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## MicroRNA-548m Suppresses Cell Migration and Invasion by Targeting Aryl Hydrocarbon Receptor in Breast Cancer Cells

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Breast cancer is the most commonly diagnosed cancer among women and one of the leading causes of cancer mortality worldwide, in which the most severe form happens when it metastasizes to other regions of the body. Metastasis is responsible for most treatment failures in advanced breast cancer. Epithelial–mesenchymal transition (EMT) plays a significant role in promoting metastatic processes in breast cancer. MicroRNAs (miRNAs) are highly conserved endogenous short noncoding RNAs that play a role in regulating a broad range of biological processes, including cancer initiation and development, by functioning as tumor promoters or tumor suppressors. Expression of miR-548m has been found in various types of cancers, but the biological function and molecular mechanisms of miR-548m in cancers have not been fully studied. Here we demonstrated the role of miR-548m in modulating EMT in the breast cancer cell lines MDA-MB-231 and MCF-7. Expression data for primary breast cancer obtained from NCBI GEO data sets showed that miR-548m expression was downregulated in breast cancer patients compared with healthy group. We hypothesize that miR-548m acts as a tumor suppressor in breast cancer. Overexpression of miR-548m in both cell lines increased E-cadherin expression and decreased the EMT-associated transcription factors SNAIL1, SNAIL2, ZEB1, and ZEB2, as well as MMP9 expression. Consequently, migration and invasion capabilities of both MDA-MB-231 and MCF-7 cells were significantly inhibited in miR-548m-overexpressing cells. Analysis of 1,059 putative target genes of miR-548m revealed common pathways involving both tight junction and the mTOR signaling pathway, which has potential impacts on cell migration and invasion. Furthermore, this study identified aryl hydrocarbon receptor (AHR) as a direct target of miR-548m in breast cancer cells. Taken together, our findings suggest a novel function of miR-548m in reversing the EMT of breast cancer by reducing their migratory and invasive potentials, at least in part via targeting AHR expression.

**Key words:** Breast cancer; MicroRNA-548m; Aryl hydrocarbon receptor; Epithelial–mesenchymal transition (EMT); Invasion; Migration

### INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women and is one of the leading causes of cancer mortality worldwide, especially in low- and middle-income countries<sup>1</sup>. Breast cancer alone is expected to account for 30% of all new cancer cases and 15% of all cancer-related deaths in women<sup>2</sup>. It is characterized by heterogeneity of genetic modification and comprises variable phenotypes that are driven by distinctive molecular alteration and variabilities<sup>3</sup>. Breast cancers could be fatal when it metastasizes to other regions of the body, which contributes to most treatment failures<sup>4</sup>. Epithelial–mesenchymal

transition (EMT) has been suggested as a key mechanism in promoting the metastatic processes in cancer cells. EMT is a biological process that allows polarized epithelial cells to undergo various biochemical modifications, including losing its junction and apical–basal polarity<sup>5</sup>. This allows the cells to adopt a mesenchymal phenotype that defines cell shape, reprogram gene expression, and, consequently, enhance the motility and acquisition of invasive properties<sup>6</sup>. Therefore, it is crucial to find a new biomarker or regulator to suppress motility and invasion processes as a potential strategy in the treatment of metastatic breast cancer.

MicroRNAs (miRNAs) are a class of highly conserved endogenous short noncoding RNAs that play a pivotal role

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in various developmental and psychological processes. These processes include cancer initiation and development<sup>7</sup>, apoptosis<sup>8</sup>, cell cycle progression<sup>9</sup>, cellular proliferation<sup>10</sup>, differentiation, and metastasis<sup>11</sup>. A miRNA may be found to be upregulated in some cancer types, and thus indicated as oncomiR, or downregulated in other type of cancers, thus displaying a tumor suppressor function<sup>12</sup>. miRNAs are important genetic regulators wherein each miRNA can possibly regulate and target the expression of hundreds of genes. On the other hand, a single transcript can be targeted by multiple miRNAs to concurrently downregulate multiple proteins in the same pathway<sup>13</sup>. Several miRNAs have been recognized in regulating EMT processes in breast cancer development by modulation of EMT-related genes. For instance, miR-138<sup>14</sup>, miR-221/222<sup>15</sup>, miR-9<sup>16</sup>, and miR-21<sup>17</sup> act as either promoters or inhibitors of EMT-related genes such as E-cadherin and vimentin, which later affect breast cancer invasion and metastasis. Understanding the mechanism and functional role of miRNAs may provide better understanding and insight into EMT regulation. In this study, we are interested to investigate the role of miR-548m in breast cancer.

miR-548 is a newly discovered miRNA family, which are poorly conserved in their sequences<sup>18</sup>. Recent evidence has shown that members of the miR-548 family have a close correlation with metastatic behaviors<sup>19–22</sup> and are more commonly downregulated in breast cancer<sup>22–25</sup>. From our previous study, miR-548m was identified as an inducer of mesenchymal phenotype in bladder cancer using multiparametric screening for EMT modulators<sup>26</sup>. However, the biological function and molecular mechanisms of miR-548m in cancers have not been fully elucidated. Moreover, a particular miRNA may also be found to be upregulated in some cancer types, and thus supposedly oncogenic, but downregulated in other cancers, which is indicative of tumor suppressor function<sup>12</sup>. In breast cancer, treatment with 5-fluorouracil<sup>27</sup> and polyphenon-60<sup>28</sup> in MCF-7 cell lines showed upregulation of miR-548m expression upon treatment, suggesting possible tumor suppressor function of miR-548m. In this present study, we assessed the expression levels of miR-548m in human breast cancer and examined its effects on cell proliferation, migration, invasion, and EMT-related gene expression. Furthermore, we explored the target of miR-548m in breast cancer cells and the possible mechanism of its function.

## MATERIALS AND METHODS

### *Gene Expression Analysis Using the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) Database*

Microarray profiles of selected breast cancers were obtained from the publicly available GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). In the GEO data set search of miR-548m, the following search terms or keywords were

applied (microRNA OR miR OR miRNA) AND (Breast) AND (tumor OR cancer OR carcinoma OR neoplas\* OR malignan\*). The microarray data sets were considered eligible if they met the criteria as follows: (1) the samples in each data set must contain breast cancer and nontumor control groups; (2) data sets consist of miR-548m expression between breast cancer and normal breast tissues; (3) miRNA–microarray data with other intervening factors were removed, such as experiments with gene knock-down and other treatments; and (4) in terms of sample size, each data set should contain at least three samples ( $n > 3$ ). GEO2R (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) was applied to screen differentially expressed miRNAs between breast cancer and normal tissue samples. The adjusted  $p$  values (adj.  $p$ ) using the Benjamini and Hochberg (BH) false discovery rate (FDR) method by default were applied to correct for the occurrence of false-positive results.

### *Cell Lines and Cell Culture*

Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO) in the presence of 1% penicillin–streptomycin (GIBCO). The cells were maintained at 37°C humidified atmosphere in an incubator with 5% CO<sub>2</sub> saturation. Transfections of miRNA and starvation media were deprived of penicillin–streptomycin and FBS, respectively.

### *Cell Transfection*

The miRIDIAN miR-548m mimic (C-301392-00, mature miRNA sequence), miRIDIAN mimic negative control #1 (CN-001000-01, mature miRNA sequence), and miRIDIAN miRNA mimic transfection control with Dy547 (fluorescently labeled mimic control) were purchased from Dharmacon (Lafayette, CO, USA). The breast cancer cell lines MDA-MB-231 and MCF-7 were reverse transfected at a final concentration of 25 nM using Lipofectamine® RNAiMAX (Life Technologies, Carlsbad, CA, USA) transfection reagent per the manufacturer's protocols. The efficiency of miR-548m overexpression was verified by visualization with a fluorescently labeled Dy547 mimic control. These controls were used under the same experimental conditions as miR-548m mimics, which allowed to assess cell transfection efficiency during each assay. All subsequent functional assays were performed 72-h posttransfection.

### *Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)*

Large RNA and small RNA from breast cancer cell lines were isolated using a NucleoSpin® miRNA Kit

(Macherey-Nagel, Düren, Germany). The concentration and purity of the mRNA and miRNA samples were measured using a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). cDNA from mRNA was generated using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The expression levels of mRNA were assessed with RT-qPCR using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Oligonucleotide primers as shown in Table 1 were synthesized by Integrated DNA Technologies (IDT). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene. The small RNA sample was reverse transcribed with TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. cDNA was quantitated using a TaqMan microRNA assay specific (Applied Biosystem) for miR-548m and RNU48 according to the manufacturer's protocol. The expression level of RNU48 was used as reference gene.

#### Western Blot Analysis

Total protein was isolated in parallel with large RNA and small RNA with the NucleoSpin® miRNA kit (Macherey-Nagel) according to the manufacturer's protocol. Protein pellets were resuspended in protein solving buffer set PSB/TCEP (Macherey-Nagel). Proteins were loaded and separated in a 10% acrylamide gel (100v) and transferred onto a 0.2-µm nitrocellulose membrane (Amersham Protran, Buckinghamshire, UK) using the Thermo-Pierce Fast Semi-Dry Blotter apparatus. The membrane was blocked with 5% nonfat milk for 1 h at room temperature with agitation, followed by overnight incubation at 4°C with primary antibodies against aryl hydrocarbon receptor (AHR) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1,000; Santa Cruz Biotechnology) in BSA with tris-buffered saline-Tween 2 (TBS-T; Santa Cruz Biotechnology). Subsequently, the membrane was washed and incubated with secondary

antibody (1:2,000; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Protein signal was detected with enhanced chemiluminescence horseradish peroxidase (HRP) substrate (Radiance Plus; Azure Biosystems, Dublin, CA, USA) and recorded using the Azure Biosystems c600 quantitative Western blot imaging system (Azure Biosystems).

#### Transwell Invasion Assay

The invasive potential of MDA-MB-231 and MCF-7 was measured using Boyden Transwell 0.8-µm pore polycarbonate membrane insert (Transwell-Costar, Corning, NY, USA) and Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning) according to the manufacturer's protocol. Briefly, the filter of the top chamber was coated with diluted Matrigel (0.3 mg/ml) following standard procedure and incubated at 37°C for 5 h to allow the Matrigel to solidify. First, miR-548m mimics and its control were reverse transfected into the breast cancer cells in a six-well plate for 72 h. They were then reseeded onto the membrane of the upper chamber coated with Matrigel at  $5 \times 10^4$  cells/well for MDA-MB-231 and at  $1 \times 10^5$  cells/well for MCF-7 in 300 µl serum-free media. DMEM (500 µl) supplemented with 10% FBS were used as a chemoattractant in the lower chamber. After 24 h, the lower chamber was washed with Dulbecco's phosphate-buffered saline (DPBS; GIBCO). Invaded cells on the lower membrane surface were fixed in 3.7% formaldehyde (Sigma-Aldrich) for 2 min. Noninvasive cells in the top chamber were removed with cotton swabs. The membrane in the lower chamber was stained with 0.2% crystal violet (Sigma-Aldrich). The number of invaded cells was viewed with a microscope and counted in at least five fields.

#### Wound Healing Assay

For the in vitro wound healing assay (scratch migration analysis), MDA-MB-231 and MCF-7 cells transfected with miR-548m mimics and negative control were allowed to grow in a 24-well plate until cells became

**Table 1.** Sequence of Primers for RT-qPCR

| Gene  | Forward Sequence (5'–3') | Reverse Sequence (5'–3') |
|-------|--------------------------|--------------------------|
| GAPDH | CCCATCACCATCTTCCAGGAG    | GTTGTCATGGATGACCTTGGC    |
| AHR   | ACATCACCTACGCCAGTGG      | CTCTATGCCGCTTGGAAGGAT    |
| SNAI1 | GGTTCTTCTGCGCTACTGCT     | TAGGGCTGCTGGAAGGTAA      |
| SNAI2 | GGGGAGGAGGGAAAGATTAG     | GCACTTGGAAGGGGTATTGT     |
| ZEB1  | GCACAACCAAGTGCAAGA       | CATTTGCAGATTGAGGCTGA     |
| ZEB2  | ATCTGCTCAGAGTCCAATGC     | TTGTTCCCTCAGGTTGAGAGC    |
| VIM   | TGGCACGTCTTGACCTTGAA     | GGTCATCGTGATGCTGAGAA     |
| ZO1   | CGGTCCTCTGAGCCTGTAAG     | GGATCTACATGCGACGACAA     |
| CDH1  | CGACCAACCCAAGAATCTA      | AGGCTGTGCCTTCCTACAGA     |
| MMP9  | TGTACCGCTATGGTTACTCTCG   | GGCAGGGACAGTTGCTTC       |

confluent after 72 h posttransfection. Briefly, a scratch was made by scraping the bottom of each well in straight lines using a sterile 10- $\mu$ l pipette tip and washed with DPBS to remove cell debris. Cells were then maintained in the incubator at 37°C with 5% CO<sub>2</sub>. Cell images were captured every 2 h using an Olympus microscope (Olympus America Inc., Waltham, MA, USA) by live imaging for 24 h. Cells were maintained in a plate incubator at 37°C with 5% CO<sub>2</sub>. The area of uncovered wound was measured by creating an area at the wound margin at given time points, and a migration was reported as percentage of wound closure at each time point.

#### Cell Proliferation Assay

Cell proliferation of MDA-MB-231 and MCF-7 was monitored by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. Briefly, cells were seeded onto 96-well plates, and the cell proliferation after transfection was documented every 24 h for 3 days. After every 24 h posttransfection, the cells were incubated with 20  $\mu$ l MTT (Sigma-Aldrich, Germany) into each well at a final concentration of 0.5 mg/ml. The incubation was continued for 4 h at 37°C and 5% CO<sub>2</sub>, allowing viable cells to bio-reduce the MTT reagent into a colored formazan product. SDS (100  $\mu$ l) was added to each well to dissolve the formazan crystals overnight. The staining intensity in the medium was measured using a SpectraMax M3® spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) by determining the absorbance at 570 nm. The absorbance at 650 nm was used as a reference.

#### Computational miRNA Target Prediction

For identification of miR-548m targets, bioinformatics analysis was performed to predict the possible target genes of miR-548m. Three different software algorithms were used to find conserved target regions, including TargetScan release 7.1 (<http://www.targetscan.org>), microRNA.org (miRanda) database, and DIANA-microT database (<http://diana.imis.athena-innovation.gr>) as reviewed by Rizzo-Campos et al.<sup>29</sup>. The predicted target genes from each software algorithms were merged, and specific cutoff point from each prediction software was applied. Finally, target genes were further narrowed down to breast cancer-associated genes (hsa-miR-548m-associated broad phenotype) using the miR-Ontology database (miRò2; [www.ferrolab.dmi.unict.it/miro](http://www.ferrolab.dmi.unict.it/miro)). Selected target genes were submitted to online tools for functional annotation analysis, Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>). The significant enrichment analysis of target genes was assessed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/kegg2.html>) with a value of  $p < 0.05$  considered as a statistically significant difference.

html) with a value of  $p < 0.05$  considered as a statistically significant difference.

#### Dual-Luciferase Reporter Assay

For construction of the AHR 3'-UTR-Luc reporter plasmid, a fragment of the 3'-untranslated region (3'-UTR) of the AHR gene was synthesized and cloned into pUC57 (Bio-Basic Inc., Amherst, NY, USA) within the *SacI* and *SalI* restriction sites. The pmirGLO Dual-Luciferase miRNA target expression vector (pmirGLO vector) containing both the firefly luciferase (*Fluc*) gene and the *Renilla* luciferase (*Rluc*) gene was purchased from Promega (Madison, WI, USA). The AHR 3'-UTR was then subcloned by 1st BASE Services (First BASE Laboratories, Selangor, Malaysia), directly downstream from a *Fluc* gene under the control of the PGK promoter, and *Rluc* gene under the control of an SV40 promoter (as a transfection control) within the *SacI* and *SalI* restriction sites. MDA-MB-231 and MCF-7 cells were cotransfected with 50 ng reporter plasmids and 25 nM miR-548m mimics and its control for 48 h. The luciferase activity was determined with Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instruction. Luciferase activity was measured with SpectraMax L luminometer (Molecular Devices). Normalized data were calculated as the ratio of *Rluc/Fluc* activities.

#### Statistical Analysis

Each experiment was performed three times at least. The results were expressed as the mean  $\pm$  standard error of the mean (SEM). Differences between values were statistically analyzed using Student's *t*-test or one-way analysis of variance (ANOVA). All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

#### miR-548m Expression in Human Breast Cancer Tissues and Cell Lines

To examine the relevance of miR-548m in human breast cancer tissues and their expression, data analysis was performed using GEO2R on publicly available data sets obtained from the NCBI GEO. Nine data sets published in GEO reference series that met the entry criteria were queried for the expression of miR-548m in breast cancer<sup>30-36</sup>. The features of the included GEO data sets are described in Table 2. The expression data of miR-548m are shown on the scatter plots and receiver operating characteristic (ROC) curves in Figures 1 and 2. miR-548m expression was significantly downregulated in breast cancer tissues compared with normal breast tissues in two GEO microarrays (GSE45666 and GSE44899, all  $p < 0.05$ ) (Fig. 1). The ROC curve of these two



**Table 2.** Information on Selected Gene Express Omnibus (GEO) Breast Cancer Microarrays Data Sets

| GEO Accession | Platform | Year | Country   | Number of Samples |       |            |          | Clinicopathological Characteristics in Patients (n) |                 |                 |                 |                 |                   |                   |      |  |  |
|---------------|----------|------|-----------|-------------------|-------|------------|----------|-----------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-------------------|-------------------|------|--|--|
|               |          |      |           | Normal            | Tumor | Metastasis | PMID     | Sources                                             | ER <sup>+</sup> | ER <sup>-</sup> | PR <sup>+</sup> | PR <sup>-</sup> | Her2 <sup>+</sup> | Her2 <sup>-</sup> | TNBC |  |  |
| GSE80038      | GPL18044 | 2016 | China     | 4                 | 4     | 4          | 28629464 | Tissue                                              | -               | -               | -               | -               | -                 | -                 | 12   |  |  |
| GSE44899      | GPL7723  | 2014 | Serbia    | 4                 | 76    | -          | 24104964 | Tissue                                              | 27              | 33              | 23              | 38              | 11                | 50                | 26   |  |  |
| GSE45666      | GPL14767 | 2013 | China     | 15                | 100   | -          | 23722663 | Tissue                                              | 52              | 63              | 39              | 76              | 41                | 74                | 36   |  |  |
| GSE57897      | GPL18722 | 2018 | China     | 31                | 422   | -          | 31209222 | Tissue                                              | 246             | 176             | 234             | 188             | 120               | 302               | 46   |  |  |
| GSE38167      | GPL14943 | 2014 | Australia | 23                | 31    | 13         | 24479446 | Tissue                                              | -               | -               | -               | -               | -                 | -                 | 31   |  |  |
| GSE48088      | GPL14613 | 2014 | Spain     | 3                 | 33    | -          | 25047087 | Tissue                                              | 26              | 8               | 22              | 12              | 13                | 21                | 4    |  |  |
| GSE44124      | GPL14767 | 2014 | Spain     | 3                 | 50    | -          | 24098452 | Tissue                                              | -               | -               | -               | -               | -                 | -                 | 7    |  |  |
| GSE32922      | GPL7723  | 2013 | Spain     | 16                | 22    | -          | -        | Tissue                                              | -               | -               | -               | -               | -                 | -                 | -    |  |  |
| GSE37407      | GPL13703 | 2012 | Sweden    | 6                 | 19    | 31         | 22294488 | Tissue                                              | 24              | 6               | 4               | 19              | 1                 | 9                 | -    |  |  |

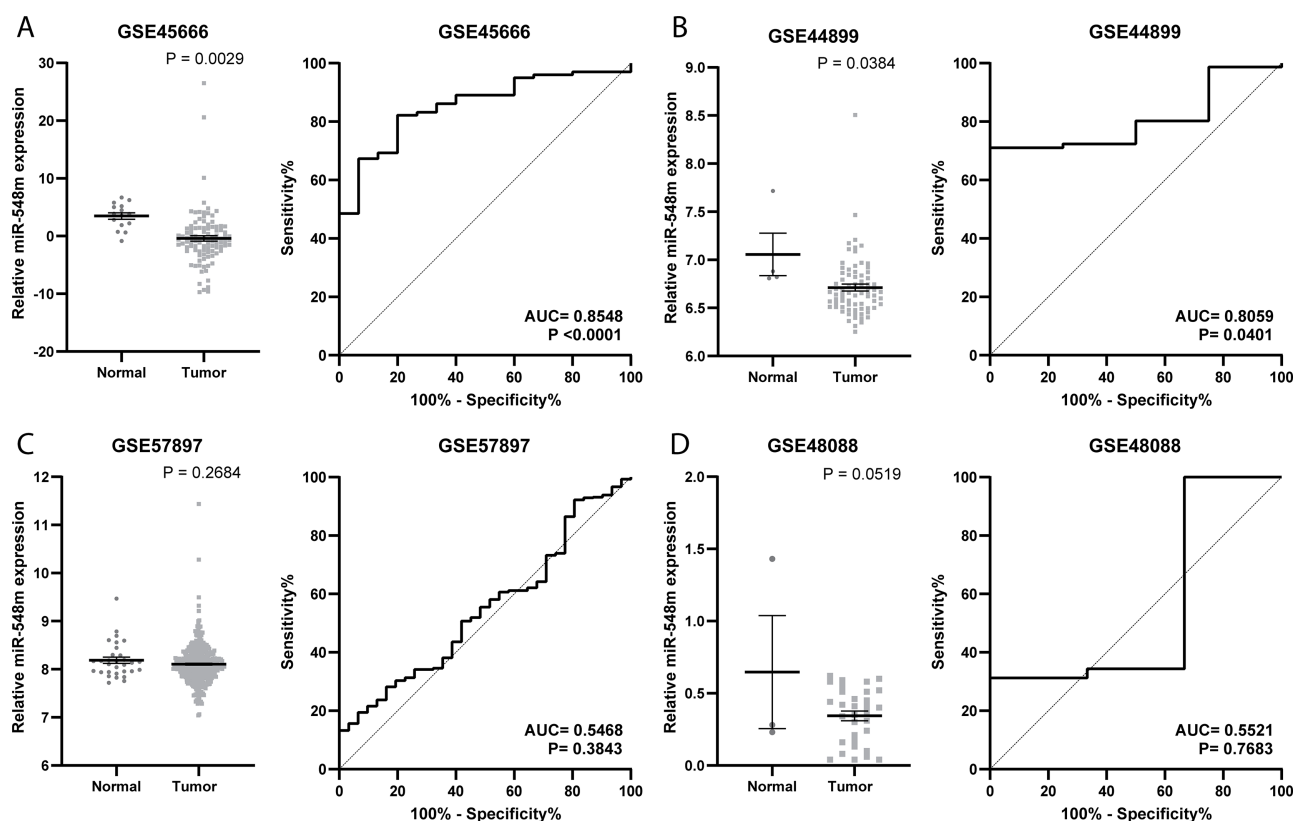
Abbreviations: GEO, Gene Express Omnibus; PMID, PubMed unique identifier; -, not available; n, number of patients; ER<sup>+</sup>, estrogen receptor positive; ER<sup>-</sup>, estrogen receptor negative; PR<sup>+</sup>, progesterone receptor positive; PR<sup>-</sup>, progesterone receptor negative; HER2<sup>+</sup>, human epidermal growth factor receptor 2 positive; HER2<sup>-</sup>, human epidermal growth factor receptor 2 negative; TNBC, triple negative breast cancer.

microarrays also implied that miR-548m exerted discriminatory capabilities that could be used to differentiate between breast cancer tissue and normal breast tissue (Fig. 1). By contrast, no significant difference in miR-548m expression was detected between breast cancer tissue and normal breast tissue in other microarrays (GSE57897, GSE48088, GSE44124, and GSE32922) (Fig. 1). In addition, the expression data of different types of breast cancer and normal breast tissue group were also collected on the GEO database. The expression of miR-548m was significantly downregulated in tissues of breast cancer with lung metastasis and primary breast tumor, compared to normal adjacent breast tissue (GSE80038;  $p < 0.05$ ) (Fig. 2). By contrast, no significant difference in miR-548m expression was detected between metastatic breast cancer tissue, primary breast cancer tissue, and normal breast tissue group in other microarrays (GSE37407 and GSE38167) (Fig. 2).

Next, we analyzed miR-548m expression in a GEO microarray data set (GSE146477) generated from primary mammary epithelial cells and a panel of breast cancer cell lines that range in tumorigenic and invasive potential<sup>37</sup>. miR-548m expression was higher in the less invasive MCF-7 cells and nontumorigenic human mammary epithelial cells (HMEC) and 184A1 cells, compared with the more aggressive breast cancer cell lines (MDA-MB-231, SKBR3, and T-47D) (Fig. 2D). However, when the endogenous expression of miR-548m in different types of breast cancer cell lines (MCF-7, HS578T, and MDA-MB-231) and nontumorigenic mammary cell line (MCF-10A) was assessed, our lab could not detect the expression of miR-548m in either breast cancer cell lines or normal human mammary cell (data not shown).

#### *miR-548m Modulates Expression of EMT Markers in Breast Cancer Cells*

To investigate the involvement of miR-548m in regulating EMT, we analyzed the expression of several EMT-related genes in MDA-MB-231 and MCF-7 cells following transfection of miR-548m mimics. The transfection resulted in significant upregulation of miR-548m in mimic-transfected cell lines compared to the negative controls (data not shown). As shown in Figure 3A and B, in both MDA-MB-231 and MCF-7 cells transfected with miR-548m mimics, E-cadherin (CDH1) expression was upregulated 1.23-fold and 1.35-fold, respectively, while no significant changes were seen on vimentin expression. In MDA-MB-231 cells, the expressions of SNAI1, ZEB2, and matrix metalloproteinase 9 (MMP9) were downregulated 1.32-, 1.30-, and 1.50-fold, respectively, following miR-548m mimics treatment (Fig. 3A). In MCF-7 cells, the upregulation of E-cadherin (CDH1) was accompanied by downregulation of SNAI2, ZEB2, and MMP9 1.45-, 2.12-, and 1.58-fold, respectively (Fig. 3B).



### *miR-548m Reduces the Migration and Invasion of Breast Cancer Cells*

We hypothesized that overexpression of miR-548m would inhibit cancer cell migration and invasion. To test this postulation, wound healing assays and Transwell invasion assay were performed on MDA-MB-231 and MCF-7 cell lines transfected with miR-548m mimics. Overexpression of miR-548m significantly inhibited the potential of both breast cancer cell migration. Transfection with miR-548m mimics caused only a 47% reduction in wound closure in MDA-MB-231, compared with 62% reduction in negative control (Fig. 4A). In MCF-7 cells, miR-548m mimics caused a 37% reduction in wound closure compared with 75% reduction in negative control after 24 h. Transwell invasion assay with Matrigel demonstrated that miR-548m mimics reduced invasion of MDA-MB-231 and MCF-7 by 45% (Fig. 4B). However, the MTT assay showed that MDA-MB-231 and MCF-7 cells transfected with negative control or miR-548m mimics for 24, 48, and 72 h had no significant effects on proliferation (Fig. 4C). These results suggested that overexpression of miR-548m reduces migration and invasion capabilities of breast cancer cells.

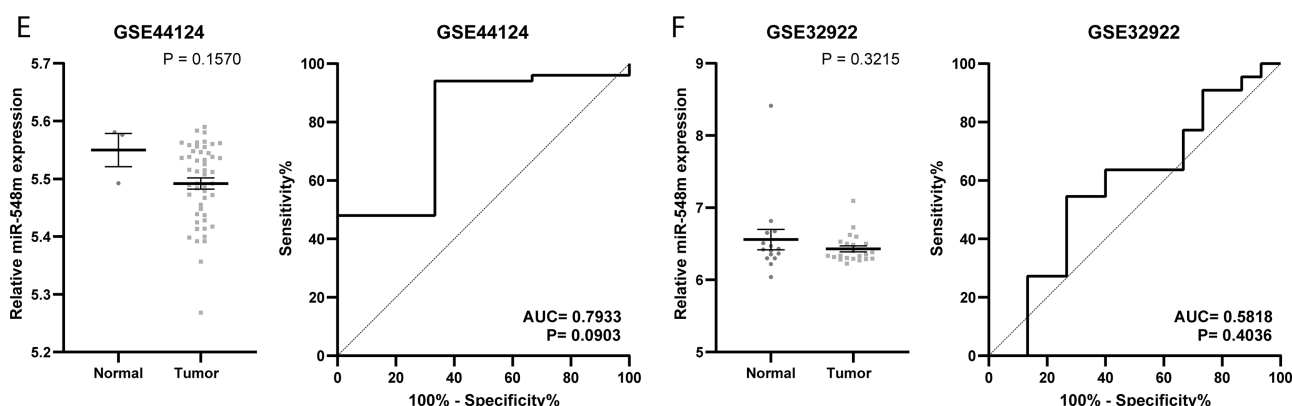
### *miR-548m Is Involved in Key Signaling Pathways and Alters its Target Gene Expression*

To identify the potential target genes of miR-548m whose modulations resulted in the decrease in migration

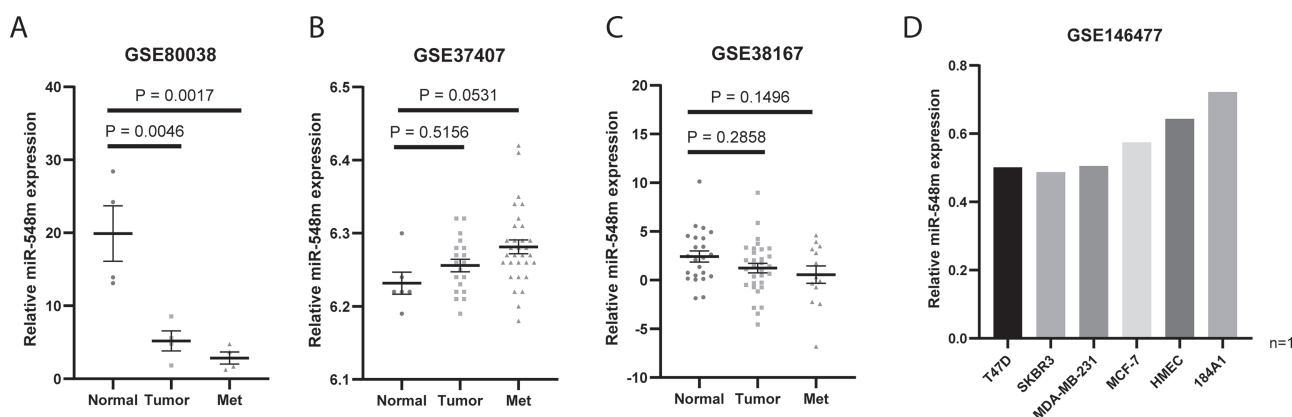
and invasion, we systematically screened through bioinformatic tools: TargetScan, microRNA.org (miRanda), and DIANA-microT-CDS. A total of 1,059 commonly predicted target genes were identified. The functional enrichment tool was employed to identify common pathways and the molecular network of the 1,059 potential target genes using KEGG analysis within the DAVID tools. The “tight junction” was found to be the most enriched pathway, followed by the “mTOR signaling pathway” (Fig. 5B). These predicted targets were eventually cross-checked with the miR-Ontology 2 (miR02) database to identify the potential targets associated with breast cancer (Fig. 5A). A total of 31 genes were identified as potential targets of miR-548m in breast cancer, and we further categorized them into the pathobiological processes involved. As a result, six of them were more closely related to EMT, cell migration, and invasion in breast cancer based on published literature (Table 3). Subsequent gene profiling on those six selected potential targets revealed AHR to be consistently and significantly downregulated in both MDA-MB-231 and MCF-7 cells (data not shown).

### *AHR as a Direct Downstream Target of miR-548m in Breast Cancer Cells*

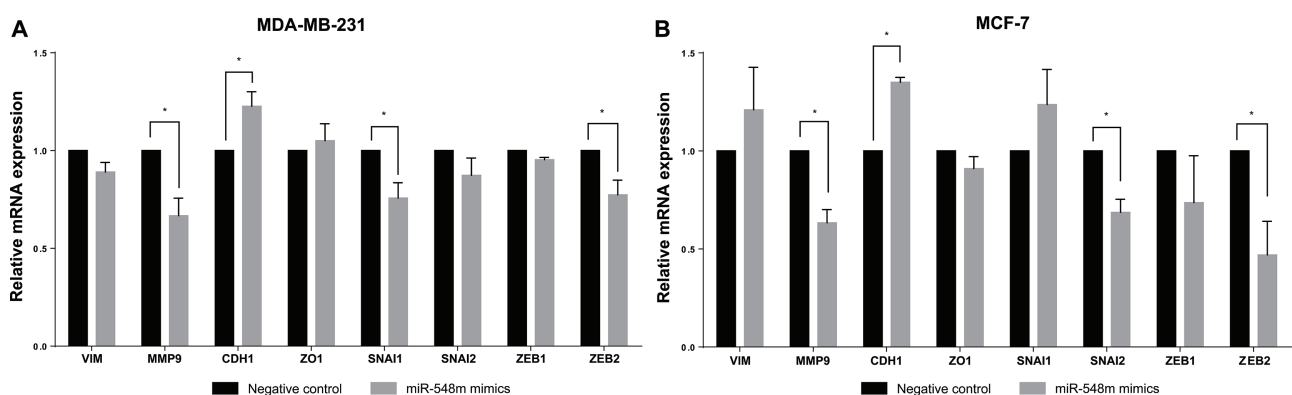
Among the genes explored, AHR (SVR score:  $-0.8462$ ) showed consistent downregulation in both cell lines at



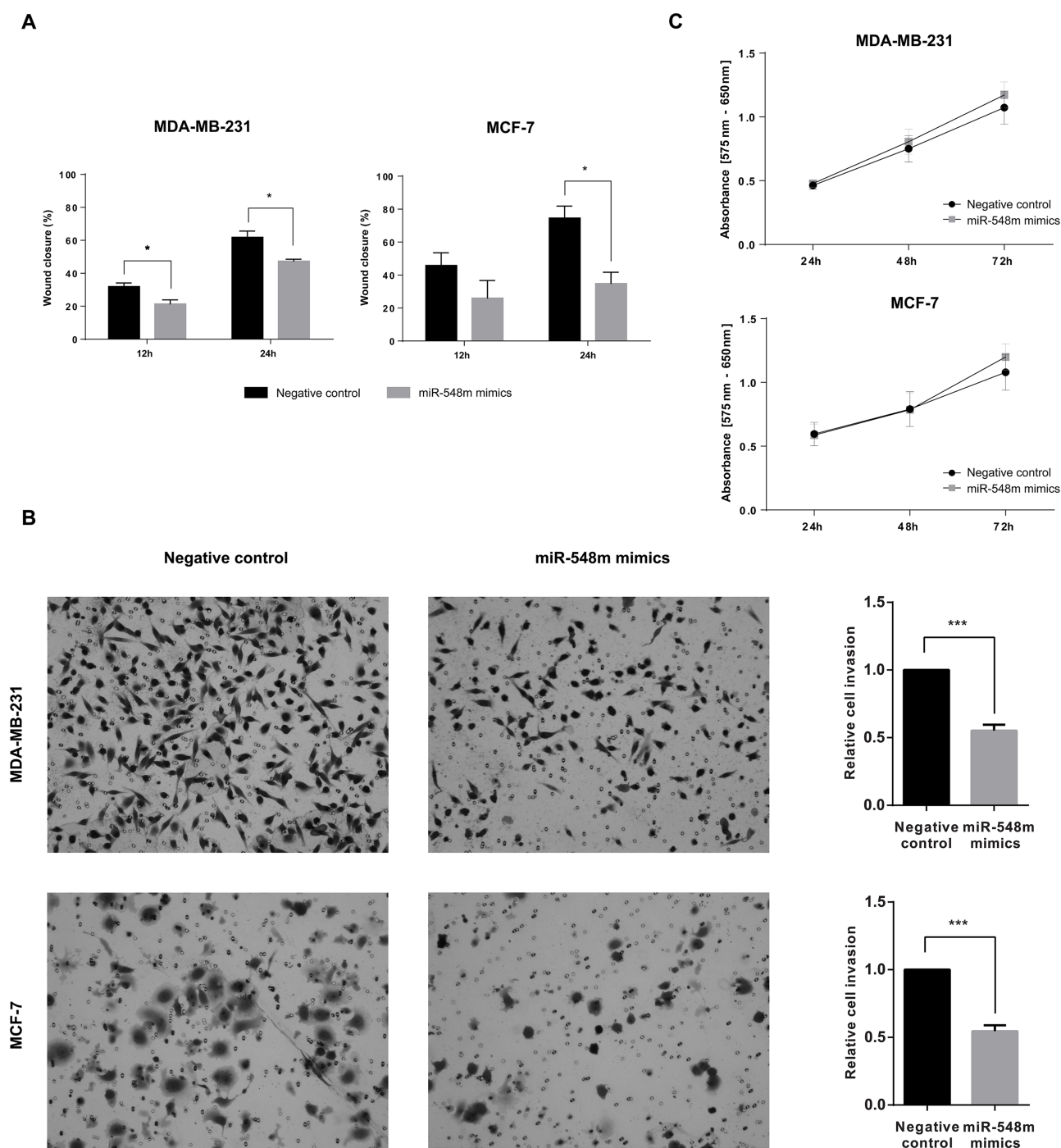
**Figure 1.** Above and facing page. Expression of miR-548m in normal breast and breast cancer tissues based on the Gene Expression Omnibus (GEO) database. The miR-548m expression and the ROC curve from (A) GSE45666, (B) GSE44899, (C) GSE57897, (D) GSE48088, (E) GSE44124, and (F) GSE32922. All data are presented as the means  $\pm$  standard error of the mean (SEM).



**Figure 2.** Expression of miR-548m in breast cancer tissues and cell lines based on the GEO database. The miR-548m expression and the ROC curve from (A) GSE80038, (B) GSE37407, (C) GSE38167, and (D) GSE146477. All data are presented as the means  $\pm$  SEM, except for (D) where  $n = 1$ .

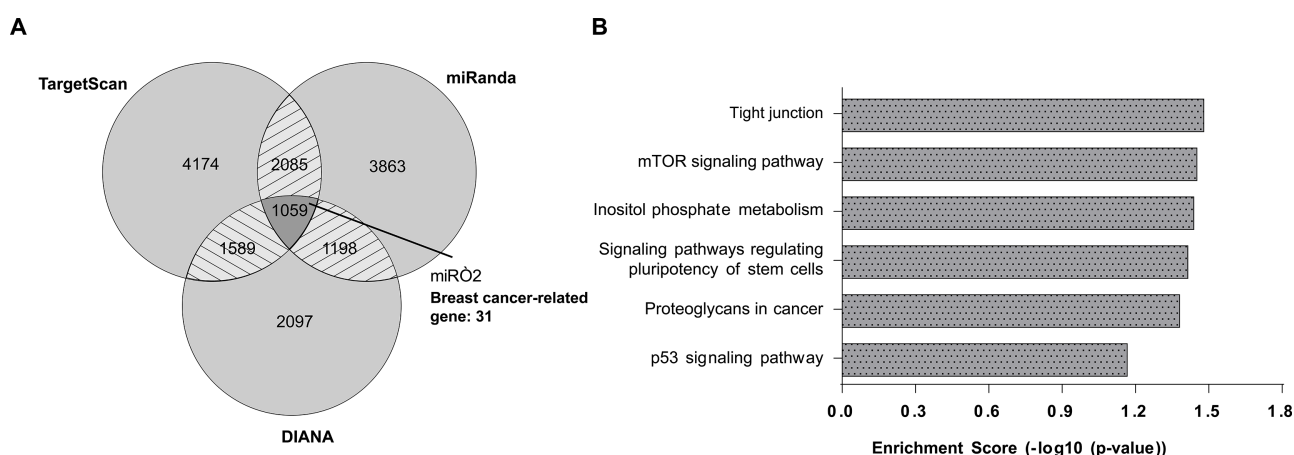


**Figure 3.** miR-548m modulates the expression of epithelial-mesenchymal transition (EMT) markers. Quantitative real-time polymerase chain reaction (RT-qPCR) analyses of relative selected EMT marker expression in (A) MDA-MB-231 and (B) MCF-7 cells transfected with miR-548m mimics compared with negative control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the results to 1. Graphs are displayed as mean  $\pm$  SEM,  $n = 3$ .  $*p < 0.05$ .



**Figure 4.** miR-548m reduces the migration and invasion of breast cancer cells. (A) The migration abilities of MDA-MB-231 and MCF-7 cells were measured by wound healing analysis between miR-548m mimics compared with negative control. For migration area quantification, the cell-free areas of the images were defined manually by NIS-Elements packages. (B) The invasion abilities of MDA-MB-231 and MCF-7 cells were measured using Transwell coated with Matrigel and stained with crystal violet (magnification: 10 $\times$ ). The graph was normalized to negative control. Representative images (left) and quantification of invasive cells (right) are shown. (C) The effects of miR-548m on proliferation of MDA-MB-231 and MCF-7 were evaluated as the value of absorbance at 575 nm with reference at 650-nm wavelength. Graphs are displayed as mean  $\pm$  SEM,  $n = 3$ . \* $p < 0.05$ ; \*\*\* $p < 0.001$ .





**Figure 5.** Possible target genes of miR-548m analysis. (A) miR-548m target genes were predicted using TargetScan release 7.1, DIANA-microT-CDS database and microRNA.org (miRanda) database, and the lists of predicted target genes were merged. Subsequently, those predicted target genes were adopted into the miR-Ontology 2 (miR02) database to identify the potential targets within breast cancer-associated classification. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of predicted miR-548m target genes within the Database for Annotation, Visualization and Integrated Discovery (DAVID) tools.

the mRNA and protein levels following introduction of miR548m mimic (Fig. 6A). The predicted binding sites of miR-548m in AHR was identified using TargetScan. The miRNA:mRNA alignment analysis showed that the 3'-UTR of AHR contains one putative binding site for miR-548m located from nucleotide position (np) 4,089 to np 4,096, 5'-UACCUUUA-3' (Fig. 6B). To validate the 3'-UTR of AHR as a direct target of miR-548m, dual-luciferase assays were performed. Overexpression of miR-548m significantly reduced the luciferase activity of the reporter vector containing the 3'-UTR of AHR, by 27% and 23% in MDA-MB-231 and MCF-7, respectively, indicating the putative binding sites were functional in both cells and confirming AHR as a direct target of miR-548m (Fig. 6C).

## DISCUSSION

Functional identification of miRNAs and their target genes is crucial in understanding their role in cancer progression. The role of miRNAs in breast cancer has drawn much interest due to their critical roles in cancer development including initiation and progression, cell proliferation, apoptosis, differentiation, invasion, and metastasis. In the present study, we identified that miR-548m regulates EMT through inhibiting migration and invasion in breast cancer cell lines. Data mining from the NCBI GEO database suggests the possible role of miR-548m as a tumor-suppressive miRNA, although the variation could be attributed to miRNA-specific function in different breast cancer histological group and subtype. There were also very low available numbers of normal tissues to be compared with tumor tissues in these data sets, affecting the differential expression and proper breast tumor

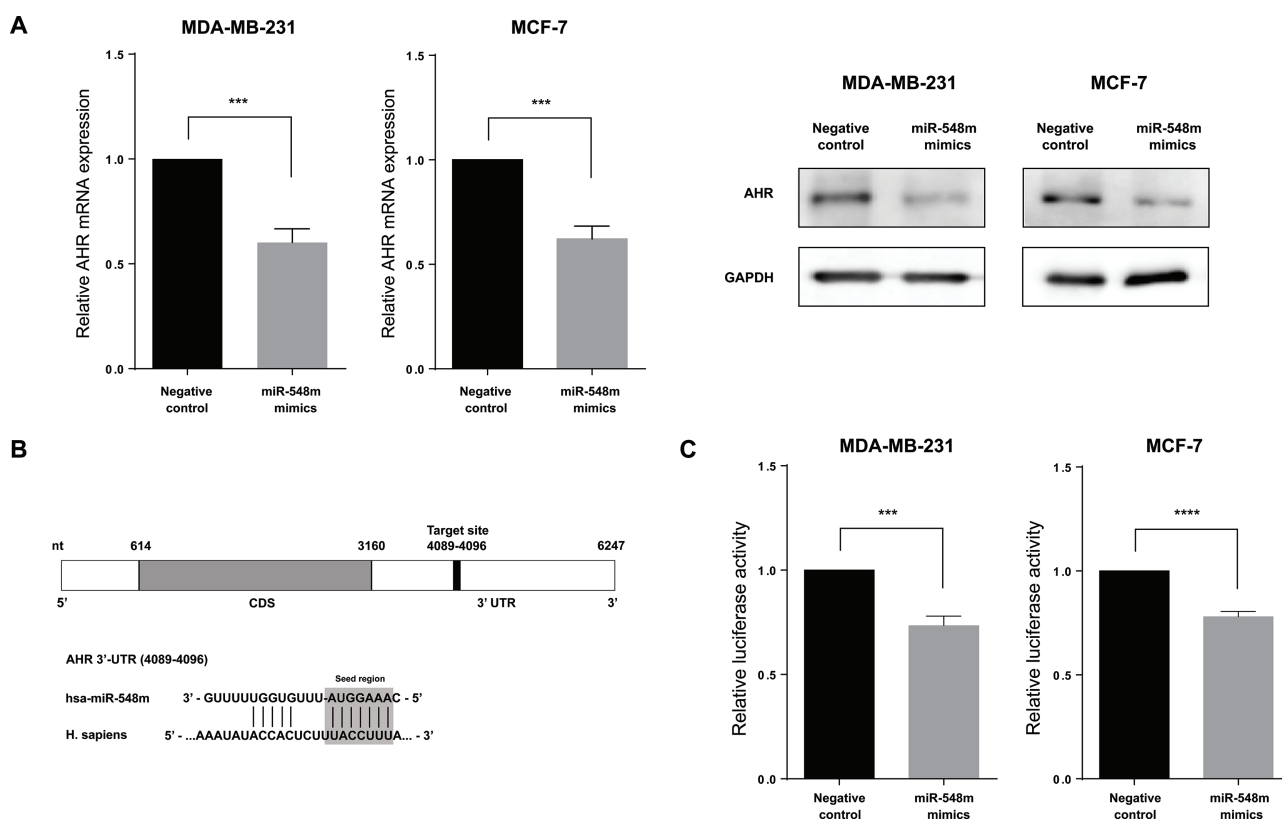
miR-548m-related analyses. Interestingly, in the GSE80038 data set, which consists of triple-negative breast cancer (TNBC) subtypes, expression of miR-548m was found to be significantly downregulated in this subtype of breast cancer with lung metastasis and primary breast tumor, compared to normal adjacent breast tissue ( $p < 0.05$ ). In parallel to our in vitro work in the TNBC cell line MDA-MB-231, overexpression of miR-548m was able to reduce the expression of several EMT markers including MMP9, SNAI1, and ZEB2, as well as inhibit migration and invasion of the cells. This further supports the role of miR-548m as a tumor suppressor miRNA in breast cancer, especially TNBC subtypes. Although in this study we were unable to detect the in vitro expression of miR-548m, this miRNA has been shown to be expressed in the same cell lines in other studies<sup>27,28,37</sup>. This discrepancy might be attributed by the phenotypic and genotypic variation in the cell line over a certain period of time as well as heterogeneity caused by genetic drifts in cultures at a single point in time<sup>38</sup>.

Nevertheless, the introduction of miR-548m mimics in MDA-MB-231 and MCF-7 resulted in increased expression of E-cadherin and downregulation of several EMT markers. Having identified the role of miR-548m in reducing breast cancer cell migration and invasion while having no effect on cell proliferation, we searched possible target genes that participated in the following processes. KEGG pathway analysis demonstrated that the putative target genes of miR-548m are enriched in several pathways, including the tight junction and mTOR signaling pathways, which are both involved in regulating migration and invasion in cancer<sup>39,40</sup>. AHR was then further identified as a direct target of miR-548m. Taken together, our study

**Table 3.** List of Selected Putative miR-548m Target Genes Related to Breast Cancer

| Gene Symbol | Description                                            | miTG score* | Cumulative Weighted Score† | miR-SVR Score‡ | Pathobiological Processes Involved                 |
|-------------|--------------------------------------------------------|-------------|----------------------------|----------------|----------------------------------------------------|
| PTEN        | Phosphatase and tensin homolog                         | 0.9986      | -0.29                      | -0.9921        | Breast tumorigenesis and tumor progression         |
| CALCR       | Calcitonin receptor                                    | 0.9487      | -0.15                      | -1.0759        | Migration, metastasis                              |
| RRP1B       | Ribosomal RNA processing 1B                            | 0.9452      | -0.26                      | -1.0334        | Polymorphism, gene for BRCA progression            |
| DNASE1      | Deoxyribonuclease I                                    | 0.9222      | -0.62                      | -1.1741        | Cell death                                         |
| FCGR2A      | Fc fragment of IgG, low affinity IIa, receptor (CD32)  | 0.9219      | -0.20                      | -1.1450        | Polymorphism                                       |
| TFRC        | Transferrin receptor                                   | 0.9181      | -0.63                      | -0.5357        | Iron metabolisms                                   |
| GADD45A     | Growth arrest and DNA damage-inducible, alpha          | 0.9098      | -0.24                      | -1.0404        | Stress response gene                               |
| PPP1CB      | Protein phosphatase 1, catalytic subunit, beta isozyme | 0.8792      | -0.12                      | -1.0589        | Cell morphology                                    |
| LATS2       | Large tumor suppressor kinase 2                        | 0.8650      | -0.19                      | -0.4257        | Cell death and differentiation                     |
| NUF2        | NUF2, NDC80 kinetochore complex component              | 0.8472      | -0.14                      | -1.0979        | Tumor growth and apoptosis                         |
| ATF1        | Activating transcription factor 1                      | 0.8459      | -0.38                      | -0.9174        | Associated with BRCA1                              |
| TGFBR1      | Transforming growth factor, beta receptor 1            | 0.8438      | -0.21                      | -0.1888        | Metastasis and invasiveness                        |
| KIF2A       | Kinesin heavy chain member 2A                          | 0.8019      | -0.19                      | -1.2142        | Proliferation and cell cycle                       |
| TNKS2       | Tankyrase                                              | 0.7955      | -0.15                      | -0.7630        | Cell growth and invasiveness                       |
| TNFRSF10B   | Tumor necrosis factor receptor superfamily, member 10b | 0.7763      | -0.20                      | -0.1971        | Cell death and differentiation                     |
| IGF1R       | Insulin-like growth factor 1 receptor                  | 0.7714      | -0.24                      | -0.1377        | Survival, cell growth and migration                |
| GNB3        | Guanine nucleotide binding protein, beta polypeptide 3 | 0.7681      | -0.26                      | -0.8943        | Polymorphism                                       |
| KLF11       | Kruppel-like factor 11                                 | 0.7411      | -0.26                      | -0.9554        | EMT, invasion and metastasis                       |
| MCM6        | Minichromosome maintenance complex component 6         | 0.7198      | -0.20                      | -0.8081        | Independent predictor of survival                  |
| RRAS2       | Related RAS viral (r-ras) oncogene homolog 2           | 0.7095      | -0.33                      | -0.3065        | Malignant transformation                           |
| AHR         | Aryl hydrocarbon receptor                              | 0.6780      | -0.23                      | -0.8462        | EMT, invasion and invasive potential               |
| STAT1       | Signal transducer and activator of transcription 1     | 0.6461      | -0.14                      | -0.8471        | Pro- or antiapoptotic signaling molecule           |
| NCOA3       | Nuclear receptor coactivator 3                         | 0.6364      | -0.12                      | -1.0889        | Cancer initiation and progression, drug resistance |
| MBL2        | Mannose-binding lectin (protein C) 2                   | 0.6296      | -0.25                      | -0.5422        | Polymorphisms, key genes of the innate immunity    |
| CDK4        | Cyclin-dependent kinase 4                              | 0.6150      | -0.32                      | -0.9499        | Cell proliferation                                 |
| TNFRSF10D   | Tumor necrosis factor receptor superfamily 10d         | 0.6144      | -0.10                      | -0.4242        | Cell apoptosis                                     |
| ZWINT       | ZW10 interacting kinetochore protein                   | 0.6108      | -0.12                      | -0.5552        | Cell growth                                        |
| FCGR2B      | Fc fragment of igg, low affinity IIb, receptor         | 0.6090      | -0.30                      | -0.2650        | Polymorphisms and chemotherapy                     |
| TBP         | TATA box binding protein                               | 0.6038      | -0.28                      | -0.5106        | Cellular transformation                            |
| DIRAS3      | DIRAS family, GTP-binding RAS-like 3                   | 0.6037      | -0.41                      | -1.1800        | Tumor suppressor                                   |
| DYPD        | Dihydropyrimidine dehydrogenase                        | 0.6036      | -0.24                      | -1.3142        | Biomarker of toxicity                              |

\*Prediction score from DIANA-microT v5.0: the higher the score, the higher the probability of targeting with high precision; value more than 0.6. †Score from TargetScan 7.2: cumulative weighted context++ score cutoff level less than -0.1. ‡Score from miRANDA (microRNA.org): predicted targets with less than -0.1 score refers as "good" score.



**Figure 6.** Validation of aryl hydrocarbon receptor (AHR) as a direct target of miR-548m. (A) MDA-MB-231 and MCF-7 cell lines were transfected with miR-548m mimics and its negative control and then subjected to RT-qPCR and Western blot analysis for the detection of AHR mRNA and protein expression, respectively. GAPDH was used as an internal control, respectively, to normalize the results to 1. (B) The predicted binding site of miR-548m and 3'-UTR of AHR as well as the potential binding sites of AHR with the seed region of miR-548m. (C) The luciferase reporter vector containing AHR 3'-UTR was cotransfected with miR-548m mimics or negative control into MDA-MB-231 and MCF-7 cells. The firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. GAPDH was used as an internal control, respectively, to normalize the results. Graphs are displayed as mean  $\pm$  SEM,  $n = 3$ . \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

identified miR-548m as a tumor suppressor miRNA in breast cancer cell lines, and its inhibitory effect on migration and invasion can be mediated through AHR.

During EMT, polarized epithelial cells undergo multiple biochemical and morphological changes, altering their phenotype from epithelial to mesenchymal. EMT processes are marked by the loss of epithelial markers such as E-cadherin and acquisition of mesenchymal markers such as vimentin. Following overexpression of miR-548m, we observed an increased expression of E-cadherin, while no significant changes were seen in vimentin expression in both MDA-MB-231 and MCF-7 cells. Indeed, in partial mesenchymal-epithelial transition (MET), studies showed that upregulation of epithelial markers were seen in metastatic tumor samples, whereas expression of mesenchymal markers such as vimentin was mostly unaltered<sup>41</sup>. However, the increase in E-cadherin expression alone could be insufficient to induce epithelial-like morphological transition in both cell lines. In our studies,

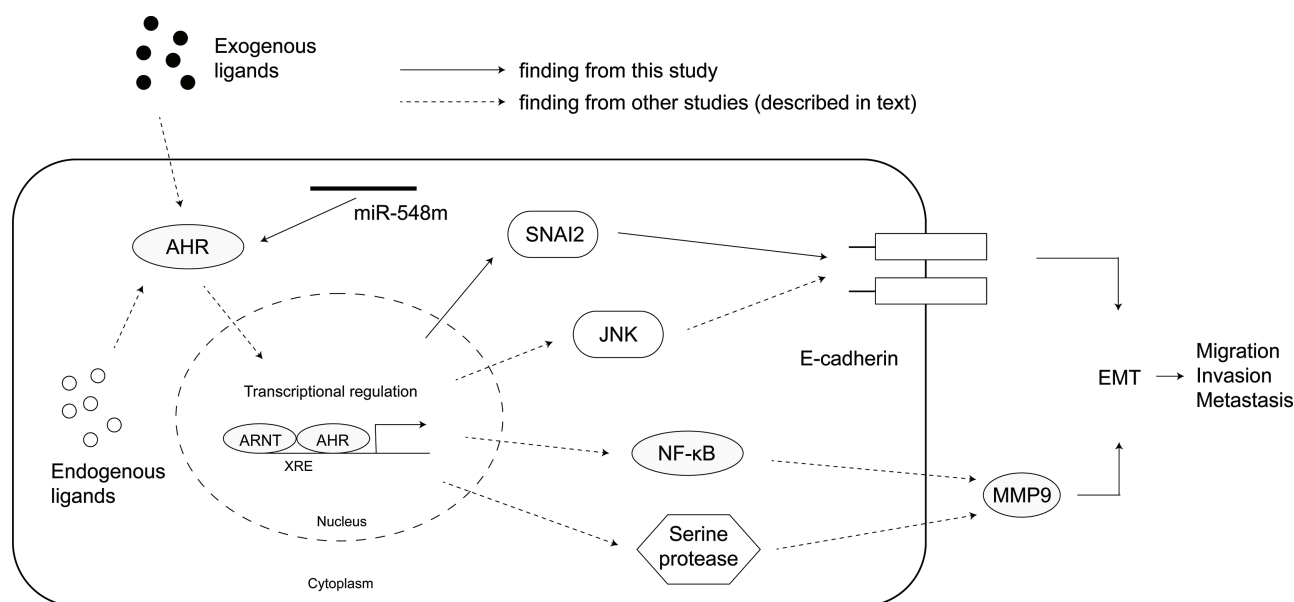
downregulation of ZEB2, but not ZEB1, in both breast cancer cells upon transfection of miR-548m mimics might lead to the increase in E-cadherin expression. This is consistent with earlier reports by Comijn et al., which suggested direct downregulation of E-cadherin by ZEB2 alone<sup>42</sup>. Other genes frequently associated with EMT in breast cancer are SNAIL1 and SNAIL2, from the *Snail* family of transcription factors. Expression of SNAIL1, but not SNAIL2, was downregulated in MDA-MB-231 following overexpression with miR-548m. By contrast, in MCF-7 cells, the expression of SNAIL2 but not SNAIL1 was downregulated. This disparity could be attributed to intrinsic regulatory network modulating SNAIL1 and SNAIL2, despite both being E-cadherin repressors. This is especially the case of the miR-548m/AHR axis, since activation of AHR has been shown to induce the transcriptional activation of its direct target, SNAIL2<sup>43</sup>. In addition, protein expression analysis could be done to detect proteins of EMT markers in breast cancer cells.

EMT is also largely discussed as a promoter of metastasis, enabling motility and invasion of epithelial cells in cancer progression. In this study, miR-548m significantly reduces migration and invasion of breast cancer cells with no effect on cell proliferation. However, there have been a limited number of researches that directly address the biological role of miR-548m specifically. Lwin et al. showed the biological function of miR-548m in lymphoma progression, where miR-548m was found acting as a tumor suppressor by directly targeting HDAC6 and formed a feed-forward loop with c-Myc, which later contributed to stroma-mediated c-Myc activation and miR-548m downregulation in a lymphoma microenvironment<sup>44</sup>. HDAC6 is related to several cell functions, including tubulin stabilization, cell motility, and cell cycle progression<sup>45</sup>. Interestingly, recent studies have shown that miR-548 family members are generally involved in regulating cell proliferation, apoptosis, migration, and invasion, where they act as tumor suppressor miRNA<sup>20,22,46</sup> or oncogenic miRNA<sup>21,47</sup> in different types of cancers. These suggested that miR-548 family members might take on different functions in different types of cancers. The differential effects could have been contributed by the ability of a particular miRNA to target multiple mRNAs, thus consequently different pathways and functions in tumorigenesis<sup>48–51</sup>. Indeed, in a screening for EMT modulators in bladder cancer, miR-548m was identified as an inducer of mesenchymal phenotype<sup>52</sup>. This is in contrast with other conserved miRNA families, such as let-7, that have an exact “seed sequence” that leads to

similar targets, functions, and molecular mechanisms<sup>53</sup>. Taken together, these results indicate that the inhibitory effect of miR-548m on migration and invasion in breast cancer cells might be associated with EMT.

A number of studies reported the involvement of miRNAs to promote or suppress tumor metastasis<sup>54,55</sup>, where some miRNAs are consistently downregulated or eliminated<sup>56,57</sup>, whereas other miRNAs are significantly accumulated in metastatic breast cancer<sup>58</sup>. miRNAs perform biological functions by directly binding to the 3'-UTR of mRNAs and by impairing protein translation<sup>59</sup>. Screening putative target genes via bioinformatic prediction demonstrates that the miR-548m target sequence within the 3'-UTR of several gene mRNAs is closely related to cell migration, cell invasion, and EMT in breast cancer. We established that AHR mRNA and protein expression were significantly downregulated upon the introduction of miR-548m in the MDA-MB-231 and MCF-7 cell lines and validated AHR as a direct target gene of miR-548m.

AHR is a member of the basic helix-loop-helix-ARNT-SIM (bHLH-PAS) subgroup of the bHLH superfamily of transcription factors<sup>60</sup>. AHR is known to be involved in transforming growth factor- $\beta$  (TGF- $\beta$ ), receptor tyrosine kinases, Notch, and Wnt signaling pathways, which associates AHR with cell proliferation, differentiation, and migration<sup>61</sup>. AHR is a ligand-activated transcription factor that could be mainly induced by aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo(a)pyrene (BaP)<sup>62</sup>. Even without the presence of exogenous or environmental



**Figure 7.** Potential role of miR-548m in regulating EMT in breast cancer. The transcriptional upregulation of E-cadherin may either be mediated by downregulation of AHR-dependent activation of SNAI2 and/or JNK. Downregulation of matrix metalloproteinase 9 (MMP9) may be mediated by serine protease and/or NF- $\kappa$ B, which are also targets of the AHR pathway.



ligands such as TCDD and BaP in our study, which is commonly known to increase AHR activities<sup>63</sup>, excessive production of endogenous ligands in breast cancer cells is also known to contribute to the overexpression and constitutive activation of AHR<sup>64,65</sup>. Several studies have proposed the correlation between endogenous ligand-activated AHR and breast cancer progression including growth and cell motility<sup>64,66</sup>. Activation of the AHR pathway in response to TCDD has been shown to modulate the expression and activity of MMP9 in prostate cancer<sup>67</sup> and melanoma cells<sup>68</sup>, while silencing of AHR expression decreased cell invasion and MMP9 expression in urothelial cancer<sup>69</sup>. In breast cancer, AHR expression in human breast cancer tissues was correlated with expression of MMP genes, including MMP1, MMP2, and MMP9, and is involved in signaling pathways related to cell motility<sup>70</sup>. This supported our findings whereby overexpression of miR-548m downregulates AHR and MMP9 in both MDA-MB-231 and MCF-7 cells, and thus suggesting that AHR signaling might regulate EMT through MMP9 in our cell lines. MMPs are predominantly expressed by stromal cells and has the ability to degrade and modify the ECM components and facilitate detachment of epithelial cells from the surrounding tissue<sup>71</sup>. Alteration of serine proteases and NF- $\kappa$ B signaling, directly associated with the AHR pathway, might be able to modify the proteolytic activation of MMP9<sup>72</sup>.

AHR activation has also been shown to potentially trigger the breakdown or downregulation of E-cadherin<sup>73,74</sup>. This transcriptional inhibition might be mediated by AHR-dependent activation of transcriptional factor SNAI2 and/or activation of JNK. In breast cancer cells, activation of AHR induces the transcriptional activation of its direct target, SNAI2<sup>43</sup>. Constitutive expression and activation of AHR have been found to lead the activation of JNK genes, which led to loss of cell–cell adhesion and increased migration in MCF-7 cells<sup>75</sup>. This supports our findings in which SNAI2 was downregulated in MCF-7 cells, while E-cadherin expression was upregulated in both MDA-MB-231 and MCF-7 upon miR-548m overexpression. Although we did not examine whether AHR is a substantial target of miR-548m in regulating the migration and invasion of breast cancer cell lines, previous reports have shown the inhibition of breast cancer cell line migration, invasion, and metastasis following AHR knockdown<sup>64</sup>. Thus, decrease in AHR expression exerted by miR-548m could inhibit EMT by upregulating E-cadherin, leading to reduced cell migration and invasion of breast cancer. It is clearly shown that regulated activities of miRNAs are able to alter or change the target gene expression program to control metastatic processes in breast cancer cells.

Taken together, this study identified miR-548m to be a potential tumor suppressor miRNA in breast cancer by

inhibiting cell invasion and migration. We have shown that AHR is a direct downstream target of miR-548m in breast cancer cells, and other studies have correlated the AHR pathway with migration and invasion<sup>64,70,74,75</sup> (Fig. 7). To the best of our knowledge, published studies in breast cancer<sup>27,28</sup> only detect miR-548m expression through miRNA array analysis without looking at their potential functions and mechanisms. Thus, our current study provides a novel insight into molecular mechanisms of miR-548m in regulating breast cancer progression and new understanding into potential therapeutic strategies and clinical classification markers for metastatic breast cancer.

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