

Progerin regulates actin cytoskeletal remodeling and inhibits EMT and metastasis in triple-negative breast cancer cells

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Abstract. Triple-negative breast cancer (TNBC) is a subtype of breast cancer, known for its poor prognosis due to its high invasiveness, strong metastatic tendencies and propensity for recurrence. Epithelial to mesenchymal transition (EMT) is a crucial process in tumor invasion and metastasis and in the formation of cancer-initiating cells. Hutchinson-Gilford progeria is a rare condition characterized by accelerated aging, caused by a mutated form of lamin A, known as progerin. The present study aimed to investigate the effect of progerin overexpression on TNBC and uncover its underlying mechanisms of action. Therefore, cell senescence was assessed using senescence-associated β -galactosidase staining, while cell proliferation was measured by colony formation, Cell Counting Kit-8 and EdU assays. Additionally, cell metastasis was evaluated using wound-healing, Transwell and cell adhesion assays. Immunofluorescence staining was carried out to observe actin cytoskeleton and nuclear morphology. The results showed that progerin markedly suppressed the colony formation, migration, invasion and adhesion abilities of BT-549 and MDA-MB-231 TNBC cell lines, without affecting cell senescence or proliferation. In addition, progerin overexpression altered nuclear morphology and actin cytoskeleton organization in TNBC cells. Furthermore, the expression levels of the mesenchymal markers, N-cadherin, vimentin, Snail and Slug, were reduced, while those of the epithelial

marker, E-cadherin, were enhanced in TNBC cells. Overall, the results of the present study suggested that progerin overexpression could inhibit TNBC cell metastasis, probably via actin cytoskeleton remodeling and regulate the expression levels of the cytoskeletal-related proteins, anillin and β -catenin, and those of the EMT-related ones. The aforementioned findings could provide novel insights into the identification of potential molecular targets for breast cancer therapy.

Introduction

Aging and cancer are two consequences that gradually occur due to molecular changes over time. The processes can actually be closely associated. However, this association is complex and dual and remains to be fully elucidated (1,2).

Hutchinson Gilford Progeria Syndrome (HGPS) is a devastating accelerated aging disease, which is caused by mutation in *LMNA*. It has been reported that the production of progerin is markedly associated with the onset of HGPS (3-5). In 90% of individuals with HGPS, a substitution (C to T) at position 1824 in *LMNA*, which encodes lamin A/C, commonly occurs (6). This mutation can lead to the activation of a cryptic splicing site, thus resulting in the production of mutant nuclei, lacking 50 amino acids (7). These missing 50 amino acids, found in the lamin A precursor progerin, include the enzymatic cleavage site for metalloproteases. Consequently, this process cannot further proceed, thus promoting the permanent maintenance of farnesylation and carboxymethylation (8). Normally, mature lamin A can translocate into the nucleus after cleavage of the farnesyl group by metalloenzymes. However, due to the permanent retention of farnesylation in progerin, its interaction with the inner nuclear membrane becomes more stable (9), thus resulting in the accumulation of progerin in the inner nuclear membrane, alteration of the nuclear lamin A structure and chromatin spatial distribution (10). This alteration can affect several cellular functions, such as genome instability, DNA repair, replication stress, telomere shortening, mitochondrial dysfunction and cellular senescence (11).

Despite lung cancer's overall numerical lead, according to the latest global cancer statistics from 2022, breast cancer dominates female-specific incidence, affecting 1 in 12 women globally (12). Triple-negative breast cancer (TNBC) is a

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biologically aggressive type of tumor, which is characterized by significant invasiveness, strong metastatic potential, easy recurrence and metastasis and limited treatment options. Compared with other cancer subtypes (luminal A/B, HER2+), TNBC exhibits distinct actin cytoskeletal dynamics that contribute to its aggressive metastatic behavior and predispose to therapy resistance. Therefore, TNBC is considered as the breast cancer subtype with the worst prognosis (13). Epidemiological data has suggested that TNBC commonly occurs in premenopausal young women <40 years of age and accounts for 15-20% of all breast cancer cases (14). Patients with TNBC commonly exhibit a shorter survival time compared with patients with other breast cancer subtypes, with a mortality rate of 40% within 5 years of diagnosis. TNBC is highly invasive and ~46% of patients with TNBC will experience distant metastases. The most common sites of recurrence are the lungs, lymph nodes and brain, with early or late involvement estimated to at ~10 and 40%, respectively (15). The postoperative recurrence rate can reach 25%, with a median survival time of 13.3 months, following surgery. The majority of patients with TNBC who respond to standard therapeutic options are in the nonmetastatic stage. However, these standard therapeutic options have not markedly improved the overall survival rate (16). Therefore, defining the molecular characteristics of TNBC and developing novel treatment options are of significant importance.

Since early TNBC is prone to recurrence and distant metastasis, much attention has been paid to the treatment of metastatic TNBC. Several treatment approaches have been proven to improve the survival rate of patients with metastatic TNBC. Therefore, focusing on prevention is crucial for addressing recurrence and metastasis. Epithelial to mesenchymal transition (EMT) is a critical process, which is involved in tumor invasion, metastasis and formation of cancer-initiating cells. Previous results from our research group demonstrated that progerin overexpression in melanoma cells attenuated cell proliferation and migration via inhibiting EMT (17). However, whether progerin has similar effects in TNBC remains elusive.

Confronting TNBC's critical therapeutic challenge of hyper-invasiveness, it was hypothesized that progerin, through its capacity to suppress EMT-driven metastasis, represents a mechanistically grounded therapeutic target uniquely suited to immobilize the aggressive phenotype of TNBC. The present study represents a mechanistic exploratory investigation focused on elucidating the functional consequences of progerin overexpression in TNBC and delineating its associated molecular pathways. Verification assays were performed to investigate this hypothesis at the cellular level and determine whether progerin overexpression could efficiently inhibit TNBC cell proliferation, migration, invasion and EMT.

Materials and methods

Cell lines and cultures. MDA-MB-231 and BT-549 cell lines were selected as TNBC models due to their well-established hyper-invasive phenotypes and representation of the genomic heterogeneity and major metastatic routes of TNBC. The MDA-MB-231 and BT-549 cell lines were a generous gift from Associate Professor Hua Zhang at Guangdong Medical University, Dongguan, China. MDA-MB-231 cells were

maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Newzerum, Ltd.). BT-549 cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 0.023 U/ml insulin (Beyotime Institute of Biotechnology) and 10% FBS. All cells were cultivated in an incubator at 37°C with 5% CO₂.

Cell infection. A lentiviral expression system using third-generation packaging systems was purchased from Shanghai GeneChem Co., Ltd and was used according to the manufacturer's instructions. On the day prior transfection, cells in medium supplemented with 10% FBS were plated into 24-well plates at a density of 5x10⁴ cells/well. The cells were then infected with the same virus titer obtained from the preliminary experimental MOI (infection MOI=100). After three days, GFP expression was observed under an inverted fluorescence microscope (Olympus Corporation). The cells were then replated in 6-well plates and after adherence, progerin-overexpressing cells were selected with 2 µg/ml puromycin. NC (Negative Control): Cells transduced with the identical lentiviral backbone (pLVX-puro) without progerin insert, were processed in parallel to account for vector effects. The Blank group was untransduced parental cells cultured under identical conditions to establish baseline characteristics.

Western blot analysis. Total protein was extracted using RIPA Lysis Buffer (Strong) (Beyotime Institute of Biotechnology; cat. no. P0013K) composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS. Protein loading was normalized to endogenous GAPDH levels through pre-experimental calibration. The cell protein lysates were separated by 10 or 12.5% SDS-PAGE and were then electrophoretically transferred onto PVDF membranes with a pore size of 0.22 µm (EMD Millipore). Membranes were blocked with 5% (w/v) non-fat dry milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h at 25°C with continuous shaking (50 rpm). The membranes were then incubated with primary antibodies against lamin A/C (dilution, 1:10,000; anti-rabbit; cat. no. ab169532), cyclin E1 (dilution, 1:1,000; anti-rabbit; cat. no. ab133266), cyclin-dependent kinase 4 (CDK4; dilution, 1:1,000; anti-mouse; cat. no. ab131197), anillin (dilution, 1:500; anti-mouse; cat. no. ab211872), flag (dilution, 1:3,000; anti-mouse; cat. no. TA180144), p16 (dilution, 1:500; anti-mouse; cat. no. sc-1661), p21 (dilution, 1:1,000; anti-rabbit; cat. no. 2947), pRb (dilution, 1:1,000; anti-rabbit; cat. no. 8516), p53 (dilution, 1:2,000; anti-rabbit; cat. no. 10442-1-AP), N-cadherin (dilution, 1:2000; anti-mouse; cat. no. 1D8B3), E-cadherin (dilution, 1:5,000; anti-rabbit; cat. no. 20874-1-AP), vimentin (dilution, 1:10,000; anti-mouse; cat. no. 3H9D1), Snail (dilution, 1:1,000; anti-rabbit; cat. no. 13099-1-AP), Slug (dilution, 1:1,000; anti-rabbit; cat. no. 12129-1-AP), metalloproteinase 7 (MMP7; dilution, 1:1,000; anti-rabbit; cat. no. 10374-2-AP), MMP9 (dilution, 1:2,000; anti-rabbit; cat. no. 10375-2-AP), GAPDH (dilution, 1:10,000; anti-mouse; cat. no. 60004-1-Ig) and β-catenin (dilution, 1:2,000; anti-rabbit; cat. no. M24002) at 4°C overnight. The blots were then incubated with the corresponding goat anti-mouse or anti-rabbit secondary antibodies (HRP, dilution, 1:2,000; cat. nos. A0216 and A0208) at room temperature for 1 h. The blots were

visualized utilizing the Super Sensitive ECL Luminescence Reagent (MilliporeSigma) on the Azure c400 instrument (Azure Biosystems, Inc.). Band densitometry was performed using ImageJ (Version 1.52; National Institutes of Health). All target protein intensities were normalized to GAPDH.

Senescence-associated (SA)- β -galactosidase staining. When a confluency of ~60% was reached, cells were rinsed with PBS and fixed in 4% paraformaldehyde at room temperature for 15 min. Cell senescence was then evaluated using a β -galactosidase staining kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Cells were incubated at 37°C for at least seven days and the SA- β -galactosidase-positive cells were observed under a microscope (magnification, 100x; Nikon Corporation).

Clone formation assays. Cells in the logarithmic growth phase were seeded into 6-well plates at a density of 1×10^3 cells per well. Following a 2-week incubation, cells were fixed using 4% paraformaldehyde at room temperature for 30 min and were then stained with 0.1% crystal violet at room temperature for 60 min. Images of the colonies were captured and their count was determined under a microscope (Olympus Corporation).

Cell viability assay. BT-549 (density, 2×10^3 cells/well) and MDA-MB-231 (density, 3×10^3 cells/well) cells at logarithmic growth stage were seeded into 96-well plates for 4 h. Following incubation for an additional 0, 24, 48 and 72 h, 100 μ l complete medium supplemented with 10% Cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Inc.) was added into each well, followed by incubation at 37°C for an additional 2 h. To assess cell proliferation, the absorbance at a wavelength of 450 nm was measured in each well using a microplate absorbance reader (BioTek Instruments, Inc.). Cell viability was assessed via constructing a cell survival curve.

Cell proliferation assay. Cells at a density of 7×10^4 were inoculated into 12-well plates at the logarithmic growth stage and grown to a normal growth stage. Cell proliferation was then assessed using the BeyoClick EdU-555 Cell Proliferation Kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. EdU-positive cells were observed and analyzed under an inverted fluorescence microscopy (Invitrogen; Thermo Fisher Scientific, Inc.).

Cell cycle analysis. Cells were harvested at a density of 70-80%, washed with PBS and fixed with 70% ethanol at 4°C overnight. Cell cycle was analyzed utilizing the Cell Cycle and Apoptosis Analysis Kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The stained cells were analyzed on the FACSVerse flow cytometer (BD Biosciences) using FlowJo software v 10.8.1 (FlowJo LLC).

Wound-healing assay. Cells at a density of $\sim 1 \times 10^6$ were plated in 6-well plates and incubated at 37°C until they reached a confluency of ~90%. Subsequently, wounds were created via lightly scratching a straight-line across the cell monolayer using a 200- μ l plastic pipette tip. Cells were incubated in fresh medium with 1% FBS. Cells were maintained in 1% FBS-containing medium during the assay based on: i)

Viability necessity: Serum-free conditions induced rapid detachment in MDA-MB-231 and BT549 cells, which may be related to the sensitivity of metastatic TNBC lines to anoikis. ii) Proliferation control: Progerin expression showed no effect on proliferation rates. The migrated cells into the wounded area were observed and images were captured at 0, 24 and 48 h under an inverted phase-contrast light microscope (Nikon Corporation) with total magnification. x100.

Transwell assay. BT-549 (density, 15×10^4) and MDA-MB-231 (density, 12.5×10^4) cells in 200 ml serum-free medium were seeded onto the upper chamber of a Transwell chamber (pore size, 8 μ m; Corning; Corning, Inc.) with Matrigel-uncoated or -coated (37°C; 2 h) membrane. The lower chamber was supplemented with 800 μ l medium containing 10% FBS. The following day, cells migrated to the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet at room temperature for 30 min. Migrated or invaded cells were counted and images were captured at five randomly selected fields under an inverted phase-contrast light microscope (Nikon Corporation) with total magnification. x100.

Cell adhesion assay. A total of 100 μ l Matrigel (Corning; Corning, Inc.) diluted in PBS at a ratio of 1:9 was added into each well of a 96-well plate, followed by gently shaking and solidification in an incubator for 2 h. After digestion and centrifugation (60.1 x g, room temperature, 4 min), the cells in the logarithmic growth phase were rinsed once with PBS, centrifuged (60.1 x g, room temperature, 4 min) and were then resuspended in serum-free medium at a density of 1×10^4 cells/ml. Following centrifugation (60.1 x g, room temperature, 4 min), the supernatant was removed and 100 μ l of the cell suspension was inoculated into each well of the 96-well plate. The cells were incubated in an incubator at 37°C and 5% CO₂ for 1 h to attach the wall. Subsequently, the unattached cells were washed with PBS for three times. BT-549 cells in 100 μ l complete medium supplemented with 10% CCK-8 solution (Dojindo Laboratories, Inc.) were added into each well, followed by incubation at 37°C for 2 h. Subsequently, the optical density at a wavelength of 450 nm was measured using a microplate absorbance reader (BioTek Instruments, Inc.). MDA-MB-231 cells, were first washed with PBS, followed by fixing with 100 μ l 4% paraformaldehyde for 30 min and staining with 0.1% crystal violet for 15 min, both at room temperature. Finally, cells were washed again with tap water until clear and transparent. Finally, the cells were allowed to dry, images captured and the cells counted.

Immunofluorescence staining. Cells were plated onto 12-mm diameter glass coverslips in 24-well plates at a confluency of >40%. Subsequently, all samples were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized in PBS supplemented with 0.3% Triton X-100 for 10 min at room temperature, blocked with PBS containing 5% normal goat serum (Sangon Biotech Co., Ltd.; cat. no. E510009) for 1 h at room temperature and were then incubated with anti-lamin A (dilution, 1:250; cat. no. ab26300; Abcam) and anti-flag (dilution, 1:400; cat. no. ab26300; OriGene Technologies, Inc.) antibodies at 4°C overnight. The samples were then washed with PBS thrice, followed by incubation with goat anti-mouse IgG (H&L)- Alexa

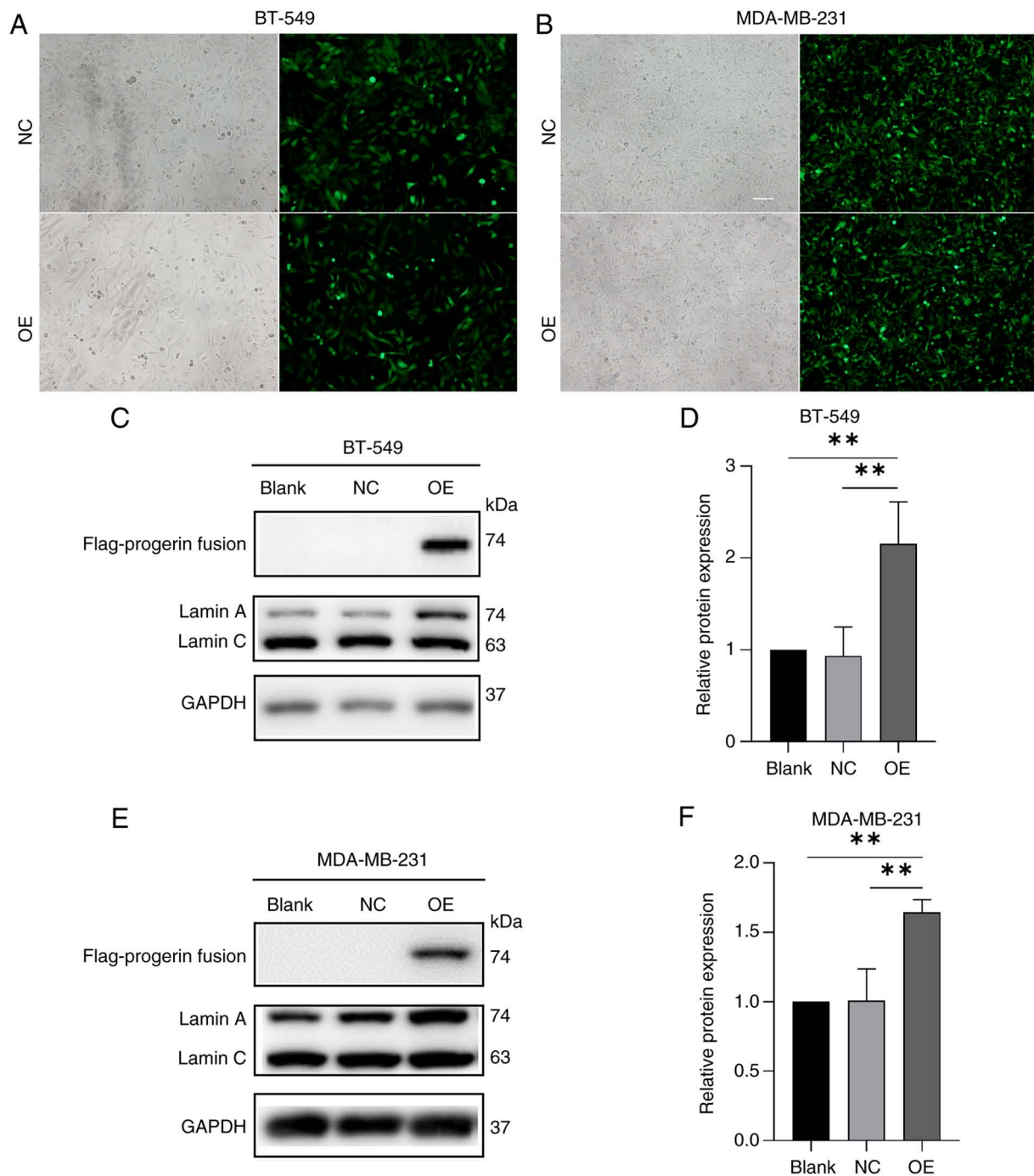


Figure 1. Stable progerin expression in BT-549 and MDA-MB-231 cells. The GFP fluorescence of (A) BT-549 and (B) MDA-MB-231 cell lines were detected 72 h after lentivirus infection. Magnification, $\times 100$. (C-F) Western blot analysis demonstrated the overexpressing efficiency of progerin. $^{**}P < 0.01$. Error bars as mean $\pm$ SEM. BT-549: $\eta^2 = 0.804$, MDA-MB-231: $\eta^2 = 0.871$. NC, negative control; OE, overexpression.

Fluor 555 (dilution, 1:1,000; Thermo Fisher Scientific, Inc.) and goat anti-rabbit IgG (H&L)- Alexa Fluor 633 (dilution, 1:1,000; ImmunoWay Biotechnology Company) for 1 h at room temperature. Additionally, for cytoskeleton assessment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with PBS containing 0.3% Triton X-100 for 10 min at room temperature and were finally incubated with the Cell Navigator F-Actin Labeling Kit *Red Fluorescence* (AAT Bioquest, Inc.) for 1 h at room temperature. All washing steps were performed with 1XPBS (pH 7.4). Subsequently, coverslips were placed onto glass microscope slides using an antifade

mounting medium supplemented with DAPI (Beyotime Institute of Biotechnology). Images were acquired under a confocal laser scanning microscope (TCS SP8; Leica Microsystems, Inc.) with LAS X software and were processed with Adobe Photoshop 2022 (Adobe Systems, Inc.). Image scale bar acquisition parameters: LaminA: original magnification: 50 μm , zoom: 10 μm ; Phalloidin: original magnification: 25 μm , zoom: 10 μm .

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. To elucidate the association of the differentially expressed genes (DEGs), screened by

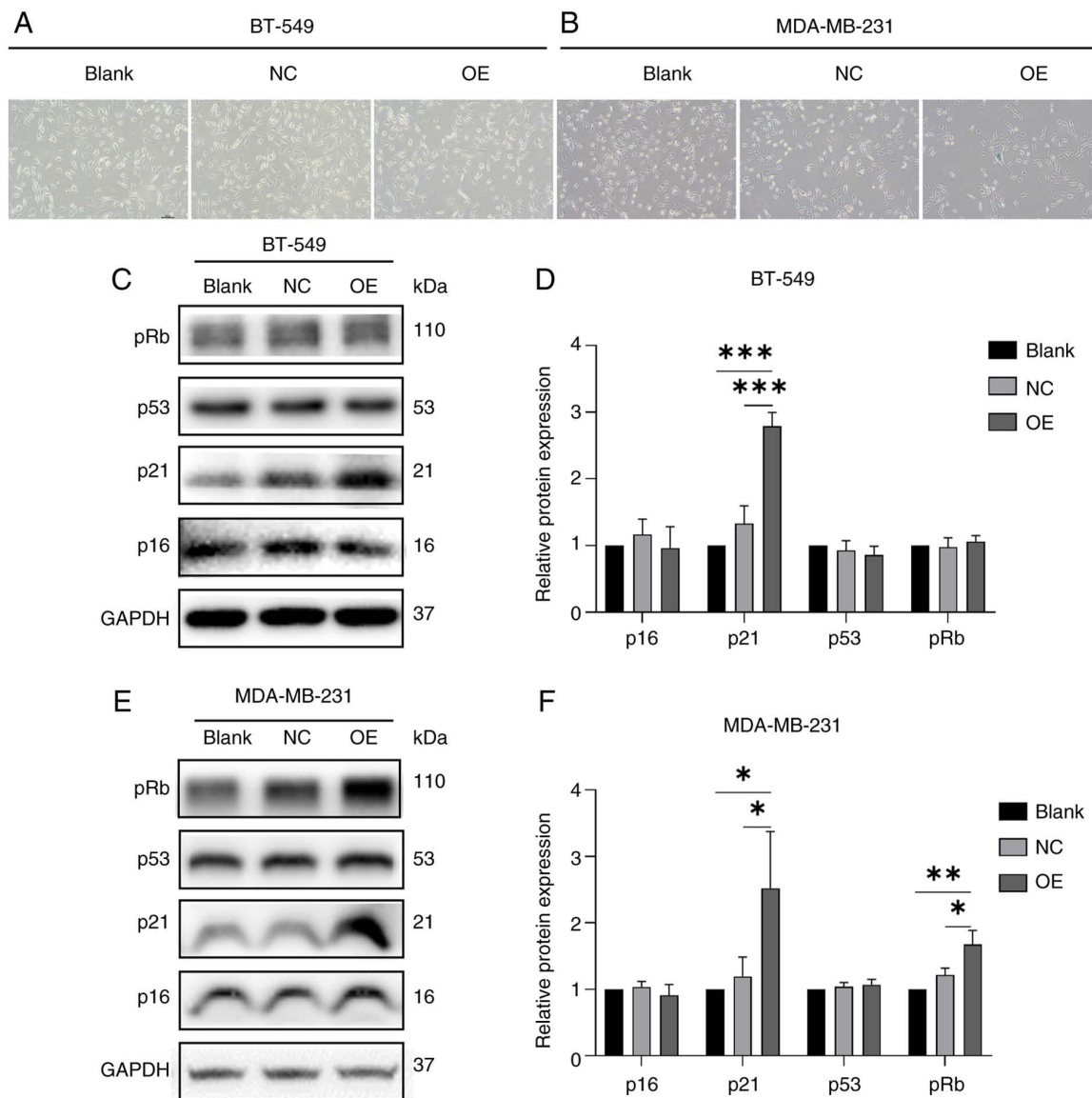


Figure 2. Progerin was unable to induce significant cellular senescence. (A and B) No significant difference in SA-β-Gal senescence staining. Magnification, x200. (C-F) Western blot analysis showed the expression of aging markers. *P<0.05, **P<0.01, ***P<0.001. Error bars as mean ± SEM. NC, negative control; OE, overexpression.

protein chips, between the progerin and control groups (fold change >0.5 or <0.67), GO classifications were used. To illustrate the biological roles of DEGs using GO analysis, a total of three key areas were examined, namely biological processes, molecular functions and cellular components. Subsequently, to predict the roles of these genes in different pathways, bioinformatics analysis using KEGG enrichment analysis was performed. The aforementioned analysis was carried out using the RStudio software packages (Posit PBC; RStudio 4.1.1) and the results obtained were uploaded to a website (https://bioinformatics.com.cn/basic_local_go_pathway_enrichment_analysis_122) for visual analysis.

Statistical analysis. Statistical analysis was based on n=3 biological replicates unless otherwise noted. The statistical differences between the experimental and control groups were determined via one-way ANOVA with Tukey's post hoc test using GraphPad Prism 8 (Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

Progerin overexpression in BT-549 and MDA-MB-231 TNBC cells. While basal-like breast cancer cell lines are highly metastatic and invasive, the TNBC BT-549 and MDA-MB-231 cell lines were selected to establish a stable progerin overexpression *in vitro* model. Following transfection of TNBC cells with progerin-overexpressing and control lentivirus for 72 h, >80% of cells expressed green fluorescent protein (Fig. 1A and B), indicating successful viral DNA integration into cell chromatin. In addition, the transfection efficacy was verified by western blot analysis (Fig. 1C-F).

Progerin cannot markedly promote TNBC cell senescence. To evaluate cell senescence, the expression of β-galactosidase in senescent cells was verified by β-gal staining. As shown in Fig. 2A and B, all cells in both the progerin-overexpression and control groups were negative for β-gal staining. In addition, the

expression levels of the senescence-related markers, p16, pRb, p21 and p53, were assessed by western blot analysis. In both MDA-MB-231 and BT-549 cells, no difference in the expression levels of p16 and p53 were obtained between the progerin overexpression and control groups (Fig. 2C-F). However, pRb was upregulated in the progerin overexpression group in MDA-MB-231 cells, while p21 was markedly upregulated in the progerin overexpression group compared with the control group in both cell lines (Fig. 2C-F).

Progerin overexpression in TNBC cells inhibits cell colony formation, but not cell proliferation and cell cycle. Cell senescence is a type of programmed growth arrest that prevents cell proliferation. In the present study, the results demonstrated that progerin overexpression resulted in the formation of fewer and smaller colonies (Fig. 3A-C). However, the CCK-8 and EdU assays revealed that progerin overexpression did not inhibit cell proliferation and reduce the proportion of EdU-positive cells (Fig. 3D-I). BT-549 and MDA-MB-231 cell lines were subjected to flow cytometric analysis to ascertain the effect of progerin on TNBC cell growth. As shown in Fig. 3J-M, there was no discernible variation in the quantity of cells in any phase of the cell cycle. In addition, no obvious changes were noted in the expression levels of G1/S phase-related checkpoints CDK4 and Cyclin E1 between the progerin overexpression and control groups (Fig. 3N-Q). These results indicated that progerin could not affect the proliferation of TNBC cells *in vitro*.

Progerin markedly impairs the metastasis of TNBC cells. Subsequently, the present study focused on the functionalities of progerin in the metastatic potential of TNBC cells. As expected, the wound healing assays showed that progerin clearly prevented TNBC cells from migrating (Fig. 4A-D). The Transwell assays also revealed that progerin overexpression inhibited BT-549 and MDA-MB-231 cell invasion (Fig. 4E-H). Subsequently, the effects of progerin on the adhesion ability of TNBC cells were also investigated. Therefore, the attachment rates between progerin expressing and control cells on Matrigel-coated surfaces were compared. The attachment rates of progerin-overexpressing cells were notably lower compared with those of control cells (Fig. 4I-K). The aforementioned results suggested that progerin overexpression not only attenuated the migratory and invasive abilities of TNBC cells, but also inhibited their adhesion ability.

Progerin overexpression can alter the morphology of the nucleus and affect cytoskeleton remodeling in TNBC cells. Since progerin is a mutant of lamin A, which is distributed on the nuclear fiber layer of the nucleus, immunofluorescence staining for lamin A was performed to observe the cellular sublocalization of progerin and the nuclear structure of lamin A in TNBC cells. The immunofluorescence staining results demonstrated that the staining of the nuclear membrane in progerin-overexpressing BT-549 and MDA-MB-231 cells was more potent, thus indicating that progerin was mainly located in the nuclear fiber layer. In addition, the morphology of the nucleus changed in this group of cells, which was characterized by nuclear enlargement, nuclear membrane invagination and even pathological mitotic figure (Fig. 5A). These nuclear structural abnormalities were accompanied by significant

changes in the organization of the cytoskeleton surrounding the nucleus. Cytoskeleton reorganization is closely associated with cell adhesion and migration. Therefore, in the present study, the actin cytoskeleton was stained with phalloidin. The staining results revealed that the arrangement of the cytoskeleton was also changed, cell pseudopodia were shortened, the number of stress fibers were reduced, while the distribution of actin filaments became irregular (Fig. 5B).

Possible mechanisms of progerin overexpression in affecting the function of TNBC cells. A previous study from the Institute of Aging Research (Guangdong Medical University, China) demonstrated that progerin could inhibit the biological function of non-small cell lung cancer cells and analyzed DEGs in progerin-overexpressing and control A549 cells by protein chip (18). In the current study, to investigate the potential mechanism of progerin overexpression, GO and KEGG pathway enrichment analysis on DEGs was conducted. The KEGG pathway analysis revealed that the top three ranking pathways were 'endocytosis', 'N-proteoglycan synthesis' and 'nucleoplasmic transport pathway' (Fig. 6), which were all apparently associated with the cytoskeleton. Additionally, GO enrichment analysis indicated that the cytoskeletal-related proteins, anillin and β -catenin, could be involved in the regulation of several signaling pathways (Fig. 7). The western blot results verified that progerin overexpression in BT-549 and MDA-MB-231 cells markedly downregulated anillin and β -catenin compared with the control groups (Fig. 8A and B). The aforementioned results were consistent with those of the protein chips. Cytoskeletal remodeling is involved in the EMT process and is considered as a significant marker of EMT, which serves a key role in tumor metastasis. Therefore, western blot analysis was performed to detect the expression levels of EMT-related proteins (Fig. 8B and C). Therefore, the results demonstrated that the expression levels of the epithelial marker E-cadherin were increased, while those of the mesenchymal markers N-cadherin and vimentin were reduced in progerin-overexpressing cells. Additionally, Snail and Slug were downregulated in these cells. Furthermore, the protein expression levels of MMP7 and MMP9 were also markedly reduced. In conclusion, these findings suggested that progerin could regulate EMT in TNBC cells.

Discussion

TNBC commonly occurs in premenopausal young women, <40 years of age and is characterized by short survival time (14). Due to its aggressive invasion ability, high recurrence rate and ease of metastasis, the prognosis of TNBC is relatively poor. For the majority of patients only few targeted therapies are available, particularly once chemoresistant metastatic disease develops. Therefore, the development of additional therapeutic strategies for TNBC is of great importance.

The present study demonstrated that although progerin could alter the expression levels of several aging-related markers, it could not promote TNBC cell senescence. This was practically consistent with previous research results. Studies from our research group also showed that progerin overexpression in non-small cell lung cancer A549 and melanoma M14 cells did not markedly promote cell senescence (17,18).

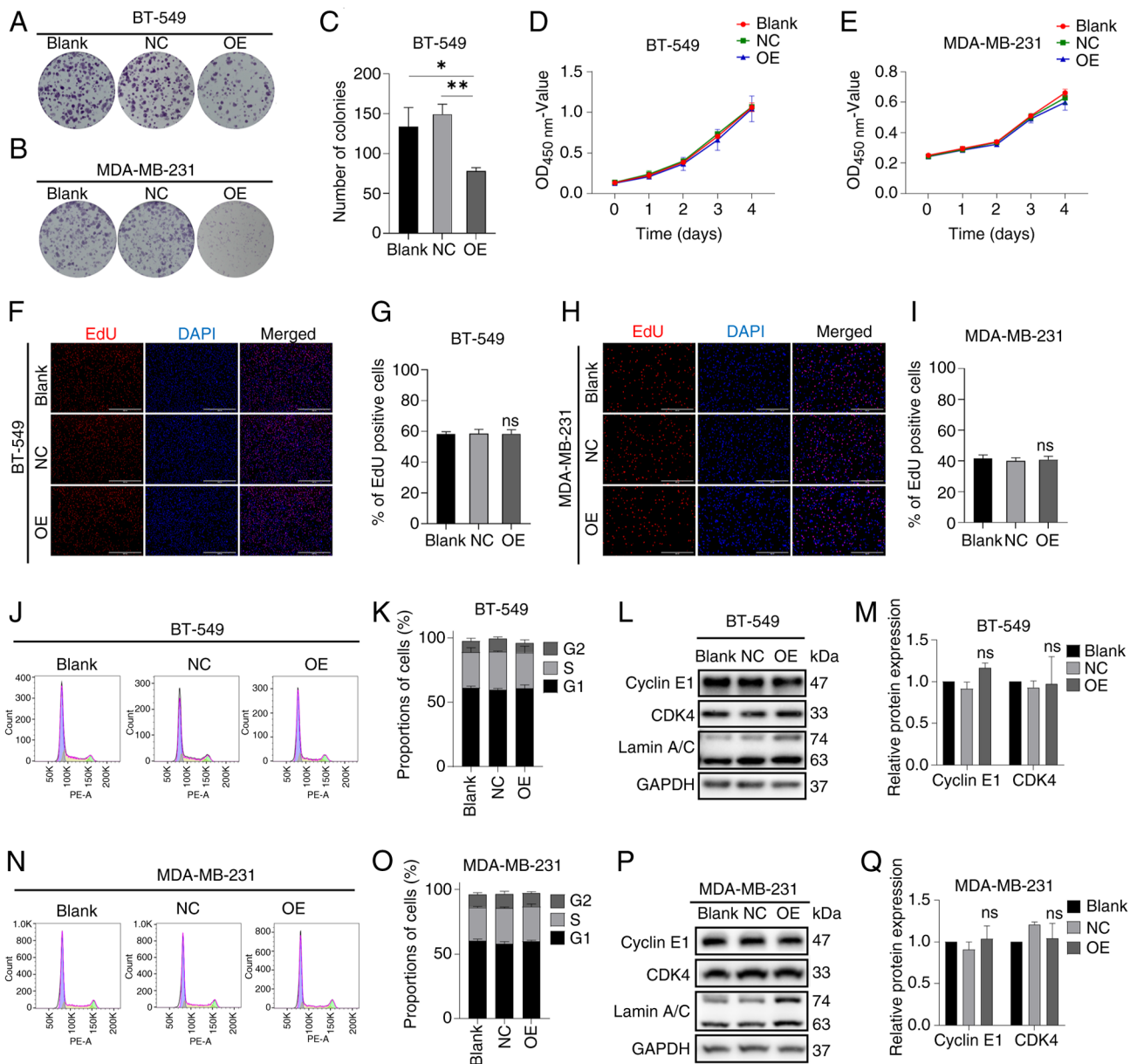


Figure 3. Progerin did not affect cell proliferation. (A-C) Clone formation assay was conducted for 15 days. (D and E) Cell viability assay was conducted for 1/2/3/4 days. (F-I) Cell proliferation assay was conducted for 2 h. Magnification, BT-549 cells x40, MDA-MB-231 cells, x100. (J, K and N, O) Cell cycle analysis was conducted by flow cytometry. (L, M and P, Q) Western blot analysis demonstrated the expression of G₁/S phase checkpoint protein Cyclin E1 and CDK4. ns P>0.05, *P<0.05, **P<0.01. Error bars as mean $\pm$ SEM. NC, negative control; OE, overexpression.

In addition, in the study by Tang *et al* (19), β -gal senescence staining indicated that progerin overexpression could not induce cellular senescence in PC-3 or MCF7 cells. In terms of cell proliferation, results of proliferation assays showed no difference between the progerin overexpression and control groups, even though the colony formation ability of TNBC cells was markedly inhibited following progerin overexpression. It was therefore hypothesized that the difference in the number of the colonies could be caused by the combination of density-dependent growth characteristics and adhesion differences of TNBC cells. The study by Tang *et al* (19) also showed that progerin could not affect the proliferation ability of MCF7 cells, which was also consistent with the results of the current study. Critically, the current study demonstrated that progerin's function is not universally conserved across

cancer types. While it suppresses proliferation in lung cancer and melanoma (17,18), it exhibits no anti-proliferative effects in TNBC. This divergence probably arises from cancer-specific genetic heterogeneity and growth dependency.

For highly aggressive TNBC, the effect of progerin overexpression on cell migration, invasion and adhesion gained our attention. The results of the present study demonstrated that progerin could markedly attenuate the migration, invasion and adhesion abilities of TNBC cells. Furthermore, EMT was inhibited, accompanied by the reorganization of the cytoskeleton and the reduced expression levels of the cytoskeleton-related proteins, anillin and β -catenin. The protein expression levels of the epithelial marker, E-cadherin, were enhanced, while those of the mesenchymal markers, N-cadherin and vimentin, were reduced in progerin-overexpressing TNBC

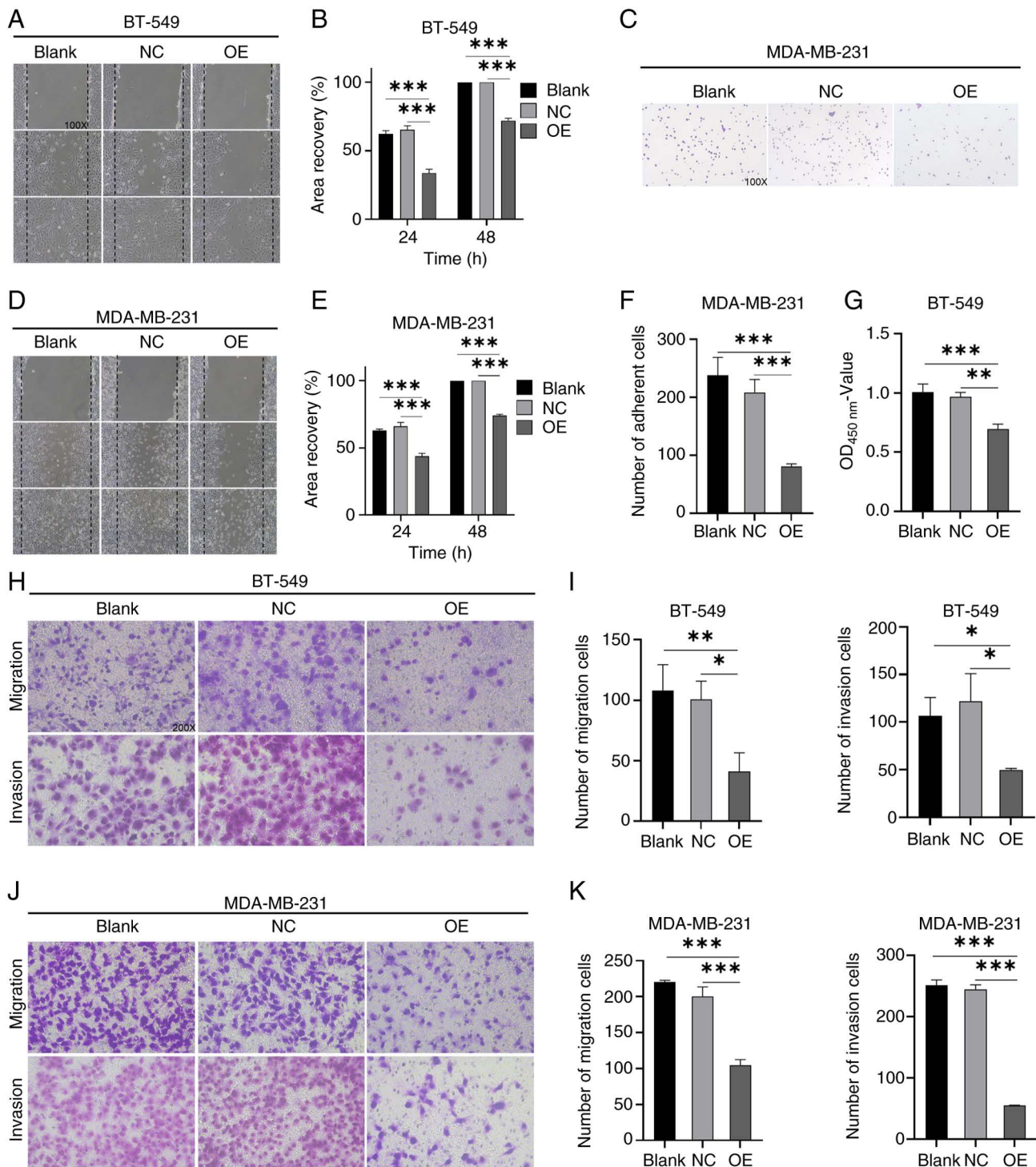


Figure 4. Cell migration, invasion and adhesion were inhibited, (A, B and D, E) Scratch wound-healing assay was conducted for 24/48 h. Magnification, x100. (H-K) Transwell assay was conducted for 24 h. Magnification, x200. Migration BT-549: $\eta^2=0.810$, MDA-MB-231: $\eta^2=0.973$; invasion BT-549: $\eta^2=0.780$, MDA-MB-231: $\eta^2=0.996$. (C and F, G) Cell adhesion assay was conducted for 1 h. Magnification, x100. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Error bars as mean $\pm$ SEM. NC, negative control; OE, overexpression.

cells. It has been reported that the transcription program of EMT is controlled by six 'core EMT-transcription factors', namely Snail/Snail, Snai2/Slug, Twist1, Twist2, zinc finger E-box-binding homeobox (ZEB) 1 and ZEB2, that can both inhibit and enhance the expression of particular genes associated with epithelial and mesenchymal phenotypes, respectively (20). In the present study, E-cadherin was upregulated, while both Snail and Slug were downregulated following

progerin overexpression in TNBC cells. Therefore, progerin overexpression could inhibit the process of EMT. It is well known that EMT/MET is a dynamic reversible state. It has been reported that TNBC cells can shift to more mesenchymal states during both invasion and colony formation, with invasion displaying a greater variety of EMT patterns compared with colony formation (21,22). The aforementioned finding could be due to the fact that the activation of an EMT program

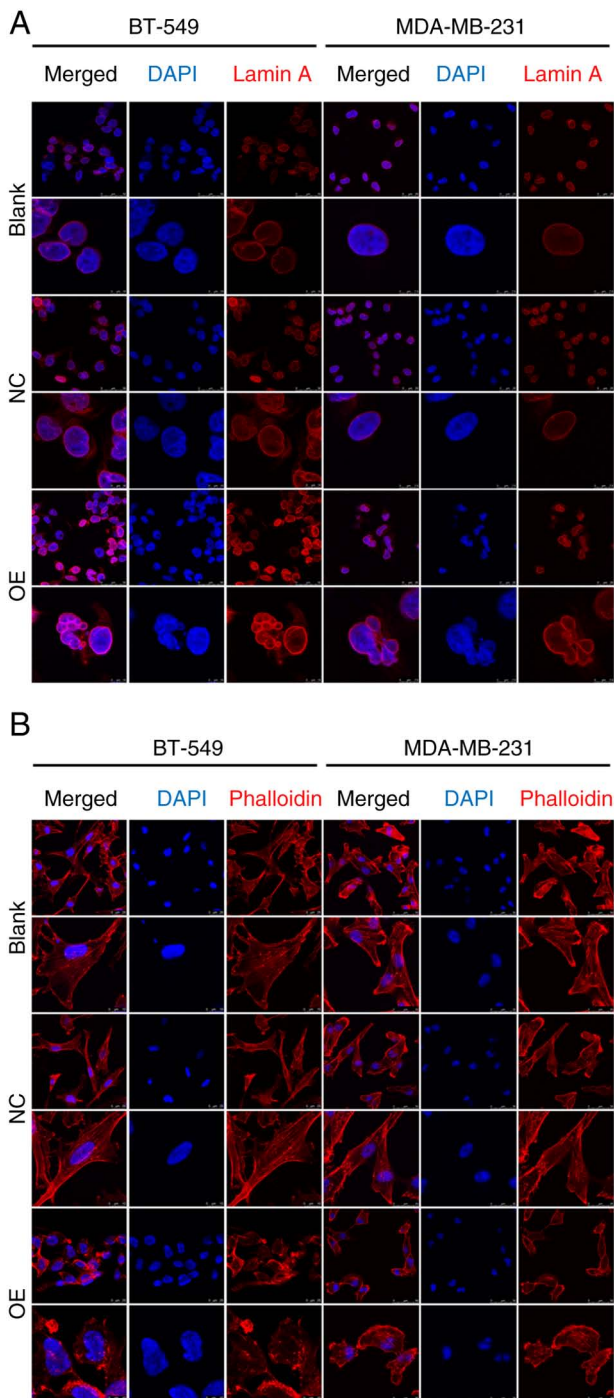


Figure 5. Cell immunofluorescence staining. (A) LaminA staining showed the structure of the nuclear lamina. (B) The F-actin cytoskeleton was stained with phalloidin. A scale bar is added to each micrograph and uniformly placed in the lower right corner. (A) Original magnification: 50 μm , Zoom in: 10 μm . (B) Original magnification: 25 μm , Zoom in: 10 μm . NC, negative control; OE, overexpression.

could give carcinoma cells the ability to initiate tumors (23), while also providing non-cancerous epithelial cells with the characteristics needed to migrate to metastatic locations and subsequently kickstart the formation of secondary tumors (24). Additionally, inhibiting EMT could effectively prevent the recurrence and distant metastasis of the primary tumor during cancer progression (25). Emerging evidence has suggested that carcinoma cells experiencing EMT can show increased

resilience against a range of currently available anti-cancer therapies (26,27). In addition, EMT blocking can also increase the sensitivity of cancer cells to standard cytotoxic therapy or immunotherapy (28-30). The aforementioned findings suggest that the combination of neoadjuvant chemotherapy with EMT inhibitors could diagnose TNBC effectively and early, thus reducing the probability of postoperative recurrence and metastasis and improve the prognosis of TNBC.

During EMT, cancer cells not only alter their adhesive properties, but also utilize developmental mechanisms to acquire migratory and invasive characteristics. This transformation entails a significant restructuring of the actin cytoskeleton, thus leading to the formation of membrane protrusions that are essential for their invasive growth (31). As a nucleoskeleton protein of the nuclear lamina, mature lamin A is required for the mechanical support of the nucleus and is considered as a significant component of the structural connection between the nucleoskeleton and the cytoskeleton. The abnormal accumulation of progerin in the inner nuclear membrane can lead to drastic changes in the nuclear structure, such as thickening of the nuclear fiber layer, increased nuclear hardness, irregular nuclear morphology (nuclear bubble) and impaired nuclear deformation ability, thus completely altering the nuclear mechanics and rearranging the entire skeleton of the cell (32). Most importantly, the present study identified a previously unreported role for progerin in TNBC: The reorganization of nuclear architecture and cytoskeleton. Progerin disrupts nuclear lamina integrity, directly impairing nuclear deformation capacity, a prerequisite for metastasis. This mechanical perturbation propagates to the actin cytoskeleton, reducing stress fiber density and invadopodia formation. This dual nuclear-cytoskeletal remodeling represents a TNBC-specific vulnerability, aligning with TNBC's unique dependence on mechanotransduction pathways for invasion. Although the findings demonstrated high target specificity, the potential for compensatory lamin A/C downregulation represents a biologically plausible off-target effect that requires consideration. Quantitative immunoblotting revealed no significant alterations in total lamin C levels, effectively mitigating this concern. A previous study by de la Rosa *et al* (33) indicated that the Zmpste24 silencing-mediated accumulation of prelamin A both *in vivo* and *in vitro* resulted in a significant reduction in its invasive potential. By analyzing genome-wide expression profiles, the results revealed that genes were mainly enriched in biological processes associated with 'extracellular matrix (ECM) synthesis' and 'interaction of ECM', as well as 'proteoglycan synthesis'. Similarly, in the present study, KEGG pathway enrichment analysis of the protein microarrays demonstrated that DEGs were mainly enriched in the terms 'endocytosis', 'N-proteoglycan synthesis' and 'nucleoplasmic transport pathways'. Obviously, these pathways were associated with the cytoskeleton, thus indicating that changes in the cytoskeleton could promote changes in several related signaling pathways. Furthermore, GO functional enrichment analysis showed that the cytoskeleton-related proteins, anillin and β -catenin, were also involved in the regulation of numerous related pathways. Experiments are being designed to investigate potential molecular interactions between progerin and core EMT regulatory proteins using co-immunoprecipitation followed by mass spectrometry analysis.

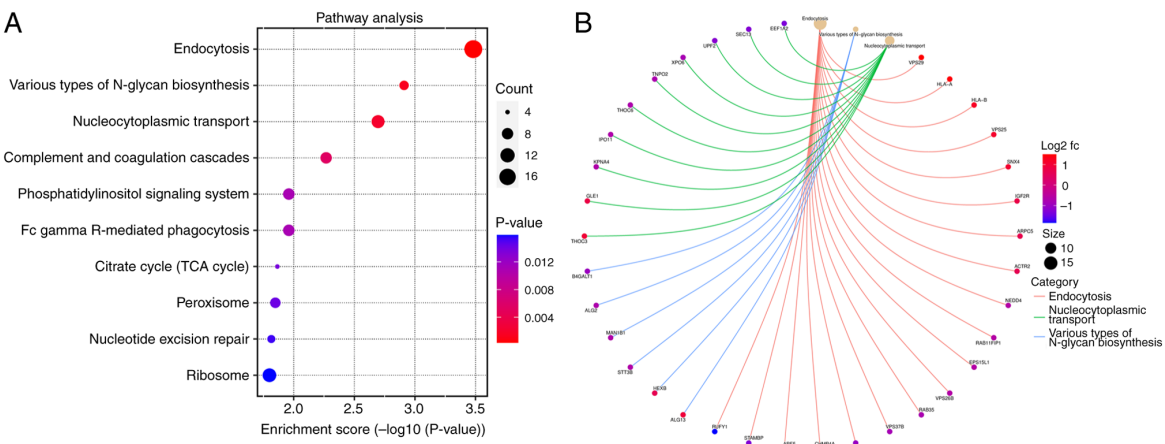


Figure 6. KEGG analysis results of A549 cells overexpressing progerin and control cells. (A) The enrichment of KEGG pathway analysis. (B) Key proteins of the top three pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes.

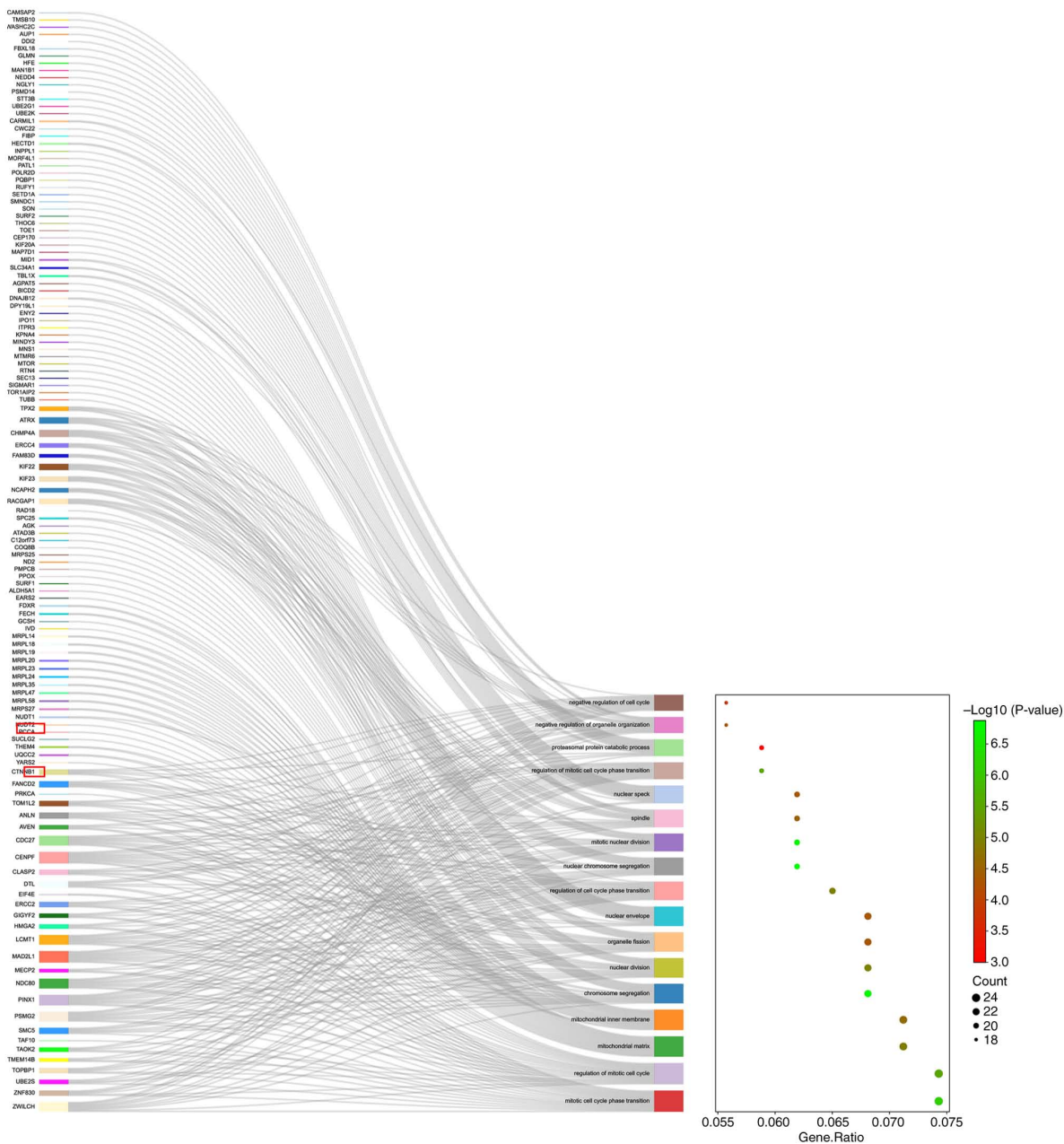


Figure 7. GO analysis results of A549 cells overexpressing progerin and control cells. GO, Gene Ontology.

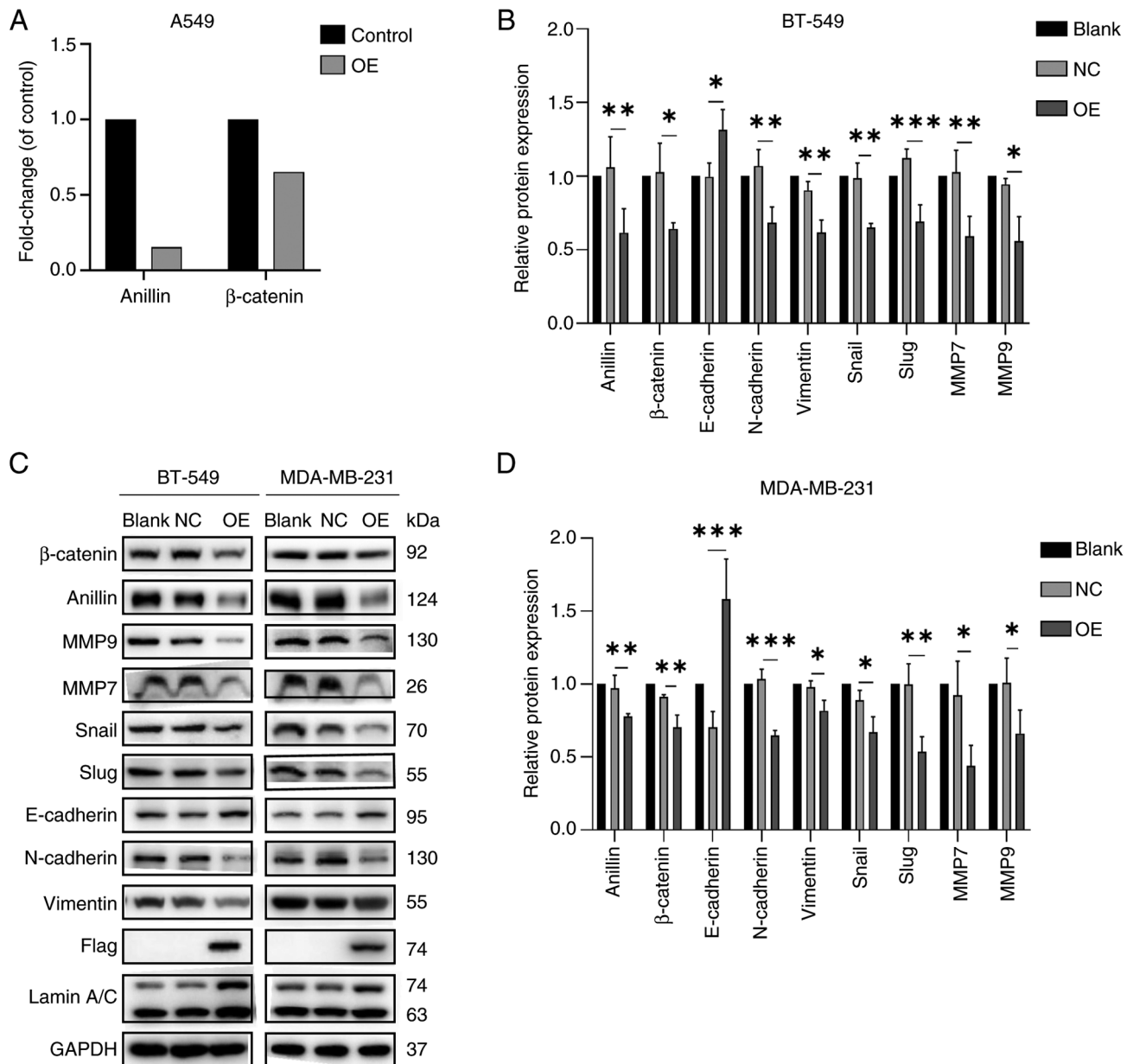


Figure 8. Underlying molecular mechanisms. (A) Fold change of Anillin and β-catenin in protein microarray of A549 cells overexpressing progerin and control cells. (C) Western blot analysis demonstrated the expression of Anillin (B and D) BT-549: $\eta^2=0.687$, MDA-MB-231: $\eta^2=0.844$, β-catenin BT-549: $\eta^2=0.773$, MDA-MB-231: $\eta^2=0.905$ and related pathway proteins. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Error bars as mean $\pm$ SEM. NC, negative control; OE, overexpression.

Anillin is an actin binding protein that can adjust the main cytoskeleton structure. It has been indicated that anillin regulates the assembly of actin cytoskeleton and the distribution of focal adhesion. In a previous study, loss of anillin, a significant factor driving collective migration and invasion, attenuated the motility of TNBC *in vivo* through MET (34). β-catenin regulates the establishment of E-cadherin-dependent cell-cell contacts via linking adherens junction proteins to the actin cytoskeleton. This processes occurs when β-catenin localizes to the cell membrane (35). A study revealed that AECHL-1 inhibited breast cancer metastasis via suppressing the Wnt/β-catenin pathway and the nuclear translocation of β-catenin (36). These findings propose that progerin-associated pathways may serve as candidate targets for clinical development in TNBC treatment.

While the present study provides novel insights into progerin's role in TNBC cytoskeletal regulation, it is important to note that these findings are based on *in vitro* experiments. Future studies employing xenograft models and clinical samples will be essential to validate these mechanisms *in vitro* and assess their therapeutic potential. Future directions for clinical translation may include: i) Developing nanoparticle-based delivery systems to achieve precise dose control of progerin, ii) screening small-molecule progerin modulators to establish pharmacological regulation systems and iii) conducting systematic *in vivo* pharmacokinetic studies to determine therapeutic windows. These strategies will overcome the 'all-or-none' expression limitations of lentiviral systems and accelerate clinical translation.

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Availability of data and material

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XH and WC developed the study's framework. XH, WLu and WLi performed the formal analysis and investigation. XH was responsible for drafting the initial manuscript. XH, XL and WC were involved in manuscript writing, reviewing and refining. XH, XL and WC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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