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Research article

Progesterone modulates cell growth via integrin αvβ3-dependent pathway in progesterone receptor-negative MDA-MB-231 cells

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ABSTRACT

Progesterone (P_4) plays a pivotal role in regulating the cancer progression of various types, including breast cancer, primarily through its interaction with the P_4 receptor (PR). In PR-negative breast cancer cells, P_4 appears to function in mediating cancer progression, such as cell growth. However, the mechanisms underlying the roles of P_4 in PR-negative breast cancer cells remain incompletely understood. This study aimed to investigate the effects of P_4 on cell proliferation, gene expression, and signal transduction in PR-negative MDA-MB-231 breast cancer cells. P_4-activated genes, associated with proliferation in breast cancer cells, exhibit a stimulating effect on cell growth in PR-negative MDA-MB-231 cells, while demonstrating an inhibitory impact in PR-positive MCF-7 cells. The use of arginine-glycine-aspartate (RGD) peptide successfully blocked P_4-induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation, aligning with computational models of P_4 binding to integrin αvβ3. Disrupting integrin αvβ3 binding with RGD peptide or anti-integrin αvβ3 antibody altered P_4-induced expression of proliferative genes and modified P_4-induced cell growth in breast cancer cells. In conclusion, integrin αvβ3 appears to mediate P_4-induced ERK1/2 signal pathway to regulate proliferation via alteration of proliferation-related gene expression in PR-negative breast cancer cells.

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1. Introduction

Breast cancer stands as one of the most frequently diagnosed cancers globally, with an estimated 2.3 million new cases reported worldwide [1]. Additionally, it ranks as the fifth leading cause of cancer-related deaths, responsible for over 0.6 million fatalities annually [2]. Alarming trends indicate that both the incidence and mortality rates of breast cancer have steadily risen over the past three decades. Current estimates suggest a further increase, with an anticipated 2.7 million new cases and 870,000 deaths globally each year by 2030 [3]. Triple-negative breast cancer (TNBC), constituting approximately 15 %–20 % of cases, presents as a distinct subtype characterized by adverse immunohistochemical reactions for estrogen receptor (ER), progesterone (P_4) receptor (PR), and human epidermal growth factor 2 [4,5]. Previous studies reported that recurrence and metastasis are the main reasons for TNBC mortality [6]. However, the intricate mechanisms underlying TNBC metastasis remain complex and poorly understood.

P_4, an ovarian sex steroid hormone synthesized in the placenta, ovaries, and adrenal glands, is crucial for breast development during puberty, primarily mediated through paracrine mechanisms [7–9]. It plays an essential role in the proliferation and differentiation of breast epithelial cells [10]. The physiological actions of P_4 are mediated by classical nuclear PR, which recruits accessory proteins through a ligand-receptor interaction mechanism [11]. P_4 binds with the nuclear PR in the cytosol, and this P_4/PR complex is subsequently translocated into the nucleus, activating target gene expressions [10,12]. In addition to classical signaling, P_4 can also activate non-classical signaling, such as growth receptor signaling pathways, to alter intracellular cyclic adenosine monophosphate (cAMP) levels and induce calcium/calmodulin-dependent protein kinase II activity [13,14]. P_4 often combines with estradiol, a type of estrogen, furthering the proliferation of mammary gland cells [7–9,15], and has the capability to activate the tyrosine kinase Src, the extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (MAPK) through interaction with the ER [16]. This unconventional interaction among steroid hormones plays a role in regulating the proliferation of both normal cells and potentially cancer cells.

P_4 has been reported to promote and maintain the growth of various types of cancers, including ovarian, uterine, glioblastomas, and breast cancers [17–19]. For example, P_4 and its metabolites, 5α-dihydroprogesterone and allopregnanolone, stimulate proliferation and migration in human glioblastoma-derived cell lines through activation of PR [19,20]. Under PR activation, the effects of proliferation and migration are related to Src activation and matrix metallopeptidase-9 (MMP-9) expression [21,22]. Allopregnanolone also induces cancer progression at low physiological concentrations but inhibits proliferation at high concentrations in breast cancer [20]. Thus, P_4 can regulate breast cancer cell progression. Epidemiological evidence suggests that exogenous synthetic progestins taken with estrogen as a menopausal hormonal treatment or contraceptive treatment increase the risk of breast cancer [23]. Stem cells are a potential source of breast cancer and can determine tumor phenotype. Breast cancer is thought to be caused, at least in part, by cancer stem cells (CSCs), which mimic the self-renewal and proliferation properties of normal stem cells and can confer drug resistance. P_4 has been recognized as a crucial hormone in governing the normal populations of mouse mammary stem cells, normal human mammary stem cells, and breast CSCs [24]. This regulation is associated with receptor activator of nuclear factor-κB ligand (RANKL) and its receptor (RANK), linking the pro-oncogenic role of P_4 in breast cancer. Additionally, it increases mammary CSCs in established breast cancer cell lines [24]. This increase is partly due to regulating transcription factors, signal transduction pathways, and microRNAs by P_4.

Integrin αvβ3, a cell-surface anchor protein, plays diverse roles in cell mobilization, anchoring, interactions with extracellular proteins, and signaling various cellular activities [25,26]. Its interaction with matrix proteins or ligands occurs through the arginine-glycine-aspartate (RGD) peptide binding domain [27]. Most hormones, such as P_4, androgen, estrogen, and thyroxine (T_4), have the potential to bind to the RGD peptide binding domain of the cell surface receptor. T_4, a kind of thyroid hormones, bind to integrin αvβ3, initiating downstream signal transduction pathways that stimulate cancer cell growth and metastasis [28–31]. In ERα-negative breast cancer MDA-MB-231 cells, integrin αvβ3 serves as a pivotal receptor in regulating cell proliferation under dihydrotestosterone (DHT) stimulation [32,33]. This proliferation is facilitated through the activation of ERK1/2 and phosphoinositide 3-kinase (PI3K) pathways [34–36]. Exploring the molecular pathways triggered by P_4 in TNBC for proliferation and metastasis is an urgent research frontier, crucial for advancing cancer therapies. Although the role of P_4 as a risk factor in the pathogenesis of TNBC remains controversial [16,37], targeting PR may not be applicable for therapeutic purposes in PR-negative TNBC. Thus, finding alternative receptors that P_4 could potentially bind to in PR-negative TNBC may serve as a promising new treatment strategy for the disease.

Although it has been previously reported that P_4 induces proliferation of breast cancer cells, the mechanism underlying this event is still unclear. In this study, we explored the mechanisms driving P_4-induced biological effects in a PR-negative breast cancer cell line through the binding of integrin αvβ3. Additionally, we examined whether changes in integrin αvβ3 signal transduction pathways altered P_4-induced growth. Gaining insights into the interplay between integrins and P_4 in TNBC could pave the way for advancing breast cancer research and identifying novel treatment avenues when hormonal therapies prove ineffective.

2. Materials and Methods

2.1. Cell culture and P_4 preparation

The human breast cancer cell lines, MCF-7 (HTB-22) and MDA-MB-231 (HTB-26) were obtained from the American Type Culture Collection (Manassas, VA, USA). These two cell lines were grown in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT, USA) and 1 % penicillin/streptomycin mixture

(Gibco-BRL, Grand Island, NY, USA), and maintained in a humidified atmosphere of 5 % CO_2 at 37 °C. P_4 (Cat. No.: SI–P8783, Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving it in absolute ethanol to a stock concentration of 1 mg/ml. Before adding P_4, cells were starved with a 0.25 % hormone-depleted serum-supplemented medium for 48 h. The starved-cells were refed with a 5 % hormone-depleted serum-supplemented medium prior to the experiment. All experiments were performed in triplicate and repeated three times.

2.2. Cell viability assay

For investigating the effect of P_4 on cell viability, MCF-7 and MDA-MB-231 cells were seeded in 96-well plates at a density of 3000 cells/well. After serum starvation, cells were stimulated with various concentrations of P_4 (10^{-8} to 10^{-5} M) in a 5 % hormone-stripped serum-containing medium. Both cell lines were separated into two groups: one with a 72-h treatment and the other with a 120-h treatment. Medium and reagents were refreshed every other day. For integrin αvβ3 interfering studies, MDA-MB-231 cells were treated with 10^{-5} M P_4 in the presence or absence of anti-integrin αvβ3 antibody (2 μg/ml, Cat. No.: sc-7312, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or the RGD peptide (500 nM, Cat. No.: HY-P0023, MedChemExpress, Monmouth Junction, NJ, USA) for 72 h. Cell viability was evaluated using the Cell Counting Kit-8 (Cat. No.: 96992, Sigma-Aldrich) according to the manufacturer's instructions.

2.3. Cell cycle analysis

MCF-7 and MDA-MB-231 cells were seeded at 6 cm dish with the number of 7.5×10^5 cells. After serum starvation, cells were treated with 10^{-5} M P_4 in the presence or absence of anti-integrin αvβ3 antibody (2 μg/ml) or RGD peptide (500 nM) for 72 h in a 5 % hormone-stripped serum-containing medium. Before staining with propidium iodide (PI), cells were trypsinized and washed by phosphate-buffered saline at room temperature, and then these cells were fixed and permeabilized with 70 % ethanol for 1 h at 4 °C. To quantify cellular DNA contents, cells were stained with PI/RNase Staining Buffer (BD Bioscience, San Jose, CA, USA) in the dark at room temperature for 30 min. Around 10000 cells were analyzed by flow cytometry on a BD FACS-Canto II flow cytometer equipped with BD FACSDivia software (BD Bioscience). Percentages of DNA contents were analyzed using FlowJo V.10.8.1 software to determine fractions in each phase of the cell cycle (G0/G1, S, and G2/M).

2.4. Reverse transcription real-time Polymerase Chain reaction (PCR)

MCF-7 and MDA-MB-231 cells were seeded at a density of 2×10^5 cells/well in 6-well plates. Serum-starved cells were stimulated with 10^{-7} M or 10^{-5} M of P_4 for 24 h in a 5 % hormone-stripped serum-containing medium. For integrin αvβ3 interfering studies, MDA-MB-231 cells were stimulated with 10^{-5} M P_4 in the presence or absence of anti-integrin αvβ3 antibody (2 μg/ml) or the RGD peptide (500 nM) for 24 h. Total RNA was extracted, and genomic DNA was removed with an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). DNase I-treated total RNA (1 μg) was reverse-transcribed using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) into complementary (c)DNA. cDNAs were used as the template for the real-time PCR and analysis. Real-time PCRs were conducted using a QuantiNovaTM SYBR® Green PCR Kit (Qiagen, Hilden, Germany) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences were as follows: *Homo sapiens integrin αv*, forward 5′-GAC TGT GGT GAA GAC AAT GTC TGT AAA CCC-3′ and reverse 5′-CCA GCT AAG AGT TGA GTT CCA GCC-3' (Accession No.: NM_001145000.3); *Homo sapiens integrin β3*, forward 5′-CTG GTG TTT ACC ACT GAT GCC AAG-3′ and reverse 5′-TGT TGA GGC AGG TGG CAT TGA AGG-3' (Accession No.: NM_000212.2); *Homo sapiens integrin β5*, forward 5′-AAC TCG CGG AGG AGA TGA G-3′ and reverse 5′-GGT GCC GTG TAG GAG AAA GG-3' (Accession No.: NM_002213.5); *Homo sapiens* cyclin D1 (*CCND1*), forward 5′-CAA GGC CTG AAC CTG AGG AG-3′ and reverse 5′-GAT CAC TCT GGA GAG GAA GCG-3' (Accession No.: NM_053056); *Homo sapiens* proliferating cell nuclear antigen (*PCNA*), forward 5′-TCTGAGGGCTTCGACACCTA-3′ and reverse 5′-TCA TTG CCG GCG CAT TTT AG-3' (Accession No.: BC062439.1); *Homo sapiens MMP-9*, forward 5′-TGT ACC GCT ATG GTT ACA CTC G-3′ and reverse 5′-GGC AGG GAC AGT TGC TTC T 3' (Accession No.: NM_004994.3); *Homo sapiens* cyclin-dependent kinase inhibitor 1A (*p21*), forward 5′-CTG GGG ATG TCC GTC AGA AC-3′ and reverse 5′-CAT TAG CGC ATC ACA GTC GC-3' (Accession No.: NM_000389.5); *Homo sapiens* programmed death ligand 1 (*PD-L1*), forward: 5′-GTT GAA GGA CCA GCT CTC CC-3′ and reverse 5′-ACC CCT GCA TCC TGC AAT TT-3' (Accession No.: NM_014143.4); and *Homo sapiens β-actin*, forward 5′-CGG CGC CCT ATA AAA CCC A-3′ and reverse 5′-ATC ATC CAT GGT GAG CTG GC-3′(Accession No.: NM_001101.5). Calculations of relative gene expressions (normalized to the *β-actin* reference gene) were performed according to the ΔΔCT method. The fidelity of the PCR was determined with a melting temperature analysis.

2.5. Confocal microscopy

MDA-MB-231 cells were seeded on sterilized cover glasses (Paul Marienfeld, Lauda-Königshofen, Germany). Serum-starved cells were stimulated with 10^{-5} M P_4 in the presence or absence of a 500 nM RGD for another 24 h in a 10 % hormone-stripped serum-containing medium. Cells were immediately fixed with 4 % paraformaldehyde in Tris-buffered saline (TBS) for 10 min and then permeabilized in 0.1 % Triton X-100 in TBS for 20 min. After 1 h of 1 % bovine serum albumin blocking, cells on the slides were incubated with an anti-integrin αvβ3 antibody (Cat. No.: GTX111672, GeneTex, Hsinchu City, Taiwan) or anti-phosphorylated ERK1/2 (T202/Y204) antibody (Cat. No.: 4377, Cell Signaling, Beverly, MA, USA) overnight at 4 °C. Then, cells were incubated with secondary antibodies conjugated with Alexa Fluor 647 (Cat. No.: ab150079, Abcam, Cambridge, MA, USA), Alexa Fluor 488 (Cat. No.:

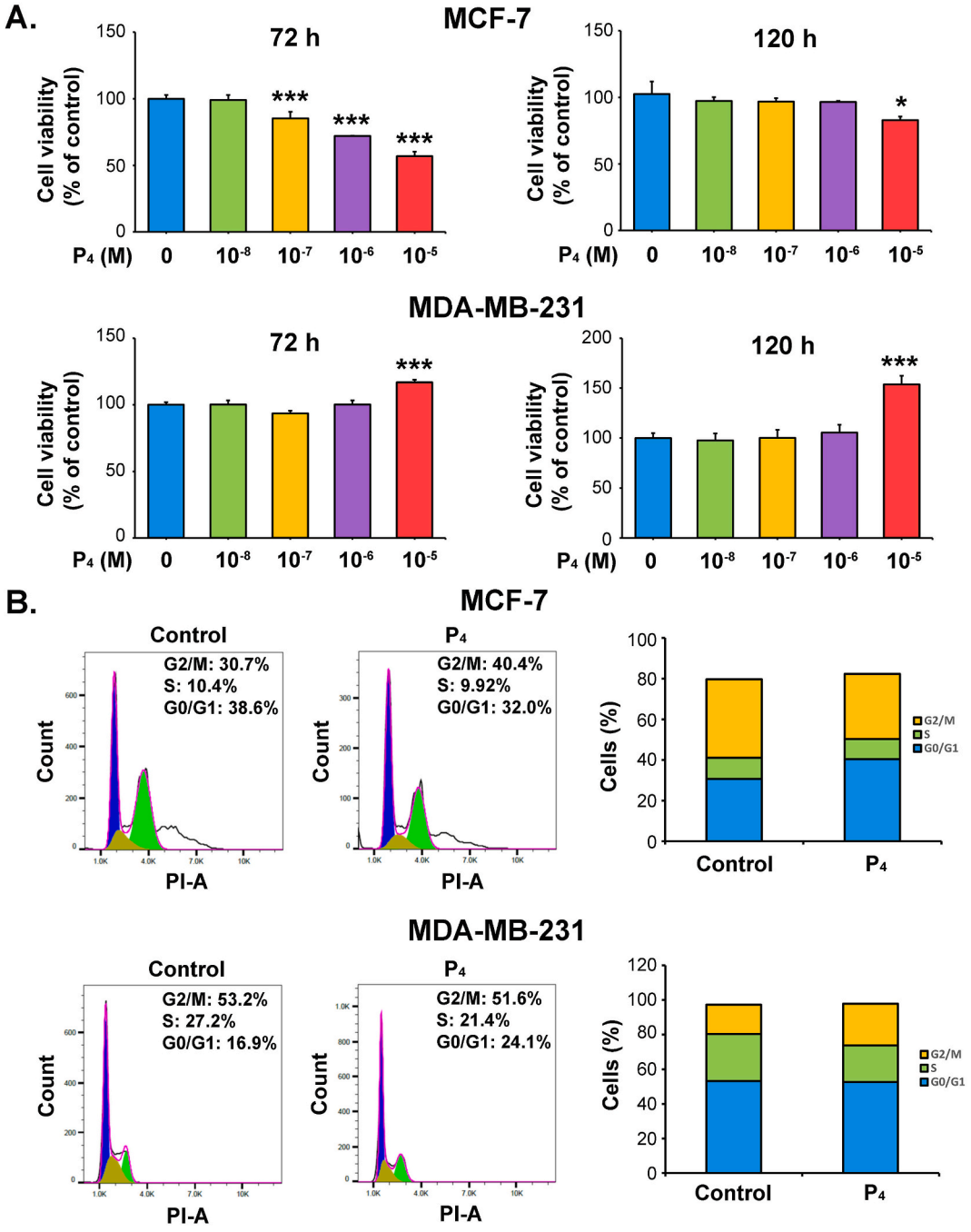

Fig. 1. P_4 induces different growth patterns in PR-positive MCF-7 and PR-negative MDA-MB-231 breast cancer cells. (A) Serum-starved cells were left unstimulated or stimulated with different concentrations of P_4 (10^{-8} to 10^{-5} M) for 72 h or 120 h. The cells were then subjected to the cell viability assay. Data are represented normalized to the unstimulated group of each cell line and presented as the mean ± standard deviation of triplicate cultures in three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the unstimulated group. (B) Serum-starved cells were left unstimulated or stimulated with 10^{-5} M P_4 for 72 h. The cells were then subjected to the flow cytometric analysis. The presented histograms and bar graphs show the percentage of cell populations in each cell cycle phase, as measured by DNA content stained with PI. The blue area represents the G0/G1 fraction, the dark yellow area represents the S fraction, and the green area represents the G2/M fraction. Similar results were obtained in three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

GTX213110-04, GeneTex), or Alexa Fluor 594 (Cat. No.: GTX213111-05, GeneTex), and stained with DAPI (Cat. No.: S36938, Thermo Fisher Scientific) for nuclei. The fluorescent signals of integrin αvβ3 antibody or *p*-ERK1/2 were recorded and analyzed with a TCS SP5 Confocal Spectral Microscope Imaging System (Leica Microsystems, Wetzlar, Germany).

2.6. Molecular docking

The docked protocol was according to a previous report [38]. Briefly, the crystal structure of integrin αvβ3 was obtained from the

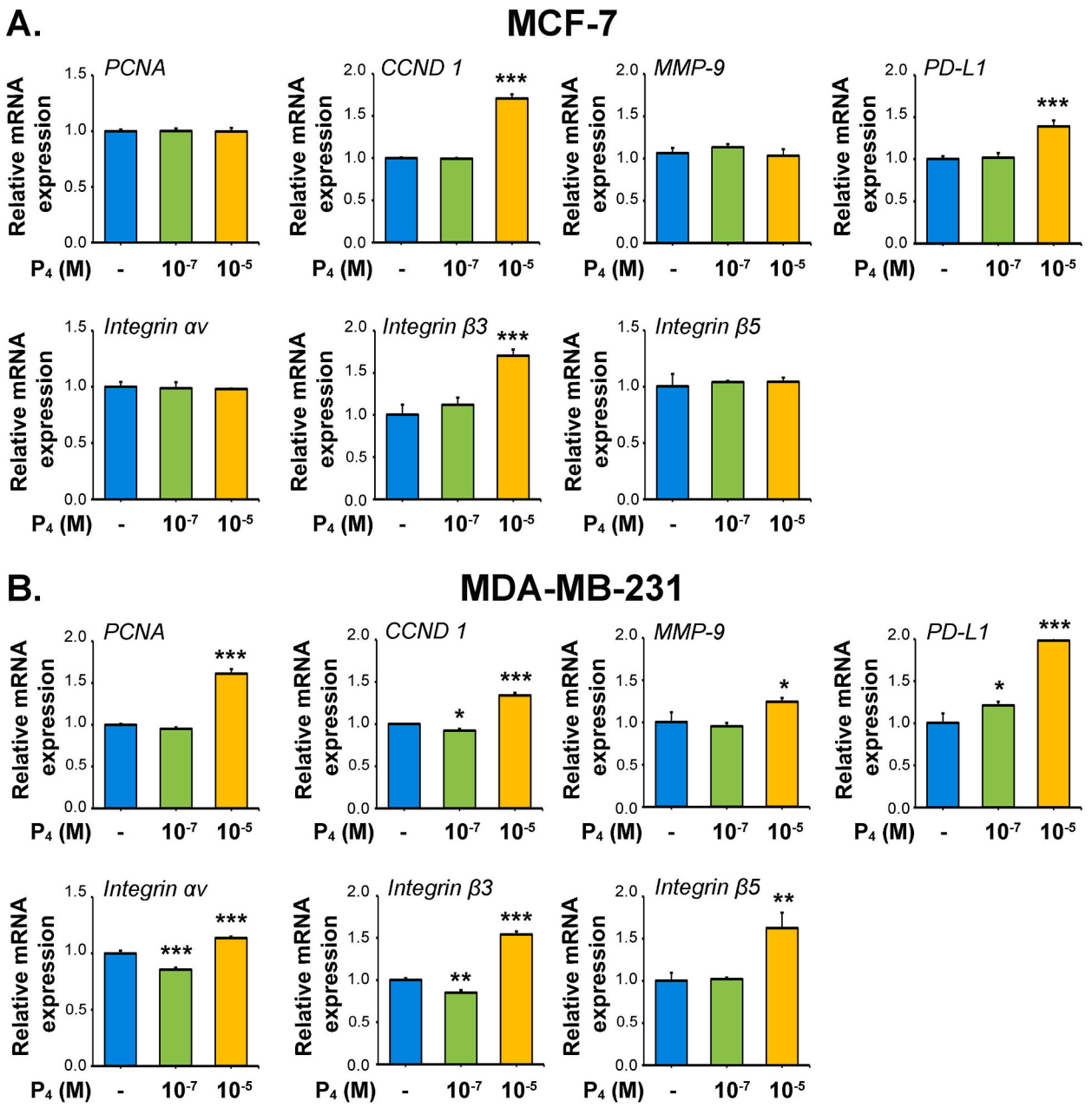

Fig. 2. P_4 regulates gene expression in PR-positive MCF-7 and PR-negative MDA-MB-231 breast cancer cells. (A, B) Serum-starved cells were left unstimulated (**−**) or stimulated with different concentrations of P_4 (10^{-7} and 10^{-5} M) for 24 h. The cells were lysed and the mRNAs extracted from cell lysates were subjected to the reverse transcription reaction. The mRNA expression of *PCNA*, *CCND1*, *MMP-9*, *PD-L1*, *integrin αv*, *integrin β3*, *integrin β5*, and *β-actin*, as a loading control, was quantified by qRT-PCR. The mRNA expression of these genes was normalized to that of *β-actin*. The quantitative values were expressed as relative mRNA levels by defining the amounts of gene expression in unstimulated group as 1. Data are represented as the mean ± standard deviation of triplicate cultures in three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the unstimulated group.

Protein Data Bank (PDB ID: 1L5G) [38]. The chemical structures of cyclic RGD and P_4 were drawn by ChemBio3D ultra 12.0. Molecules were docked with integrin protein using AutoDock Vina [39]. The grid box parameters were built for integrin αvβ3 (center: x = 16, y = 43, z = 47; size: x × y × z = 45 × 45 × 45). The binding free energies (ΔG, kcal/mol) were calculated for each protein-ligand binding affinities. The docking results were visualized using PyMOL and analyzed by BIOVIA Discovery Studio Visualizer to show protein-ligand interactions.

2.7. Western blotting analysis

The Western blot analysis were conducted as described in the previous studies [30,32]. Serum-starved MCF-7 or MDA-MB-231 cells were stimulated with different concentrations of P_4 in the presence or absence of anti-integrin αvβ3 antibody (2 μg/ml) or RGD peptide (500 nM) for 72 h. Cells were harvested, and total proteins were extracted. The primary antibodies of integrin β3 (Cat. No.: GTX111672, GeneTex), integrin β5 (Cat. No.: sc-6627, Santa Cruz Biotechnology), focal adhesion kinase (FAK) (Cat. No.: 3629, Cell signaling), *p*-FAK (Y397) (Cat. No.: 8556, Cell signaling), ERK1/2 (Cat. No.: 9102, Cell signaling), *p*-ERK1/2 (T202/Y204) (Cat. No.: 4377, Cell signaling), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. No.: GTX100118, GeneTex) were incubated with membranes overnight at 4 °C. Proteins were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies and detected using an Immobilon Western HRP Substrate Luminol Reagent (Millipore, Burlington, MA, USA). The bands were imaged and recorded with the Amersham Imager 600 system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The densitometric analysis was performed using ImageJ 1.47 software (National Institutes of Health, Bethesda, MD, USA).

2.8. Statistical analysis

Collected data were analyzed by IBM®SPSS® Statistics software version 19.0 (SPSS Inc., Chicago, IL, USA). A Two-tails Student's *t*-test was conducted, and results were considered significant at $P < 0.05$ (* or $^{\#}$), $P < 0.01$ (** or $^{\#\#}$), and $P < 0.001$ (*** or $^{\#\#\#}$).

3. Results

3.1. P_4 induces differential growth patterns in PR-positive and PR-negative breast cancer cells

Steroid hormones induce biological activities via classical or non-classical signaling in specific hormone receptor-positive or -negative cells, respectively [40,41]. The growth effect of P_4 on PR-positive breast cancer MCF-7 cells differs from those on PR-negative breast cancer MDA-MB-231 cells [42,43]. To confirm the action of P_4 on the viability of MCF-7 and MDA-MB-231 cells, three concentrations of P_4 (10^{-7}, 10^{-6}, and 10^{-5} M) were stimulated with these two cell lines for 72 h and 120 h. The viability of MCF-7 cells decreased in a dose-dependent manner after 72 h of P_4 stimulation ranging from 10^{-7} to 10^{-5} M (Fig. 1A). Notably, at a P_4 concentration of 10^{-5} M, the viability of MCF-7 cells was reduced by 43 % compared to the control group (Fig. 1A). However, when stimulating MCF-7 cells with the same concentration of P_4 for 120 h, the cell viability exhibited a 20 % reduction (Fig. 1A). Within the PR-negative MDA-MB-231 cells, treatment with 10^{-5} M P_4 significantly stimulated cell viability over 72- and 120-h periods, whereas concentrations of 10^{-6}, 10^{-7}, and 10^{-8} M P_4 did not produce a noticeable effect (Fig. 1A). At a P_4 concentration of 10^{-5} M, there was a notable suppression of MCF-7 cell growth and a significant stimulation of MDA-MB-231 cell growth (Fig. 1A). To further investigate whether P_4 affects cell growth through regulation of the cell cycle in breast cancer cells, MCF-7 and MDA-MB-231 cells were stimulated with 10^{-5} M P_4 for 72 h. In MCF-7 cells, P_4 decreased the proportion of cells in the G2/M phase, whereas in MDA-MB-231 cells, it increased the proportion of cells in this phase (Fig. 1B). These results indicate that P_4 has distinct effects on cell growth in PR-positive and PR-negative breast cancer cells. To further explore the distinct growth effects of P_4 on PR-positive and PR-negative breast cancer cells, particularly related to the differential expression of proliferation-related genes *PCNA*, *CCND1*, *MMP-9*, and *PD-L1*, MCF-7 and MDA-MB-231 cells were stimulated with 10^{-7} and 10^{-5} M P_4 for 24 h. In MCF-7 cells, the higher concentration (10^{-5} M) of P_4 increased *PD-L1* and *CCND1* expression by 1.4- and 1.5-fold, respectively, while *PCNA* and *MMP-9* expression remained unchanged (Fig. 2A). Conversely, in PR-negative MDA-MB-231 cells, this concentration notably elevated the expression of all four proliferation-related genes (Fig. 2B). Moreover, even at the lower concentration (10^{-7} M) of P_4, a considerable induction of *PD-L1* expression was observed (Fig. 2B). Steroid hormones, such as estrogen and androgen, are recognized for their role in regulating breast cancer progression through the non-classical receptor integrin αvβ3 [41,44,45]. Considering this, P_4 could potentially have a similar impact on breast cancer growth. In MCF-7 cells stimulated with 10^{-5} M P_4, only *integrin β3* expression increased (Fig. 2A). Notably, in MDA-MB-231 cells stimulated with a lower concentration of P_4 (10^{-7} M), both *integrin αv* and *integrin β3* gene expressions were elevated (Fig. 2B). These results indicate that P_4 regulates gene expressions of integrin αvβ3, suggesting its involvement in the signaling pathway mediated by P_4 in PR-negative breast cancer cells.

3.2. P_4 interacts with the RGD binding site of integrin αvβ3

Despite the absence of nuclear PR in TNBC cells, P_4 exhibited an impact on cell growth. As indicated in Fig. 2B, the upregulation of *integrin αvβ3* expression induced by P_4 prompts further investigation into whether integrin αvβ3 serves as the non-classical cell surface receptor for P_4 binding and participates in P_4-induced signal transduction and subsequent biological activity. Integrin αvβ3 elicits signaling transduction via its RGD binding site once ligand binding. Earlier studies have indicated that this binding site accommodates compounds like heteronemin and derivatives of thyroid hormones [27,46]. Based on these findings, there is a suggestion that P_4 might

also have compatibility within the RGD binding site of integrin αvβ3. To figure out the interaction between P_4 and the RGD binding site of integrin αvβ3, molecular docking was employed. The analysis revealed two distinct binding models for P_4. Model 1 exhibited a lower binding free energy (−6.7 kcal/mol) (Fig. 3A) compared to docking model 2 (−6.5 kcal/mol) (Fig. 3B). This suggests a higher affinity of model 1 (depicted in blue stick) for integrin αvβ3 receptor relative to model 2 (depicted in white stick). Both models, despite their different orientations, occupied the same region below the cyclic RGD (cRGD) molecule (purple sticks) in integrin αvβ3 (Fig. 3C). In model 1, a metal interaction between the C3-carbonyl group of P_4 and Mg^{2+} was observed (Fig. 3D). Furthermore, the C20-carbonyl group of P_4 in model 1 formed a hydrogen bond with ARG248 of the αv subunit. However, model 2 exhibited no significant interactions, only some alkyl interactions within the P_4 steroid backbone (Fig. 3E). These findings suggest a specific binding affinity of P_4 to integrin αvβ3 and provide insights into potential interaction sites crucial for future studies.

3.3. *Integrin αvβ3 is involved in P_4-induced signal transduction in PR-negative breast cancer cells*

To confirm whether P_4 affects the protein expression of integrin β3, MCF-7 and MDA-MB-231 cells were stimulated with various concentrations of P_4 for 120 h. No obvious band was detected by anti-integrin β3 antibody in MCF-7 cells (Fig. S1). Stimulating this cell with 10^{-5} M P_4, the expression of integrin β5 was decreased (Fig. S1). In MDA-MB-231 cells, an induction of integrin β3, rather than integrin β5, was observed following stimulation with 10^{-5} M P_4. (Fig. S1). These results shows that P_4 induces the protein expression of integrin β3 only in PR-negative MDA-MB-231 cells. To investigate the involvement of integrin αvβ3, serving as a non-classical receptor for P_4, in mediating the impacts of P_4 on the protein expression of integrin β3 and the gene expression of *integrin αv*, *integrin β3*, and *integrin β5*, MDA-MB-231 cells were treated separately with an anti-integrin αvβ3 blocking antibody and the RGD peptide, which inhibits integrin-ligand interactions, for 72 h. Although both the blocking antibody and the RGD peptide treatments significantly downregulate the protein expression of integrin β3, with no observable effect on the protein expression of integrin β5, there was no obvious reduction in the P_4-induced gene expressions of *integrin αv*, *integrin β3*, and *integrin β5* (Fig. 4; Fig. 5A–C). Additionally,

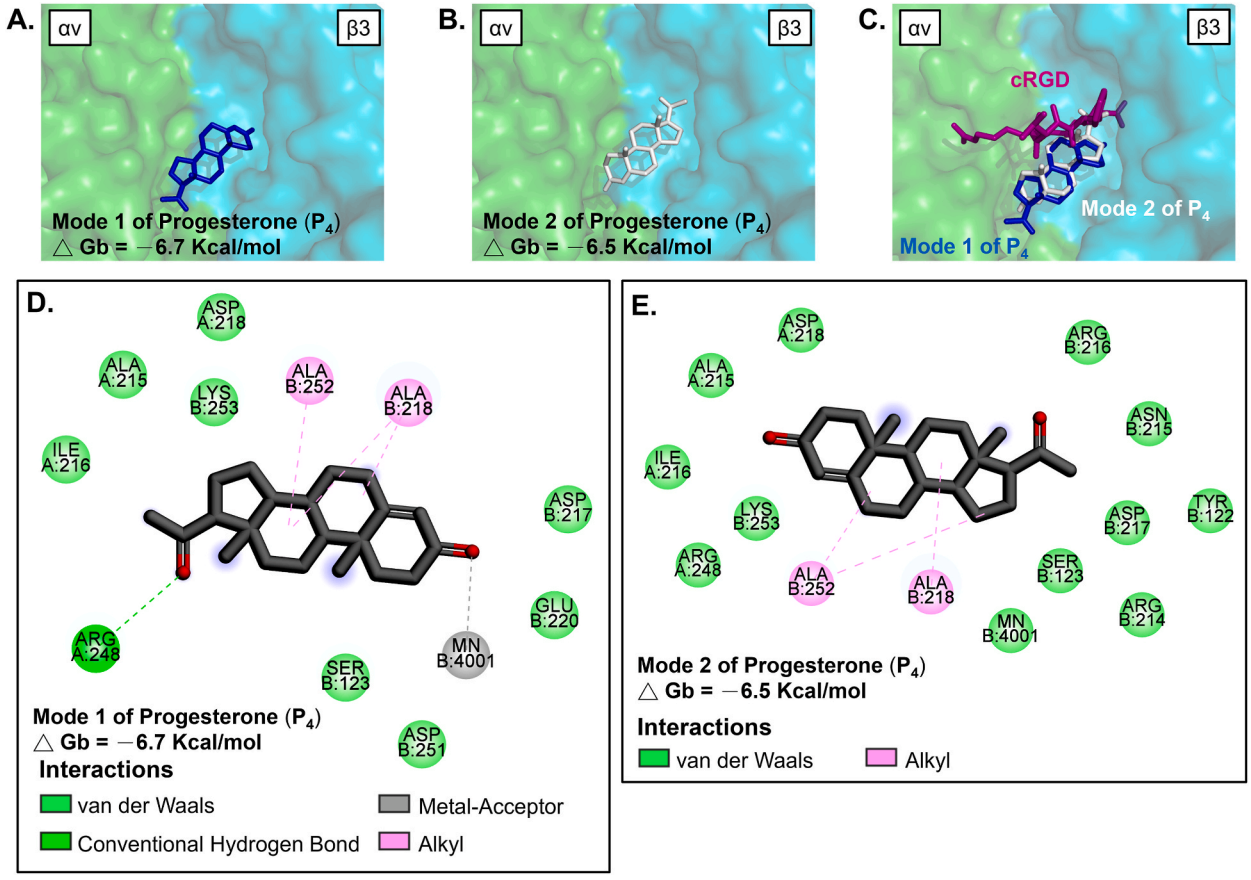

Fig. 3. Predicted docking poses of P_4 bound at the cRGD-binding site of integrin αvβ3. (A, B) Docking models 1 and models 2 of P_4 are respectively colored in blue and white, and the free binding energies are anticipated to be −6.7 -and −6.5 kcal/mol, respectively. (C) Superimpositions of binding models for modes 1 (blue) and 2 (white) mapped into cRGD peptide (purple) of αvβ3 integrin subunits. (D, E) Binding mode 1 and mode 2 of P_4 are illustrated within integrin αvβ3 and their corresponding 2D interaction plots by the BIOVIA Discovery Studio Visualizer (http://accelrys.com). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

blocking the RGD binding site of integrin αvβ3 also suppressed the P_4-induced proliferation-related genes *CCND1*, *p21*, and *PCNA* (Fig. 5D–F). These observations indicate that integrin αvβ3, rather than integrin αvβ5, mediates the effects of P_4 on integrin β3 protein expression and the associated regulation of proliferation-related genes in PR-negative MDA-MB-231 cells.

ERK1/2 plays an important role in transducing P_4-induced signaling [47]. Upon P_4 stimulation, the translocation of phosphorylated ERK1/2 from the cytoplasm to the nucleus regulates downstream gene expression [48]. To further examine integrin αvβ3 is the non-classical receptor for P_4 to elicit downstream signaling pathway, P_4-stimulated MDA-MB-231 cells were treated with or without the RGD peptide for 24 h. The differential expression of integrin αvβ3 was detected on the cell surface or within the cytoplasm of MDA-MB-231 cells (Fig. 6A and B, Integrin αvβ3 panel). P_4 stimulation did induce nuclear translocation of *p*-ERK1/2 compared to the control group (Fig. 6A and B, *p*-ERK1/2 panel; Fig. 6C). Notably, treatment with the RGD peptide significantly suppressed P_4-induced expression of *p*-ERK1/2 and the accumulation of nuclear *p*-ERK1/2 (Fig. 6A and B, *p*-ERK1/2 panel; Fig. 6C). These findings suggest a dependency of P_4-induced nuclear accumulation of *p*-ERK1/2 on integrin αvβ3 in MDA-MB-231 cells.

In our previous studies, activation of FAK mainly regulates integrin αvβ3-mediated cell proliferation through ERK1/2 signaling pathway [49]. We further investigated whether blocking P_4 binding to integrin αvβ3 affected FAK activity in MDA-MB-231 cells. An anti-integrin αvβ3 blocking antibody and the RGD peptide were used to treat these cells with or without P_4 stimulation for 24 h. P_4 significantly increased phosphorylation of FAK and ERK1/2 without altering their total protein levels (Fig. 7). Treatment with either anti-integrin αvβ3 antibody or the RGD peptide markedly reduced P_4-induced phosphorylation of FAK and ERK1/2 (Fig. 7). Notably, specific inhibition of integrin αvβ3 function suppressed FAK activity more than ERK1/2, suggesting FAK as the primary effector of integrin αvβ3 rather than other integrin types.

3.4. *Integrin αvβ3 affects P_4-induced cell proliferation in PR-negative breast cancer cells*

To examine whether integrin αvβ3 is involved in P_4-induced cell viability, P_4-stimulated MDA-MB-231 cells were treated with an anti-integrin αvβ3 blocking antibody and the RGD peptide for 72 h. The treatment of the RGD peptide significantly inhibited P_4-induced cell viability within 72 h of treatment (Fig. 8A). However, the anti-integrin αvβ3 antibody stimulated cell viability by 1.5-fold compared to the control. After P_4 stimulation, antibody-treated cells also showed significantly higher cell viability than P_4-stimulated cells (Fig. 8A). Furthermore, we examined the cell cycle profiles of P_4-stimulated MDA-MB-231 cells under treatment with an anti-

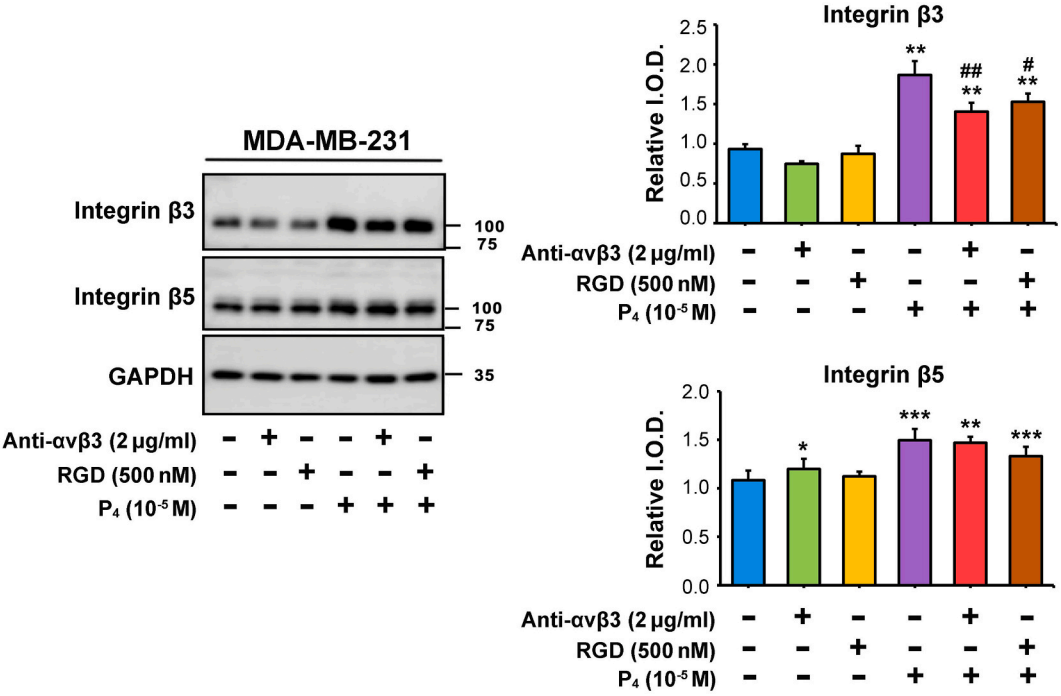

Fig. 4. Blocking of integrin αvβ3 activity down-regulates P_4-induced integrin β3 expression in MDA-MB-231 cells. Serum-starved cells were left unpretreated or pretreated with anti-integrin αvβ3 antibody (2 μg/ml) or the RGD peptide (500 nM) for 1 h and then were left unstimulated or stimulated with10^{-5} M P_4 for 72 h. The cells were then lysed and cell lysates were subjected to Western blotting for the detection of the indicated integrin β3, integrin β5, and GAPDH, as a loading control (These original blot images are provided in the Supplementary file). Similar results were obtained in three independent experiments. The quantitative results were expressed as fold increase by defining the amounts of the indicated detected proteins in untreated cells, where the absence of P_4 stimulation was considered as 1. Data are represented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells, where the absence of P_4 stimulation; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the P_4-stimulated unpretreated cells.

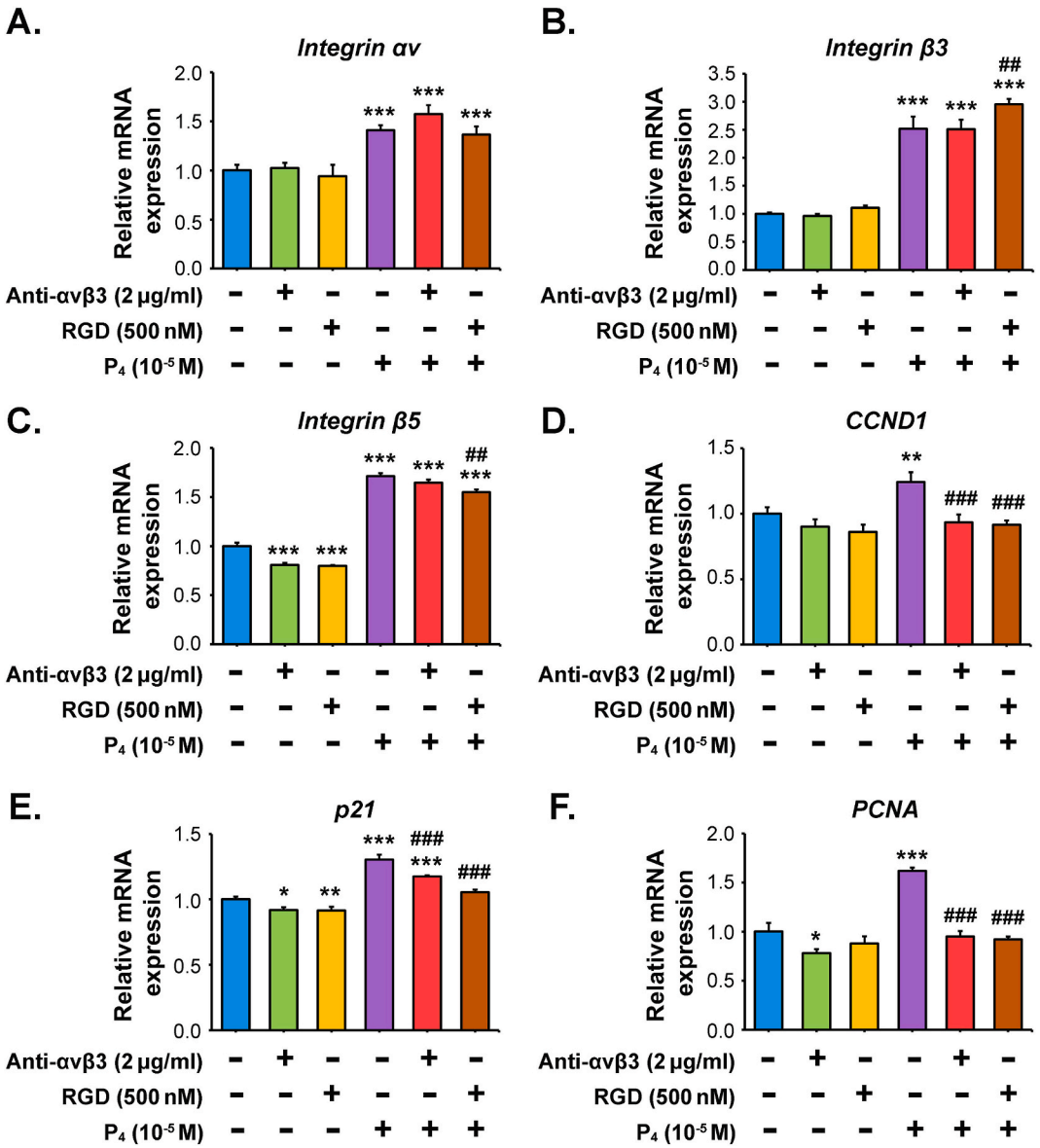

Fig. 5. Blocking of Integrin αvβ3 activity affects P_4-induced gene expressions in MDA-MB-231 cells. (A–F) Serum-starved cells were left unpretreated or pretreated with anti-integrin αvβ3 antibody (2 μg/ml) or the RGD peptide (500 nM) for 1 h and then were left unstimulated or stimulated with10^{-5} M P_4 for 72 h. The cells were lysed and the mRNAs extracted from cell lysates were subjected to the reverse transcription reaction. The mRNA expression of *integrin αv*, *integrin β3*, *integrin β5*, *CCND1*, *p21*, *PCNA*, and *β-actin*, as a loading control, was quantified by qRT-PCR. The mRNA expression of these genes was normalized to that of *β-actin*. The quantitative values were expressed as relative mRNA levels by defining the amounts of gene expression in untreated cells, where the absence of P_4 stimulation as 1. Data are represented as the mean ± standard deviation of triplicate cultures in three-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells, where the absence of P_4 stimulation; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the P_4-stimulated unpretreated cells.

integrin αvβ3 blocking antibody and the RGD peptide. Consistent with the cell viability results from the Cell Counting Kit-8 assay, inhibiting integrin αvβ3 activity reduced the proportion of P_4-stimulated cells in the G2/M phase (Fig. 8B). These data suggest that integrin αvβ3 plays a role in regulating the proliferation of PR-negative breast cancer cells induced by P_4.

4. Discussion

Our study reveals the impact of P_4 on both PR-positive (MCF-7) and PR-negative (MDA-MB-231) breast cancer cells. P_4 exhibits distinct effects on cell viability and the expression of proliferation-related genes in these cells. It reduces viability and selectively modulates the expressions of only *CCND1* and *PD-L1* genes in MCF-7 cells, while notably stimulating growth and upregulating the

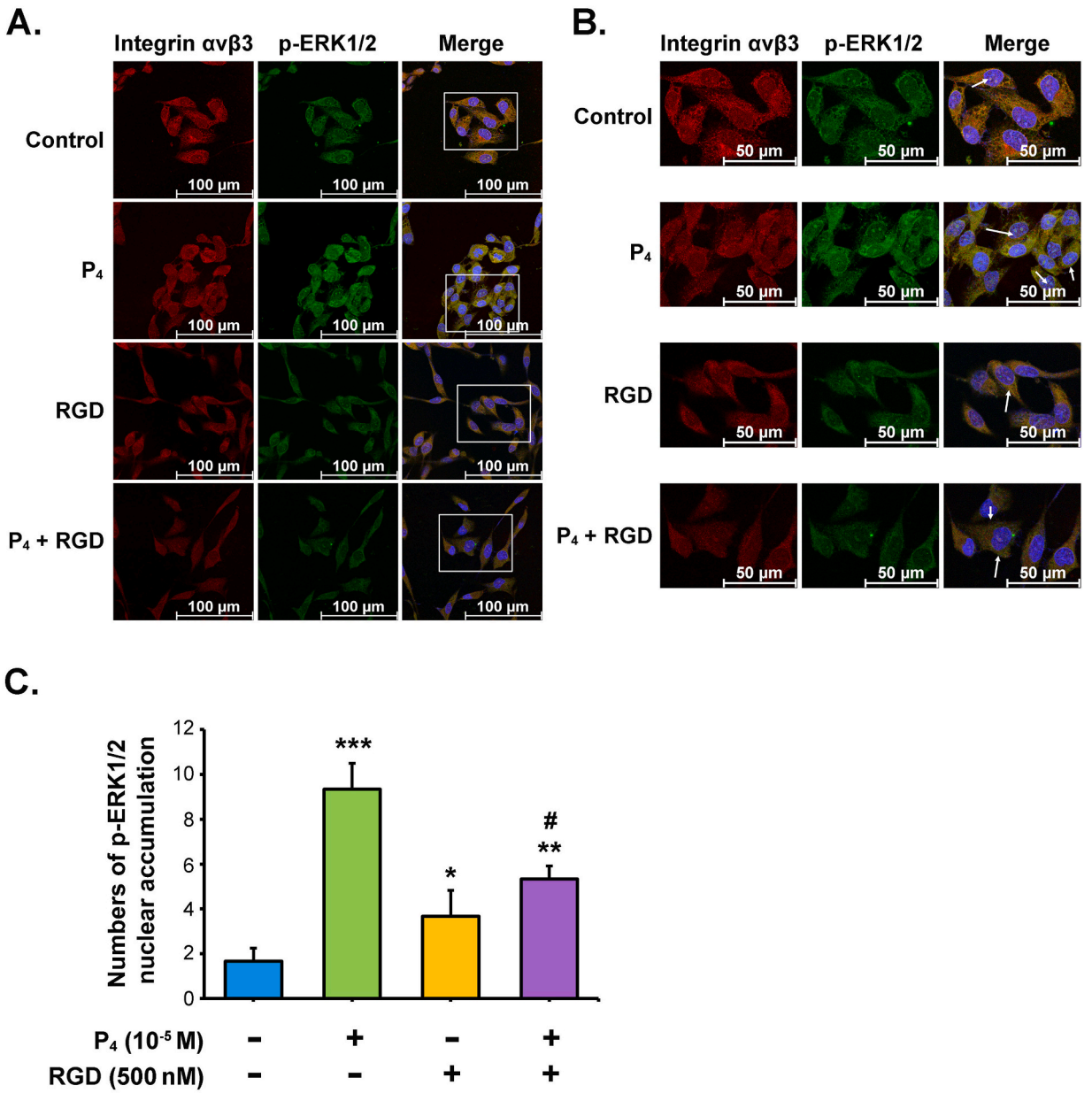

Fig. 6. P_4-induced ERK1/2 activation is integrin αvβ3-dependent in MDA-MB-231 breast cancer cells. (A, B) Cells were seeded on a cover glass and starved for 48 h. Different combinations of treatment were described in the Confocal microscopy section of Materials and Methods. Cells then were fixed for confocal microscopy. The cells were fixed, permeabilized and immunostained with antibodies against integrin αvβ3 (red color) and *p*-ERK1/2 (green color). The merge image shows colocalization (yellow color) of these two proteins. Nuclei were counterstained with DAPI (blue color). Accumulation of *p*-ERK1/2 in the nucleus was showed as white arrows. The right panel (B) shows a zoom-in image of the left panel (A) to present cells in a more focused manner. (C) Quantification of the number of *p*-ERK1/2 accumulation in the nucleus. Data are represented as the mean ± standard deviation of triplicate cultures in three-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells, where the absence of P_4 stimulation; $^{\#}P < 0.01$ compared to the P_4-stimulated unpretreated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expressions of *PCNA*, *CCND1*, *MMP-9*, and *PD-L1* genes in MDA-MB-231 cells. The study delves into the interaction between P_4 and integrin αvβ3, suggesting its role as a non-classical receptor in PR-negative breast cancer cells. This interaction finds support in molecular docking, alterations in protein expression, and the investigation of signaling pathways, particularly ERK1/2 activation and FAK activity. Notably, integrin αvβ3 emerges as pivotal in mediating P_4-induced cell proliferation in PR-negative breast cancer, highlighting its potential as a therapeutic target.

Distinct effects of P_4-regulated growth emerged in PR-positive and PR-negative breast cancer cells (Figs. 1 and 2). At a

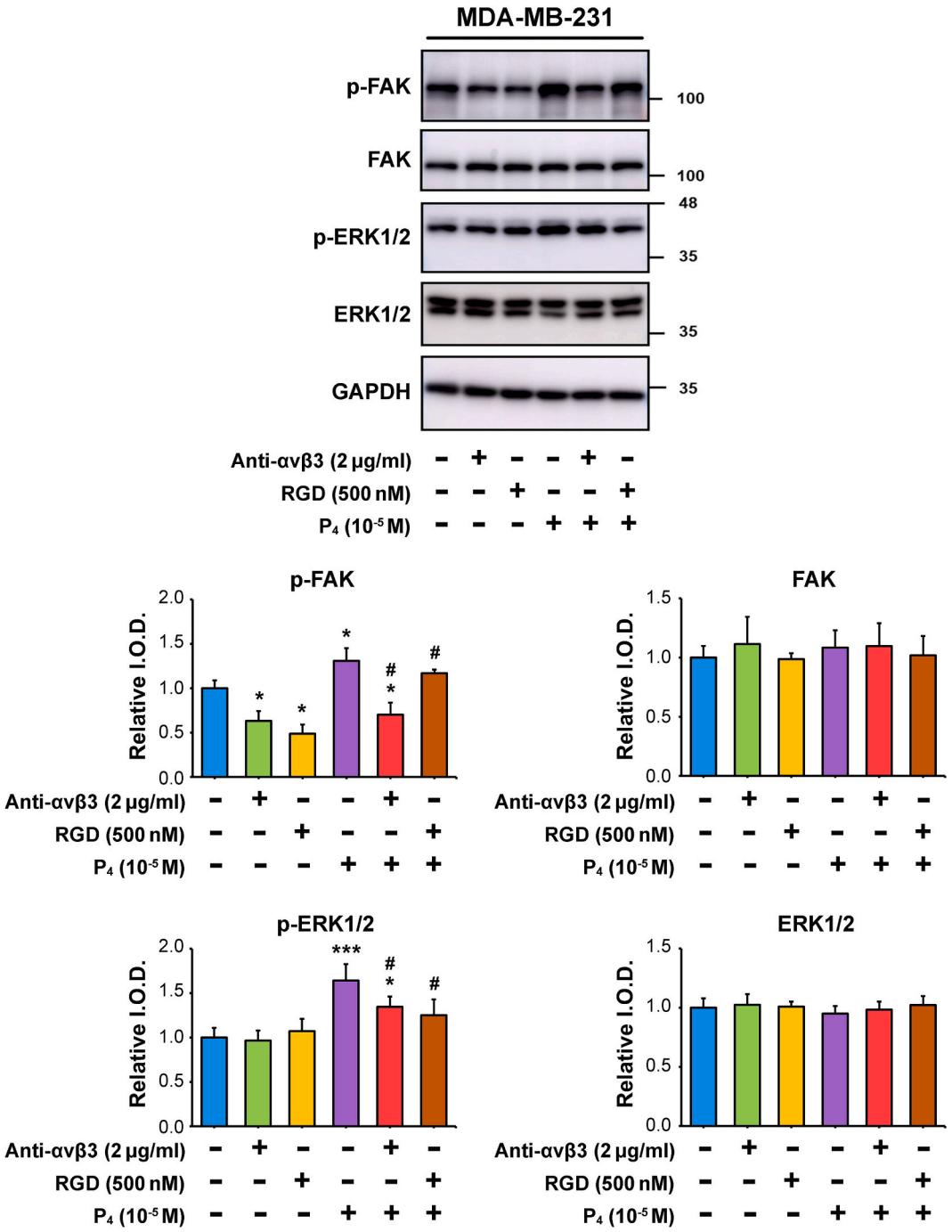

Fig. 7. P_4 regulates signal transduction protein profiles in MDA-MB-231 cells. Serum-starved cells were left unpretreated or pretreated with anti-integrin αvβ3 antibody (2 μg/ml) or the RGD peptide (500 nM) for 1 h and then were left unstimulated or stimulated with 10^{-5} M P_4 for 72 h. The cells were then lysed and cell lysates were subjected to Western blotting for the detection of the indicated *p*-FAK (Y397), FAK, *p*-ERK1/2 (T202/Y204), ERK1/2, and GAPDH, as a loading control (These original blot images are provided in the Supplementary file). Similar results were obtained in three independent experiments. The quantitative results were expressed as fold increase by defining the amounts of the indicated detected proteins in untreated cells, where the absence of P_4 stimulation was considered as 1. Data are represented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to untreated cells, where the absence of P_4 stimulation; $^{\#}P < 0.05$ compared to the P_4-stimulated unpretreated cells.

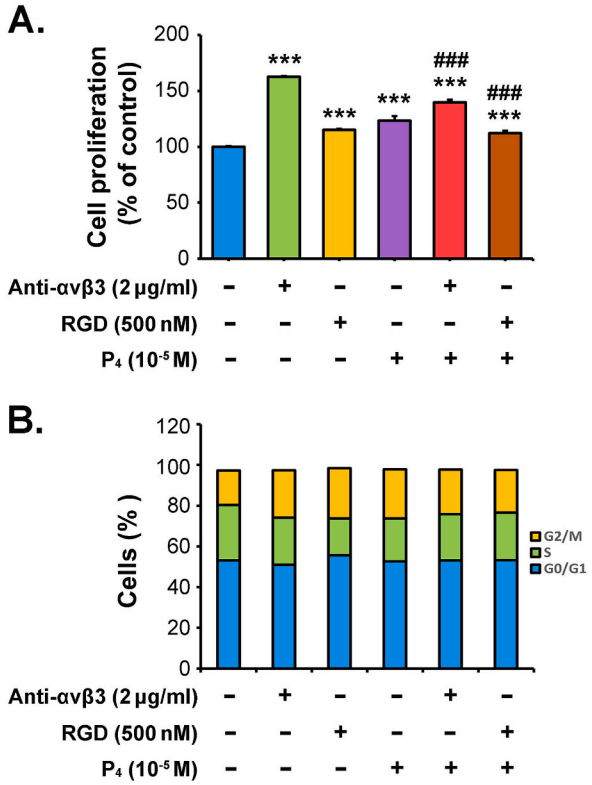

Fig. 8. Blocking of RGD binding site affects P_4-induced cell growth in MDA-MB-231 cells. (A, B) Serum-starved cells were left unpretreated or pretreated with anti-integrin αvβ3 antibody (2 μg/ml) or the RGD peptide (500 nM) for 1 h and then were left unstimulated or stimulated with 10^{-5} M P_4 for 72 h. (A) The cells were then subjected to the cell viability assay. Data are represented normalized to the untreated cells, where the absence of P_4 stimulation and presented as the mean ± standard deviation of triplicate cultures in three-independent experiments. ***$P < 0.001$ compared to untreated cells, where the absence of P_4 stimulation; $^{###}P < 0.001$, compared to the P_4-stimulated unpretreated cells. (B) The cells were then subjected to the flow cytometric analysis. The bar graphs represent the percentage of cell populations in each cell cycle phase, as measured by DNA content stained with PI. Similar results were obtained in three-independent experiments.

concentration of 10^{-5} M, P_4 notably stimulated growth in PR-negative breast cancer MDA-MB-231 cells while inhibiting growth in PR-positive breast cancer MCF-7 cells (Fig. 1). Beyond cell growth, 10^{-5} M P_4 induced the expression of several genes associated with proliferation in MDA-MB-231 cells (Fig. 2). However, at the same concentration, P_4 only influenced the expression of *CCND1* and *PD-L1* genes (Fig. 2), concurrently inhibiting cell growth in MCF-7 cells (Fig. 1). P_4 influenced the gene expression of *integrin αv*, *integrin β3*, and *integrin β5* in MDA-MB-231 cells (Fig. 2) but had no impact on *integrin αv*, *integrin β5*, *PCNA*, or *MMP-9* expressions in MCF-7 cells. These findings suggest a potential influence of P_4 on integrin-dependent signaling pathways specifically in MDA-MB-231 cells. Moreover, at different concentrations, P_4 stimulated *PD-L1* expression in both cell lines (Fig. 2). Specifically, only 10^{-7} M P_4 activated *PD-L1* expression in MDA-MB-231 cells (Fig. 2). Previous studies have linked thyroid hormone-induced PD-L1 expression in various cancer cell types [50], and the role of estrogen in promoting PD-L1 expression, specifically in MCF-7 cells [41]. Notably, the induction of PD-L1 by T_4 is integrin αvβ3-dependent [29], which plays a pivotal role in cancer cell growth [51]. Therefore, the P_4-induced PD-L1 expression might involve a similar signaling pathway implicated in breast cancer cell proliferation in MDA-MB-231 cells.

The activation of integrin αvβ3-dependent signals leads to downstream FAK and subsequent MAPK cascades [52–54]. P_4 has been known to activate several signaling pathways, including the ERK1/2 signaling, cAMP/PKA signaling, cGMP activates protein kinase G (PKG) signaling [55], and PI3K/Akt pathway [56]. Activation of the ERK1/2 pathway regulates cell growth, differentiation, motility, and survival in breast cancer cells and other types of cancers [32,50,57–61]. In the previous studies, several small molecules or hormones have been shown to bind to the RGD-binding domain in integrin αvβ3, such as resveratrol [62], thyroid hormones [9], DHT [62], doxycycline [49], estrogen [35], and heteronemin [27]. In the computational docking modeling, the position of the RGD peptide partially overlapped with the P_4 binding site on the RGD pocket (Fig. 3). Our study shows that interfere with P_4 binding to integrin αvβ3 either by anti-integrin αvβ3 blocking antibody or RGD peptide down-regulates proliferation-related genes *CCND1*, *p21*, and *PCNA* and suppresses ERK1/2 signaling pathways in MDA-MB-231 cells (Figs. 5–7). These results suggest that P_4 plays roles in biological activities is integrin αvβ3-dependent in PR-negative breast cancer cells.

Integrins exhibit different functions in cellular processes through their activation. As demonstrated in our study, treating cells with an anti-integrin αvβ3 blocking antibody or RGD peptide induced the proliferation of MDA-MB-231 cells. Surprisingly, while P_4-induced cell proliferation was suppressed after RGD peptide treatment, it is unexpected that inhibiting integrin αvβ3 activity would enhance the

growth of MDA-MB-231 cells. In addition to influencing cell proliferation, integrin αvβ3 also manipulates various other cellular processes in cancer, including metastasis and angiogenesis [63]. When a cancer cell faces the choice of migrating or invading (migration/invasion) or seeking additional oxygen and nutrients (angiogenesis), it may not be concurrently engaged in proliferation. Thus, the activation of integrin αvβ3 might primarily regulate cancer cell metastasis and angiogenesis rather than proliferation. Indeed, activation of another subtype integrin α5β1 increases cell adhesion to fibronectin but decreases the fraction of K562 cells in S phase [64]. Hence, P_4 may primarily induces cell proliferation but not the migration or invasion regulated by integrin αvβ3.

In summary, P_4 exerts distinct regulatory effects on cell growth in PR-positive and PR-negative breast cancer cells. It stimulates cell proliferation in PR-negative MDA-MB-231 cells while inhibiting proliferation in PR-positive MCF-7 cells. Within MDA-MB-231 cells, P_4 binding to integrin αvβ3 triggers ERK1/2 activation, further amplifying the expression of proliferation-related genes. This demonstrates the association between P_4-induced cell proliferation in PR-negative MDA-MB-231 cells and the involvement of integrin αvβ3.

5. Ethics approval and consent to participate

Not applicable.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Chung-Che Tsai: Supervision, Conceptualization. **Yung-Ning Yang:** Writing – review & editing. **Kuan Wang:** Funding acquisition. **Yu-Chun E. Chen:** Formal analysis. **Yi-Fong Chen:** Formal analysis. **Jen-Chang Yang:** Formal analysis. **Zi-Lin Li:** Supervision, Conceptualization. **Haw-Ming Huang:** Supervision, Conceptualization. **Jens Z. Pedersen:** Methodology. **Sandra Incerpi:** Methodology. **Sheng-Yang Lee:** Writing – review & editing. **Hung-Yun Lin:** Writing – original draft. **Jaqueline Whang-Peng:** Funding acquisition.

Declaration of competing interest

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

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List of abbreviations

cAMP	cyclic adenosine monophosphate
CCND1	cyclin D1
cDNA	complementary DNA
CSCs	cancer stem cells
cRGD	cyclic RGD
DHT	dihydrotestosterone
ER	estrogen receptor
ERK1/2	extracellular signal-regulated kinase 1/2
FAK	focal adhesion kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HRP	horseradish peroxidase
ITGAV	integrin αv

MAPK	mitogen-activated protein kinase
MMP-9	matrix metallopeptidase-9
P_4	Progesterone
PCNA	proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
p	phosphorylated
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PKG	protein kinase G
PR	P_4 receptor
RANKL	Receptor activator of nuclear factor-κB ligand
RGD	arginine-glycine-aspartate
T_4	Thyroxine
TBS	tris-buffered saline
TNBC	Triple-negative breast cancer

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34006.

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