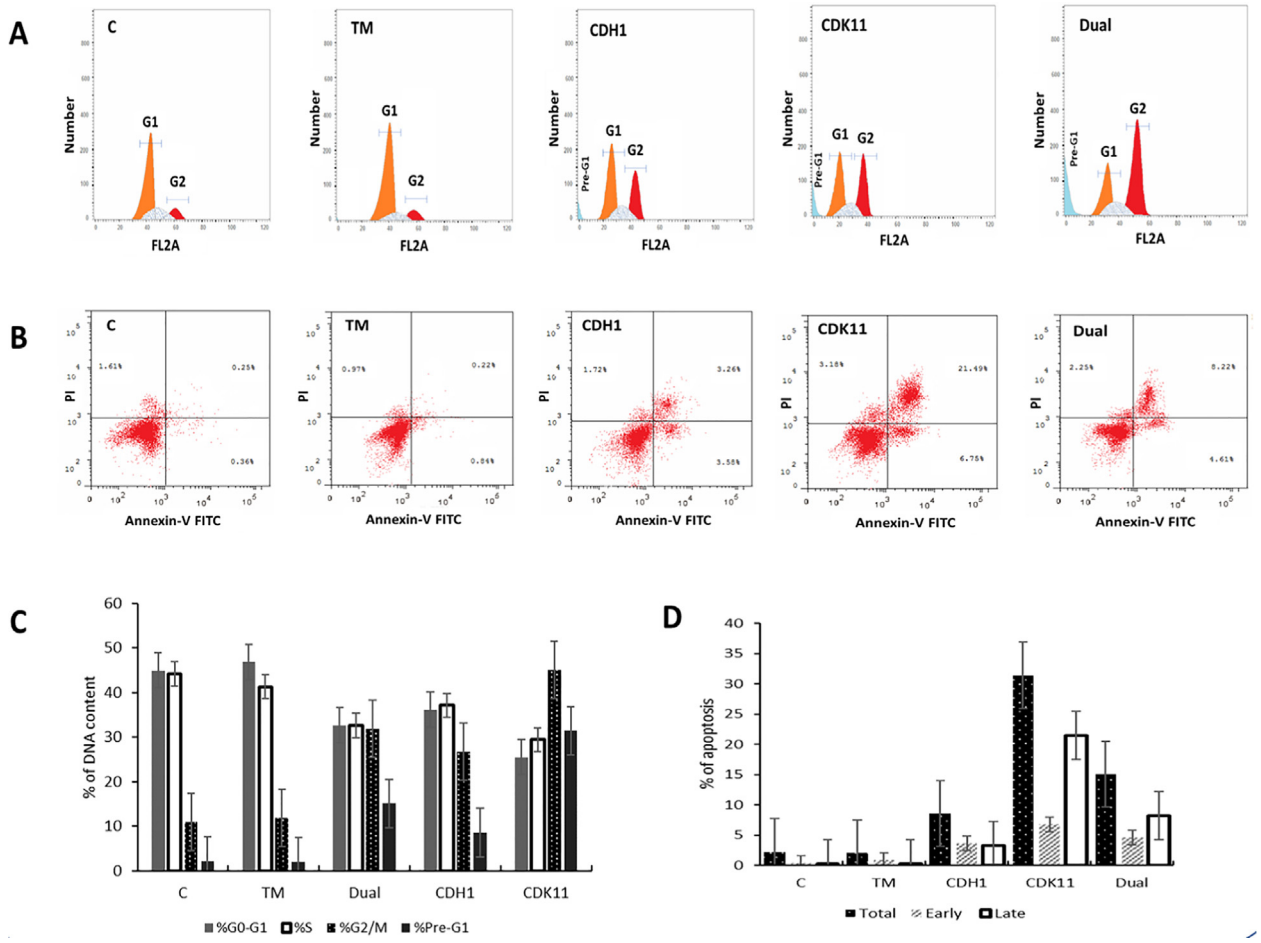

Fig. 1. Cell cycle distribution and apoptosis detection using flow cytometry. (A) Cell phases' distribution analysis indicates an arrest of the cell cycle at G2/M phase when MCF-7 cells treated with dual CRISPR targeting *CDH1* and *CDK11* compared with the single hit with CRISPR plasmids. (B) Apoptosis detection using flow cytometry. An increase in the late apoptosis has been observed in *CDH1*-targeting CRISPR/Cas9 plasmid, *CDK11*-targeting CRISPR/Cas9 plasmid treatments. (C) The percentages of DNA content of MCF-7 cell treated with *CDH1*-targeting CRISPR/Cas9 plasmid, *CDK11*-targeting CRISPR/Cas9 plasmid, and dual transfection. (D) The percentages of total, early, and late apoptosis. Data showed that challenging MCF-7 cells with *CDK11*-targeting CRISPR/Cas9 plasmid increased the percentage of total apoptosis compared to other treatments and control. C: Control; TM: Transfection Media.

3.6. Tumor metastasis

For further detection of tumor metastasis, the lung tissues from control group and rodents treated with different treatments were examined. Sections were stained with H&E and examined under light microscope. Data showed that the TM group showed peribronchial aggregation of the tumor cells (Fig. 3-IV-C and D), while the control group showed normal lung tissue architecture (Fig. 3-IV-A). Moreover, lung tissues of rodents that received cells transfected with CRISPR/Cas9 showed normal tissue architecture with absence of the tumor cells (Fig. 3-IV-B).

4. Discussion

Breast cancer is a global life-threatening disease that affects women and men although in a smaller proportion [24,25]. To date, several chemotherapeutic drugs have been developed to treat breast cancer, albeit with side effects [26,27]. Therefore, the search for novel treatment for breast cancer is a demand.

Genome editing via CRISPR/Cas9 might offer a potential strategy to treat several diseases, including breast cancer [26,28]. The present study aimed at evaluating the efficiency/efficacy of the CRISPR/Cas9- edited breast cancer cells to control the invasion, metastasis of mammary gland tumor in rats. Animals treated with MCF-7 breast adenocarcinoma cells and primarily challenged with CRISPR/Cas9 to either activate *CDH1* or knock out *CDK11* showed less tumor cells infiltration and invasion. As the cell adhesion glycoprotein E-cadherin (encoded by *CDH1*) is inactivated in breast cancer [18,29,30], the activation of this gene in MCF-7 cells could restrict the spread of tumor cells in mammary gland of rats received these cells, while in untreated cells, data showed new blood vessel formation as an indication of angiogenesis. The loss of *CDH1* is an essential mark of the epithelial to mesenchymal transition (EMT), the process through which the metastatic potential of malignant cells is increased [17]. Our data suggested that upregulating *CDH1* controlled the angiogenesis potential of breast cancer cells. Reports indicated that the expression of *CDH1* is disrupted in almost 90% of all epithelial carcinomas [31], and this disruption increases the metastatic potential of malignant cells [17], meaning that it is quite crucial to activate this gene as a primary line of molecular treatment in different types of cancers, including breast carcinoma. Our data also indicated that the negative and positive controls could not inhibit angiogenesis, where *CDH1* was in its inactivated form.

We further knocked out *CDK11*, which is a member of the serine/threonine protein kinase family that plays crucial roles in tumor cell proliferation and growth by controlling cell-cycle

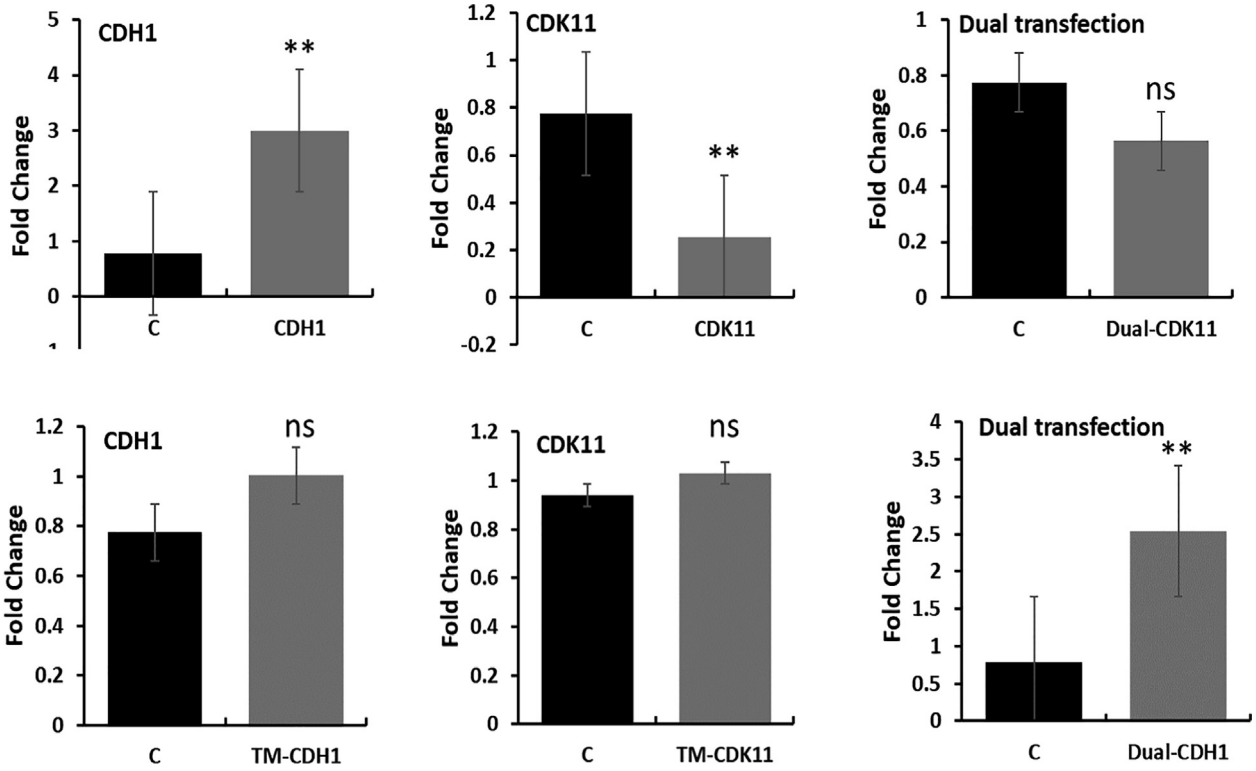

Fig. 2. Gene expression analysis. CRISPR/cas9 was used to activate *CDH1* and KO *CDK11*. Data indicate that treatments upregulated and downregulated *CDH1* and *CDK11*, respectively. Treatment of MCF-7 with transfection media had non-significant effect on the expression profile of both genes. C: Control; TM: Transfection Media.

[21,32]. It has been indicated that the KO of *CDK11* leads to a dramatic decrease (>75%) in its expression, and this results in also in significant down-regulation of the MMP-9 and VEGF involved in cancer progression and metastasis as well as up-regulation of p53 [21]. Because *CDK11* protein is a catalytic subunit of M-phase promoting factor (MPF); which is essential for G1/S and G2/M phase transitions of cell cycle [33,34], knock out of this gene leads to disruption of cells cycle. This was observed in this study; where cells challenged with *CDK11*-targeting CRISPR/Cas9 KO plasmid were arrested in G2/M phase. Moreover, KO of *CDK11* strongly triggered apoptosis, as it is required for cancer cell growth in vitro and in vivo, and its inhibition leads to apoptosis [21].

In the present study, dual targeting (*i.e.,* activation of *CDH1* and KO of *CDK11*) resulted in minimal colonization in the mammary tissue of rats, indicating that the double hits of CRISPR/Cas9 represent a more efficient intervention to control the spread and metastasis of breast cancer in vivo. This profile was observed in several previous works [35,36]. This treatment also arrested MCF-7 cells at G2/M phase, although further investigation is needed to elucidate the molecular interactions that lead to this cytostatic effect.

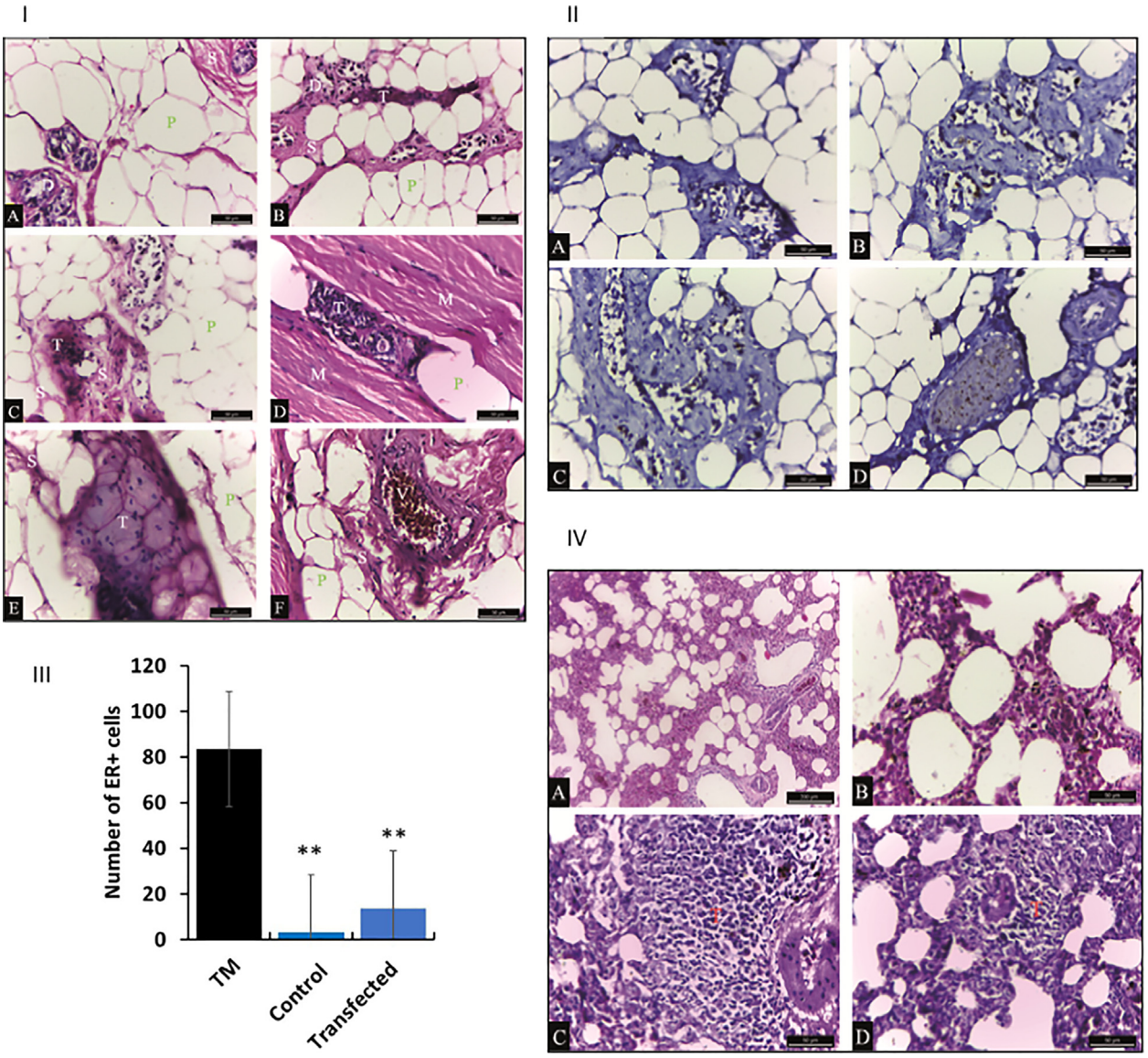

Fig. 3. The immunohistochemistry and histopathology of tissues removed from BC cells challenged with CRISPR/Cas9. I: Light micrograph of mammary tissue from different groups (H&E 400×, Scale bar: 50 μm); (A) control group showing normal architecture of the rat mammary tissue with stromal connective tissue surrounding the ducts and embedded in adipose tissue. (B) Transfected group showing minimal colonization of tumor cells. (C–F) TM group showing colonization of the MCF-7 cancer cell within the adipose tissue and invasion of the surrounding stroma and muscles, and formation of new blood vessels with tumor cells in their lumen. (Adipose tissue (P), Ducts (D), Stromal connective tissue (S), Tumor cells (T), Muscles (M), Blood vessels (V)). II: Immunohistochemical detection of estrogen receptors of mammary tissue from different groups (H&E 400×, Scale bar: 50 μm); (A) Control group showing negative staining of the mammary tissue. (B and C) Transfected group showing few positive cells (Brown color). (D) TM group showing multiple positive staining of the tumor cells (Brown color) within the mammary tissue. III: Histogram showing the mean values ± SE of Number of ER + cells. Data represented as Mean ± SEM. IV: Light micrograph of lung tissue from different groups; (A) Control group showing normal architecture of the rat lung tissue. B: Transfected group showing normal structure of the lung tissue. (C and D) TM group showing colonization of the MCF-7 cancer cell within the lung tissue. (Tumor cells: T). (H&E 100×, Scale bar: 200 μm A; H&E 400×, Scale bar: 50 μm (B–D)).

5. Conclusions

Finding a reliable treatment for breast cancer is of great importance to save millions of patients that are suffering from the burden of this disease. Traditional chemotherapies have been implemented to solve this dilemma albeit ineffective due to their side effects. Restoring the normal functions of mutated genes in breast cancer represents a more promising approach, taking into consideration its advantage of having minimal to no adverse effects. CRISPR/Cas9 might be a potential approach for controlling several diseases, including breast cancer. Data obtained in the present study indicated that targeting *CDH1* using CRISPR/Cas9 activation plasmid and *CDK11* using CRISPR/Cas9 KO plasmid helped in controlling cancer progression, invasion, and metastasis in mammary gland of rats treated with the CRISPRized cells. Genome editing using CRISPR/Cas9, although needs further investigations, seems to be an effective therapy for breast cancer.

Conflict of interest

All authors declare that they have no conflicts of interest.

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