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Original Article

Transforming Growth Factor-β1-mediated Regulation of circ_DISP3 and ATF3 in Human Triple-negative Breast Cancer Cells

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Abstract

Background and objectives: We previously reported that transforming growth factor-beta 1 (TGF-β1) promoted breast cancer progression and metastasis through inhibiting the expression of miR-4638-3p via directly targeting activating transcription factor 3 (ATF3). The present study aimed to elucidate the mechanisms of TGF-β1 downregulating ATF3 targeting miR-4638-3p via circRNA in MDA-MB231 cells.

Methods: Three triple-negative human breast cancer cells (MDA-MB231, MDA-MB468, and MDA-MB453) were employed. In-silico analyses were used to identify the list of circRNAs targeting miR-4638-3p. RT-qPCR was performed to determine the expression of shortlisted circRNAs. Transient transfection and western blot analyses were carried out to identify the functional role of circ_DISP3. A dual-luciferase reporter assay was used to identify the direct binding of circ_DISP3 and miR-4638-3p.

Results: There was an inverse correlation between the expression of circ_DISP3 and miR-4638-3p in these cells. Antisense oligos-mediated silencing of circ_DISP3 restored the function of miR-4638-3p, and downregulated ATF3 in MDA-MB231 cells. The luciferase reporter assay identified the direct binding of circ_DISP3 to miR-4638-3p in these cells.

Conclusions: TGF-β1 influences the expression of ATF3 to stimulate circ_DISP3 to sponge miR-4638-3p in hBC cells. Hence, we suggest that the circ_DISP3/miR-4638-3p/ATF3 axis regulated by TGF-β1 may have potential applications for bone-metastatic breast cancer.

Keywords: Breast cancer; Transforming growth factor-beta; circRNA; miRNA; Activating transcription factor 3.

Abbreviations: ASO, antisense oligonucleotides; ATF3, activating transcription factor 3; BC, breast cancer; cDNA, complementary DNA; ceRNA, competing endogenous RNA; circRNA, circular RNA; CSCD, cancer-specific CircRNA database; lncRNA, long noncoding RNAs; miRNA, microRNA; MRE, miRNA response element; MMP13, matrix metalloproteinase 13; MW, molecular weight; ncRNA, noncoding RNA; PCR, polymerase chain reaction; qPCR, real-time PCR; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; S. no, serial number; TGF-β, transforming growth factor-beta; TNBC, triple-negative breast cancer.

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Introduction

According to recent statistics, breast cancer (BC) is the most common cause of death in women worldwide.[1] Among the various BC subtypes, triple-negative breast cancer (TNBC) accounts for around 15% of all BC cases. TNBC is characterized by its aggressive histology, poor prognosis, shorter survival, and unresponsiveness to most therapies.[2] Despite advances in the therapeutic field, TNBC shows an increased risk of early metastasis, causing high morbidity and mortality.[3] Metastasis is a complex multistep process that starts with the invasion of a tumor from the primary site through the vasculature to a distant target site. The predominant sites of BC metastasis include bone, lung, and liver.[4] The activation of several transcription factors and cytokines by the tumor microenvironment initiates metastasis to various organs.[5] Such transcription factors are prevalent in BC, and one example is activating transcription factor-3 (ATF3).[6,7]

ATF3 is a stress-inducible gene belonging to the activating protein 1 family, whose expression is associated with multiple diseases ranging from cancer to cardiac hypertrophy.[8] ATF3 has been implicated in regulating oncogenic activities through various mechanisms that either promote or suppress cancer progression depending on the cellular environment and the signaling pathways involved.[9] ATF3 also plays an immunoregulatory role in the tumor microenvironment. For instance, reports provided by Chang *et al.* demonstrated that ATF3 promoted a microenvironment at tumor metastatic sites that aid monocyte recruitment and immunosuppression.[10]

Transforming growth factor-beta (TGF-β) is an essential pluripotent cytokine that regulates normal mammary gland development and breast carcinogenesis.[11] Studies have implicated the regulatory role of TGF-β in exhibiting high percentages of lymphovascular invasion and lymph node invasion of micropapillary breast carcinoma.[12] Studies have also shown that the multifunctional cytokine, TGF-β1, regulates the expression of ATF3 and promotes the growth and metastasis of BC.[13,14] It was identified that genes for cell proliferation (cyclin A1), invasion (matrix metalloproteinase 13; MMP13), and bone metastasis (Runx2) were found to be ATF3 target genes.[15–17] Therefore, targeting ATF3 may help control BC development and the ensuing bone metastases. Since ATF3 has few druggable sites like other transcription factors, direct targeting ATF3 for translational research is difficult.[18]

Increasing evidence suggests the involvement of noncoding RNAs (ncRNAs), such as microRNAs (miRNAs) and circular RNAs (circRNAs), in regulating BC pathogenesis.[19–21] MiRNAs are small endogenous ncRNAs of 20–25 nucleotides in length. They regulate various biological processes, including angiogenesis, proliferation, migration, apoptosis, and differentiation, by binding to the complementary target mRNA.[22] For instance, overexpression of miR-21 downregulated the expression of Leucine zipper transcription factor-like 1, a key tumor suppressor gene, in BALB/c nude mice, facilitating BC cell proliferation and metastasis to the lungs and liver.[23] A study by Hunter *et al.* reported that the overexpression of miR-526b and miR655 positively regulates the angiogenesis of BC cells.[24] CircRNAs are long noncoding RNAs (lncRNAs) with the characteristic feature of a covalent close-loop structure lacking 5′ and 3′ ends.[25–28] There is emerging evidence of circRNAs that possess a miRNA response element (MRE) and function as competing endogenous RNAs (ceRNAs) that sponge and sequester the endogenous activity of miRNAs. For instance, a study by Sun *et al.* reported that upregulation of circRNA-0001361 increased the expression of FGFR4, a potential epithelial-to-mesenchymal transition and metastasis inducer, by sponging miR-491-5p.[28] Silencing of this circRNA downregulated the expression of FGFR4, reducing the axillary response after neoadjuvant chemotherapy in BC. A study by Song *et al.* showed that the upregulation of circ_ATAD3B sponged miR-570-3p, which significantly increased the expression level of MX2, a gene linked to the progression of human cancer.[27] circ_ATAD3B is currently being studied as a possible diagnostic biomarker of BC.

We previously reported that TGF-β1 promoted BC progression and metastasis via down-regulating the expression of miR-4638-3p, which directly targets ATF3 in hBC cells.[18] Therefore, in this study, we aim to investigate the mechanism by which TGF-β1 regulates miR-4638-3p and ATF3 in hBC cells.

Material and methods

Cell culture

Triple-negative human breast cancer cells (MDA-MB231, MDA-MB468, and MDA-MB453) were procured from the National Centre for Cell Science (Pune, India). The cells were maintained in Dulbecco's modified eagle's medium (Lonza Bioscience, Milan, Italy) supplemented with 10% fetal bovine serum and 1× antibiotics (penicillin, streptomycin, and amphotericin B) (Lonza Bioscience). The cells were treated with TGF-β1 (R&D Systems, Minneapolis, MN, USA) at a final concentration of 5 ng/mL for 1, 2, 4, 8, and 24 h).

Table 1. circRNAs that putatively target miR-4638-3p

S. no	circRNA ID	Score	Host gene
1.	chr12:11885925|11891342	142	ETV6
2.	chr21:10454221|10499540	144	BAGE2
3.	chr18:48962211|48965520	154	–
4.	chr3:12584517|12590905	140	RAF1
5.	chr20:20391916|20392672	145	RALGAPA2
6.	chr5:113082859|113143360	154	MCC
7.	chr16:67280201|67280692	150	PLEKHG4
8.	chr1:11530006|11530386	142	DISP3
9.	chr7:99393741|99394320	144	AC004922.1
10.	chr17:50862690|50863606	160	TOB1

circRNA, circular RNA; S. no, serial number.

In-silico analysis

A list of circRNAs predicted to target miR-4638-3p was retrieved from the cancer-specific CircRNA database (CSCD) 2.0 (http://gb.whu.edu.cn/CSCD2/#). The list was then sorted using filters such as circRNA present only in BC and MCF cells and seed sequence type (7mer-m8 and 8mer-1a). The validated circRNAs were eliminated from using TarBase and manual web search. A unique list of 10 unvalidated circRNAs was selected for further analysis (Table 1). The antisense oligonucleotide (ASO) for circ_DISP3 (5′-GGACAGGTCCCTTGGTACAGGTCGA-3′) was custom-designed and procured from Eurofins Genomics (Louisville, KY, USA).

Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

MDA-MB231, MDA-MB453, and MDA-MB468 cells were cultured and treated with 5 ng/mL TGF-β1 for 1, 2, 4, 8, or 24 h) or were not treated (control). RNA was isolated using Trizol reagent (Takara, Japan) and converted to cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qPCR) was performed with primers for circRNAs (Eurofins, Augsburg, Germany) (Table 2) using Genious 2× SYBR Green Fast qPCR Mix (ABclonal, Woburn, MA, USA) in Applied Biosystems QuantStudio 3 instrument (Thermo Fisher Scientific, Waltham, MA, USA). The relative expression of circRNAs was calculated using the ΔΔCt method, with RPL13AB as an internal control.

Transient transfection

Cells at 70–80% confluency were transiently transfected with negative control or antisense oligonucleotides (ASO) of circ_DISP3 (60 nM) (Eurofins Genomics) using X-treme gene transfection reagent (Roche, Basel, Switzerland). After 24 h transfection, cells were treated with TGF-β1 or left untreated. Protein

Table 2. Primers used in qPCR analysis of circRNAs

S. no	Name	Primer, 5′– 3′
1	circ_ETV6	F: AAATCTCACAAAGTGCGGCAA
		R: AGAGGAACCTGAACCCCTC
2	chr18:48962211|48965520	F: GACTCCCAGCTCTTGCTGT
		R: GGACAACCCAGGAATGTGG
3	circ_BAGE2	F: AGGCTGGAAGTGAAGTGCAA
		R: AGGAGCGCACTGTGTGTAAT
4	circ_PLEKHG4	F: GGGCCTTGGAGACCCTTTA
		R: CTTCCCAGACTTCCCTAGC
5	circ_TOB1	F: CGCCTCCTTTTGGTCACTC
		R: GGGAGAAGTACGTGCAACC
6	circ_DISP3	F: GGGGGTCCGGTTTTTCTTG
		R: CCATTCCAGTCCCTCACAGG
7	circ_RALGAPA2	F: CTAGAGGTTTGCTCCACCC
		R: CTGTGAGAACAAAGCCTGCG
8	circ_RAF1	F: CCCCAGAGGTGATCCGAAT
		R: TAAGGAAGCTCCCCCGTCA
9	circ_Mcc	F: TAGACCGTCTGCAAGGCAC
		R: CCTCGTTGACCTCGTGTTG
10	circ_Ac004992.1	F: GGCATGAGTATCTGGGATGT
		R: GAGTGAAGGATGGGGGTCA

circRNA, circular RNA; F, forward; qPCR, real-time polymerase chain reaction; R, reverse; S. no, serial number.

samples were collected using RIPA buffer and subjected to western blotting.

Western blot assay

Total cell protein lysates were collected using RIPA buffer (Bio Basic; Markham, ON, Canada), comprising phosphatase and protease inhibitors, and western blotting was performed as previously described.[29] Briefly, proteins were resolved by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes, blocked, and incubated with antibodies. The protein bands were visualized using the Chemidoc system (Bio-Rad). The blots were stripped and probed. Antibodies against ATF3 (Cat #sc-81189; Santa Cruz Biotechnology, Dallas, TX, USA) and alpha tubulin (Cat #2125; Cell Signaling Technology, Danvers, MA, USA) were probed. alpha tubulin was used as an endogenous control.

Luciferase reporter assay

The luciferase reporter assay was performed using the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Fitchburg, WI, USA), as previously described.[30,31] The sense and antisense oligonucleotides containing the wild-type or mutant MRE of the circ_DISP3 were synthesized. They were annealed and ligated into the pmirGLO vector. Cells were transfected with pCMV-MIR (empty vector; cat #PCMVMIR; Origene, Rockville, MD, USA) or pCMV-MIR4638 (mir-4638 overexpressing vector; cat #SC401776; Origene) along with the wild-type or mutant constructs of circ_DISP3 using the transfection reagent, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h transfection, the cells were lysed, and relative luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity. *Renilla* luciferase activity was used as an internal control.

Statistical analysis

Experiments were conducted in triplicate, and the results were analyzed by one-way analysis. p-values of <0.05 were considered statistically significant.

Results

Identification of circRNAs that putatively target miR-4638-3p

The CSCD 2.0 bioinformatics tool was used to identify circRNAs that putatively target miR-4638-3p (Fig. 1). We retrieved 8125 circRNAs predicted to target miR-4638-3p, and filtering by cell type (BC or MCF-7), number of algorithms (two), and site type (7mer-m8 or 8mer-1A), the number of circRNAs was reduced 20. WebBase and TarBase were used to identify an unvalidated list of 10 circRNAs that putatively target miR-4638-3p (Table 1).

TGF-β1 regulated the expression of circRNA that putatively targets miR-4638-3p in hBC cells

RT-qPCR was performed to determine the presence and expression pattern of the above-shortlisted circRNAs in hBC cells. Following TGF-β1 stimulation, several circRNAs were upregulated or down-

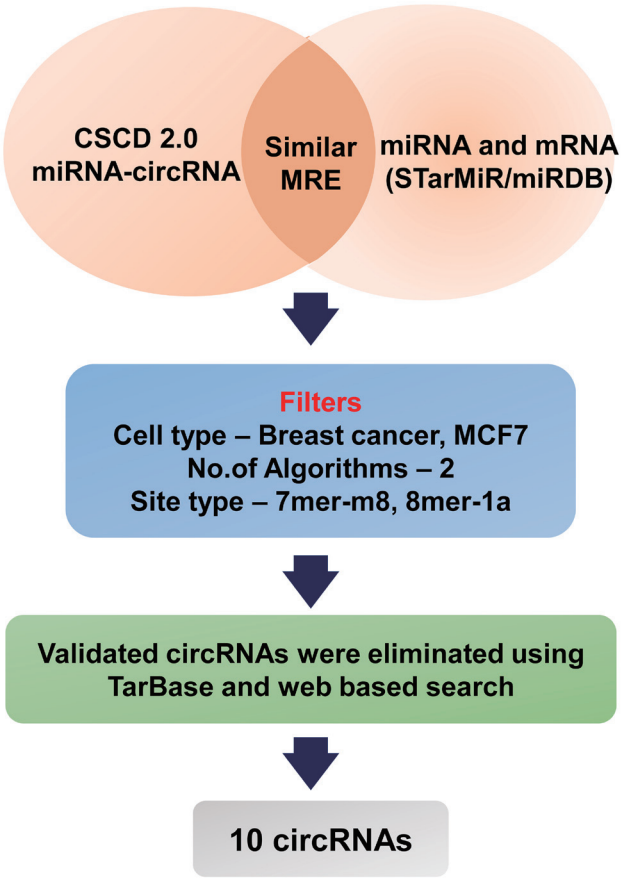

Fig. 1. *In-silico* identification of circRNAs that putatively target miR-4638-3p. CSCD, cancer-specific circRNA database; circRNA, circular RNA; mRNA, messenger RNA; miRNAs, microRNAs; MRE, miRNA response element.

regulated in MDA-MB231 cells (Fig. 2). Of the expression profiles, circ_MCC, circ_PLEKHGH, circ_TOB1, circ_ETV6, and circ_BAGE2 were downregulated at the tested times of TGF-β1-treatment, compared with the MDA-MB231 control cells. In contrast, circ_DISP3, circ_RALGAPA2, circ_AC004922.1, circ_chr18_48962211-48965520, and circ_RAF1 were significantly upregulated at each TGF-β1 treatment time compared with their control cells (Fig. 2). Circ_DISP3 was significantly upregulated at 2 and 8 h of TGF-β1-treatment compared with their respective control (Fig. 2c).

In addition to MDA-MB231 cells, we also determined the expression of circ_DISP3 in other TNBC cell lines. Upregulation of circ_DISP3 was observed in both MDA-MB453 and MDA-MB468 cells 1, 2, 4 and 24 h) (Fig. 3a, b). The results were consistent with the expression profiles of circ_DISP3, especially at 2 h of TGF-β1-treatment in MDA-MB231 cells (Fig. 2c).

ASO-mediated silencing of circ_DISP3 downregulated ATF3 expression in hBC cells

As TGF-β1 upregulated the expression of circ_DISP3 in hBC cells (Figs. 2 and 3), its functional role was determined. MDA-MB231 cells were transiently transfected with negative control or ASO for circ_DISP3, followed by control or TGF-β1 treatment. Whole-cell lysates were collected and assayed by western blotting the antibodies against ATF3 and alpha tubulin. TGF-β1 increased ATF3 expression in MDA-MB231 cells compared to the transfection. negative controls. The silencing of circ_DISP3 with ASO decreased the ATF3 expression seen in TGF-β1-stimulated cells, compared with their negative transfection control groups (Fig. 4). Therefore, the silencing of circ_DISP3 caused a decrease in TGF-β1-stimulated ATF3 expression in hBCs, and the effect may have been caused loss of the targeting of miR-4638-3p cells.

circ_DISP3 directly interacted with miR-4638-3p in hBC cells

We previously reported that miR-4638-3p directly targeted ATF3.[18] In this study, silencing of circ_DISP3 decreases ATF3 expression in hBC cells (Fig. 4). Hence, we hypothesized that circ_DISP3 acted as a sponge toward miR-4638-3p, resulting in increased ATF3 expression in hBC cells. We next determined whether circ_DISP3 directly interacted with miR-4638-3p using a dual-luciferase reporter assay system. Various bioinformatics tools, such as miRBase and CSCD 2.0, STarMiR, were used to identify the presence of MREs in circ_DISP3 toward miR-4638-3p. Of four predicted sites, (5–25) had excellent logit prob scores for circ_DISP3 and miR-4638-3p (Fig. 5a). To determine their direct interaction, MDA-MB-231 cells were transiently cotransfected with pCMV-MIR or pCMV-MIR4638 in the presence of pmirGLO or wild-type MRE or mutant MRE constructs of circ_DISP3 in pmirGLO. We observed a significant decrease in luciferase activity when the cells were cotransfected with wild-type circ_DISP3 constructs and pCMV-MIR4638. In contrast, there were no substantial changes in luciferase activity following cotransfection of mutant circ_DISP3 or pmirGLO constructs with pCMV-MIR or pCMV-MIR4638 (Fig. 5b). The results confirm the direct binding between miR-4638-3p and circ_DISP3 in hBC cells.

Discussion

Several studies have found that cytokines and growth factors such as interleukins, TNF-α, TGF-β, colony-stimulating factor 1, and VEGF have a key role in inducing BC metastasis. In the early stages of tumorigenesis, TGF-β has tumor suppression activity such as cell cycle arrest and apoptosis.[32] In contrast in later stages of cancer, TGF-β increases tumor progression and metastasis by increasing cell invasion, migration, and resistance to treatment.[33]

Like TGF-β, ATF3 has a dichotomous role in hBC cells. In untransformed mammary epithelial cells, ATF3 expression was observed to be low, and it stimulated cell apoptosis. In. contrast ATF3 expression enhanced cell proliferation and invasion in BC cells.[34,35] Our previous studies demonstrated that TGF-β1-stimulation in MDA-MB231 cells results in persistent and continuous ATF3 expression. This effect could be due to its interaction with factors like SMAD4 and NFATC2 in stabilizing ATF3 in these cells.[6] ATF3 was also seen to influence the expression of its direct downstream target genes, such as cyclin A, Runx2, and MMP-13 to facilitate BC development and bone metastasis.[15–17]

In addition to cytokines and stress signals, ATF3 is regulated by several short ncRNAs (miRNA, siRNA, shRNA) and lncRNAs (circRNA, linear long ncRNA).[19,21] Recent studies have attempted post-transcriptional regulation of ATF3 using many ncRNAs. For example, Rohini *et al.* reported that the downregulation of miR-590-3p was responsible for the increased expression of ATF3 and Runx2 in hBC cells, thereby inhibiting cell apoptosis and promoting cell proliferation.[8] Song *et al.* found that miR-590-3p controlled ATF3, thus regulating carcinogenesis.[36] MiR-590-3p influenced BC by targeting GOLPH3, a Golgi-related gene, in addition to ATF3, which in turn affected the ATF3/miR-590/GOLPH3

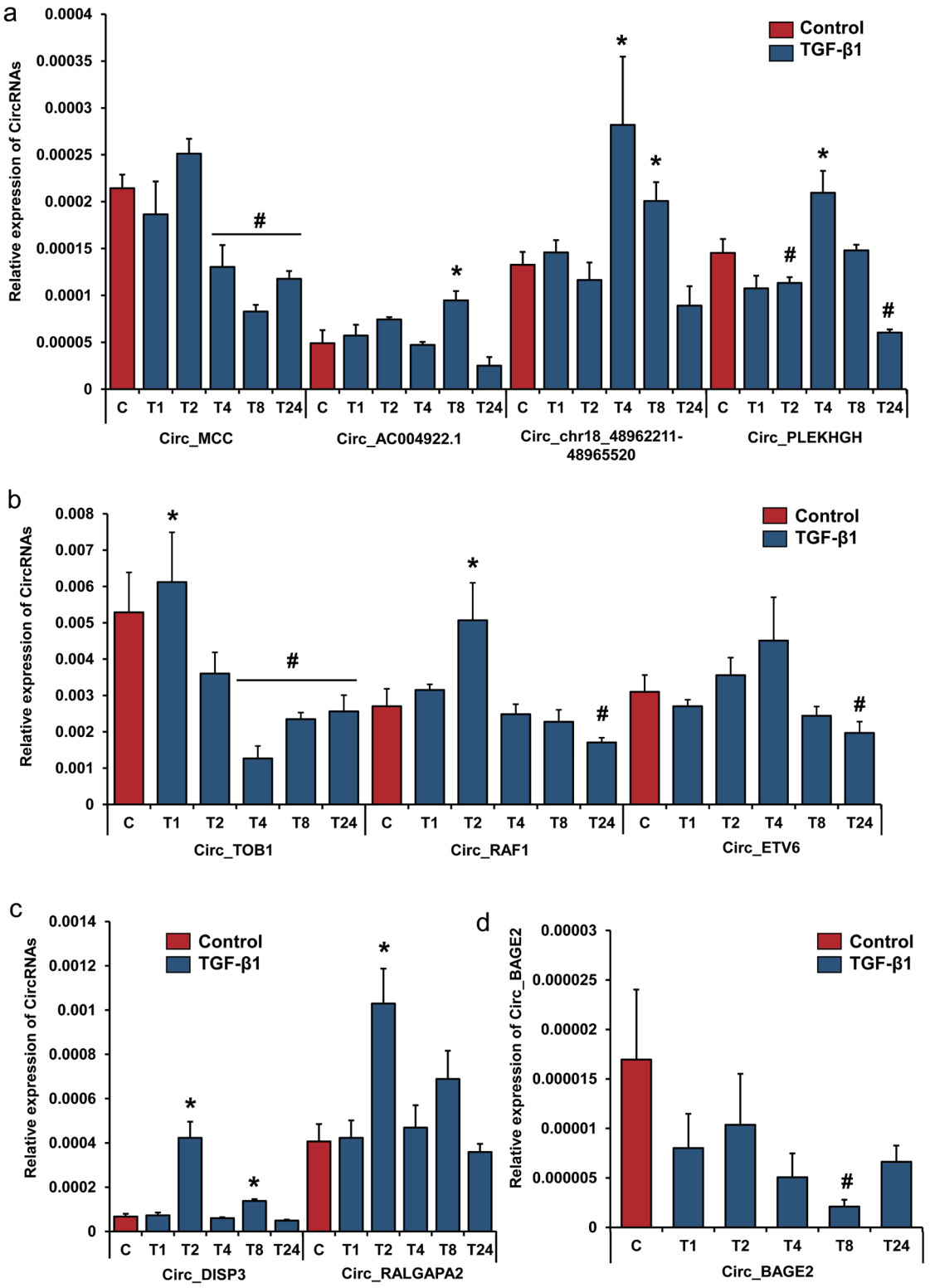

Fig. 2. Differential expression profiles of circRNAs in hBC cells upon TGF-β1-treatment. MDA-MB231 cells were either left untreated or treated with TGF-β1 (5 ng/mL) for 1, 2, 4, 8, or 24 h. Total RNA was isolated, cDNA synthesized, and subjected to qPCR analysis with primers for circRNAs and RPL13AB. Relative expression profiles of (a) circ_MCC; circ_AC004922.1; circ_chr18_48962211-48965520; circ_PLEKHGH, (b) circ_TOB1; circ_RAF1; circ_ETV6, (c) circ_DISP3; circ_RALGAPA2, and (d) circ_BAGE2. RPL13AB was used for normalization. *$p < 0.05$; #$p < 0.05$ compared with the control. cDNA, complementary DNA; circRNAs, circularRNAs; qPCR, real-time PCR; TGF-β1, transforming growth factor-beta 1.

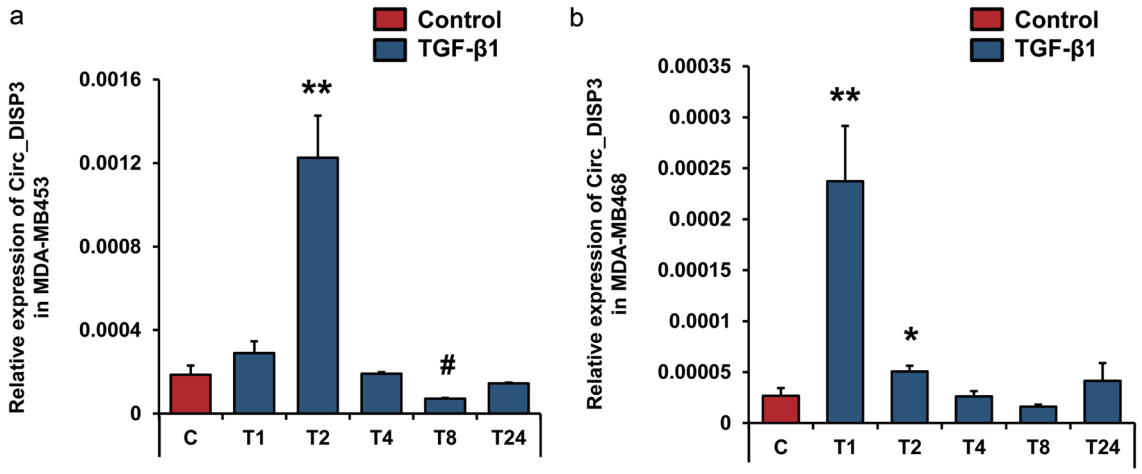

Fig. 3. TGF-β1 regulated the expression of circ_DISP3 in MDA-MB453 and MDA-MB468 cells. MDA-MB453 and MDA-MB468 cells were treated with TGF-β1 (5 ng/mL) for 1, 2, 4, 8, or 24 h or untreated (control). Total RNA was isolated, cDNA was synthesized, and qPCR analysis was carried out. Relative expression of (a) circ_DISP3 in MDA-MB453 and (b) circ_DISP3 in MDA-MB468. RPL13AB was used as an internal control for normalization. *$p < 0.05$ compared with the control, #$p < 0.05$ compared with the control, **$p < 0.01$ compared with the control. cDNA, complementary DNA; circRNAs, circularRNAs; qPCR, real-time PCR; TGF-β1, transforming growth factor-beta 1; TNBC, triple-negative breast cancer.

signaling cascade, and inhibited proliferation of BC cells. Akshaya *et al.* reported that TGF-β1 treatment substantially reduced the expression of miR-4638-3p in BC cells and that overexpression suppressed BC progression and invasion and induced apoptosis by targeting ATF3.[18] This study emphasizes the role of miR-4638-3p as a therapeutic target for bone-metastatic BC.

CircRNAs have gained interest in recent years as potential new cancer diagnostic and prognostic biomarkers.[37] circRNAs regulate gene transcription by acting as miRNA sponges to mediate biological functions.[38] For example, Chen *et al.* previously reported that circ-MALAT1 increased the expression of the oncogene JAK2 by sponging miR-6887-3p to enhance self-renewal of liver cancer stem cells.[39] Zheng *et al.* found a positive correlation of the expression levels of circSEPT9 and leukemia inhibitory factor.[40] Increased circSEPT9 expression upregulated the expression levels of cancer progression genes, LIF, phosphor-STAT3, ID1, and MDM2 by sponging miR-637. Upregulation of circSEPT9 in TNBC tissue spontaneously increased lung metastasis with an increase in the number of metastatic nodules. Li *et al.* reported that upregulation of circMMP11 indirectly regulated the expression level of MMP11 (matrix metalloproteinase-11), a paracrine factor in invasive BC.[41] The increased expression of circMMP11 was found to be associated with the proliferation, migration, and invasion of BC cells.

In this study, we identified the molecular mechanism by which these ncRNAs (miRNA and circRNAs) regulated the expression pattern of ATF3 in hBC cells. *In-silico* analysis using CSCD 2.0 identified circRNAs that putatively target miR-4638-3p (Fig. 1 and Table 1). Of those circRNAs, TGF-β1-stimulation increased the expression of circ_DISP3 in hBC cells (Figs. 2 and 3). Previous studies reported that upregulated circRNA acted as a sponge for miRNAs. For example, Huang *et al.* reported that overexpression of circRPPH1 in MDA-MB231 acted as sponges for miR-512-5p targeting STAT1 and leading to BC progression and invasion.[42] More convincingly, circFBXW7 overexpression reduced tumor growth by preventing cell migration and cancer cell proliferation both *in vitro* and *in vivo*.[42] CircFBXW7 inhibited the growth and metastasis of TNBC by upregulating the expression of FBXW7 and sponging miR-197-3p.[43] In addition to the highly invasive cell line MDA-MB231, we have also checked the expression of circ_DISP3 in other TNBC cells, MDA-MB453, and MDA-MB468.[44–46] The circ_DISP3 expression pattern was consistent in all three hBC cell lines. There is evidence of the difference in the expression pattern of genes in different cell lines, but that was not the case for the circRNA in our study.[47]

As TGF-β1 upregulated the expression of circ_DISP3, its functional role was determined by ASO-mediated silencing of circ_DISP3. Our results suggest that the silencing of circ_DISP3 by ASO transfection downregulated TGF-β1-stimulated ATF3 expression in MDA-MB231 cells (Fig. 4). This might be attributed to the increase of miR-4638-3p expression level due to the lack of miRNA targeting by the circRNA. Løvendorf *et al.* reported that the knockdown of circRNA using ASO led to the functional loss of circRNA *in vitro* and *in vivo*.[48] Xu *et al.* reported that targeting

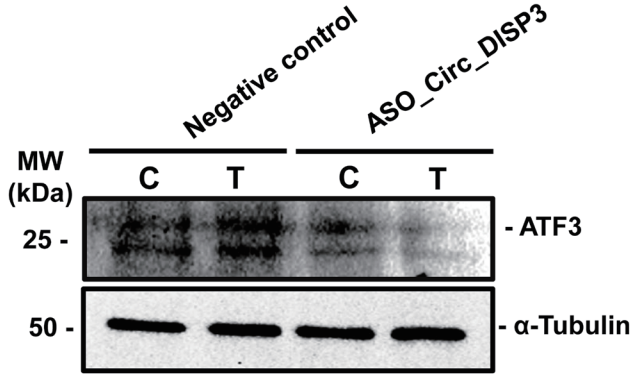

Fig. 4. ASO-mediated silencing of circ_DISP3 decreased TGF-β1-stimulated ATF3 expression in hBC cells. MDA-MB231 cells were transiently transfected with negative control or ASO_circ_DISP3, followed by 2 h TGF-β1-treatment. Whole-cell lysates were collected and subjected to western blot assays using anti-ATF3 antibody. Alpha tubulin was the normalization control. ASO, antisense oligonucleotide; ATF3, activating transcription factor 3; MW, molecular weight; TGF-β1, transforming growth factor-beta 1.

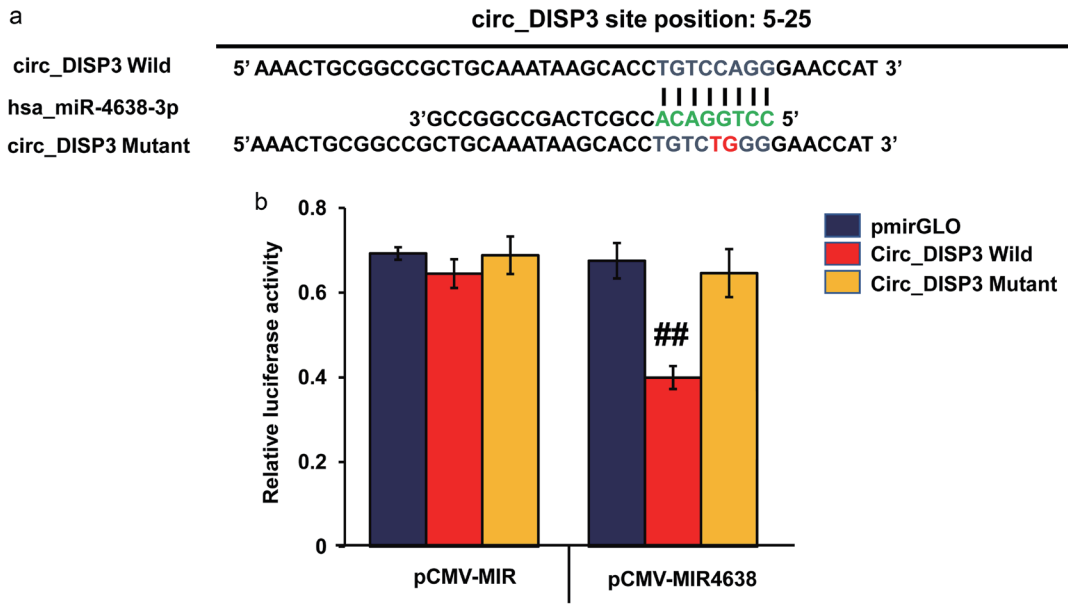

Fig. 5. Direct targeting of miR-4638-3p with circ_DISP3 in MDA-MB231 cells. (a) A pictorial representation of binding between miR-4638-3p seed site and MRE of circ_DISP3 (wild-type and mutant binding sites). Nucleotides in blue, green, and red indicate circ_DISP3 MRE, seed site of miR-4638-3p, and mutated circ_DISP3 MRE, respectively. (b) Cells were cotransfected with pCMV-MIR or pCMV-MIR4638 along with pmirGLO or wild-type or mutant constructs of circ_DISP3. Lysates were collected 24 h after transfection, and the relative luciferase activity was measured after normalization with *Renilla* luciferase activity. ##p< 0.01 compared with pmirGLO or mutant circ_DISP3 under negative control (pCMV-MIR) or pCMV-MIR4638 transfection. MRE, miRNA response element.

circIKBKB via ASO delayed the incidence of bone metastasis in BC cells.[49] Similarly, in female BALB/C nude mice, ASO-mediated silencing of circRNA progesterone receptor was observed to suppress estrogen receptor-positive BC cell growth by sponging miR-301a-5p.[50]

A luciferase assay was performed to further understand the mechanism of miR-4638-3p and circ_DISP3 binding. The results revealed the direct interaction of miR-4638-3p with the MRE of circ_DISP3 (Fig. 5). The direct interaction of circRNAs with miRNAs has previously been identified by luciferase reporter assays.[51,52] CircRNAs serve as miRNA sponges to inhibit miRNA activity and regulate the expression of their target genes. For example, circ_CUX1 regulates p300-mediated Runx2 acetylation in rat osteoblastic cells by acting as a sponge for miR-130b-5p. The knockdown of circ_CUX1 led to the upregulation of miR-130b-5p, thereby reducing the expression level of p300.[53]

Conclusion

Overall, our results show that TGF-β1 stimulated the expression of ATF3 via the stimulation and sponging activity of circ_DISP3 toward miR-4638-3p in hBC cells. The knockdown of circ_DISP3 reversed the effect of TGF-β1, *i.e.,* ATF3 expression decreased via lack of targeting of miR-4638-3p in those cells. TGF-β1-regulation of circ_DISP3/miR-4638-3p/ATF3 axis thus had a pivotal role in BC progression and bone metastasis (Fig. 6). Further, *in vitro* and *in vivo* studies need to be carried out to validate and support these findings for clinical application.

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There is nothing to declare.

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Conflict of interest

There are no known conflicting financial or personal interests that could have affected the research described in the study.

Author contributions

Study concept and design (NS, IS), *in-silico* analysis (SM, ASMR), performing the experiments (IS, RLA, SM, IM, RK), technical assistance (SG, SK), manuscript preparation (SM, ASMR, RK, IM, KS, IS), and study supervision, content assessment and funding (NS). All authors made a significant contribution to this study and approved the final manuscript.

Data availability

The corresponding author will provide the results produced during the current investigation upon reasonable request.

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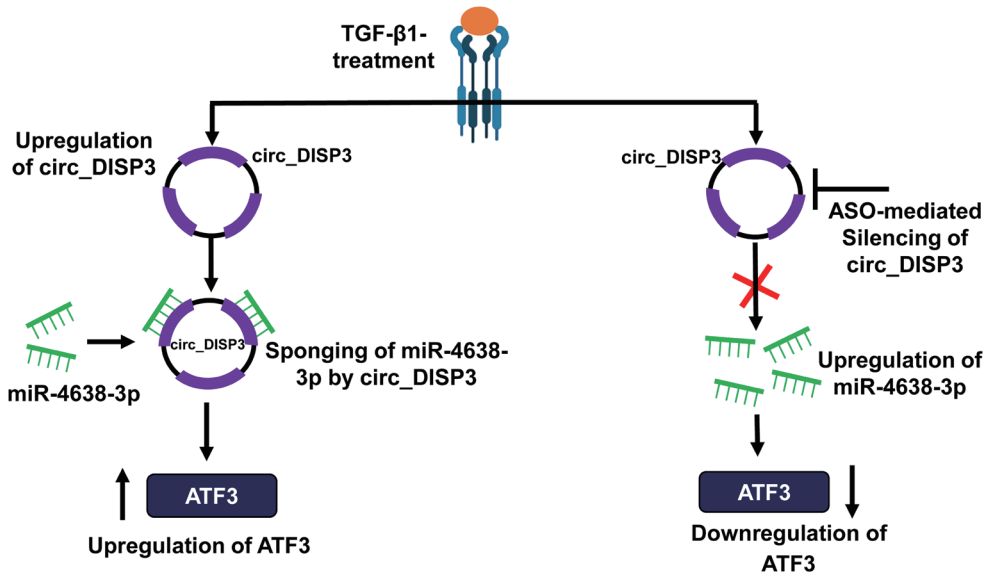

Fig. 6. Schematic representation of TGF-β1-mediated ATF3 expression in hBC cells. TGF-β1 increased the expression level of circ_DISP3, causing the sponging activity toward miR-4638-3p and increasing ATF3 expression in hBC cells. Whereas the ASO-mediated silencing of circ_DISP3 facilitated the nontargeting of miR-4638-3p, decreasing ATF3 expression in these cells. ASO, antisense oligonucleotides; ATF3, activating transcription factor 3; BC, breast cancer; TGF-β1, transforming growth factor-beta 1.

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