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Hypophosphorylation of retinoic acid receptor alpha inhibits triple-negative breast cancer cell migration and invasion

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Abstract: Retinoic acid receptor α (RARα) is a transcription factor that plays an essential role in tumor progression. Triple-negative breast cancer (TNBC) is a subtype of breast carcinoma with a poor prognosis due to early therapeutic escape from conventional treatments and aggressive metastatic relapse by the occurrence of an epithelial-mesenchymal transition (EMT). However, as the expression level of RARα does not correlate with the overall survival of TNBC patients, we speculate that post-translational modification such as phosphorylation of RARα may be involved in EMT and TNBC metastasis. After overexpressing a phosphorylation-defective mutant of RARα at serine 77 residue (RARαS77A), we found that RARα hypophosphorylation inhibited MDA-MB-231 cell motility and migration *in vitro* while reducing the lung metastatic potential *in vivo.* This was accompanied by increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal markers β-catenin and zinc finger E-box-binding homeobox 1 (ZEB1) in agreement with the suppression of EMT. Interestingly, the overexpression of wild-type RARα in the presence of the RARα agonist AM580 failed to suppress EMT and cell migration. These results indicate that hypophosphorylated RARαS77 can directly mimic activated RARα to inhibit EMT and migration/invasion of cells, thus providing a novel target in the therapeutic intervention of TNBC.

Keywords: retinoic acid receptor α (RARα); triple-negative breast cancer (TNBC); epithelial-mesenchymal transition (EMT); RARα agonist AM580; phosphorylation

INTRODUCTION

Triple-negative breast carcinoma (TNBC) is an aggressive subgroup of breast cancer characterized by the lack of estrogen receptor (ER) and progesterone receptor (PR) expression, and human epidermal growth factor receptor 2 (HER2) amplification, and accounts for about 15% of breast carcinomas [1]. Due to the lack of targeted therapy, TNBC has a high incidence of distant disease recurrence within three years of diagnosis and a high frequency of visceral metastases, which remains a clinical challenge with limited therapeutic options [2].

Metastasis of TNBC is correlated with aberrant activation of epithelial-mesenchymal transition (EMT), a process by which epithelial cells acquire a mesenchymal phenotype by gaining migratory and invasive properties and modifying cell adhesion molecules. EMT allows cancer cells to break free from their primary tumor site into circulation and eventually settle in distant organs to form metastasis [3]. The molecular aspect during EMT is loss of epithelial cell markers E-cadherin, claudin and cytokeratin expression while mesenchymal cell markers N-cadherin and vimentin are overexpressed. Several transcription factors such as zinc finger protein SNAI1 (SNAIL), SNAI2 (SLUG) and ZEB1/2 play a critical role during EMT by modulating the expression of E-cadherin [4]. Therefore, finding ways to regulate genes involved in EMT may inhibit the acquisition of a mesenchymal phenotype and help in the design of new therapies against metastatic TNBC.

Retinoic acid receptors (RARs) are members of the nuclear receptor superfamily of transcription factors. Among the three different RAR genes (RARα, RARβ and RARγ) that have been characterized, RARα interacts with its target genes to participate in tumor growth, metastasis, drug resistance and other processes [5]. According to the canonical model of gene regulation by RARs, in the absence of ligand, RARα form heterodimers with retinoid X receptor (RXR), which

can bind constitutively specific response elements (RAREs) located in the promoters of target genes. This DNA-bound RARα is associated with corepressors nuclear receptor corepressor 1 (NCoR1) and NCoR2, which serve as adaptors recruiting other subcomplexes endowed with histone deacetylase (HDAC) activity, thus leading to transcriptional repression [6,7]. Upon ligand binding, RARα undergoes conformational changes, which disassociates from the corepressors and recruits coactivators such as nuclear receptor coactivator (NCoA)1/2/3 and histone acetyltransferase (HAT), thus paving the way for the recruitment of the transcription machinery and activation of target genes [8]. It has been reported that RARα directly activates matrix metalloproteinase (MMP)2 expression, a critical regulatory gene that aids tumor invasion by destroying the basement membrane and extracellular matrix to increase the migrative and invasive potential of colorectal cancer cells [9]. RARα also transcriptionally activates follistatin-like 3 (FSTL3) to hasten the migration and invasion of thyroid cancer cells [10]. In addition, short-period treatment of RARα- and RARβ-selective agonists activates membrane-organizing extension spike protein (MOESIN), focal adhesion kinase (FAK) and paxillin, an intracellular adaptor protein, to reduce cellular adhesion of T-47D human breast cancer cells in a non-genomic fashion [11]. However, a possible antimetastatic property of RARα was revealed, as all-*trans*-retinoic acid (ATRA) activated a RARα-dependent epithelial differentiation program via downregulation of neurogenic locus notch homolog protein 1 (NOTCH1) and stimulation of transforming growth factor beta (TGFβ) to inhibit the motility of HER2-positive breast cancer cells [12]. Thus, it seems that the role of RARα in tumor migration is time- and tissue-dependent, which inspired us to investigate the function of RARα in the migration of TNBC cells.

As a phosphoprotein, RARα is phosphorylated at S369 located in the ligand-binding domain by mitogen- and stress-activated kinase 1 (MSK1), thus allowing the binding of transcription factor II Human (TFIIH) and thereby phosphorylation of the N-terminal domain at S77 by cyclin-dependent kinase 7 (CDK7)/cyclin H. This controls the recruitment of RARα/TFIIH complexes to the response elements of specific gene subsets and subsequently targets gene activation [13]. Further studies show that RA-suppressed CDK7/cyclin H phosphorylation of RARα at S77 is associated with cell cycle arrest and transcription of RA-target genes [14-16]. Therefore, we aimed to explore the roles and potential mechanisms of RARα phosphorylation in TNBC migration/invasion.

In this study, we demonstrated that hypophosphorylated RARα inhibits the process of EMT and cell migration in TNBC cells via increasing E-cadherin while decreasing β-catenin, ZEB1 and MMP2 expression, and point to RARα as a target in the therapeutic intervention of TNBC.

MATERIALS AND METHODS

Ethics statement

All procedures, care and handling of animals were approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University and conducted in compliance with the standard guidelines.

Chemicals and reagents

The RARα agonist AM580 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Antibodies against RARα (sc-515796) and ZEB1(sc-25388) were from Santa Cruz Biotechnology (CA, USA). The antibody against RARα (p-Ser77) was from Sigma-Aldrich (St. Louis, MO, USA). Antibody against E-cadherin (ab40772) was purchased from Abcam (Cambridge, UK). Antibodies against β-Catenin (#9562), matrix metallopeptidase 2 (MMP2; #13132), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5174) and β-tubulin (#2128) were purchased from Cell Signaling Technologies (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad (Hercules, CA, USA).

Cell line and cell culture

The human breast cancer cell line MDA-MB-231 was purchased from the cell bank of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

(DMEM/F12; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

Plasmid transfection and lentiviral production

pLVX-ACGFP-N1-RARα plasmids were purchased from Shanghai Nuoyue Biotechnology Co., Ltd. China. The pLVX-ACGFP-N1-RARα-S77A were constructed using the QuickMutation™ Plus gene site-directed mutation kit (Beyotime, Shanghai, China). Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Virion production and lentiviral transduction were performed as described before [17]. The lentivirus packaged with the empty pLVX-ACGFP-N1 vector served as the vector control.

MTT analysis

MDA-MB-231 cells overexpressing either RARαS77A, RARα or empty vector were seeded on 96-well plates at 3000 cells/well overnight and treated with or without 1 μM of AM580 for the indicated times. Twenty μL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) were added to each well at the end of the treatment and incubated for another 4 h. Formed formazan crystals were dissolved in 100 μL dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm on a microplate reader (Bio Tek, CA, USA).

Wound healing assays

MDA-MB-231 cells were cultured in 24-well plates. When the cells reached nearly 100% confluence, cells were scratched with a pipette tip (200 μL) and then washed twice using phosphate buffered saline (PBS) to remove the floating cells. The cell culture medium was replaced with serum-free DMEM. Cell migration was monitored for 12 h and the distance traversed by the cells was quantified.

Cell migration assays

Cells (1.5×10^5) in 200 μL of serum-free DMEM/F12 medium were seeded on a polycarbonate membrane inserted in a 24-well transwell device (Costar, Cambridge, MA, USA) in the presence or absence of AM580. Cells were allowed to migrate toward the same growth medium supplemented with fetal bovine serum (FBS) in the bottom reservoir. The transwells were fixed with 4% paraformaldehyde and stained with hematoxylin. Migrated and invaded cells were photographed and counted under an inverted microscope.

Western blotting analysis

Cells were harvested and lysed on ice in radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase inhibitors (NaF and Na_3VO_4), protease inhibitors phenylmethylsulfonyl fluoride (PMSF), and aprotinin. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). An equal quantity of proteins was then separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were then blocked with 5% nonfat milk at room temperature for 1 h and incubated with primary antibodies overnight at 4°C. Next, the membranes were washed three times with TBS-T (Tris-buffered saline-5% Tween 20) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Chemiluminescent detection was performed by electrochemiluminescence (ECL; BIO-RAD, USA).

Experimental lung metastasis model

MDA-MB-231-luc or MDA-MB-231-RARαS77A-luc cells (5×10^5 cells/mouse) were injected into female nude mice (BALB/c nu/nu; 5 weeks old) through the tail vein to establish a lung metastasis model. After 10 days, the lungs were removed to measure luminescence using an *in vivo* imaging system (IVIS) (Caliper Life Sciences, Hopkinton, MA, USA).

Statistical analysis

All data are expressed as the mean±SD. Statistical significance was analyzed using Student's t-test. $P<0.05$ was considered statistically significant.

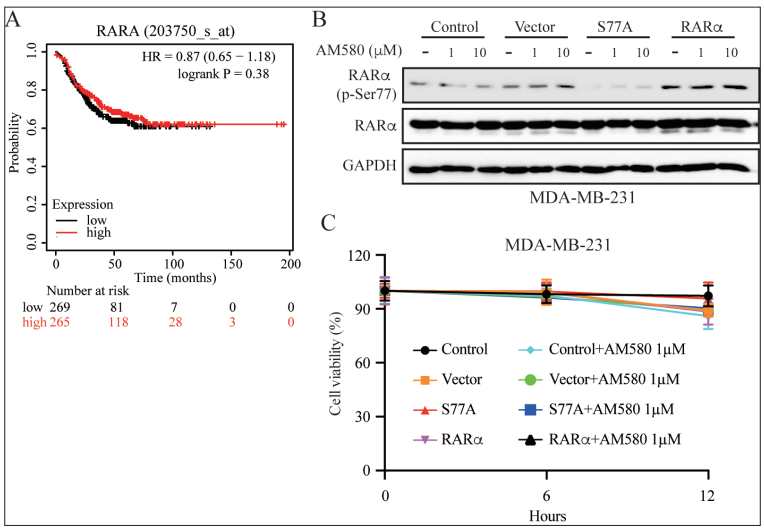

Fig. 1. Hypophosphorylation of RARα does not suppress MDA-MB-231 cell survival. **A** — Kaplan-Meier representations of the probabilities of recurrence-free survival according to the expression levels of RARα in TNBC patients. A log-rank test was used to evaluate significance. **B** — Western blotting analysis of phosphorylated and total RARα in transfected cells. **C** — After overexpressing RARαS77A, RAR or empty vector in MDA-MB-231 cells, MTT analysis was used to determine cell viability.

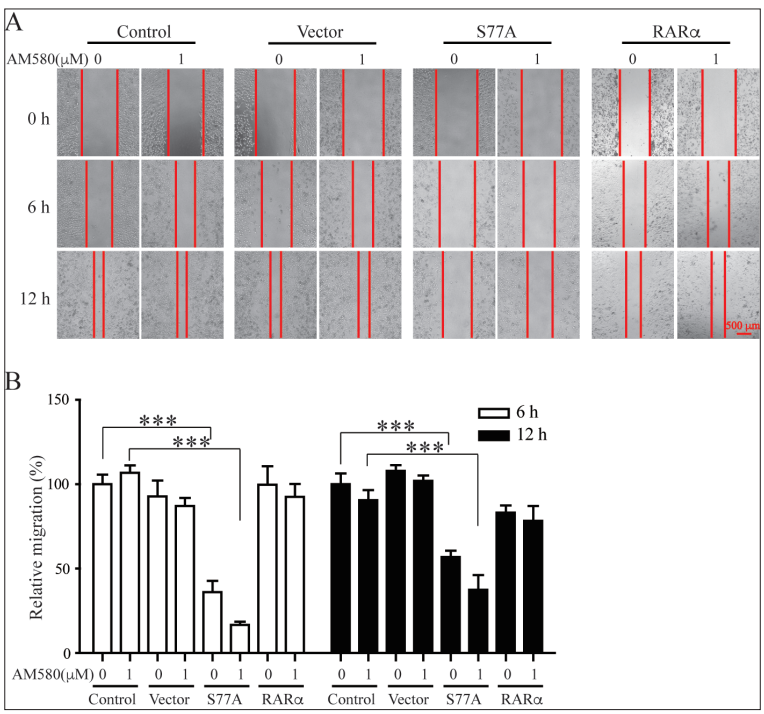

Fig. 2. RARαS77A inhibits the motility of MDA-MB-231 cells. **A** — After overexpressing RARαS77A in MDA-MB-231, the wound-healing assay was performed in the presence or absence of AM580 for up to 12 h. Scale bar, 500 μm. **B** — The distance traversed by cells in panel A was quantified. ***P<0.001.

RESULTS

Hypophosphorylation of RARα does not suppress MDA-MB-231 cell survival

As RARα is associated with progression of different tumors [5], we first analyzed whether RARα expression correlates with TNBC patient survival. Using the online tool Kaplan Meier Plotter, we found no correlation between the expression level of RARα and overall patient survival (Fig. 1A). Since a study has shown that RARα is hyperphosphorylated in TNBC patient specimens [17], we next investigated whether forced hypophosphorylation of RARα will inhibit TNBC progression. We therefore used a phosphorylation-defective mutant of RARα (RARαS77A) to mimic the hypophosphorylated RARα. The lentiviral transfection efficiency of the mutant RARαS77A, wild-type RARα and empty vector in MDA-MB-231 cells was analyzed by Western blotting. The results showed that the lentiviral transfection of RARαS77A in MDA-MB-231 cells resulted in decreased phosphorylation of RARα, while wild-type RARα and empty vector had no effect on the phosphorylation level of RARα (Fig. 1B). Next, we investigated the effect of this hypophosphorylated RARα on cell proliferation. MTT analysis was performed in MDA-MB-231 cells overexpressing RARαS77A or RARα and treated with or without AM580, the specific RARα agonist, for up to 12 h. Results showed that neither RARαS77A nor RARα suppressed MDA-MB-231 cell survival (Fig. 1C).

Hypophosphorylated RARα reduces TNBC cell motility and migration

As the aggressive nature of TNBC is reflected by an increased likelihood of distant recurrence and death within 5 years following primary intervention and a shorter survival once diagnosed with metastatic disease [18], we sought to investigate the impact of RARαS77A on TNBC cell migration. Wound

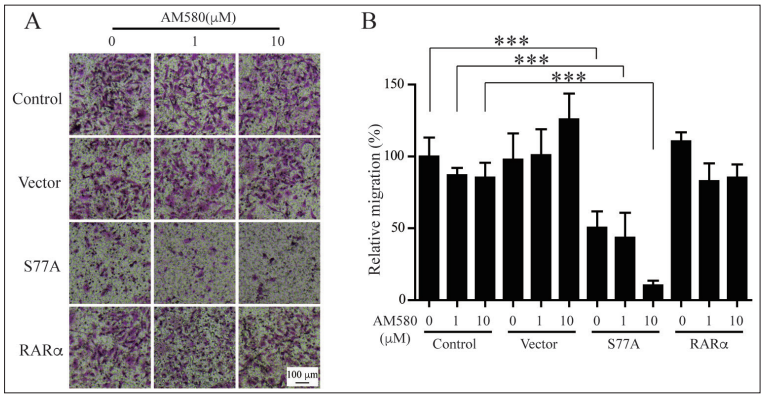

Fig. 3. RARαS77A inhibits the migration of MDA-MB-231 cells. **A** — Transwell assay performed in RARαS77A-transfected MDA-MB-231 cells with or without AM580 (1, 10 μM). Scale bar, 100 μm. **B** — Quantification of cells migrating to the lower chamber. ***P<0.001.

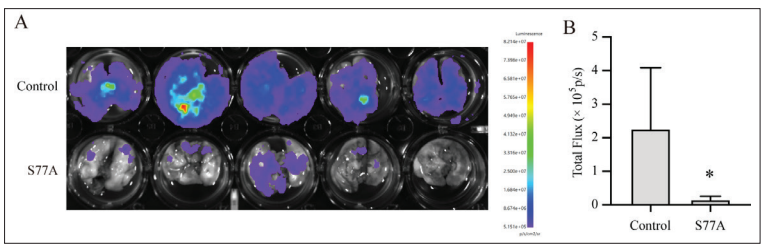

Fig. 4. RARαS77A inhibits TNBC lung metastasis. Mice were given an intravenous injection of 5×105 MDA-MB-231-luc (control) or MDA-MB-231-RARαS77A-luc (S77A) cells. Ten days after tumor inoculation, the mice were killed and lung metastasis were measured by the IVIS system. **A** — Bioluminescence imaging of the lungs. **B** — Bioluminescence intensities in each group. *P<0.05.

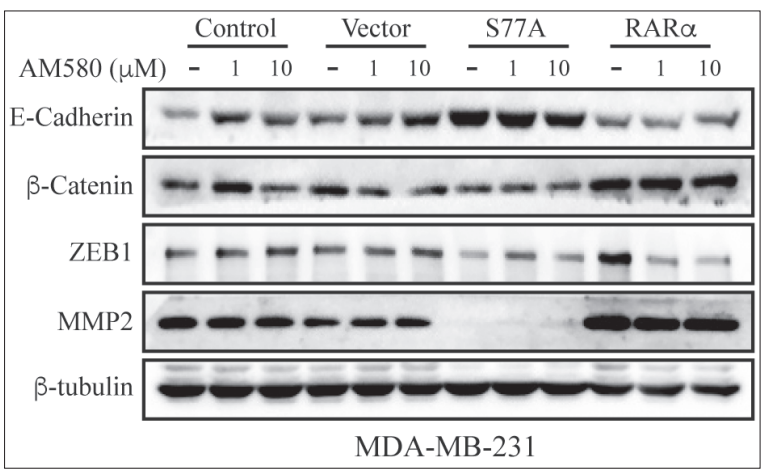

Fig. 5. RARαS77A suppresses EMT to inhibit TNBC cell migration. Western blotting analysis of proteins related to migration and metastasis.

healing analysis of MDA-MB-231 cells overexpressing either RARαS77A or RARα was performed, and the distance traversed by cells was quantified. Results showed that compared with vector or RARα, RARαS77A dramatically reduced cell motility, regardless of the addition of AM580 (Fig. 2). To further confirm the antimigratory action of RARαS77A, transwell analysis was performed. Results showed that RARαS77A strongly suppressed the motility of MDA-MB-231 cells (Fig. 3). The data suggest that hypophosphorylated RARα activated an antimigratory response in TNBC cells *in vitro*.

RARαS77A inhibits TNBC lung metastasis

To further evaluate whether RARαS77A possesses antimetastatic effects *in vivo*, a lung metastasis model was used. Mice were intravenously injected with MDA-MB-231-luc (control) or MDA-MB-231-RARαS77A-luc (S77A) cells to establish lung metastasis. After 10 days, an obvious decrease in cancer metastasis was observed in the lungs of the RARαS77A group (Fig. 4). This result demonstrated an antimetastatic potential of hypophosphorylated RARα *in vivo*.

RARαS77A suppresses EMT to inhibit TNBC cell migration

Since the aberrant regulation of EMT has been considered a fundamental event in cancer cell motility and metastasis [3,19], we further investigated the effects of RARαS77A on specific molecules associated with EMT by Western blotting. As shown in Fig. 5, RARαS77A increased the expression of the epithelial cell marker E-cadherin while decreasing the expression of β-catenin, ZEB1 and matrix metalloproteinase 2 (MMP2). However, overexpression of wild-type RARα did not affect the above molecules, irrespective

of the addition of AM580, which correlated with our previous results (Figs. 2-3). Together, our data demonstrated that RARαS77A attenuates the migration/invasion ability of MDA-MB-231 cells by inhibiting EMT.

DISCUSSION

Accumulating evidence indicates that aberrant RARα expression is a common phenomenon and a potential marker of prognosis in a multitude of cancers, including breast cancer [20,21]. It also plays a key role in mediating the antimetastatic activity of all-trans retinoic acid (ATRA) in breast cancer cells [22]. However, its exact role is yet to be ascertained in TNBC progression. This work confirmed that the phosphorylation status of RARα is related to TNBC cell motility.

Indeed, the post-translational modification such as phosphorylation of RARα is essential for the integrated regulation of its activity. Previous studies have shown that hyperphosphorylation of RARα by cyclin-dependent kinase-activating kinase (CAK) is associated with increased proliferation of acute myeloid leukemia (AML) and other types of cancer cells, whereas RA induced RARα hypophosphorylation or mimicked RARα hypophosphorylation by expressing RARαS77A mutant, which inhibits cancer progression [14,23,24]. Of note, when expressing RARα and RARαS77A in parallel in the RA-resistant AML cell line harboring a defective RARα-ligand-binding domain (LBD) or in embryonic teratocarcinoma RARα-/- stem cells, RARαS77A, but not RARα, induced cell differentiation without a need for RA stimulation [14]. These *in vitro* findings suggest that hypophosphorylated RARαS77 can function in a ligand-independent manner to suppress tumorigenesis. However, its effect and mechanism in TNBC migration and invasion remain to be determined.

Here, using RARαS77A that cannot be phosphorylated by CAK on S77, we demonstrated that the decrease in phosphorylation of RARαS77 has a minimal effect on MDA-MB-231 cell proliferation within 12 h while inhibiting cell migration and invasion, both *in vitro* and *in vivo*. Western blotting analysis showed that RARαS77A upregulated the epithelial marker E-cadherin while reducing the expression of ZEB1 (a transcriptional repressor that allows cancer cells to invade and spread) and the cytoskeletal protein β-catenin, both of which are reliable prognostic markers of solid tumor aggressiveness [25]. In addition, RARαS77A downregulated MMP2, a collagenase that can degrade the extracellular matrix to promote the invasion and metastasis of tumor cells [26,27]. Thus, the suppression of EMT is likely to be a major contributor to the antimetastatic action of RARαS77A. Interestingly, wild-type RARα in the presence of the agonist AM580 failed to suppress cell motility, which suggests that the agonist alone cannot activate RARα, while RARαS77A can mimic an activated RARα in TNBC cells. The inability of AM580 to activate wild-type RARα may be due to a higher expression of the fatty acid-binding protein 5 (FABP5) in TNBC, which competes with the cellular retinoic acid binding protein 2 (CRABP2) and shuttles ligands to the peroxisome proliferator activated receptor beta (PPARβ) instead of RARα to promote cell proliferation [28]. Of note, previously we reported that RARαS77A reduced cell viability at later time points (24 h and above) [17]. Therefore, to minimize the impact of the loss of viable cells on the observation of cell migration/invasion, we limited the experimental duration to within 12 h. Overall, it seems that hypophosphorylated RARαS77 inhibits TNBC progression through multiple mechanisms.

Nevertheless, as RARα is a transcription factor regulating EMT-inducing factors such as SLUG, forkhead box protein C2 (FOXC2), ZEB1 and ZEB2, and factors activating TGF-β-SMAD signaling including transforming growth factor beta receptor I (TGFBR1), TGFBR2, transforming growth factor-beta 2 (TGF-β2) and mothers against decapentaplegic homolog 3 also known as SMAD family member 3 or SMAD3 [9,27,29], further studies such as chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (qPCR) are warranted to dissect how hypophosphorylated RARαS77 activated downstream gene transcription to suppress EMT. This would, in turn, provide a new molecular platform for developing therapeutic strategies that directly mimic activated RARα to inhibit the metastasis of TNBC cells.

In summary, hypophosphorylated RARαS77 mimics activated RARα to inhibit TNBC migration and invasion by suppressing EMT. Our findings provide novel insight into the development of small

molecules, which can mimic the structure-conformation of hypophosphorylated RARαS77 and suppress TNBC metastasis.

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Conflicts of interest disclosure: The authors declare that they have no conflicts of interest.

Data availability: Data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Ying%20et%20al_7818_Data%20Report.pdf

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