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A New Gene Therapy Approach by Tenascin-C Genome Editing Induces Apoptosis and Cell Cycle Arrest in Triple-Negative Breast Cancer Cells

Halin Bareke[1], Emine Salva[2], Suna Özbaş[1]

[1]Department of Pharmaceutical Biotechnology, Marmara University Faculty of Pharmacy, Institute of Health Sciences, İstanbul, Türkiye
[2]Department of Pharmaceutical Biotechnology, İnönü University Faculty of Pharmacy, Malatya, Türkiye

Abstract

BACKGROUND/AIMS: There is a pressing need for new therapies for the most aggressive subtype of breast cancer, triple-negative breast cancer (TNBC). *Tenascin-C (TN-C)* codes for a tumor microenvironment-specific protein, which promotes apoptosis evasion and cell proliferation. The aim of this study was to knock down TN-C by using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system to induce cancer cell apoptosis and stunt cell proliferation, laying the grounds for a new gene therapy approach in TNBC.

MATERIALS and METHODS: The human TNBC cell line, MDA-MB-231 cells were transfected by TN-C-specific CRISPR/Cas9 plasmids. TN-C messenger RNA levels were assessed by real-time polymerase chain reaction to determine the knock-down efficiency. Two days after the transfection, the percentage of apoptotic cells and the proportion of cells in cell cycle phases were compared between the treatment and the control groups using flow cytometry. The resultant change in cell proliferation due to the knock-down was determined by MTT assay.

RESULTS: Transfection with the TN-C CRISPR/Cas9 plasmid reduced TN-C levels in the cells by approximately 49% relative to the scrambled-control CRISPR/Cas9 transfected cells. This TN-C downregulation increased the percentage of cells in apoptosis and induced G1-phase arrest. The combined effect of apoptosis and cell cycle arrest led to a significant decrease in the number of cancer cells in the treatment group.

CONCLUSION: Our successful preliminary study of a potential *TNBC* gene therapy based on TN-C genome editing by the CRISPR/Cas9 system led to significant decrease in TNBC cell numbers and it justifies the testing of this system in more advanced preclinical studies.

Keywords: Tenascin-C, CRISPR-cas systems, triple negative breast neoplasms, gene editing, apoptosis

INTRODUCTION

Cancer incidence is increasing, and it is predicted to result in 13 million deaths in 2030.[1] Breast cancer is the most common cancer affecting women worldwide, and its incidence is also projected to increase due to multifactorial variables, including lifestyle changes and increased average lifespan.[2] Apoptosis evasion is one of the hallmarks of cancer, including breast cancer, which enables the tumor cells to thrive despite the presence of anti-apoptotic signals.[3] Apoptosis, or programmed cell death, is induced by cell-intrinsic and extrinsic signals and is vital in tissue development, homeostasis, and the removal of dysfunctional cells, such as tumor cells, from the body.[4] This process is mediated by the serial activation of the caspase enzymes, which culminates in DNA fragmentation, the formation of apoptotic bodies, and the clearance of the apoptotic cell by phagocytic cells.

Cancer cells achieve apoptosis evasion by downregulating pro-apoptotic proteins such as Bcl-2-associated X (Bax) protein and upregulating anti-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2) protein.[5] Since

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ORCID IDs of the authors: H.B. 0000-0003-4577-3325; E.S. 0000-0002-1159-5850; S.Ö. 0000-0002-1721-7543.

Address for Correspondence: Halin Bareke
E-mail: halin.bareke@gmail.com
ORCID ID: orcid.org/0000-0003-4577-3325

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the administration of most chemotherapeutics leads to apoptosis induction, apoptosis resistance also promotes therapy resistance.[5] Therefore, anti-apoptotic mechanisms in a tumor enable its survival, its resistance to therapy, and its recurrence. Targeting molecules to reverse apoptosis evasion in tumors can restrict tumor growth and overcome therapy resistance. However, since apoptosis is a normal physiological process, the choice of target molecule must be tumor-specific to avoid deleterious side effects.

In solid tumors, the tumor microenvironment (TME) can promote tumor survival, invasiveness, and metastasis.[6] The TME can be defined as non-cancerous cells and structural components which surround the tumor cells. Although the structure of TME might vary slightly among tumor types, it generally consists of stroma cells (e.g., fibroblasts), leukocytes, blood vessels, and extracellular matrix (ECM).[6] The TME can support tumor progression in many ways, including by acting as a growth factor reservoir, modifying cell adhesion, providing nutrients through angiogenesis, and preventing immune cells from reaching the tumor.[7] Some components of the tumor TME, such as growth factors and matricellular proteins, have also been shown to be involved in apoptosis evasion of solid tumors.[8] Tenascin-C (TN-C) is one of the multimeric matricellular proteins in the TME which can promote tumor survival and spread by inducing apoptosis resistance, epithelial-mesenchymal transition, immune evasion, cell proliferation, and ECM degradation.[9,10] Normally, TN-C is found in the embryo near migrating cells, at the interaction sites of epithelial and mesenchymal cells, and in developing connective tissue.[11] TN-C expression after birth is highly restricted, with only minimal expression in tissues which withstand tensile stress, such as tendons and ligaments and in lymphoid organs (thymus, spleen, bone marrow etc.), where there is high cell turnover.[12] Despite its restricted expression in normal healthy tissues, it is consistently upregulated in solid tumors with prognostic significance.[13] TN-C has emerged as an ideal target for gene silencing oncotherapy approaches since its expression is highly restricted to tumors in adults. It is an especially optimum candidate for clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based therapies. CRISPR/Cas9 technology is the latest and the most influential genome-editing technology which can create permanent changes (i.e., knock-in, knock-out, and correction of genes) in the genome.[14] Since the created changes in the genome are irreversible, the target gene should be, like TN-C, highly specific only to the tumors.

TN-C is highly expressed and associated with a poorer prognosis in breast cancer.[13] It is expressed both by the stromal cells in the TME and breast cancer cells.[11] TN-C expression in breast cancer is correlated with lymph node and lung metastasis, tumor size, increased angiogenesis, and treatment resistance and rapid invasiveness.[15-17] TN-C can mediate these effects by decreasing cell adhesion, promoting cell survival and angiogenesis, and suppressing immune responses.[18] It has been shown that TN-C levels are especially high in the most aggressive breast cancer subtype, triple-negative breast cancer (TNBC), due to the autophagy deficiency of these cells.[19] This is important because TNBC is negative for estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 expression, meaning that treatment targets such as endocrine receptors normally exploited in other subtypes are absent in TNBC.[20] Therefore, TN-C is an important therapeutic target for TNBC, for which such targets are scarce. The TME is shown to be involved in immunomodulation and tumor progression in TNBC, and so, when using a TN-C-based gene therapy, the interaction between the TME and TNBC can potentially be reprogrammed.[21]

As a specific target for oncotherapies, TN-C has been shown to be involved in apoptosis resistance in multiple cancer types, including TNBC. One previous study showed that targeting TN-C with RNA interference induces apoptosis in human TNBC cell lines.[22] Moreover, in breast cancer cell cultures, TN-C reverses the cell cycle arrest induced by doxorubicin by inhibiting p21.[23] In a study of pancreatic cancer cells, it was shown that TN-C induced anti-apoptotic proteins Bcl-Extra-Large protein and Bcl-2 and it decreased caspase activity by activating the ERK1/2/NF-κB/p65 cascade, delineating a mechanistic link between TN-C expression and apoptosis evasion.[24]

To the best of our knowledge, no study has thus far used CRISPR/Cas9 to decrease TN-C expression in TNBC. Thus, the aim of this study was to knock down the *TN-C* gene using the effective genome-editing tool CRISPR/Cas9 in order to inhibit the growth of TNBC cells via apoptosis and hence, provide a foundation for a new gene therapy approach in TNBC.

MATERIALS AND METHODS

Materials

TN-C positive, human TNBC cell line MDA-MB-231 [American Type Cell Collection (ATCC® HTB-26™)] was purchased from ATCC (Manassas, VA, USA). The cells were maintained in a cell culture medium composed of high glucose DMEM medium (Sigma-Aldrich, St. Louis, MO, USA), 10% heat-inactivated fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), 100 I.U/mL penicillin and 100 µg/mL streptomycin (both from Capricorn Scientific, Ebsdorfergrund, Germany), and 2 mM L-glutamine (Sigma-Aldrich, St.Louis, MO, USA). The cells were passaged twice a week using trypsin/EDTA (Wisent BioProducts Quebec, Canada) for the chemical detachment of the cells from the culture flasks, and Ca^{+2}-and Mg^{+2}-free 1X PBS (Wisent BioProducts Quebec, Canada) for cell washes.

A plasmid coding for two TN-C-specific single guide RNAs (sgRNA) (CTGTTTCGAAGGCTACGCCG and CGGGAGAGGCGGGTGACAGT) and Cas9 enzyme on a single plasmid was synthesized from VectorBuilder (Chicago, IL, USA). A control plasmid containing scrambled sgRNA (sequence: GCACTACCAGAGCTAACTCA) and Cas9 enzyme was purchased from Origene (Rockville, MD, USA).

The cell transfections were carried out using a nanoparticle-based transfection agent, Lipofectamine 3000 (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA).

Cell proliferation studies were performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) and dimethylsulfoxide (DMSO) (Merck, Kenilworth, New Jersey, USA).

In vitro Transfection

Transfection of MDA-MB-231 cells by the CRISPR/Cas9 plasmids was accomplished by the reverse-transfection method, according to the manufacturer's protocol. The plasmids used in transfections were scrambled sgRNA CRISPR/Cas9 plasmid (1 µg) as a control and 1 µg of the TN-C CRISPR/Cas9 plasmid. A plasmid (µg): p3000 (µL): Lipofectamine (µL) ratio of 1:3:2 was used to complex the plasmids with the transfection reagents. The total volume was increased to 50 µL with a reduced serum medium, Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA). The complexed plasmid was mixed with 150,000 cells in 450 µL of complete medium and plated in triplicate on 24-well culture plates.

For the proliferation studies, the same plasmid-to-transfection agent ratio was adjusted for a total volume of 100 µl (200 ng of each plasmid) and the cells were plated on 96-well plates.

Real-Time PCR

The knock-down effect of the TN-C CRISPR/Cas9 plasmids on TN-C mRNA levels was assessed by real-time PCR (RT-PCR). First, RNA was isolated from the cells 3 days after the transfection according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen, Hilden, Germany). After the spectrophotometric confirmation of RNA quality (A260/A280=2), cDNA was synthesized using 0.5 µg of RNA. cDNA synthesis was performed by reverse transcription according to the manufacturer's protocol (ImProm-II Reverse Transcription System, Promega, Madison, WI, USA).

Primers specific to the human TN-C mRNA were used for the RT-PCR reaction (forward: AGAGAACCAGCCAGTGGTGT, reverse: GCCTGCTCCTGCAGTACATT). The TN-C mRNA levels were normalized using GAPDH mRNA levels as an internal control and the primers with sequences provided in a previous study were used.[25] The RT-PCR reactions were carried out in triplicate for each group, using the SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in QuantStudio™3 (Thermo Fisher Scientific, Waltham, MA, USA). TN-C mRNA levels were relatively quantified using the ΔΔCt calculation.

Apoptosis

The effect of reducing the expression of the *TN-C* gene on cell apoptosis was investigated using flow cytometry by staining with the ImmunoStep FITC Annexin-V Apoptosis Detection Kit with propidium iodide (PI) (Salamanca, Spain). The cells were detached from the plates 48 hours after transfection and washed 3 times with 1X PBS. The cells were stained according to the manufacturer's protocol. To correct for fluorescence spillover, the cells were also stained with Annexin-V only and PI only. The stained cells were visualized using the BD FACSAria cytometer (BD Biosciences, Franklin Lanes, NJ, USA) and analyzed using Infinicyt 2.0 software (Cytognos, Salamanca, Spain). Unstained untransfected cells were used to gate the quadrants in the dot plots.

Cell Cycle Analysis

A flow cytometry-based cell cycle kit containing RNAse, PI, and detergent was used to determine the effect of TN-C knockdown on the cell cycle (Cytognos, Salamanca, Spain) on day 2 post-transfection. PI enters through the cell membrane as it is permeabilized by a detergent, and then PI binds only to DNA since RNAse removes all RNA from the cell. Therefore, the amount of DNA in the cell could be determined, providing an indirect indicator of cell cycle phases. Similar to the apoptosis assay, the cells were trypsinized and washed 3 times with 1X PBS. Then, 200 µL of the ready solution was added, and after a 10-minute incubation at room temperature in the dark, the cells were immediately visualized using the BD FACSAria cytometer and analyzed using Infinicyt 2.0 software.

Cell Proliferation

The effect of transfection with TN-C CRISPR/Cas9 on cell proliferation was assessed by the formation of formazan salt by the living cells after the addition of MTT. The change in cell proliferation and hence cell number was measured as the difference in the amount of formazan formed. Three days after the transfection, the cell media was removed, and 0.5 mg/mL MTT in complete medium was added. The cells were incubated for 4 hours in a humidified incubator at 5% CO_2 and 37 °C to allow MTT to be converted into formazan. The insoluble formazan salt was subsequently dissolved by DMSO. The colorimetric reading was measured at 570 nm and 690 nm by a microplate reader (SmartSpec 300, Bio-Rad, Hercules, CA, USA). The absorbance at 690 nm was subtracted from that at 570 nm to obtain a corrected reading.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism Software version 7.00 for Windows (GraphPad Software, San Diego, California, USA). Student's t-test was used to test for statistical significance. Values of p-smaller than 0.05 were considered statistically significant ($^{*}p<0.05$; $^{**}p<0.01$).

RESULTS

CRISPR/Cas9 Plasmids Successfully Downregulate TN-C Expression

Transfection success was investigated by observing green fluorescent protein (GFP) fluorescence under a fluorescent microscope. There were GFP-positive cells 24 hours after transfection in TN-C CRISPR/Cas9 and scrambled CRISPR/Cas9 plasmid-transfected cells, confirming a successful transfection (Figure 1).

The TN-C relative expression study using RT-PCR showed that 3 days after the transfection, TN-C mRNA levels were significantly lowered ($p=0.0007$) to half of the level in the cells transfected with the scrambled control (Figure 2).

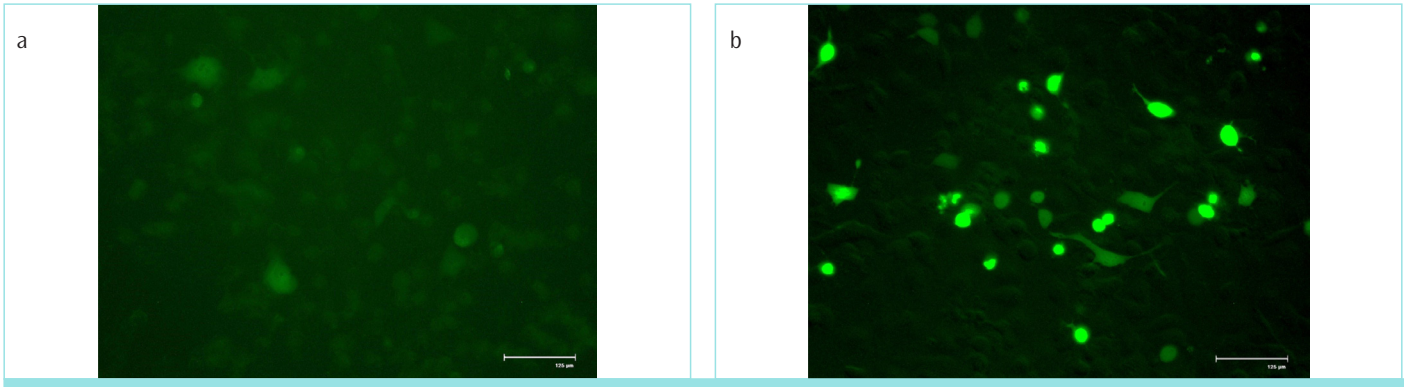

Figure 1. GFP fluorescence 24 hours after transfection with (a) TN-C CRISPR/Cas9 and (b) Scrambled CRISPR/Cas9 plasmids.

GFP: Green fluorescent protein, TN-C: Tenascin-C, CRISPR: Clustered regularly interspaced short palindromic repeats.

TN-C Genome Editing Induces Apoptosis in the Cancer Cells

The effect of TN-C genome editing by CRISPR/Cas9 on apoptosis was investigated 48 hours after transfection. With the onset of apoptosis, phosphatidyl serine, normally found in the inner layer of the cell membrane, is exposed on the outer layer, where Annexin-V can bind to it. Therefore, Annexin-V positive cells show the cell population in the early apoptotic phase. With the progression of apoptosis, PI can enter the cell due to increased cell permeability. Thus, double staining with Annexin-V and PI indicates late apoptosis. A representative result of four independent experiments is shown in Figure 3. TN-C genome editing increased the percentage of cells both in the early and late apoptotic stages, where the percentage was almost doubled for the late apoptotic cells.

Cell Cycle Distribution

As the literature shows that TN-C can affect the cell cycle, experiments were carried out to investigate whether reducing TN-C expression with the TN-C CRISPR/Cas9 plasmid alters cell distribution within the cell cycle phases. TN-C downregulation led to G0/G1 cell cycle arrest as the percentage of cells at this phase and in apoptosis (Figure 4, far left) increased, and the percentage of the cells in the later phases, namely S and G2/M phases decreased, relative to the scrambled CRISPR/Cas9 control.

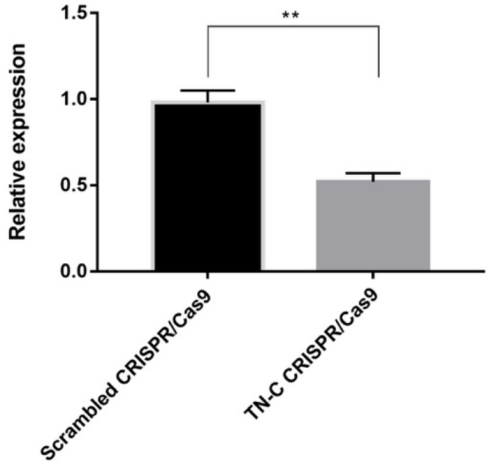

Figure 2. TN-C mRNA levels 3 days after transfection in the control and the experimental groups.

TN-C: Tenascin-C.

Transfection with the TN-C CRISPR/Cas9 Plasmids Decreased TNBC Cell Proliferation

Three days after the transfection, cell proliferation was examined using the MTT assay. Comparing the cell proliferation of the scrambled control transfected cells and TN-C CRISPR/Cas9 transfected TNBC cells revealed that the proliferation/cell numbers were halved by *TN-C* gene editing (p=0.0022) (Figure 5).

DISCUSSION

It has become increasingly apparent that the TME plays an enabling role in the maintenance and propagation of tumors. There is a two-way relationship between the tumor and its microenvironment, such that they reciprocally shape each other via chemokines, proteins, growth factors, and remodeling enzymes.[8] In light of this understanding, treatment strategies that regard the tumor as a "complex organ" and target the TME constituents have a promising therapeutic potential.

Breast cancer is projected to remain an important public health concern worldwide. TNBC specifically is an important threat to women's health globally since it has been refractory to the developments in diagnostic and therapeutic approaches. Addressing women's health issues such as breast cancer is integral to meeting the United Nation's Sustainable Development Goals (specifically Goals 3 and 5). Furthermore, research also shows that improving women's well-being alleviates the emotional and physical burden of disease and contributes to national productivity and economic advancement.[26] Therefore, we sought to provide a foundation for a new TNBC gene therapy approach by combining TME-targeting and the latest breakthrough in molecular biology, CRISPR/Cas9 technology. TN-C is one of the proteins found in the TME which sculpts the microenvironment to the benefit of the breast cancer cells, both in the primary site and the metastatic niche.[27,28] As TN-C expression is highly specific to cancer cells, it was chosen as the ideal knock-down target for the CRISPR/Cas9 system. Apoptosis evasion is a key mechanism

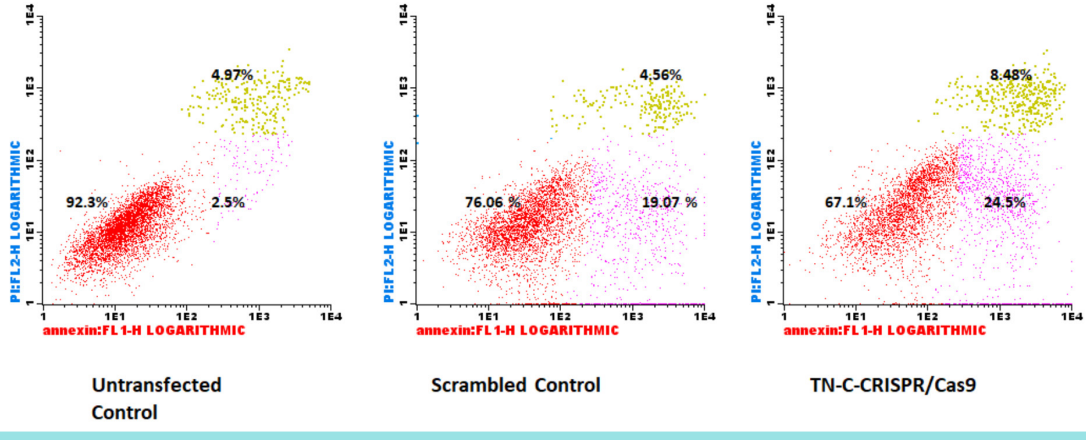

Figure 3. The percentage of cells alive (Annexin-V-, PI-) in early apoptosis (Annexin-V+, PI) and late apoptosis (Annexin-V+, PI+) in the control and the experimental groups. (Note: Annexin staining is depicted on the x-axis, and PI staining, the y-axis).

PI: Propidium iodide.

to target because it enables both tumor survival and therapy resistance, so reversing this evasion concomitantly opens up many therapeutic windows. In this study, we showed that TN-C downregulation by this system induced apoptosis in the aggressive MDA-MB-231 cell line and led to G1 arrest with a statistically significant decrease in cell proliferation. This shows that this system is viable for testing in more advanced studies, alone or in combination with other therapies.

CRISPR/Cas9 plasmids are usually challenging to transfect because of their large size due to the Cas9 enzyme and the strong promoters used. The TN-C-specific CRISPR/Cas9 plasmid and the control scrambled CRISPR/Cas9 plasmid used in this study were both large (10 kb). However, by increasing the amount of the complexation agent p3000 in the transfection kit, successful transfection was achieved, as shown by the GFP fluorescence images of the cells 24 hours post-transfection. The difference in the intensity of GFP fluorescence between the two plasmids can be attributed to the different GFPs coded on the plasmids, namely enhanced GFP on TN-C CRISPR/Cas9 plasmid and turbo GFP (tGFP) on the scrambled control under the control of CMV promoter and the promoter of the elongation factor-1 alpha (EF-1 alpha), respectively. EF-1 alpha has been shown to express the *GFP* gene more strongly than the CMV; therefore, the fluorescence from the scrambled control cells was stronger.[29]

Within the 3 days of transfection, there was approximately a 49% decrease in TN-C mRNA levels, demonstrating that two sgRNAs on the CRISPR/Cas9 plasmid can successfully target the *TN-C* gene. Two sgRNAs that target the initial exons of the large *TN-C* gene were used due to the size of the gene, which has 29 exons. The choice of exons to be targeted is especially important for genes such as *TN-C* because 9 of its exons can be alternatively spliced. Given that those sgRNAs targeting loci closer to the transcription start site result in better editing efficiency, the initial exons were successfully targeted by the sgRNAs used in this study.[30] It is known that the higher the number of cell cycles that the cell populations undergo, the higher the editing efficiency. Since MDA-MB-231 is a rapidly proliferating cell line, the high editing efficiency we observed is expected. The silencing efficiency during the later days can also be investigated to examine whether the edited cells have a selective disadvantage in the cell population pool. The latest addition to the chemotherapeutic arsenal against metastatic TNBC are poly (ADP-ribose) polymerase (PARP) inhibitors, such as Olaparib.[20] These inhibitors direct the cells to error-prone DNA damage repair (non-homologous end-joining), creating lethal mutations in the cell.[31] Since the CRISPR/Cas9-based knock-down and knock-out studies also rely on the mistakes made during the DNA-damage repair in non-homologous

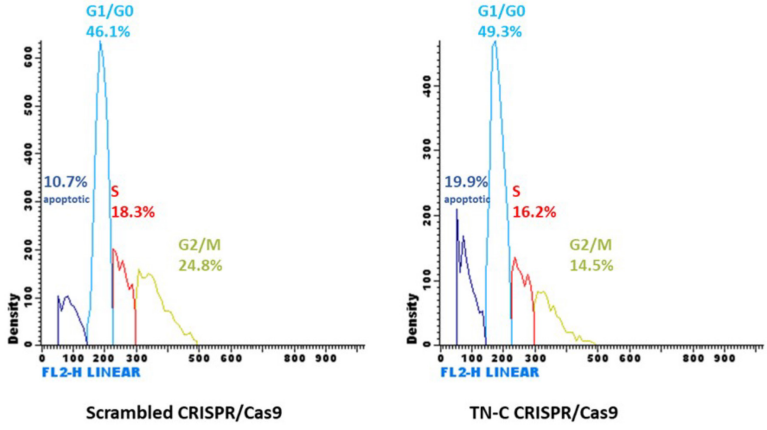

Figure 4. The distribution of the cells that are apoptotic and in different phases of the cell cycle, as evaluated by flow cytometric staining.
TN-C: Tenascin-C, CRISPR: Clustered regularly interspaced short palindromic repeats.

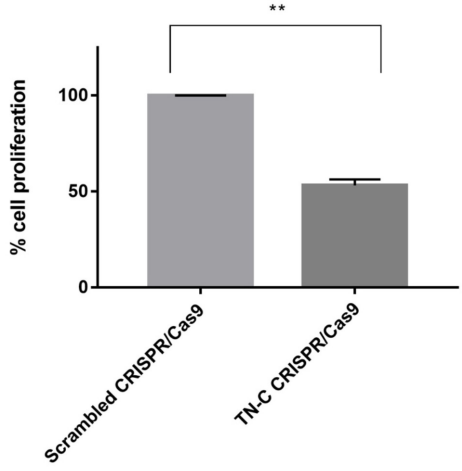

Figure 5. The effect of transfection with the control and TN-C CRISPR/Cas9 on cell proliferation.
TN-C: Tenascin-C, CRISPR: Clustered regularly interspaced short palindromic repeats.

end-joining, it is highly probable that combining PARP inhibitors with this CRISPR/Cas9-based therapy would enhance knock-down efficiency, leading to a synergistic anti-tumor response in TNBC.

A higher percentage of cells were found in early apoptosis in the scrambled control and TN-C CRISPR/Cas9 transfected cell groups than in the untransfected cells. The cell death and decrease in cell proliferation observed in the scrambled control transfected cells can be attributed to the expression of Cas9 and GFP which put extra strain on the cellular resources, which can lead to a decrease in proliferation and even apoptosis. Nevertheless, the early and late apoptotic cell percentages in the TN-C edited cell population were markedly higher than in the scrambled control cells. In future studies, using multicaspase flow cytometric staining or demonstrating caspase-3 activity would further support these findings. However, it is clear from the apoptosis and cell cycle analysis that TN-C downregulation induced apoptosis which yielded meaningful decreases in the number of TNBC cells and this is one of the ultimate goals of oncotherapies. TN-C can mediate anti-apoptotic effects by binding to growth factor receptors through its epidermal growth factor-like domain and induction of the ERK/NF-κB pathway.[24,32] This pathway can then lead to the activation of anti-apoptotic cells and cyclin D1 which reduce apoptosis and induce cell cycle progression.[33] Metastatic disease is the major cause of morbidity in TNBC, and metastatic TNBC cells have been shown to be more resistant to apoptosis. Therefore, the TN-C genome editing system could also be studied for use against metastatic TNBC.

The observed difference in cell proliferation can potentially be attributed to increased apoptosis and decreased proliferation since the pleiotropic effects of TN-C include cell cycle modulation. A study on the pancreatic ductal adenocarcinoma cells showed that TN-C addition to the cultures prompted G1/S transition.[24] Another study showed that TN-C promotes progression into the S phase via AKT signaling, leading to enhanced cyclin D1 expression. Cyclin D1 then complexes with CDK4 to free E2F from retinoblastoma protein to enable G1/S transition.[10] From these data, it could be deduced that the downregulation of TN-C would decrease G1/S transition. In the present study, this deduction has been confirmed. TN-C downregulation increased the number of cells in the G0/G1 phase, and the percentage of cells in the other phases decreased, consistent with previous studies. Drug-resistant disease is one of the primary challenges in treating TNBC, resulting in high mortality. Previous research has shown that TN-C can aid in doxorubicin resistance by reversing G1/S arrest via p21 inhibition.[23] We demonstrated that TN-C downregulation led to G1 arrest, and we propose that this approach could be assessed for reversing doxorubicin resistance and testing a doxorubicin combination therapy.

Study Limitations

The main limitation of this study is that only one TNBC cell line was used and another TN-C expressing TNBC should be used in order to confirm our findings. Evidence is also needed to show that different levels of silencing correlate with the amount of apoptosis induced. Furthermore, the silencing efficiency for the later time-points could be investigated in order to determine whether the edited cancer cell percentage would decrease due to the reduced fitness of these cells.

CONCLUSION

This study demonstrated for the first time that TN-C downregulation by the CRISPR/Cas9 system successfully induced apoptosis and G1 arrest in the human TNBC cell line. These results warrant further testing via the examination of combinatorial therapies with chemotherapeutic drugs and studies on reversing drug resistance. By formulating a viral or a non-viral vector, this approach could also be tested within *in vivo* gene therapy settings in an animal cancer model.

MAIN POINTS

- TNBC is an important problem for women's health globally.
- A tumor-promoting matricellular protein, TN-C, was successfully downregulated using CRISPR/Cas9 technology in TNBC cells, for which there is an urgent need for new therapeutic targets.
- TN-C downregulation induced apoptosis and inhibited G1/S transition, leading to a significant reduction in TNBC cell numbers.
- These data provide a strong foundation for further studies focusing on designing a new *TN-C*-based gene therapy approach using the powerful CRISPR/Cas9 system in TNBC cells.

ETHICS

Ethics Committee Approval: The study does not require ethics committee approval since it does not involve any human or animal subject.

Informed Consent: The study does not require informed consent approval since it does not involve any human or animal subject.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: H.B., E.S., S.Ö., Design: H.B., E.S., S.Ö., Data Collection and/or Processing: H.B., E.S., Analysis and/or Interpretation: H.B., E.S., S.Ö., Literature Search: H.B., Writing: H.B.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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