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## Original

# Eribulin Treatment Induces High Expression of miR-195 and Inactivates the Wnt/ $\beta$ -catenin Signaling Pathway in Triple-negative Breast Cancer

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**Abstract** : Triple-negative breast cancer (TNBC) accounts for 10–15% of all breast cancer cases and shows a poor prognosis with 30% distant metastasis. With few specific target molecules and ineffective hormonal and anti-HER2 treatment, an alternative therapeutic method for TNBC is urgently required. Recently, a non-taxane inhibitor of microtubule dynamics called eribulin was developed for breast cancer therapy. Eribulin induces irreversible mitotic mass formation in cancer cells during the G2-M phase, initiating apoptosis; however, the mechanism underlying this eribulin activity remains unclear. We reported previously that exposing non-basal-like (NBL) TNBC cells to eribulin increases miR-195 expression, which in turn decreases the expression of targeted Wnt3a. The present study sought to further clarify the mechanism of this antitumor effect by exploring how eribulin affects Wnt/ $\beta$ -catenin signaling based on miRNA expression changes in TNBC. In an NBL type of human breast cancer cell line (MDA-MB-231 cells), we compared the expression levels of Wnt/ $\beta$ -catenin signaling pathway proteins in cells exposed to an miR-195 mimic (cells transfected with miR-195 and in which Wnt3a expression was suppressed) and in cells exposed to eribulin. Expression levels of Wnt3a,  $\beta$ -catenin, and GSK-3 $\beta$  were measured by ELISA and observed by fluorescence immunostaining. Wnt3a and  $\beta$ -catenin expression was significantly lower and GSK-3 $\beta$  expression was significantly higher in the cells exposed to eribulin and transfected with miR-195 mimic than in the untreated controls, suggesting that eribulin inactivates the Wnt/ $\beta$ -catenin signaling pathway. Therefore, a novel antitumor mechanism of eribulin was determined, whereby eribulin induces high expression of miR-195 to inactivate the Wnt/ $\beta$ -catenin signaling pathway in NBL-type TNBC.

**Key words** : triple-negative breast cancer, eribulin, miR-195, GSK-3 $\beta$ , Wnt/ $\beta$ -catenin signaling pathway

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## Introduction

Breast cancer is the most common cancer in women and is classified into subtypes based on its gene expression. Triple-negative breast cancer (TNBC) is negative for estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) expression. TNBC accounts for 10–15% of all breast cancer cases and shows a poor prognosis with a 30% chance of distant metastasis; however, no specific therapeutic target molecules have been reported for TNBC, and hormone or anti-HER2 treatments are not effective. Thus, a new therapeutic strategy involving specific targets is urgently required<sup>1–3</sup>. TNBC is a disease with a poor prognosis<sup>1–3</sup> and no definite therapeutic target, other than BRCA1/2, has been identified, despite many trials. In 2011, TNBC was subdivided into six types, namely basal cell-like type 1 (BL-1), basal cell-like type 2 (BL-2), immunomodulatory type (IM), mesenchyme-like type (M), mesenchymal stem cell-like type (MSL), and luminal androgen receptor type (LAR)<sup>2, 4</sup>. Establishing a target for each subtype is also needed for the development of effective therapeutic agents.

One candidate therapeutic agent proposed for TNBC is eribulin mesilate, a non-taxane microtubule dynamics inhibitor that induces irreversible mitotic mass formation at the G2-M phase and induces apoptosis in cancer cells<sup>5–9</sup>. A purported antitumor mechanism for eribulin involves the suppression of microtubule elongation due to their polymerization without affecting shortening of microtubules, aggregation of microtubule monomers, or microtubules themselves<sup>5–10</sup>. This function of eribulin could intersect with the wingless-type MMTV integration site family (Wnt)/ $\beta$ -catenin signaling pathway, a pivotal regulator of cell proliferation and differentiation, as well as embryogenesis and tumorigenesis<sup>11, 12</sup>. Activation of Wnt/ $\beta$ -catenin signaling has been reported in several cancers, such as colorectal cancer, ovarian cancer, and breast cancer<sup>13</sup>. In particular, the activation of this pathway is frequently observed in TNBC and is reportedly related to this cancer's poor prognosis<sup>14, 15</sup>.

Another potential target of eribulin is cellular microRNA (miRNA). These regulatory non-coding RNAs (19–25 nucleotides in length) bind complementarily to a specific target nucleotide of the 3'-untranslated region (UTR) of mRNA and can inhibit the expression of both mRNA and protein<sup>16–20</sup>, although the potential antitumor mechanisms of miRNAs remain ill-defined. Our previous study<sup>21</sup> suggested an association between miRNA and eribulin in MDA-MB-231 cells, but there is no other published data regarding the miRNA-mediated antitumor mechanism of eribulin. We speculated that the miRNA that regulates Wnt3a, an upstream protein of the Wnt/ $\beta$ -catenin signaling pathway, is involved in the antitumor mechanism of eribulin.

Therefore, we aimed herein to analyze miRNA expression as a potential mechanism of eribulin's activity in TNBC cells and to clarify the influence of eribulin on the Wnt/ $\beta$ -catenin signaling pathway.

## Materials and methods

### *Cell lines and culture conditions*

The human non-basal-like (NBL) type of the TNBC cell line MDA-MB-231 (mesenchymal cell-like type) was derived from the Japanese Cancer Resources Bank (Osaka, Japan). MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) Ham/F12 (Sigma-Aldrich, Oberhaching, Germany) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA), 100 units/ml penicillin/100 mg/ml streptomycin (GIBCO penicillin-streptomycin liquid; Invitrogen, CA, USA). We also added 1 mM pyruvic acid (Sigma-Aldrich) to the DMEM. The cells were incubated at 37°C and with 5% carbon dioxide.

### *Eribulin Compound*

MDA-MB-231 cells were seeded in 6-well plates or 10 cm-diameter plates and treated with a final concentration of 1 nM eribulin mesilate (C<sub>40</sub>H<sub>59</sub>NO<sub>11</sub> · CH<sub>4</sub>O<sub>3</sub>S; MW, 826.00) (Halaven<sup>®</sup>, Eisai Co., Ltd, Shinjuku-ku, Tokyo, Japan), as set in our previous study<sup>21</sup>.

### *Transfection*

We generated miRNA mimics of miR-195-5p complementary to the 5'-UTR using the miRCURY LNA<sup>™</sup> microRNA Mimic (Exiqon Inc., Vedbaek, Denmark), with nucleic acid sequences as follows: (<http://www.exiqon.com/mirna-inhibitors>);  
has-miR-195-5p: 5'-rUrArGrCrArGrCrArCrArGrArArArUrArUrUrGrGrC-3'.

The MDA-MB-231 cells were then transfected with each miRNA mimic by lipofection. For transient transfections, the cells were seeded at low density (1×10<sup>6</sup> cells/ml) in 6-well dishes and transfected with 100 pmol of each miRNA mimic using Lipofectamine<sup>®</sup> 2,000 transfection reagent (Thermo Fisher Scientific K.K., Waltham, MA, USA). At 4 h after transfection, the cells were washed and incubated in complete DMEM for 48 h.

### *miRNA analysis*

MDA-MB-231 cells (1×10<sup>6</sup> cells/10 ml) were cultured in 10-cm dishes and treated with 1 nM eribulin for 24 h, before the miRNA extractions using miRNeasy Mini kit (Qiagen, Tokyo, Japan). Next, cDNAs were synthesized from 500 ng miRNAs by using miScript II RT kit (Qiagen, Tokyo, Japan) and miR-195 expression was measured by PCR array (miScript miRNA PCR array for human breast cancer, MIHS-109ZA; Qiagen). PCR was performed using the miRNA MiScript SYBR Green human breast cancer miRNA PCR kit (Qiagen) and ABI PRISM 7000 Sequence System (Applied Biosystems Inc., Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Data were analyzed using the  $\Delta\Delta C_t$  method (original expression level =  $2^{-\Delta\Delta C_t}$ ) and expressed as a ratio ( $\Delta\Delta C_t$ ) of Ct level in eribulin-treated cells to that in untreated MDA-MB-231 cells.

### *ELISA for WNT3a, $\beta$ -catenin, and GSK-3 $\beta$*

The expressions of wingless-type mouse mammary tumor virus (MMTV) integration site, member 3A (Wnt3a) and  $\beta$ -catenin were detected using an enzyme-linked immunosorbent assay kit (SEP155Hu; Uscn Life Science, Wuhan, Hubei, China) and catenin Beta 1 (CTNNb1) enzyme-linked immunosorbent assay kit (E91021Hu; Uscn Life Science), respectively. The expression of GSK-3 $\beta$  was detected using a GSK-3beta sandwich ELISA kit (7265CK; Cell Signaling Technology, Danvers, MA, USA) and P-GSK-3beta (S9) sandwich ELISA kit (7311CK; Cell Signaling Technology). All detection assays were analyzed using a fluorospectrophotometer ( $\lambda$  = 450 nm; Spectra Max i3; Molecular Devices Co., Sunnyvale, CA, USA).

### *Fluorescent immunohistochemistry*

The sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 5 min to remove endogenous peroxidases, before incubation in a non-specific blocking reagent (X0909, DAKO, Glostrup, DK) for 5 min. The section were then incubated with primary antibodies, anti-WNT3A rabbit antibody (1 : 100, HPA050514; Atlas Antibodies, Stockholm, Sweden) or anti P-GSK-3 beta (S9) rabbit mAb (1 : 200, 9323S; Cell Signaling Technology), for 1 h at room temperature, followed by incubation with TRIC-conjugated anti-rabbit IgG secondary antibodies (A21428, Life Technologies) in a humid chamber at 37°C for 30 min. Sequentially, sections were incubated with purified mouse anti-beta-catenin at 37°C for 1 h followed by FITC-conjugated anti-mouse IgG (A11001, Life Technologies) for 30 min. Sections were finally counterstained to visualize nuclei with bisbenzimidazole H33342 (Hoechst 33342), and imaged using a Delta Vision microscope (AIRIX Corp., Tokyo, Japan).

### *Statistical analysis*

Significant differences between the groups were determined by two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. Significance of miRNA analysis was assessed by ANOVA using the miScript miRNA PCR Array Data Analysis software (Qiagen), with the significance level set at  $P < 0.05$ .

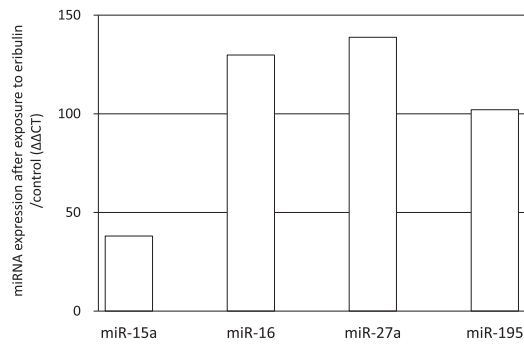
## **Results**

### *Analysis of miRNA expression*

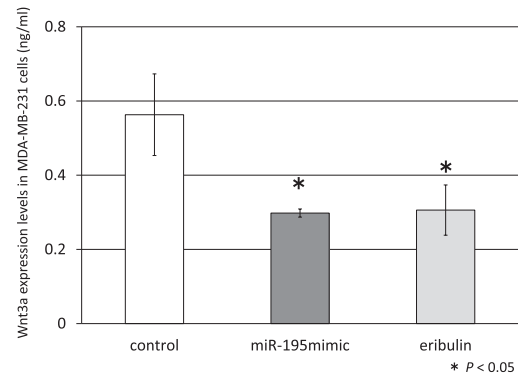
We examined the expression of miRNAs targeting Wnt3a in MDA-MB-231 cells after exposure to eribulin.  $\Delta\Delta CT$  values for miR-15a, miR-16, miR-27a, and miR-195 were 38.5, 129.79, 138.78, and 102.06, respectively, indicating higher expression of all these miRNAs after eribulin exposure compared to levels in the control cells (Fig. 1).

### *Analysis of ELISA*

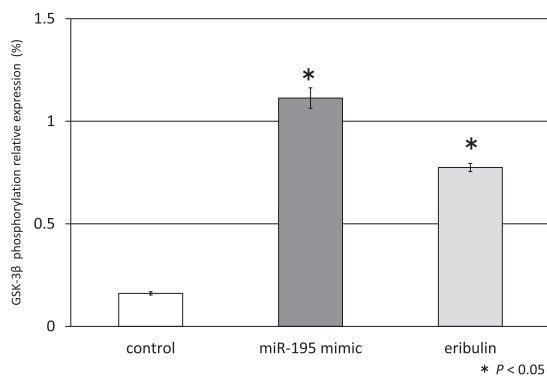
The miRNA, miR-195, was shown to target Wnt3a in breast cancer in our previous study<sup>21</sup>. In this study, we therefore compared and examined the expression levels of proteins in the Wnt/ $\beta$ -catenin signaling pathway, namely Wnt3a, GSK-3 $\beta$ , and  $\beta$ -catenin, in MDA-MB-231



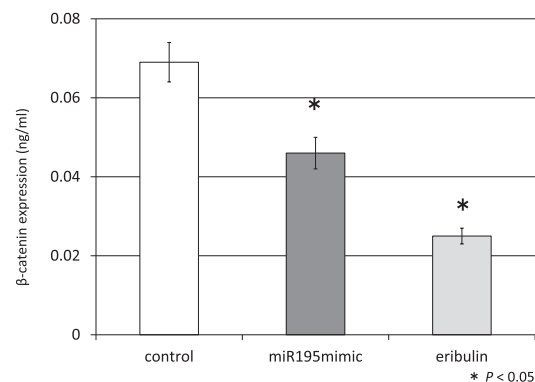
**Fig. 1.** Cells after exposure to eribulin  
The expressions of miRNAs targeting Wnt3a were analyzed in the MDA-MB-231 cells after eribulin exposure. The miR-15a, miR-16, miR-27a, and miR-195 expression levels in MDA-MB-231 cells increased after exposure to eribulin by 38.05, 129.79, 138.78, and 102.06 times, respectively.



**Fig. 2.** Comparison of Wnt3a expression levels  
Expression levels of Wnt3a were significantly lower in eribulin-exposed and miR-195 mimic-transfected MDA-MB-231 cells than in untreated controls. Data are expressed as mean  $\pm$  SD ( $n=4$ ). \* $P < 0.05$  compared with untreated controls; Bonferroni's multiple comparison test.



**Fig. 3.** Comparison of GSK-3 $\beta$  phosphorylation  
GSK-3 $\beta$  phosphorylation was significantly higher in miR-195 mimic-transfected and eribulin-exposed cells than in untreated controls. Data are expressed as mean  $\pm$  SD ( $n=4$ ). \* $P < 0.05$  compared with untreated controls; Bonferroni's multiple comparison test.



**Fig. 4.** Comparison of  $\beta$ -catenin expression levels  
Expression levels of  $\beta$ -catenin were significantly higher in MDA-MB-231 cells treated with miR-195 mimic and eribulin than in untreated controls. Data are expressed as mean  $\pm$  SD ( $n=4$ ). \* $P < 0.05$  compared with untreated controls; Bonferroni's multiple comparison test.

cells after transfection with a miR-195 mimic (miR-195 mimic group) and exposure to eribulin (eribulin group). Wnt3a expression was significantly lower in the miR-195 mimic group ( $0.298 \pm 0.01$  ng/ml) and eribulin group ( $0.306 \pm 0.067$  ng/ml) than in the untreated control group ( $0.563 \pm 0.011$  ng/ml) (Fig. 2). In contrast, phosphorylated GSK-3 $\beta$  expression was significantly higher in the miR-195 mimic group ( $1.11 \pm 0.05\%$ ) and eribulin group ( $0.774 \pm 0.02\%$ ) than in the untreated control group ( $0.212 \pm 0.015\%$ ) (Fig. 3), whereas  $\beta$ -catenin expression was significantly lower in the miR-195 mimic group ( $0.046 \pm 0.004$  ng/ml) and eribulin group ( $0.025 \pm 0.002$  ng/ml) than in the untreated controls ( $0.069 \pm 0.005$  ng/ml) (Fig. 4).

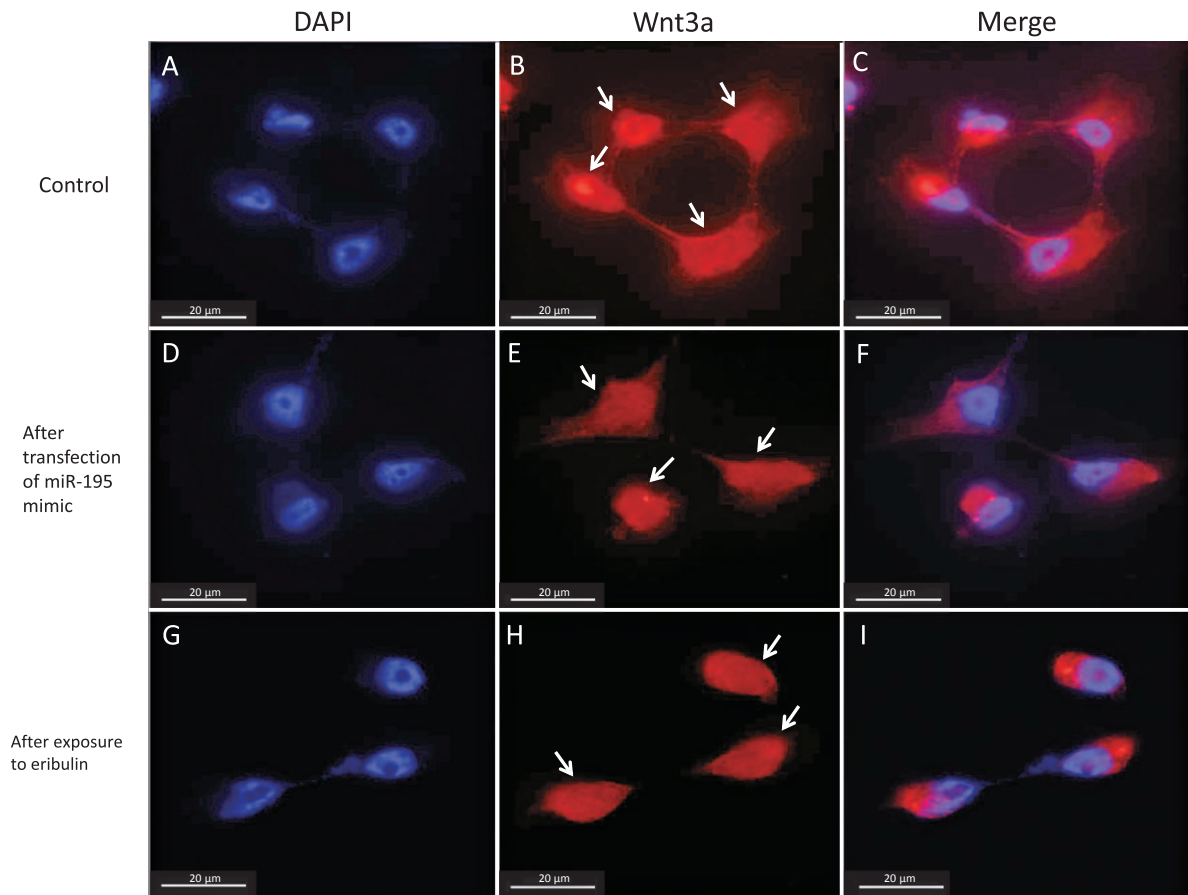


Fig. 5. Immunohistochemical staining of Wnt3a

(A) The nuclei of control cells were visualized with a blue DAPI stain. (B) The untreated MDA-MB-231 cell controls were positive for Wnt3a (red). (C) Immunofluorescence images showing the localization of Wnt3a (red) and DAPI-stained (blue) nuclei in control cells. (D) DAPI-stained nuclei of MDA-MB-231 cells after transfection with the miR-195 mimic. (E) Wnt3a was weakly expressed in MDA-MB-231 cells after transfection of the miR-195 mimic. (F) The low expression of Wnt3a in MDA-MB-231 cells after the transfection of miR-195 mimic. (G) DAPI-stained nuclei of MDA-MB-231 cells after the exposure to eribulin. (H) Wnt3a was weakly expressed. (I) Immunofluorescence images of Wnt3a (red) and DAPI-stained (blue) nuclei of MDA-MB-231 cells after the exposure to eribulin. Scale bar: 20  $\mu$ m.

### Immunohistochemical staining

We next examined the expression patterns of Wnt/ $\beta$ -catenin signaling proteins in the MDA-MB-231 cells by immunofluorescence. The untreated control group showed higher cytoplasmic expression of Wnt3a than the miR-195 mimic and eribulin groups (Fig. 5), and higher expression of  $\beta$ -catenin localized in the nucleus and cytoplasm (arrow). In contrast, the miR-195 mimic group and the eribulin group showed higher expressions of pGSK-3 $\beta$  in the cytoplasm than the untreated control group (Fig. 6).

### Discussion

First, we identified miRNAs that were involved in the Wnt/ $\beta$ -catenin signaling pathway. In eribulin-treated MDA-MB-231 cells, miR-15a, miR-16, miR-27a, and miR-195 were more highly

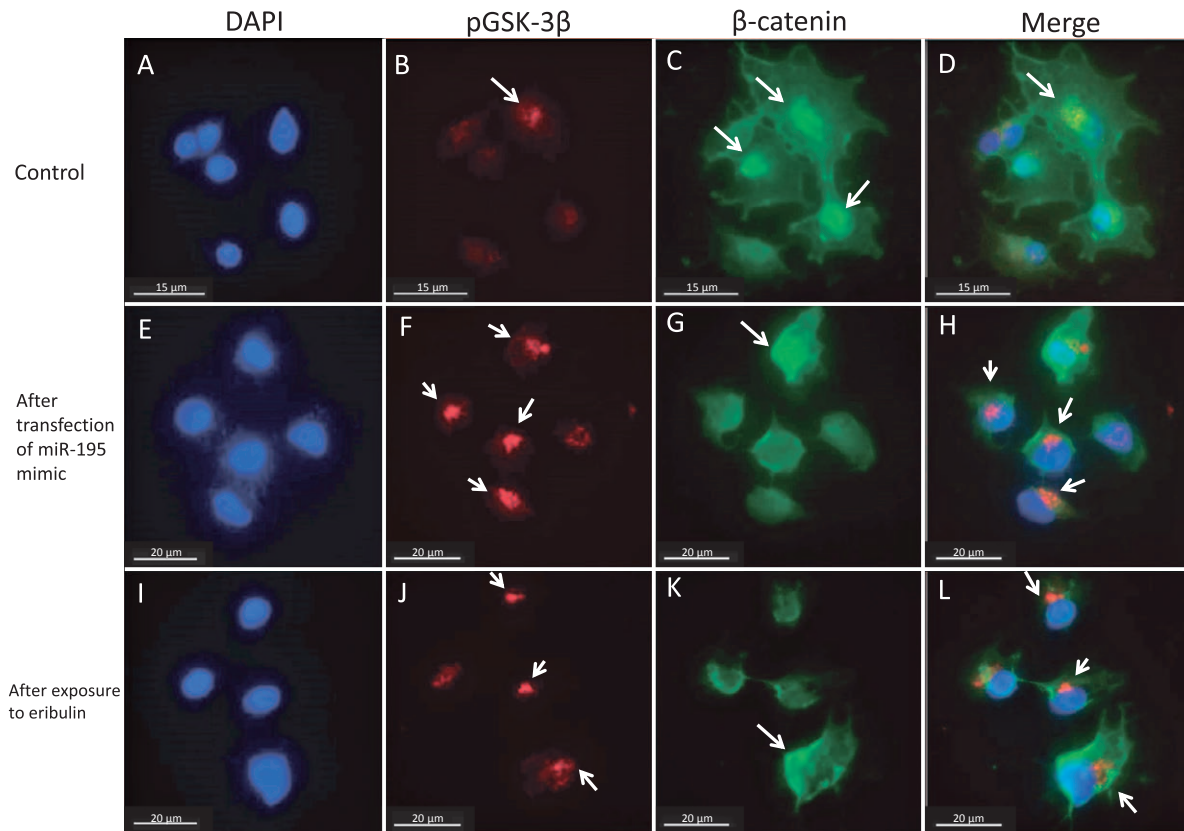


Fig. 6. Immunohistochemical staining of  $\beta$ -catenin and GSK-3 $\beta$

(A) DAPI-stained (blue) nuclei of control cells. (B) GSK-3 $\beta$  immunostaining in untreated MDA-MB-231 cells showed low expression levels. (C) Immunohistochemical staining of anti- $\beta$ -catenin in untreated MDA-MB-231 cells (arrow). (D) Double immunofluorescence images of  $\beta$ -catenin (green) and GSK-3 $\beta$  (red) in control cells, with DAPI-stained nuclei (blue). (E) DAPI-stained nuclei of MDA-MB-231 cells after eribulin exposure and miR-195 mimic transfection, with (F) GSK-3 $\beta$  expression after transfection of miR-195 mimic. (G) Immunopositive staining of  $\beta$ -catenin was localized in the cytoplasm and membrane of MDA-MB-231 cells after transfection of miR-195 mimic. (H) The number of GSK-3 $\beta$ -positive MDA-MB-231 cells increased after transfection of miR-195 mimic compared with that of control cells. (I) DAPI-stained nuclei of MDA-MB-231 cells after eribulin exposure, with (J) GSK-3 $\beta$ -positive signals localized in the cell cytoplasm. (K)  $\beta$ -catenin-positive signals were localized in the cytoplasm and membrane of MDA-MB-231 cells after eribulin exposure. (L) The double immunofluorescence imaging of  $\beta$ -catenin (green) and GSK-3 $\beta$  (red), as well as DAPI-stained (blue) nuclei of MDA-MB-231 cells after eribulin exposure. Scale bar: 20  $\mu$ m.

expressed than in the untreated control cells, and these miRNAs have been reported to target Wnt3a<sup>21-28</sup>). Moreover, miR-195 targets encoding mRNAs, such as Wnt3a, cyclinD1, CDK4/6, cyclinE1, WEE1, E2F3, and BCL2, and binds complementarily to the base sequence of their UTRs to inhibit mRNA translation and protein expression<sup>26, 29-35</sup>). In addition, miR-195 shows potential tumor suppressive action in bladder cancer<sup>30</sup>), and inhibits cell proliferation in the liver by the induction of INF<sup>31</sup>). In breast cancer, strong expression of miR-195 has been inversely correlated with tumor size, lymph node metastasis, and vascular invasion<sup>36</sup>), whereas low expression of miR-195 has been linked to a poor prognosis<sup>37</sup>).

Based on the reports above, we transfected an miR-195 mimic into MDA-MB-231 cells to

induce high expression of miR-195 and to analyze the subsequent activation of Wnt/ $\beta$ -catenin signaling. The miR-195 mimic-transfected cells showed significantly lower Wnt3a expression than control cells and miR-195 was bound complementarily to Wnt3a-encoding RNA (Wnt3A), inhibiting Wnt3a translation and protein expression. Furthermore, since Wnt3a expression was also significantly reduced after eribulin exposure, we proposed that expression of miR-195 was enhanced and expression of Wnt3a was suppressed by exposure to eribulin. In addition, immunofluorescence examination revealed that the cytoplasm of untreated control cells showed higher expression of Wnt3a than cells in the miR-195 mimic and eribulin groups. These results indicated that miR-195 mimic transfection and the exposure to eribulin attenuated the expression of Wnt3a in MDA-MB-231 cells.

Next, we examined the expression levels of GSK-3 $\beta$  and  $\beta$ -catenin in the treated cells. GSK-3 $\beta$  phosphorylation was significantly increased in the miR-195 mimic and eribulin groups compared to the untreated control group, while  $\beta$ -catenin expression was significantly decreased. These results revealed that miR-195 mimic transfection and the eribulin exposure caused the phosphorylation of  $\beta$ -catenin GSK-3 $\beta$  and the subsequent ubiquitination and degradation of  $\beta$ -catenin. Furthermore, immunofluorescence staining revealed higher and more concentrated  $\beta$ -catenin expression in the nucleus and cytoplasm of the untreated control group than in the cytoplasm of the miR-195 mimic-transfected and eribulin-exposed group. In contrast, pGSK-3 $\beta$  expressions in the cytoplasm of the miR-195 mimic group and eribulin group were higher than in the untreated control group, attributing the attenuation of  $\beta$ -catenin expression to the high expression of GSK-3 $\beta$ .

In the Wnt/ $\beta$ -catenin signaling pathway of the untreated control cells, GSK-3 $\beta$  is not phosphorylated by binding to the receptor on the cell membrane, whereas  $\beta$ -catenin is stabilized and accumulated in the cytoplasm before being transferred into the nucleus. In the nucleus,  $\beta$ -catenin binds to the transcription factor T cell factor/lymphocyte enhancing factor (Tcf/Lef) and promotes the expression of genes that regulate cell cycle progression, such as cyclin D1 and c-myc<sup>11, 12, 38</sup>. On the contrary, in the miR-195 mimic group and eribulin group, GSK-3 $\beta$  expression decreased the amount of cytoplasmic  $\beta$ -catenin, possibly via the binding of  $\beta$ -catenin to axin along with adenomatous polyposis coli (APC) and GSK-3 $\beta$ , forming an Axin complex, which is then phosphorylated by casein kinase 1 (CK1) and GSK-3 $\beta$ , ubiquitinated, and degraded by the proteasome<sup>39-41</sup>.

Activation of the Wnt/ $\beta$ -catenin signaling pathway also promotes TNBC metastasis<sup>15</sup>) as well as cell proliferation and differentiation<sup>42</sup>); however, it is possible that eribulin inactivates the Wnt/ $\beta$ -catenin signaling pathway directly and induces apoptosis in cancer cells by suppressing Wnt3a expression through high expression of miR-195<sup>43, 44</sup>) (Fig. 7).

In 2014, miR-195 was implicated as a tumor suppressor directly targeting Wnt3a<sup>26, 44</sup>). This study also revealed that exposure to eribulin enhanced the expression of miR-195, which suppressed Wnt3a expression. In the present study, miR-195 mimic transfection and exposure to eribulin group also suppressed cellular  $\beta$ -catenin expression. Taken together, these findings suggest that eribulin exposure could suppress the expression of Wnt3a by enhancing the expression



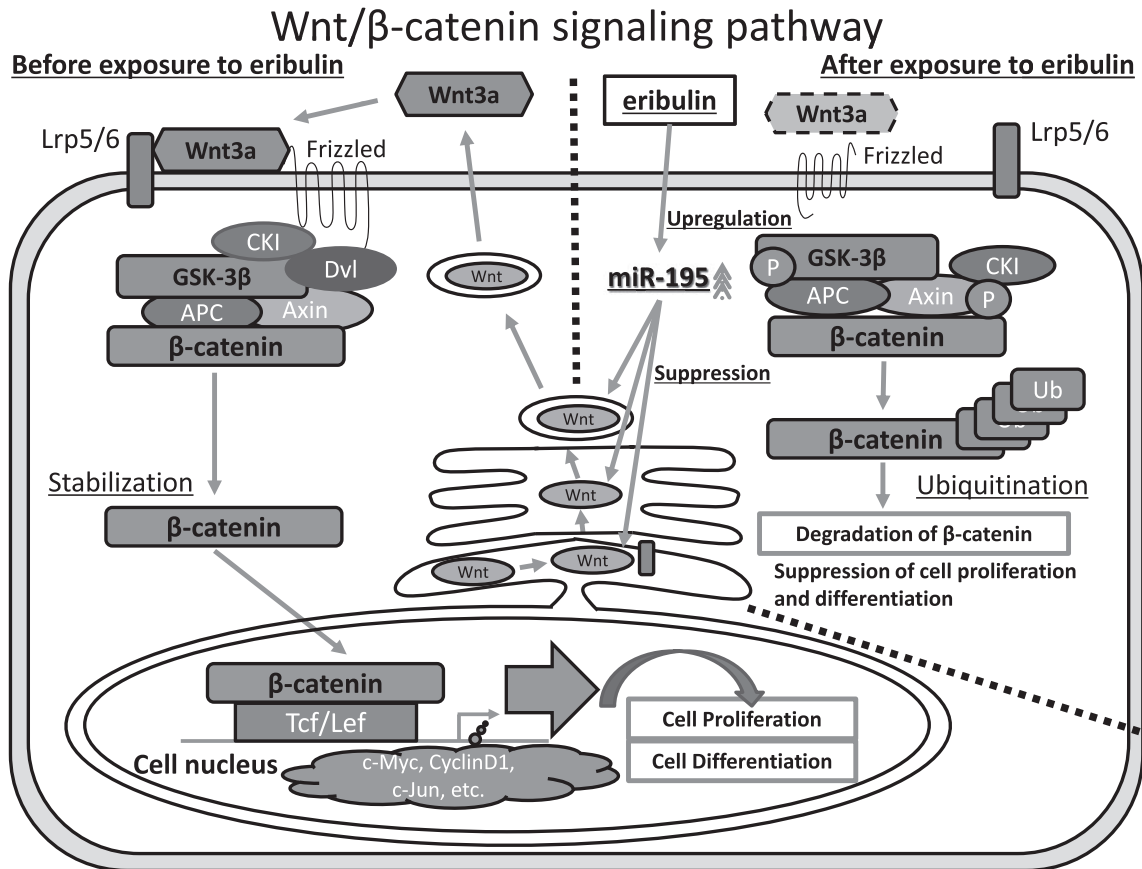


Fig. 7. Wnt/ $\beta$ -catenin signaling pathway in the absence or presence of eribulin in MDA-MB-231 cells. In the absence of eribulin, Wnt3a binds to the receptor on the cell membrane, preventing GSK-3 $\beta$  phosphorylation as well as  $\beta$ -catenin dissociation and stabilization into the nucleus. Consequently, the proteins involved in the cell cycle progression are transcribed, the cell cycle progresses, and then the cancer cells proliferate. In the presence of eribulin, miR-195 is upregulated and the expression of complementary Wnt3a is decreased.  $\beta$ -catenin binds to Axin together with APC and GSK-3 $\beta$ , forming an Axin complex, which is phosphorylated by CK1 and GSK-3 $\beta$ , ubiquitinated, and degraded by the proteasome. Consequently, protein transcription does not proceed, the cell cycle is arrested, and cell death is imminent.

of miR-195 and directly inactivating the Wnt/ $\beta$ -catenin signaling pathway, in a possible mechanism underlying its antitumor effect.

The mechanism by which eribulin inactivated the Wnt/ $\beta$ -catenin signaling pathway in TNBC cells could not be sufficiently examined in this study, because Wnt3a expression was suppressed by high expression of miR-195. However, this newly reported mechanism of eribulin action is potentially useful for similar mechanistic investigation of other anticancer agents in various types of cancer cells and the development of new drugs.

## Conclusion

The present study revealed a potential antitumor mechanism by which eribulin increased miR-195 expression and suppressed Wnt3a expression in a cell model of non-basal cell-type TNBC, thereby inactivating the Wnt/ $\beta$ -catenin signaling pathway and inhibiting cell proliferation.

**Conflict of interest disclosure**

The authors have no conflict of interest to disclose.

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