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Effect of simvastatin on gene expression varies across breast cancer cell subtypes

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Research Article

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Posted Date: November 11th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11471925/v1

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Additional Declarations: No competing interests reported.

Title: Effect of simvastatin on gene expression varies across breast cancer cell subtypes

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Background: Simvastatin has attracted interest for its potential anticancer effects, yet its molecular impact may vary among breast cancer models. We investigated its effects on genes involved in cell-cycle control, tumour progression, and immune regulation in three breast cancer cell lines.

Methods and Results: MCF-7, SK-BR-3, and MDA-MB-231 cells were exposed to simvastatin for 24 hours. Cellular metabolic activity was assessed using XTT. Based on concentration–response profiles, gene expression was examined after treatment with 50 μM simvastatin in MDA-MB-231 cells and 75 μM in MCF-7 and SK-BR-3 cells. Seven target genes were analysed by quantitative real-time PCR. TCGA-BRCA and TIMER3 data provided additional context.

Simvastatin reduced metabolic activity at higher concentrations, with differences among cell lines. *CDKN1A* expression increased significantly in all three models. *CXCR4* and *PD-L1* expression decreased significantly in MDA-MB-231 cells, whereas *PTEN* expression increased in SK-BR-3 cells while *CD44, MAPK14,* and *NOTCH1* showed no significant changes. Public datasets revealed variation in selected gene expression between breast tumours and normal tissue and across molecular subtypes.

Conclusion: *CDKN1A* upregulation was shared across the three models, while other transcriptional responses differed by cell line. These findings suggest that cellular background influences the molecular response to simvastatin and warrant validation in additional models and at the protein and functional levels.

Keywords: Breast cancer, simvastatin, gene expression, cell culture

Introduction

Breast cancer is a highly heterogeneous disease, and differences in receptor status, signalling networks and mutation profiles can strongly influence tumour behaviour and treatment response [1, 2]. This variability is also reflected in experimental models, where breast cancer cell lines with distinct molecular backgrounds may respond differently to drugs. Therefore, findings obtained in one cell line should not be assumed to represent the response of other breast cancer models [3].

Statins are widely used cholesterol-lowering drugs that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway [4, 5]. Inhibition of this pathway reduces the synthesis of both cholesterol and non-sterol isoprenoid intermediates required for the prenylation of small GTPases, including Ras and Rho proteins, thereby affecting signalling pathways involved in cell proliferation and survival [5, 6]. These pleiotropic effects have generated increasing interest in the potential use of statins as repurposed anticancer agents. In breast cancer models, simvastatin has been shown to reduce proliferation and viability, promote apoptosis and cell-cycle arrest, and interfere with oncogenic signalling pathways [7], although the magnitude of these effects appears to depend on the molecular context of the tumour model [6, 8]

However, sensitivity to statins differs markedly across breast cancer models. Recent studies have shown that luminal breast cancer cells are generally less responsive to statin treatment than triple-negative models, although substantial variability also exists within individual subtypes [6, 9]. This differential sensitivity has been linked, at least in part, to the ability of tumour cells to restore mevalonate pathway activity following HMGCR inhibition. In particular, rapid activation of SREBP2 and subsequent upregulation of HMGCR have been associated with resistance to statin treatment in ER-positive breast cancer cells, whereas this compensatory response is weaker in statin-sensitive triple-negative cells [10]. Transcriptomic analyses further indicate that statin exposure induces broader changes in cholesterol homeostasis and growth-related signalling networks, with responses differing according to cellular background [10]. Despite these observations, direct analysis of the same molecular targets across biologically distinct breast cancer models remains limited. It therefore remains unclear which transcriptional responses to simvastatin are conserved across breast cancer cells and which are determined by the molecular context of individual models.

In the present study, we examined the transcriptional response to simvastatin in MCF-7, SK-BR-3, and MDA-MB-231 breast cancer cells using the same set of target genes. We focused on *CDKN1A, CXCR4, PD-L1 , PTEN, CD44, MAPK14,* and *NOTCH1* because these genes are linked to key processes in breast cancer, including cell-cycle control, tumour-suppressor signalling, metastatic behaviour, stress responses, stemness, and immune regulation [11-18]. By comparing the same targets across three biologically distinct breast cancer models, we aimed to identify which transcriptional responses were shared and which were more dependent on the molecular background of each cell line.

2. Materials and methods

2.1. Cell culture

MCF-7 (ATCC HTB-22), SK-BR-3 (ATCC HTB-30), and MDA-MB-231 (ATCC HTB-26) human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM/F12 containing 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a humidified incubator with 5% CO_2. The medium was renewed every 48 h, and cells were subcultured at approximately 70–80% confluence using 0.25% trypsin–EDTA.

2.2. XTT cell viability assay

Cell viability after simvastatin treatment was assessed using the XTT Cell Viability Kit (Cell Signaling Technology, Danvers, MA, USA; Cat. No. 9095). Cells were seeded in 96-well plates at 1×10^4 cells per well in 100 µL of complete DMEM/F12 medium and allowed to attach for 24 h.

Simvastatin (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-200829A) was dissolved in 100% ethanol to prepare a 24 mM stock solution and then diluted in complete DMEM/F12 medium. Cells were treated with 2.5, 5, 10, 25, 50, or 100 µM simvastatin for 24 h. The final ethanol concentration at the highest simvastatin dose was approximately 0.42% (v/v), and a

vehicle control containing the same ethanol concentration without simvastatin was included. Each condition was tested in technical triplicate.

After 24 h of treatment, the XTT assay was carried out according to the manufacturer's instructions, and absorbance was measured at 450 nm. Cell viability was expressed relative to the corresponding control. Concentration–response curves were generated separately for each cell line using GraphPad Prism version 11.0 (GraphPad Software, Boston, MA, USA).

The XTT results were then used to guide the concentrations selected for the gene-expression experiments. A concentration of 50 μM simvastatin was used for MDA-MB-231 cells. For MCF-7 and SK-BR-3 cells, 75 μM was chosen as an intermediate concentration between 50 and 100 μM, where a clearer reduction in metabolic activity was observed.

2.3. Simvastatin treatment

For gene-expression analysis, MDA-MB-231 cells were treated with 50 μM simvastatin for 24 h, whereas MCF-7 and SK-BR-3 cells received 75 μM simvastatin for 24 h. The corresponding ethanol concentrations were 0.208% and 0.313% (v/v), respectively. Vehicle controls were exposed to matched ethanol concentrations without simvastatin. Three independent biological replicates were performed for each cell line.

2.4. RNA isolation and cDNA synthesis

Following treatment, total RNA was extracted using the PureLink RNA Microscale Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop spectrophotometer, and samples with A260/A280 ratios of 1.9–2.1 were used for downstream analysis.

For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with random hexamer primers. The resulting cDNA was subsequently used for quantitative real-time PCR.

2.5. Quantitative real-time PCR

Gene expression was quantified by real-time PCR using LightCycler 480 SYBR Green I Master Mix (Roche Applied Science, Mannheim, Germany) on a LightCycler 96 Real-Time PCR System. Primer sequences are provided in Supplementary Table S1.

PPIA, 18S rRNA, and *GAPDH* were evaluated as candidate reference genes using RefFinder. PPIA showed the highest expression stability and was selected for normalization.

PCR amplification consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Melting-curve analysis was performed to verify amplification specificity. Relative transcript levels were calculated using the $2^{-\Delta Ct}$ method.

2.6. Public database analyses

Publicly available breast cancer datasets were used to examine the expression patterns of selected genes in patient tumours. TCGA-BRCA data were analysed to compare gene

expression between primary breast tumours and normal breast tissue and across molecular subtypes [19]. TIMER3 was used to assess the association between *PD-L1* expression and $CD8^+$ T-cell infiltration in basal-like breast cancer [20]. These analyses were included to provide a broader clinical context for the genes examined in this study and were independent of the simvastatin treatment experiments.

2.7. Statistical analysis

Three independent biological replicates were included in the gene-expression analyses. Technical replicates were averaged within each experiment before statistical analysis. Data are shown as mean ± SEM. For each cell line, simvastatin-treated cells were compared with the corresponding vehicle controls using a two-sided Welch's *t*-test. Statistical significance was indicated as follows: $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$. Exact *p* values were shown for non-significant results approaching statistical significance. Statistical analyses were carried out using GraphPad Prism version 11.0 (GraphPad Software, Boston, MA, USA).

3.Results

3.1. Effect of simvastatin on cellular metabolic activity

Simvastatin produced a concentration-dependent reduction in cellular metabolic activity after 24 h of treatment (Figure 1). MCF-7 and SK-BR-3 cells showed minimal changes at concentrations up to 25 µM, followed by a more pronounced reduction at higher concentrations. In MDA-MB-231 cells, the decrease became evident at 50 µM, although variability among technical replicates was greater at this concentration. At 100 µM, metabolic activity was markedly reduced in all three cell lines.

The vehicle control did not show a comparable reduction in metabolic activity. Based on these concentration–response profiles, 50 µM simvastatin was selected for MDA-MB-231 cells, whereas 75 µM was used for MCF-7 and SK-BR-3 cells in the subsequent gene-expression experiments.

3.2. *CDKN1A* expression

Simvastatin significantly increased *CDKN1A* mRNA expression in all three breast cancer cell lines (Figure 2A–C). Expression increased in MDA-MB-231 ($p = 0.0320$), MCF-7 ($p = 0.00996$), and SK-BR-3 cells ($p = 0.000313$).

In the TCGA-BRCA dataset, *CDKN1A* expression was lower in primary breast tumours than in normal breast tissue (Figure 2D) and differed across molecular subtypes (Figure 2E).

3.3. *CXCR4* expression

Simvastatin significantly reduced *CXCR4* mRNA expression in MDA-MB-231 cells ($p = 0.0175$), while no significant changes were detected in MCF-7 or SK-BR-3 cells (Figure 3A–C).

Analysis of the TCGA-BRCA dataset showed higher *CXCR4* expression in primary breast tumours than in normal breast tissue (Figure 3D). *CXCR4* expression also differed across

molecular subtypes, with the highest levels observed in triple-negative breast cancer (Figure 3E).

3.4. *PD-L1* expression

Simvastatin significantly reduced *PD-L1* mRNA expression in MDA-MB-231 cells (p = 0.0128), while no significant changes were detected in MCF-7 or SK-BR-3 cells (Figure 4A–C).

In the TCGA-BRCA dataset, *PD-L1* expression differed between primary breast tumours and normal breast tissue (Figure 4D) and varied across molecular subtypes, with the highest expression observed in triple-negative breast cancer (Figure 4E). TIMER3 analysis further showed a positive association between *PD-L1* expression and $CD8^+$ T-cell infiltration in basal-like breast cancer.

3.5. *PTEN* expression

Simvastatin did not significantly alter *PTEN* mRNA expression in MDA-MB-231 or MCF-7 cells, whereas *PTEN* expression increased significantly in SK-BR-3 cells ($p < 0.05$) (Figure 5A–C).

In the TCGA-BRCA dataset, *PTEN* expression was lower in primary breast tumours than in normal breast tissue (Figure 5D) and differed across molecular subtypes (Figure 5E).

3.6. *CD44, MAPK14* and *NOTCH1* expression

Simvastatin treatment did not significantly alter *CD44*, *MAPK14*, or *NOTCH1* mRNA expression in any of the three breast cancer cell lines. These findings are presented in Supplementary Figure S1.

4. Discussion

In this study, we compared the transcriptional response to lipophilic statin simvastatin in three breast cancer cell lines with distinct receptor and gene mutation profiles. Simvastatin response differed considerably among these models. Gene expressions of *CDKN1A* increased significantly in all three cell lines, whereas *CXCR4* and *PD-L1* decreased only in MDA-MB-231 cells, and PTEN increased in SK-BR-3 cells. However, *CD44, MAPK14,* and *NOTCH1* remained unchanged in all cell lines under the conditions tested. Together, these results point to a combination of shared and cell line-specific transcriptional effects of simvastatin in breast cancer cells.

CDKN1A encodes p21, a cyclin-dependent kinase inhibitor with a central role in cell-cycle control [21]. Statins have been shown to alter cell-cycle regulation and suppress proliferation in breast cancer models [6, 10], and inhibit growth and induce apoptosis in both MCF-7 and MDA-MB-231 cells with JNK signalling contributing to this response [7]. It has also been reported that statins exert antiproliferative effect by suppressing YAP/TAZ expressions via JNK signal activation thereby downregulating *PD-L1* expression in pancreatic cancer [22]. The extent of statin sensitivity varies substantially across breast cancer models. MDA-MB-231 cells

are generally more responsive than luminal ER-positive models such as MCF-7 [23], however, statins are currently indicated particularly for the treatment of ER-positive breast cancer [24].

CDKN1A upregulation by statins blocks CDK4/6 actıvation leading to inhibition of proliferation and inducing apoptosis. In addition, statins block CDK4/6 actıvation via inhibition of YAP/TAZ –SREBP2 axis. CDK4/6 inhibitors ,e.g. palbociclib , are currently used in combination regimens in ER+ and HER2+ advanced metastatic and PIK3A-mutated breast cancer cells. Our study indicates that the efficacy of statins in these cells may be in part, due to the CDKN1A upregulation. CDK4/6 inhibitors are not indicated for the treatment of patients with TNBCs, although these cells display high sensitivity to statins in invitro assays [6, 23, 25] The downregulation of *CXCR4* and *PD-L1* gene expressions together with *CDKN1A* upregulation by simvastatin in TNBC cell line MDA-MB-231 in our study may explain the high responsiveness of these cells to statins. suggesting that lipophilic statins may be used as an adjunct therapy in triple negative breast cancer patients, while our findings should be validated in clinical trials. *PD-L1* overexpression is most common in TNBCs, and PD-L1 inhibitor drugs, eg, pembroluzimab, are commonly used for TNBCs in combination therapies.

Simvastatin has previously been reported to increase *PTEN* expression and suppress downstream AKT signalling in breast cancer models [26]. *PTEN* status may also influence statin responsiveness, as reduced sensitivity to simvastatin has been observed in PTEN-deficient TNBC cells [27]. In our study, however, the increase in PTEN expression was restricted to SK-BR-3 cells, which remains to be explored.

Simvastatin has also been reported to reduce migration and invasion in MDA-MB-231 cells through mevalonate-dependent mechanisms [28, 29]. *CXCR4* is a key regulator of breast cancer cell migration, invasion, and metastatic dissemination [30, 31]. In our TCGA-BRCA analysis, *CXCR4* expression was increased in primary breast tumours relative to normal breast tissue, with the highest expression observed in the triple-negative subgroup. This pattern is consistent with the established association between CXCR4 signalling and aggressive breast cancer phenotypes.

No significant changes were detected in *CD44*, *MAPK14*, or *NOTCH1* mRNA expression. This suggests that the transcriptional response to simvastatin was selective rather than broadly distributed across all genes examined. Such a pattern is plausible given the pleiotropic effects of statins, which can involve kinase signalling, membrane-associated processes, and protein regulation without necessarily being accompanied by parallel changes in transcript abundance [8, 32].

In our experimental models, *CDKN1A* was the only gene that responded consistently across all three cell lines, while *CXCR4, PD-L1,* and *PTEN* changed only in individual models. These patterns should not be interpreted as subtype-specific biomarkers, since a single cell line cannot capture the biological heterogeneity of luminal, HER2-positive, or triple-negative breast cancer. They instead identify responses that warrant further investigation in larger panels of models within each molecular subtype.

Several limitations should be considered when interpreting these findings. Only three breast cancer cell lines were included, so the observed responses are more appropriately viewed as model-specific rather than representative of entire molecular subtypes. The molecular analysis was also limited to mRNA expression and was not supported by protein-level or functional validation. In addition, TCGA-BRCA and TIMER3 were used to provide broader disease-related context and should not be regarded as independent validation of the simvastatin response. Future studies should confirm these findings at the protein and functional levels, include a wider range of breast cancer models defined by receptor and mutation status, and examine dose and exposure conditions that more closely reflect clinically relevant statin exposure.

5. Conclusion

Among the genes examined in response to simvastatin, *CDKN1A* showed the most consistent transcriptional response, with increased expression in all MCF-7, SK-BR-3, and MDA-MB-231 breast cancer cells, whereas *CXCR4* and PD-L1 expressions decreased more significantly only in MDA-MB-231 cells. In contrast, *CD44*, *MAPK14*, and *NOTCH1* expressions were not significantly altered under the conditions tested. These findings suggest that multiple pathways activated by statins may eliminate cancer promoting genes to counteract drug resistance in breast cancer cells.

Altogether, the consistent upregulation of *CDKN1A* gene, and the marked downregulation of *CXCR4* and *PD-L1* genes by simvastatin in our data, further highlights the anticancer properties of lipophilic statins, and support their further clinical evaluation in the combination therapies of breast cancer subtypes.

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Data availability

The raw data are available from the corresponding author upon reasonable request.

Funding

This study was supported by Scientific Research Projects Coordination Unit of Altinbas University, Project number: PB2023-TIP-3

Author Contributions

The authors have no conflicts of interest. F.K. and N.A. designed the research; F.K., B.O.K and N.A analyzed and interpreted the data and wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Figures:

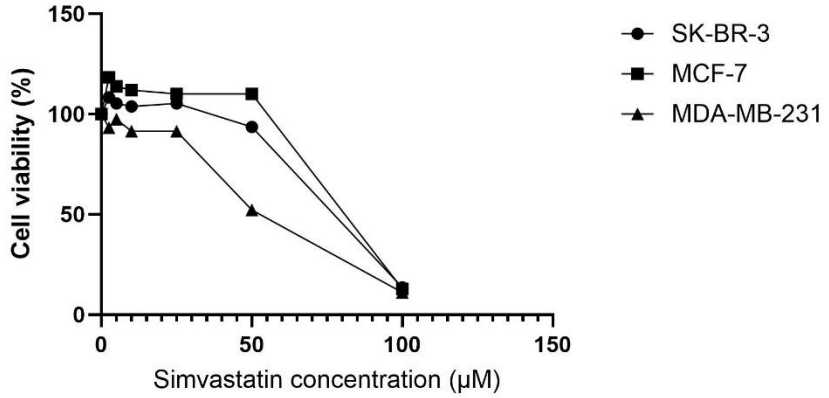


Figure 1. Effect of simvastatin on cell viability in breast cancer cell lines.

Concentration–response curves showing cell viability in SK-BR-3, MCF-7, and MDA-MB-231 cells after 24 h of simvastatin treatment. Cell viability was expressed as a percentage of the corresponding control. MDA-MB-231 cells showed a marked reduction in viability at 50 μM, whereas SK-BR-3 and MCF-7 cells remained relatively resistant up to 50 μM. At 100 μM, cell viability was markedly reduced in all three cell lines.

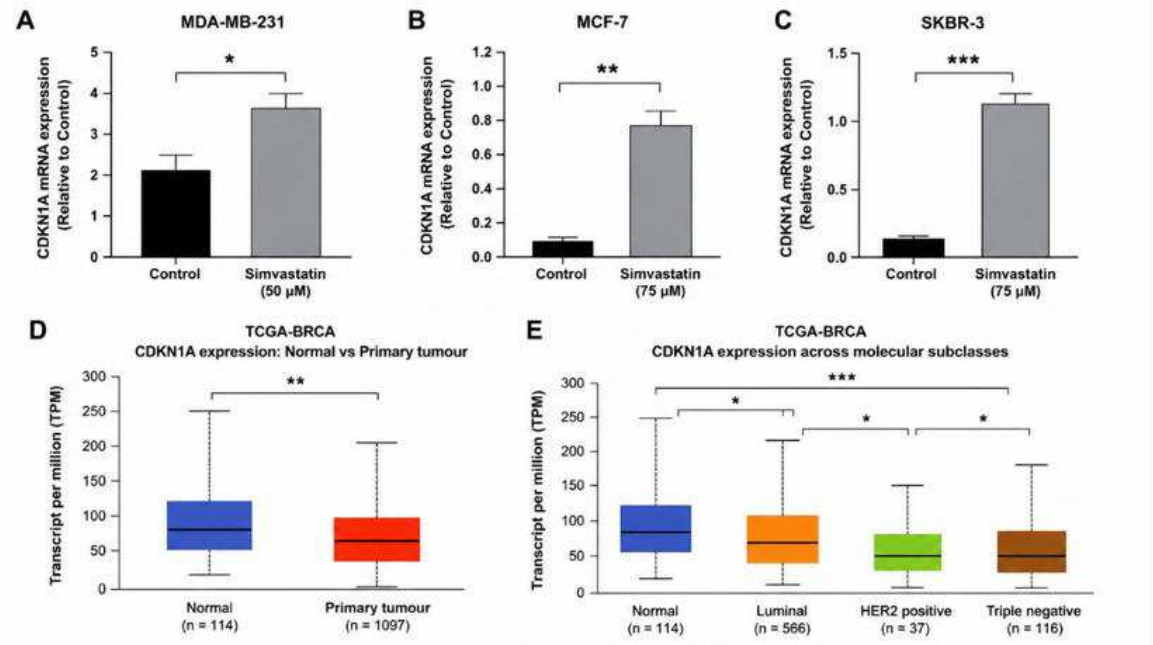


Figure 2. Effect of simvastatin on *CDKN1A* expression in breast cancer cell lines and TCGA-BRCA analysis. (A–C) *CDKN1A* mRNA expression in MDA-MB-231, MCF-7, and SK-BR-3 cells after 24 h of simvastatin treatment. (D) *CDKN1A* expression in normal breast tissue and primary breast tumours in the TCGA-BRCA dataset. (E) *CDKN1A* expression across molecular subtypes in TCGA-BRCA. Bar graphs represent mean ± SEM.Statistical significance is indicated as $p < 0.05$, $p < 0.01$, and $p < 0.001$.

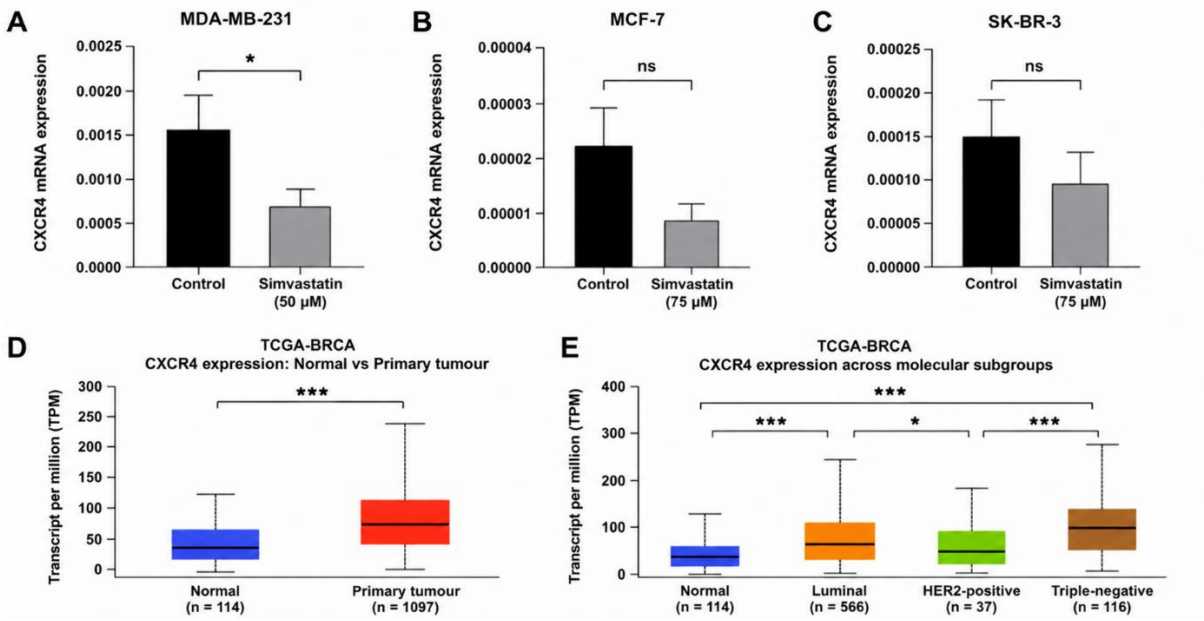


Figure 3. Effect of simvastatin on *CXCR4* expression in breast cancer cell lines and TCGA-BRCA analysis. (A–C) *CXCR4* mRNA expression in MDA-MB-231, MCF-7, and SK-BR-3 cells after 24 h of simvastatin treatment. *CXCR4* expression was significantly reduced in MDA-MB-231 cells, while no significant changes were observed in MCF-7 or SK-BR-3 cells. (D) *CXCR4* expression in normal breast tissue and primary breast tumours in the TCGA-BRCA dataset. (E) *CXCR4* expression across molecular subgroups in TCGA-BRCA. Bar graphs represent mean ± SEM. Statistical significance is indicated as $p<0.05$ and $p<0.001$; ns, not significant.

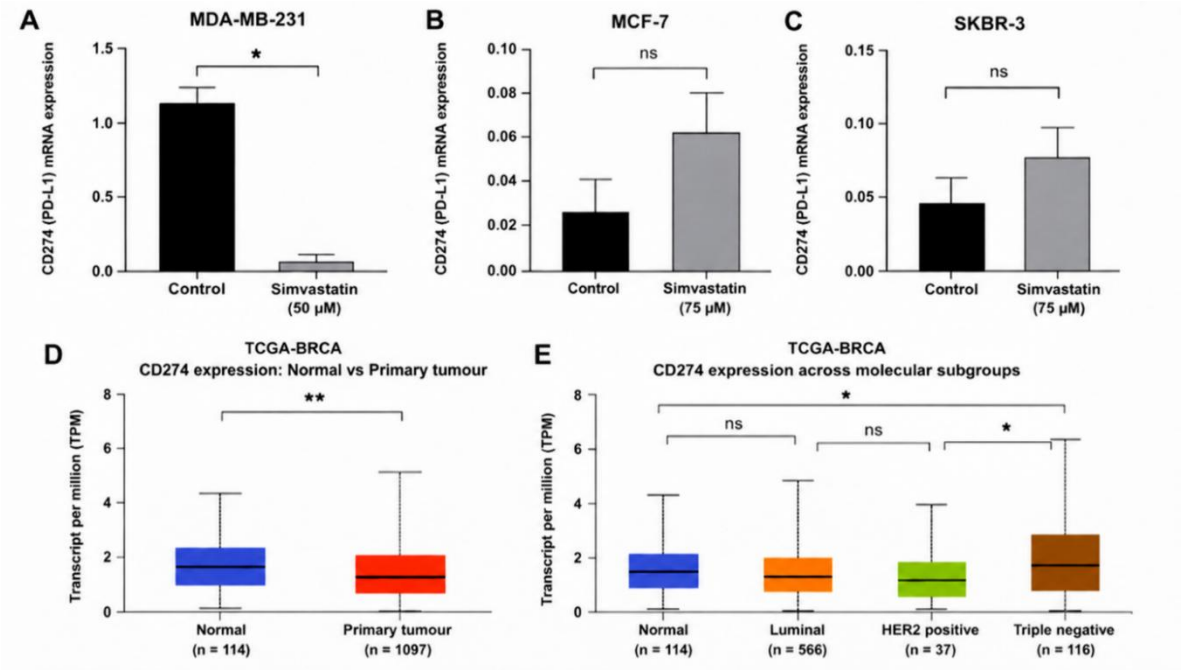


Figure 4. Effect of simvastatin on PD-L1 (CD274) expression in breast cancer cell lines and TCGA-BRCA analysis.

(A–C) *CD274 (PD-L1)* mRNA expression in MDA-MB-231, MCF-7, and SK-BR-3 cells after 24 h of simvastatin treatment. *PD-L1* expression was significantly reduced in MDA-MB-231 cells, whereas no significant changes were observed in MCF-7 or SK-BR-3 cells. (D) *PD-L1* expression in normal breast tissue and primary breast tumours in the TCGA-BRCA dataset. (E) *PD-L1* expression across molecular subgroups in TCGA-BRCA. Bar graphs represent mean ± SEM. Statistical significance is indicated as $p < 0.05$ and $p < 0.01$; ns, not significant.

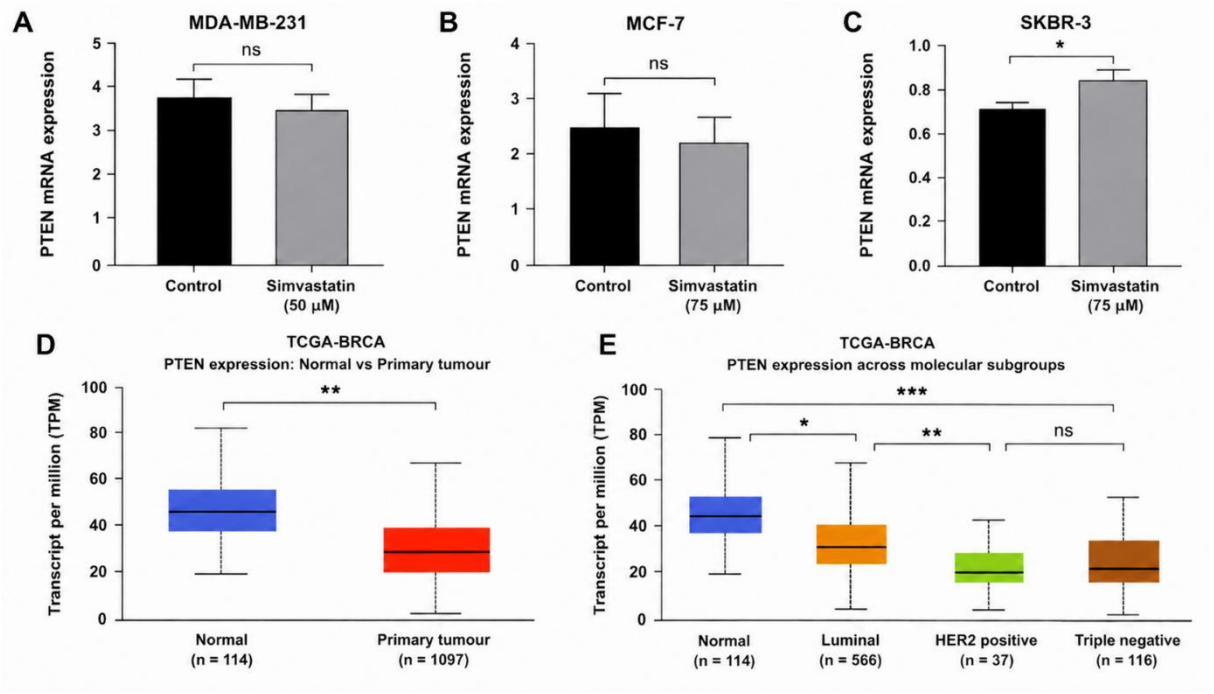


Figure 5. Effect of simvastatin on *PTEN* expression in breast cancer cell lines and TCGA-BRCA analysis.
(A–C) *A* mRNA expression in MDA-MB-231, MCF-7, and SK-BR-3 cells after 24 h of simvastatin treatment. *PTEN* expression was not significantly altered in MDA-MB-231 or MCF-7 cells, whereas a significant increase was observed in SK-BR-3 cells. (D) *PTEN* expression in normal breast tissue and primary breast tumours in the TCGA-BRCA dataset. (E) *PTEN* expression across molecular subgroups in TCGA-BRCA. Bar graphs represent mean ± SEM. Statistical significance is indicated as $p < 0.05$, $p < 0.01$, and $p < 0.001$; ns, not significant.

Supplement Table S1. Primer sequences used for quantitative RT-qPCR analysis.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
CD44	CAGGTGTATTCCACGTGGAG	CCATACCTGCAGGTCTCAAAT
CD274	ATTATTCAATTTGTGCATGGAG	CATCTGTGATCTGAAGTGCAG
CXCR4	AAGCATGACGGACAAGTACAG	GGATGAGGACACTGCTGTAGA
NOTCH1	CTCCTTCGAGTGCCAGTGTCT	TTGACCTCGCAGTGCACAC

CDKN1A	TGAGCGATGGAACTTCGACTT	CGTGGGAAGGTAGAGCTTGG
MAPK14	TTGACTCAGATGCCGAAGAT	ACTGAGCAAAGTAGGCATGTG
PTEN	ACAAGAGGCCCTAGATTTCTAT	AGTGCCACTGGTCTATAATCC
GAPDH	GTCATCCCTGAGCTGAACG	TTCTAGACGGCAGGTCAGGT
PPIA	TATCTGCACTGCCAAGACTGAGTG	CTTCTTGCTGGTCTTGCCATTCC
18S rRNA	ACCCGTTGAACCCCATTCGTGA	GCCTCACTAAACCATCCAATCGG