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Original Research

# MicroRNA-181a suppresses norethisterone-promoted tumorigenesis of breast epithelial MCF10A cells through the PGRMC1/EGFR–PI3K/Akt/mTOR signaling pathway

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ABSTRACT

*Background:* Research suggests that hormone replacement therapy may increase the risk of breast cancer, and progestins such as norethisterone (NET) play a key role in this phenomenon. We have demonstrated that microRNA-181a (miR-181a) suppresses NET-promoted breast cancer cell survival. Nonetheless, the effects of NET and miR-181a on the tumorigenesis of human breast epithelial cells have not yet been elaborated.

*Methods:* Assays of cell viability, proliferation, migration, apoptosis, and colony formation were performed to investigate the pro-tumorigenesis effect of NET and the effects of miR-181a on human breast epithelial MCF10A cells. The expressions of cell-proliferation-related genes and apoptotic factors were analyzed by quantitative RT-PCR and Western blot in MCF10A cells treated with NET and miR-181a.

*Results:* NET significantly increased MCF10A cell viability, proliferation, migration, and colony formation, but reduced cellular apoptosis. In addition, NET increased the expression of progesterone receptor membrane component 1 (PGRMC1), EGFR, B-cell lymphoma 2, cyclin D1, and proliferating cell nuclear antigen, but decreased the expression of pro-apoptosis factors, such as Bax, caspase-7, and caspase-9. Overexpression of miR-181a strongly inhibited the effects of NET on MCF10A cells and abrogated NET-stimulated PGRMC1, EGFR, and mTOR expression.

*Conclusions:* Activation of the PGRMC1/EGFR–PI3K/Akt/mTOR signaling pathway is the primary mechanism underlying the pro-tumorigenesis effects of NET on human breast epithelial MCF10A cells. Additionally, miR-181a can suppress the effects of NET on these cells. These data suggest a therapeutic potential for miR-181a in reducing or preventing the risk of breast cancer in hormone replacement therapy using NET.

## Introduction

Breast cancer (BC) is one of the most common malignant tumors in women worldwide and a leading cause of cancer-related deaths in women, with an estimated 1.9 million incident cases and 601,000 deaths reported in 2017 [1]. Used in contraception and hormone replacement therapy (HRT) among reproductive and menopausal women, synthetic progestogens (also known as progestins) may play a crucial role in the occurrence and development of BC [2]. Studies have indicated that progestins substantially increase the relative risk of BC when added to estrogens as HRT, but the mechanisms of action are not well understood [3,4].

Tumorigenesis is the process by which normal cells transform into cancer cells. Based on clinic evidence, estrogen plus progestin in-

*Abbreviations:* Akt, protein kinase; BC, breast cancer; BCL2, B-cell lymphoma 2; CASP-7, caspase 7; CASP-9, caspase 9; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HRT, hormone replacement therapy; miRNA, microRNA; mTOR, mechanistic target of rapamycin; NC, negative control; NET, norethisterone; PCNA, proliferating cell nuclear antigen; PGRMC1, progesterone receptor membrane component 1; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PR, progesterone receptor; PTEN, phosphatase and tensin homolog; qPCR, quantitative RT-PCR; VEGF, vascular endothelial growth factor.

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creases the risk of BC and promotes tumorigenesis [5]. Norethindrone (NET) is a progestin used alone or in combination with estrogen in HRT. Clinical studies have suggested that estrogen/NET HRT increases the risk of BC [6]. In addition, NET stimulates the proliferation of progesterone receptor membrane component 1 (PGRMC1)-overexpressing BC cells [7]. High expression levels of PGRMC1 in BC tissue are correlated with poor outcomes regarding cancer tumor grade, risk of metastasis, and survival time [8]. PGRMC1 is an oncogene that plays an important role in promoting BC cell proliferation, survival, and growth as well as in progestin-induced proliferation of BC cells [7,9,10]. PGRMC1 mediates a strong proliferation of BC cells induced by NET, as we previously demonstrated *in vitro* studies [11,12]. PGRMC1 co-localizes with epidermal growth factor receptor (EGFR), which is a potent transmembrane receptor-tyrosine kinase that drives tumorigenesis. Thus, the prooncogenic effects of PGRMC1 may result from binding and stabilizing EGFR at the plasma membrane [13].

MicroRNAs (miRNAs) are endogenous small non-coding regulatory RNA (~22 nucleotides). Upon binding to target mRNAs, miRNAs either trigger mRNA cleavage and decay or inhibit translation. Emerging evidence suggests that deregulation of miRNAs is often associated with human cancers. Dysregulation of microRNA-181a (miR-181a) occurs in many tumors, including human BC [14]. Compared with less-aggressive human BC cells (*e.g.*, MCF-7), aggressive cancer cells (*e.g.*, triple-negative MDA-MB-231) have significantly lower miR-181a expression levels [15]. We and others have previously demonstrated that miR-181a exerts anti-BC effects by preventing tumor invasion, promoting cancer cell apoptosis, and enhancing drug sensitivity [11,15]. In addition, miR-181a not only suppresses MCF-7 cell growth but also abrogates NET-provoked MCF-7 cell growth [11].

MCF10A is an estrogen receptor (ER) and progesterone receptor (PR) double-negative (ER-/PR-) human breast epithelial cell line and is the most commonly used normal breast cell for *in vitro* models [16]. Although miR-181a expression is found in MCF-10A cells [14], the role of miR-181a and the effects of NET on breast epithelial cells remain poorly defined. Both PGRMC1 and EGFR are expressed in MCF10A cells [17]. We hypothesized that NET promotes the tumorigenesis of breast epithelial cells by upregulating the PGRMC1/EGFR signaling pathway. Therefore, in this study, we aimed to identify alterations in the PGRMC1/EGFR signaling pathway in NET-treated MCF10A cells and to assess whether miR-181a suppresses the effects of NET and NET-upregulated signaling pathways.

## Materials and methods

### *Cell culture*

The human breast epithelial cell line MCF10A was obtained from the American Type Culture Collection (ATCC, Manassas). Cells were cultured in growth medium containing DMEM/F12 (#11,330–032, Invitrogen) supplemented with 10% horse serum (#16,050–122, Invitrogen), EGF (#CYT-217, 20 ng/mL, ProSpec), hydrocortisone (#H-0888, 0.5 mg/mL, Sigma), insulin (#I-1882, 10 $\mu$g/mL, Sigma), and 1% penicillin-streptomycin (#15,070–063, Invitrogen) in a humidified atmosphere of 5% $CO_2$ at 37 ˚C. Cells were incubated with NET for 4 weeks before use in this study.

### *NET treatment*

MCF10A cells were seeded in the wells of 24-well plates in growth medium. After 24 h, the cells were treated with synthetic progestogen (NET, 19-norethisterone, Sigma) at the indicated concentrations. Cell viability was assessed after 48 h of treatment. A NET concentration of $10^{-10}$ M was found to be the most effective low concentration and was thus used in the 4-week NET treatment.

### *Transfection of miR-181a mimic*

The mirVana miRNA mimic hsa-miR-181a-5p (Cat. 4,464,066) and a negative control miRNA mimic (Cat. 4,464,058) were purchased from Thermo Fisher Scientific. miRNA transfection was performed according to the manufacturer's protocol. Briefly, 4-week NET-treated MCF10A cells were transfected with the indicated concentration of miR-181a mimic or miRNA-negative control (NC) using lipofectamine 2000 (Invitrogen, Life Technologies) for 48 h.

### *Cell viability and proliferation assays*

Cell viability was assessed using an MTT assay as previously described [18]. [$^3$H]-thymidine DNA incorporation was used to detect cell proliferation [19]. Briefly, 50 $\mu$L of [$^3$H]-thymidine solution (#NET027 × 250UC: 1 $\mu$ci/$\mu$L, Perkin Elmer) was added to each well of a 24-well plate, and the incorporated thymidine was measured in the same number of cells from the control and NET-treated groups during a 24-hour period.

### *Colony formation assay*

NET-treated MCF10A cells were plated in a 6-well plate (100 cells/well). The medium was changed weekly, and the plates were monitored for 3 weeks for obvious countable colony formation. The colonies were fixed and stained as previously described [20]. Colonies containing more than 50 individual cells were counted using a stereomicroscope. The plating efficiency was defined as the number of colonies counted per number of cells plated.

### *Transwell migration assay*

A migration assay was performed by using Transwell inserts (#COR-3421, Corning Life Sciences). For each insert, $5 \times 10^4$ cells were added. VEGF (Cat: 50,159-MNAB, Sino Biological) was added at 10 ng/mL to the lower chamber as a positive control. After culturing for 6 h at 37 ˚C, the inserts were fixed, and the migrated cells were stained with DAPI. The migratory cells were observed by fluorescent microscopy (Leica DIM8 microscope) and counted in five different fields under a microscope using ImageJ.

### *Apoptosis assays*

Cell apoptosis was determined by flow cytometry analysis using the Annexin V Apoptosis Assay Kit (V13241, Molecular Probes, Inc.) as previously described [21]. Apoptotic rates were expressed as the percentage of apoptotic cells over the total number of cells.

### *Quantitative RT-PCR*

RNA extraction, cDNA synthesis, and reverse transcription-quantitative PCR (RT-qPCR) were performed as previously described [19]. $\beta$-actin was used as an internal control. PCR primers were synthesized by Alpha DNA (Montreal, Quebec, Canada) as follows: $\beta$-actin, forward 5′-CTGCGGCATTCACGAAACTAC-3′, reverse 5′-ATCTCTTTCTGCATCCTGTCCG-3′; PGRMC1, forward 5′-CGACGG CGTCCAGGACCC-3′, reverse 5′-TCTTCCTCATCTGAGTACACAG-3′; proliferating cell nuclear antigen (PCNA), forward 5′-GGCTCTAGCCTGA CAAATGC-3′, reverse 5′-GCCTCCAACACCTTCTTGAG-3′; cyclin D1, forward 5′- TGGAAACCATCCGCCGCGC-3′, reverse 5′- CGATCTTCCG CATGGACGGCAG-3′; B-cell lymphoma 2 (BCL2), forward 5′-GGTGAA CTGGGGGAGGATTG-3′, reverse 5′-GTGCCGGTTCAGGTACTCAG-3′; Bax, forward 5′-TGGCAGCTGACATGTTTTCTGAC-3′, reverse 5′-TCA CCCAACCACCCTGGTCTT-3′; caspase 7 (CASP-7), forward 5′-GCAGCGCC

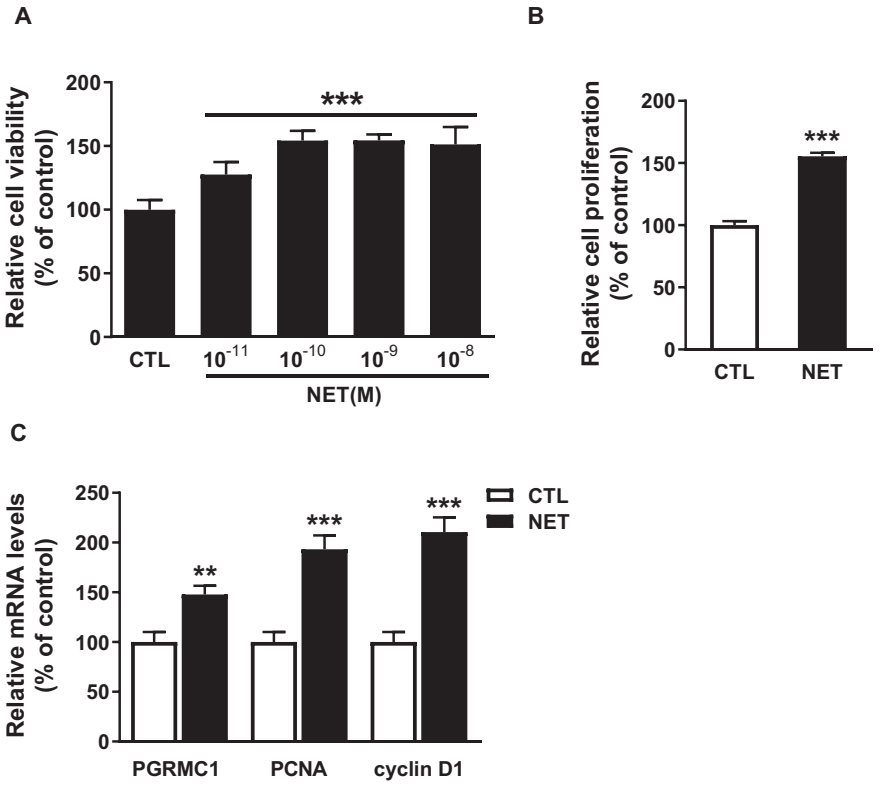



**Fig. 1.** NET increased MCF10A cell vi ability and cell proliferation. (A) MCF10A cells were in cubated with the indicated concentrations of NET for 48h. Cell vi ability was assessed-byan MTT assay, and values are presented as a percentage of the control (CTL). ***$p$<0.001 vs. CTL. (B) After a 4-week induction with NET (10–10 mol/L), proliferation of MCF10-Acells was evaluated by a [3H]-thymidine in corporation assay. There lative proliferation rates are presented as a percentage of CTL. ***$p$<0.001 vs. CTL. (C) mRNA levels of PGRMC1, PCNA, and cyclin D1 in MCF10A cells aftera 4-week NET induction were determined by q PCR. The relative mRNA level of each gene was calculated and expressed as a percentage of CTL (100%). **$p$<0.01, ***$p$<0.001 vs.CTL.

GAGACTTTTAG-3′, reverse 5′- GCTGCAGTTACCGTTCCCAC-3′; caspase 9 (CASP-9), forward 5′-TTCCCAGGTTTTGTTTCCTG-3′, reverse 5′-ACCCTAAGCAGGAGGG-3′.

### *Western blot analysis*

Total proteins were extracted from MCF10A using M-PER containing Halt™ Protease Inhibitor Cocktail, EDTA-Free (100X) (Thermo Fisher Scientific, Inc.). Western blot was performed as previously described [22]. All antibodies used in this study were purchased from Cell Signaling Technology, Inc., including anti-PGRMC1 (#13,856, l:1000), anti-EGFR (#2085, l:1000), anti-mechanistic target of rapamycin (mTOR) (#2983, l:1000), anti-phosphatase and tensin homolog (PTEN) (#9188, l:1000), and anti-$\beta$-actin (#3700, l:1000). $\beta$-actin was used as a loading control. Proteins were visualized using an ECL Western blotting detection system (PerkinElmer, Inc.). Densitometry values were measured in terms of pixel intensity by ImageJ.

### *Statistical analyses*

Experiments were performed at least three times independently. Results were analyzed with GraphPad Prism 8 statistical software. Values are presented as means ± SEM. Student's $t$-test and one-way ANOVA followed by post-hoc Bonferroni tests were used to analyze differences between groups. P-values $<$ 0.05 were considered to be statistically significant.

## Results

### *NET increased MCF10A cell viability and cell proliferation*

BC causation has been linked to sustained exposure of the breast to progestin [6]. Progestin NET is commonly used in HRT, and it promotes the growth of BC cells [10]. To investigate the effect of NET on mammary epithelial cells, the human mammary epithelial cell line MCF10A was used in this study. MCF10A cells exhibit some features of normal breast epithelium, including dependence on hormones and growth factors for cell growth [16]. This cell line is widely used as an in vitro model for studying normal breast cell function and transformation. Four concentrations of NET ($10^{-11}$, $10^{-10}$, $10^{-9}$, and $10^{-8}$ M) were used to treat MCF10A cells for 48 h. Cell viability analysis revealed that NET significantly increased cell viability ($p < 0.001$) in a dose-dependent manner, with $10^{-10}$ M NET being the most effective lower dose (**Fig. 1A**).

In clinical practice, one cycle of HRT treatment lasts 4 weeks. *In vitro*, morphologic changes in MCF10A were observed after 3 weeks in continuous culture with NET, and the changes were similar to the morphologic transformation observed under an oxidative microenvironment and estrogen/EGFR treatment [23]. Based on these observations, a 4-week incubation with NET was applied to investigate the long-term effect of NET on MCF10A cells. After a 4-week incubation with NET at $10^{-10}$ M, MCF10A cell proliferation increased significantly (**Fig. 1B**).

PGRMC1 plays an important role in progestin-induced BC cell proliferation; moreover, we have found that PGRMC1 expression increases in NET-treated MCF-7 cells [11]. To investigate the altered expression of cell-cycle-related genes in NET-pre-treated MCF10A cells, we performed

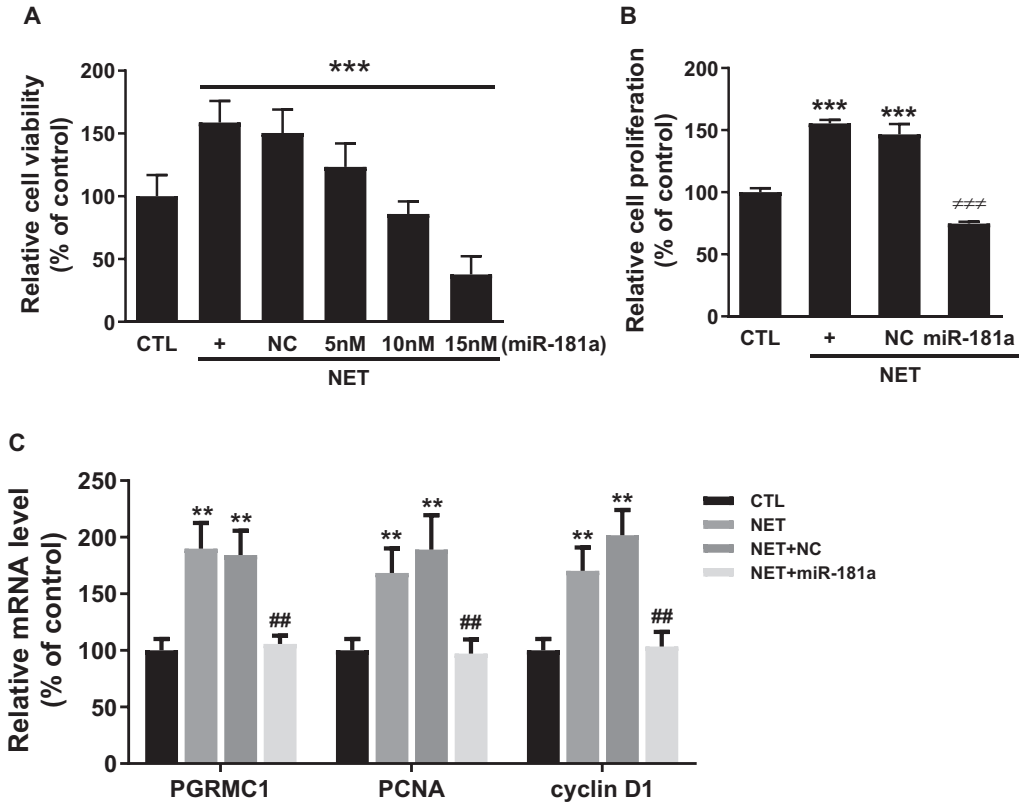



**Fig. 2.** *MiR-181a abrogated the pro-growth effects of NET.* NET-treated MCF10A cells were treated with the indicated concentration so fmiR-181a ornegative control miRNA (NC) for 48h (10nMof each miRNA was used in BandC). Cell vi ability was assessed by an MTT assay (A), and cell proliferation was evaluated by a[3H]-thymidine incorporation assay (B). Them RNA levels of PGRMC1, PCNA, and cyclin D1 were evaluated by qPCR (C). The relative values are presented as a percentage of CTL. -$^{**}p<0.01$, $^{***}p<0.001$ vs. CTL;$\#\#p<0.01$, $\#\#\#p<0.001$ vs. NC.

quantitative PCR (qPCR). We observed a significant increase in the expression of PGRMC1, PCNA, and cyclin D1 (Fig. 1**C**). This result indicates that long-term NET treatment may cause breast epithelial MCF10A cells to undergo oncogenic transformation/tumorigenesis.

### *MiR-181a abrogated the pro-growth effects of NET*

We previously discovered that miR-181a decreases BC cell viability and NET-stimulated BC cell viability [11]. To determine whether miR-181a interferes with the effects of NET on mammary epithelial cells, NET-pre-treated MCF10A cells were transfected with miR-181a mimic at different doses (5 nM, 10 nM, and 15 nM), and cell viability was analyzed 48 h after transfection. MiR-181a abrogated the effect of NET on cell viability in a dose-dependent manner (Fig. 2**A**). miR-181a at a concentration of 10 nM was able to effectively interfere with the effect of NET on cell viability without significantly reducing cell viability. Hence, this concentration of miR-181a (10 nM) was chosen for subsequent *in vitro* experiments. As predicted, miR-181a (10 nM) significantly abrogated the effect of NET on MCF10A cell proliferation (Fig. 2**B**). Moreover, miR-181a attenuated the effect of NET on the expression of proliferation-related genes PGRMC1, PCNA, and cyclin D1 (Fig. 2**C**).

### *MiR-181a abolished the effect of NET on cell survival*

Although NET increases MCF10A cell growth, its effect in preventing MCF10A cell survival has not yet been investigated. To address this issue, a cell apoptosis assay was performed on NET-treated MCF10A cells. The resulting data showed that NET slightly but significantly reduced the cell apoptosis rate (Fig. 3**A and 3B,** $p < 0.01$**)**. qPCR also revealed that NET increased the expression of the anti-apoptotic protein BCL2, but inhibited the expression of the apoptotic factor Bax and the apoptotic effectors CASP-7 and CASP-9. Moreover, miR-181a completely reversed the anti-apoptotic effect of NET on MCF10A cells and dysregulated gene expressions (Fig. 3**C**).

### *MiR-181a prevented the formation of NET-induced colony formation*

To analyze the clonogenic potential and transformation of NET-treated MCF10A cells, a colony formation assay was performed. We found that NET significantly increased the colony formation of MCF10A, with the plating efficiency increasing to 58.3% ± 7.1% compared to 31% ± 6.2% for the control group (Fig. 4**A**, $p < 0.01$). However, miR-181a abrogated the clonogenicity of MCF10A cells induced by NET (Fig. 4). These results further confirm the pro-survival effect of NET on MCF10A cells.

### *MiR-181a suppressed NET-increased cell migration*

The migration of NET-treated MCF10A cells was assessed using a Transwell assay. The results showed that NET significantly increased cell migration compared with the control groups (Fig. 5**,** $p < 0.001$), with 538.8% ± 33.1% migrated cells in the NET-treated group and only 200.5% ± 28.8% in the control group. Nonetheless, in the miR-181a group, migrated NET-treated MCF10A cells decreased to 386.3% ± 36.4% (Fig. 5**,** $p < 0.001$). These data further support the effect of

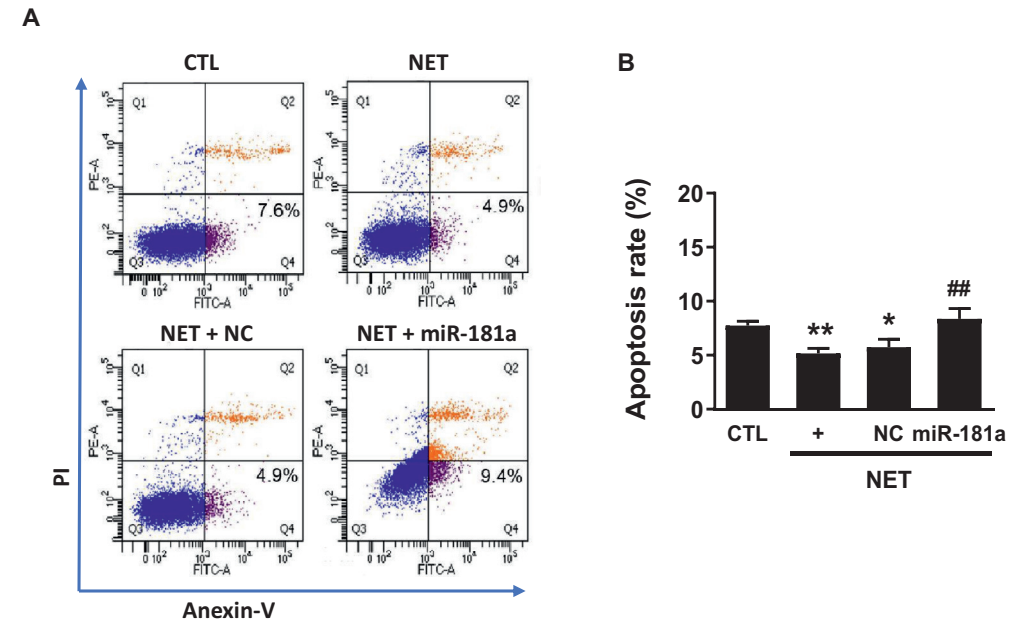
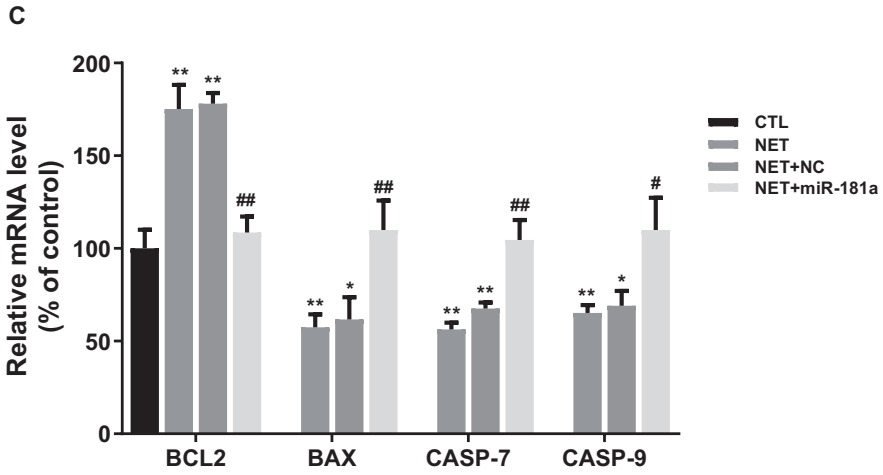



**Fig. 3.** *MiR-181a abolished the effect of NET on cell survival.* (A) Representative figures of flow cytometry results and apoptoticrates of MCF10A cells after 48-h treatment with 10nMmiR-181a or negative control miRNA (NC). MCF10A cells were stained with Annex in-FITC and propidium iodideusing the Annex in VA poptosis Assay Kit. (B) The percentage of apoptotic cells was quantified. $^{*}p<0.05$, $^{**}p<0.01$ vs. CTL. $\#\#p<0.01$ vs.NC. (C) mRNA levels of BCL2, Bax, CASP-7, and CASP-9 were quantified by qPCR after NET-treated MCF10A cells were transfected with 10nMmiR-181a or negative control miRNA for 48h. The values are presented as fold changes relative to the control group (CTL, seta s100%). $^{*}p<0.05$, $^{**}p<0.01$ vs. CTL; $\#p<0.05$, $\#\#p<0.01$ vs. NC.




NET on MCF10A cell migration and indicate that miR-181a decreases the pro-migration effect of NET.

### *MiR-181a attenuated the NET-dysregulated PI3K/Akt/mTOR signaling pathway*

The phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt)/mTOR pathway is an important intracellular signaling pathway, playing a pivotal role in regulating cell cycle and survival [24]. This pathway can be antagonized by various factors including PTEN, a tumor suppressor gene. To understand the mechanisms underlying the pro-oncogenic effect of NET and the inhibitory effects of miR-181a on the proliferation, migration, and survival of MCF10A cells, we investigated alterations in the PI3K/Akt/mTOR pathway in NET-treated MCF10A cells. Western blot was performed to evaluate the expression of key players in the PI3K/Akt/mTOR pathway, including PGRMC1, EGFR, mTOR, and PTEN. NET significantly increased the expression of PGRMC1, EGFR, and mTOR, but reduced the expression of PTEN (**Fig. 6**, $p < 0.05$). Consistently, miR-181a completely reversed the effect of NET on the expression of these four genes (**Fig. 6**). These data suggest that upregulating PGRMC1/EGFR–PI3K/Akt/mTOR signal pathway may contribute to the pro-tumorigenic effect of NET on breast epithelial MCF10A cells. However, overexpression of miR-181a strongly abrogated the effects of NET.

## Discussion

This study demonstrated that NET exhibits a pro-tumorigenic effect on breast epithelial MCF10A cells supported by increased cell proliferation, colony formation, and migration and decreased cellular apoptosis. This effect of NET was accompanied by an increased expression of oncogenes (PGRMC1 and EGFR) and stimulation of the PI3K/Akt/mTOR pathway. Moreover, overexpression of miR-181a can efficiently suppress NET-induced cell proliferation and migration and can promote cell apoptosis. These data strongly indicate that miR-181a may function as a tumor suppressor by attenuating the NET-provoked oncogenic transformation in breast epithelial MCF10A cells.

Tumorigenesis occurs as normal cells gain malignant properties, including dedifferentiation, rapid proliferation, metastasis, and evasion of apoptosis, which have been generalized as the hallmarks of cancer. Clinical trials of HRT have demonstrated that NET increases the risk of developing BC [6]. This study demonstrated the following pro-tumorigenesis effects of NET on breast epithelial MCF10A cells: 1) pro-proliferation with upregulated expression of cell-proliferation-related genes (**Fig. 1**), 2) pro-survival with downregulated apoptotic genes (**Fig. 3**), 3) pro-colony formation, an apparent feature or process of cell tumorigenesis (**Fig. 4**), and 4) pro-migration (**Fig. 5**). NET upregulates the expression of PCNA and cyclin D1 in breast epithelial cells. The cyclin D1 pro-

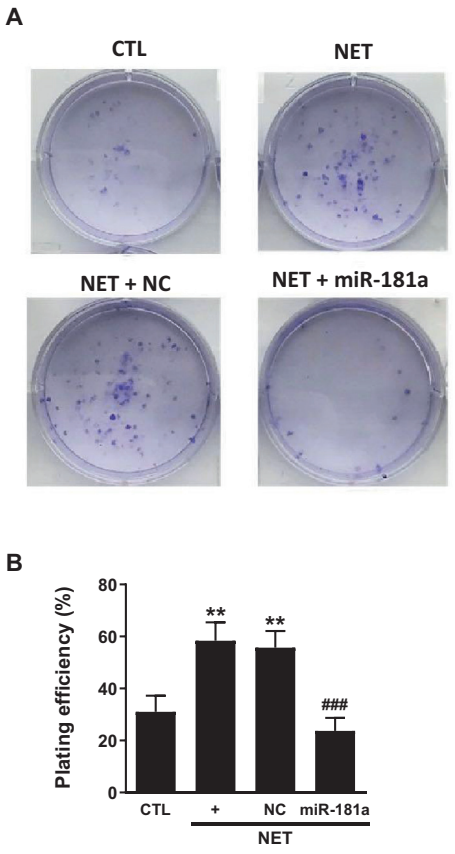



**Fig. 4.** *MiR-181a impeded NET-induced colony formation.* NET-treated MCF10A cells were transfected with 10nMmiR-181a or negative control miRNA (NC) and then plated in a 6-wellplate (100cells/well) for 3 weeks. (A) Representative image so f colony formation. (B) Colonies consisting of more than 50 cells were counted to determine the plating efficiency. Values are averages from three independent experiments. $^{**}p<0.01$ vs. CTL; $###p<0.001$ vs. NC.

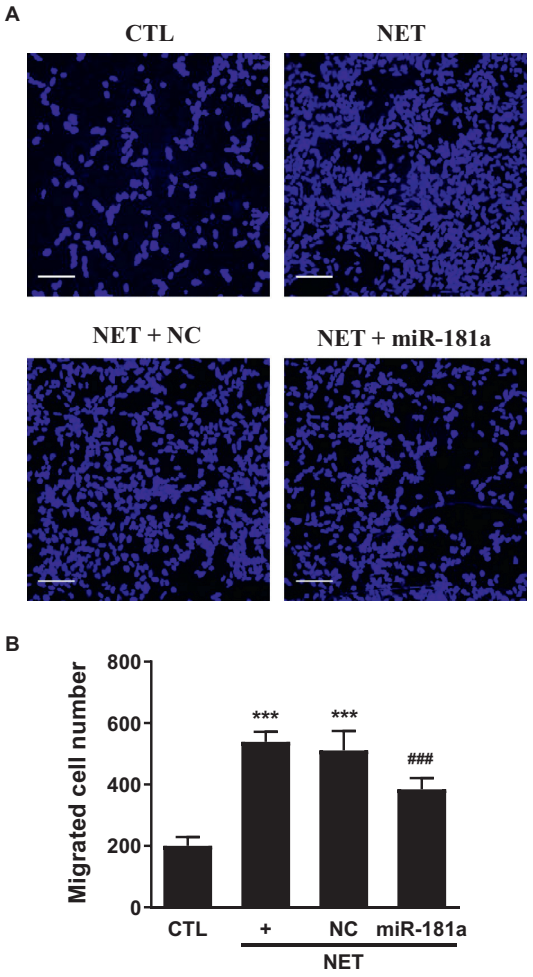



**Fig. 5.** *MiR-181a suppressed NET-increased cell migration.* (A) Representative images of migrated cells detected by a Trans well migration assay. Magnification: 200x; scalebar: 25 $\mu$m. (B) Count so f migrated cells. $^{***}p<0.001$ vs.CTL; $###p<0.001$ vs. NC.

tein is required for progression through the G1 phase of the cell cycle. PCNA is a cyclin-D1-associated protein that is essential for DNA replication, repair, and recombination and other cellular processes. In addition, NET increases the expression of PGRMC1 in MCF10A cells. PGRMC1 is recognized as an oncogene that is expressed in mammalian tissues and breast epithelial cells [17]. PGRMC1 enhances BC cell proliferation in a NET-dependent manner, and the pro-tumor effect of PGRMC1 has been confirmed in mouse xenograft models [10]. At the molecular level, NET activates PGRMC1 activity by inducing the phosphorylation of PGRMC1 at the casein kinase 2 phosphorylation site Ser181 [25]. Therefore, NET-induced breast epithelial MCF10A cell growth may result from upregulation of the PGRMC1 signaling pathway, which may be responsible for the increased tumorigenesis risk of BC observed during clinical treatment with NET.

NET stimulated the expression of not only PGRMC1, but also EGFR (**Fig. 6**). EGFR is a potent receptor-tyrosine kinase that drives tumorigenesis and is upregulated in a variety of tumors. Upregulated EGFR signaling pathway has been linked to increased cell proliferation, angiogenesis, and metastasis as well as decreased apoptosis, which is closely associated with cancer progression and tumorigenesis [26]. Studies have shown that PGRMC1 increases plasma-membrane-associated EGFR levels, co-localizes with EGFR in cytoplasmic vesicles, and cofractionates with EGFR in high-density microsomes [13]. Our data further indicate that NET plays a pro-tumorigenesis role in breast epithelial MCF10A cells by upregulating the PGRMC1/EGFR signaling pathway.

EGFR is implicated in the regulation of the PI3K/Akt/mTOR pathway [27]. This intracellular signaling pathway plays a critical and multifaceted role in cancer pathogenesis and progression [28]. The PI3K/Akt/mTOR pathway is often constitutively activated in various malignancies, including BC, by genomic abnormalities. In addition to EGFR, inactivation or dysregulation of PTEN can also activate the PI3K/Akt/mTOR pathway. PTEN, a tumor suppressor gene, evokes its phosphatase activity and antagonizes the activity of PI3K, which leads to dephosphorylation of the serine/threonine protein kinase Akt. mTOR is a downstream effector for many signaling pathways, including the PI3K/Akt pathway. Moreover, mTOR is involved in multiple signaling pathways, fundamental cell processes, and disease processes. In particular, overactivation of mTOR can promote tumor formation, proliferation, and metastasis [29]. We found that PTEN expression was downregulated, but mTOR was upregulated in MCF10A cells by NET treatment (**Fig. 6**), suggesting that NET may modulate the activity of the PI3K/Akt/mTOR signal pathway by regulating the expression of PTEN and mTOR. Taken together, these results indicate that NET upregulates the expression of PGRMC1/EGFR and the PI3K/Akt/mTOR pathway to promote tumorigenesis of human breast epithelial MCF10A cells (**Fig. 7**). Notably, as only one human breast epithelial MCF10A cell line was used in this study, to have greater external validity of the effect of NET in breast tumorigenesis, further investigation is required to assess whether NET exhibits the similar effect on other nontumorigenic breast cell lines (*e.g.*, MCF12A, an immortalized non-cancerous human epithelial breast cell line), or to validate these findings with other methods such as *in vivo* models.

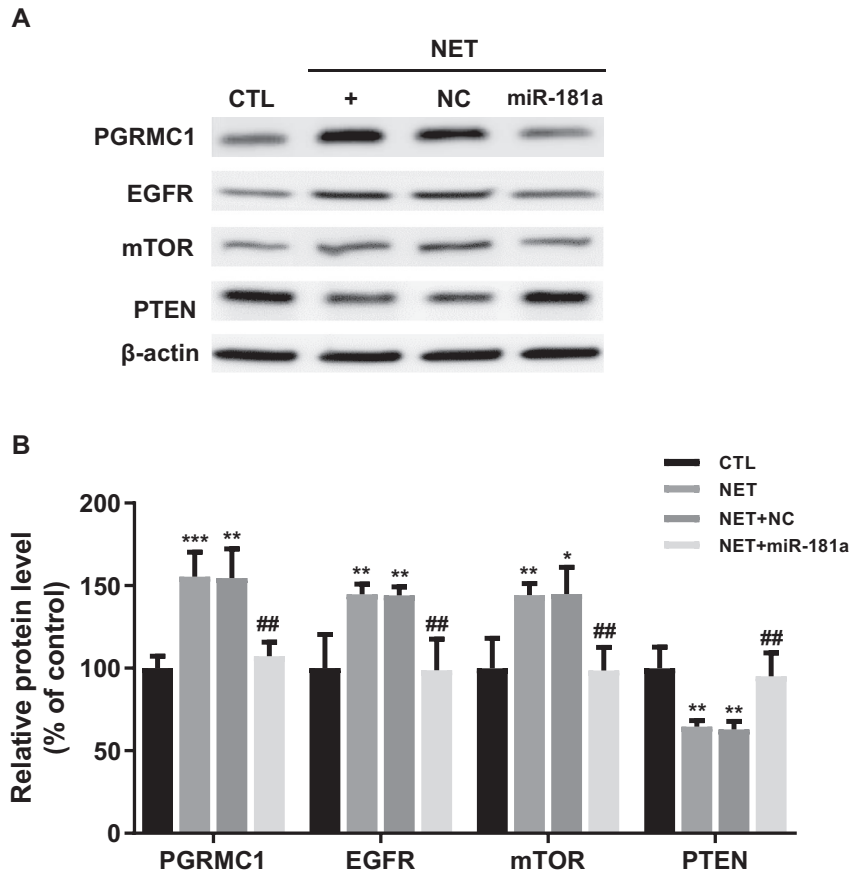



**Fig. 6.** *MiR-181a attenuated the NET-dys regulated PI3K/Akt/mTOR signaling pathway.* (A) Representative Western blotof protein levels for PGRMC1, EGFR, mTOR, and PTEN in NET-treated MCF10A cells with or without transfection of miR-181a. (B) Quantified protein levels for each gene, presented as a percentage of CTL (setas100%). Values are means±SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. CTL; $\#\#p<0.01$ vs. NC.

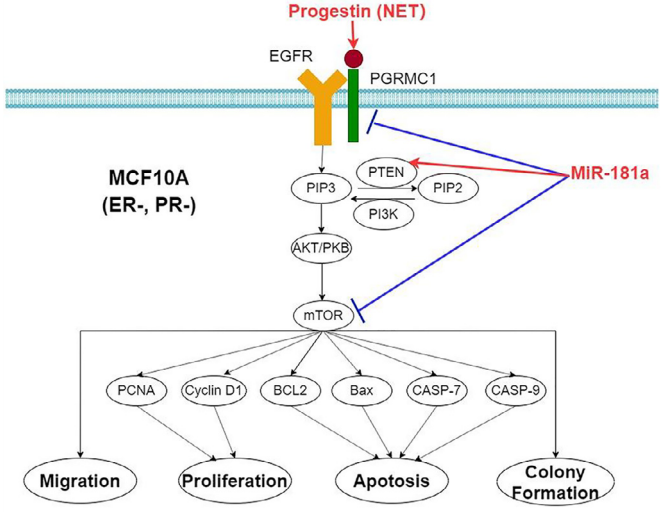



**Fig. 7.** *Schematic depicting possiblem echanisms governing the pro-tumorigenesis effect of NET and miR181a targets in the PGRMC1/EGFR–PI3K/Akt/mTOR signaling pathway in breastepithelialMCF10Acells.* Akt, proteinkinaseB(PKB); BCL2, B-celllymphoma2; CASP-7, caspase7; CASP-9, caspase9; EGFR, epidermal growth factor receptor; ER, estrogen receptor; mTOR, mechanistic target of rapamycin; NET, norethister one; PCNA, proliferating cell nuclear antigen; PGRMC1, progesterone receptor membrane component 1; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PR, progesteronereceptor; PTEN, phosphatase and tensinhomolog.

MiR-181a is involved in important fundamental cell functions such as growth, proliferation, death, and survival. Despite conflicting reports on the roles of miR-181a in BC [14], the finding that miR-181a inhibits NET-provoked cell proliferation in MCF10A cells (**Fig. 2**) is in agreement with previous observations in BC cells and glioma cells [11,30]. In addition, recent studies have verified that miR-181a can modulate cell apoptosis by targeting apoptosis-related genes, including the oncogenic gene BCL2 [11,14]. MiR-181a downregulates BCL2 expression by forming an imperfect base pairing with the 3′-untranslated region of this gene. In this study, we have demonstrated that miR-181a completely abolishes the NET-increased expression of BCL2 in MCF10A cells (**Fig. 3B**), which further indicates that anti-apoptotic BCL2 is a direct target of miR-181a. Bax is a BCL2 family member that exhibits a pro-apoptotic function by binding to and antagonizing BCL2. The caspase-mediated apoptosis pathway is closely related to the occurrence of BC. CASP-7 and CASP-9 are expressed in MCF10A cells and play an important role in the survival of breast epithelial cells. Our data suggest that the upregulated expression of Bax, CASP-7, and CASP-9 caused by miR-181a may abrogate the pro-apoptosis effect of NET on breast epithelial MCF10A cells.

We previously revealed that miR-181a suppresses BC cell viability regardless of the hormonal receptor phenotype [11]. The growth of MCF-7 cells (ER+/PR+), MDA-MB-231 cells (triple-negative, ER-/PR-/HER2-), and MCF10A cells (ER-/PR-) is suppressed by miR-181a. Interestingly, all of these cells express PGRMC1. Although there is no evidence to show that PGRMC1 is a direct target of miR-181a, miR-181a has been shown to modulate its expression in BC cells [11]. In this study, miR-181a completely blocked NET-stimulated expression of PGRMC1 and EGFR (**Fig. 6**), indicating that PGRMC1/EGFR mediates progestin-dependent cell survival signals in breast epithelial MCF10A cells, with the exis-

tence of functional interactions between PGRMC1/EGFR and miR-181a. Furthermore, miR-181a decreased the NET-induced increase in mTOR expression, which is consistent with the observation that miR-181a reduces NET-promoted cell colony formation, cell invasion, and migration. Thus, miR-181a may reduce the risk of tumorigenesis in MCF10A cells provoked by NET. As miR-181a usually has multiple targets, the interaction of miR-181a with other targets or signaling pathways in human breast epithelial cells cannot be excluded. Therefore, future studies should investigate the genes potentially regulated by miR-181a.

In conclusion, this study revealed the pro-tumorigenesis effect of NET on human breast epithelial MCF10A cells through upregulating PGRMC1/EGFR- PI3K/Akt/mTOR signal pathway and suppressing PTEN expression. Moreover, overexpression of miR-181a dramatically abrogated the effects of NET by restoring the dysregulated PI3K/Akt/mTOR pathway. These findings warrant further investigation of the pro-tumorigenesis effect of NET on human breast epithelial cells *in vivo* animal models and may lead to an insightful approach for preventing or reducing the risk of oncogenic transformation during HRT.

## Ethical approval

No ethical approval was required for this study.

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## Disclosures

There is no conflict of interest (either financial or personal). This manuscript has not been previously published and is not being considered concurrently for another publication. All authors and acknowledged contributors have read and approved the manuscript.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101068.

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