



# Role of Integrin $\alpha\beta 3$ in Doxycycline-Induced Anti-Proliferation in Breast Cancer Cells

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Doxycycline, an antibiotic, displays the inhibition of different signal transduction pathways, such as anti-inflammation and anti-proliferation, in different types of cancers. However, the anti-cancer mechanisms of doxycycline *via* integrin  $\alpha\beta 3$  are incompletely understood. Integrin  $\alpha\beta 3$  is a cell-surface anchor protein. It is the target for estrogen, androgen, and thyroid hormone and plays a pivotal role in the proliferation, migration, and angiogenic process in cancer cells. In our previous study, thyroxine hormones can interact with integrin  $\alpha\beta 3$  to activate the extracellular signal-regulated kinase 1/2 (ERK1/2), and upregulate programmed death-ligand 1 (*PD-L1*) expression. In the current study, we investigated the inhibitory effects of doxycycline on proliferation in two breast cancer cell lines, MCF-7 and MDA-MB-231 cells. Doxycycline induces concentration-dependent anti-proliferation in both breast cancer cell lines. It regulates gene expressions involved in proliferation, pro-apoptosis, and angiogenesis. Doxycycline suppresses cell cyclin D1 (*CCND1*) and *c-Myc* which play crucial roles in proliferation. It also inhibits *PD-L1* gene expression. Our findings show that modulation on integrin  $\alpha\beta 3$  binding activities changed both thyroxine- and doxycycline-induced signal transductions by an integrin  $\alpha\beta 3$  inhibitor (HSDVHK-NH<sub>2</sub>). Doxycycline activates phosphorylation of focal adhesion kinase (FAK), a downstream of integrin, but inhibits the ERK1/2 phosphorylation. Regardless, doxycycline-induced FAK phosphorylation is blocked by HSDVHK-NH<sub>2</sub>. In addition, the specific mechanism of action associated with pERK1/2 inhibition *via* integrin  $\alpha\beta 3$  is unknown for doxycycline treatment. On the other hand, our findings indicated that inhibiting ERK1/2 activation leads to suppression of *PD-L1* expression by doxycycline treatment. Furthermore, doxycycline-induced gene expressions are disturbed by a specific integrin  $\alpha\beta 3$  inhibitor (HSDVHK-NH<sub>2</sub>) or a mitogen-activated protein kinase (MAPK)/

extracellular signal-regulated kinases (ERK) kinase (MAPK/ERK, MEK) inhibitor (PD98059). The results imply that doxycycline may interact with integrin  $\alpha\beta3$  and inhibits ERK1/2 activation, thereby regulating cell proliferation and downregulating *PD-L1* gene expression in estrogen receptor (ER)-negative breast cancer MDA-MB-231 cells.

**Keywords:** doxycycline, anti-proliferation, integrin  $\alpha\beta3$ , signal transduction, breast cancer

## HIGHLIGHTS

- The doxycycline inhibits proliferative genes including a cell-cycle related gene (cell cyclin D1, *CCND1*) and the oncogene (*c-Myc*) in breast cancer cells.
- The doxycycline may interact with integrin  $\alpha\beta3$  in ER-negative MDA-MB-231 breast cancer to down-regulate ERK1/2 activity and suppresses cancer cell growth sequentially.
- Integrin  $\alpha\beta3$  plays an important role in several gene expressions and antiproliferation in MDA-MB-231 cells through doxycycline-induced signal transduction.

## 1 INTRODUCTION

Doxycycline is an antibiotic for bacterial infections. However, previous studies indicate that doxycycline can promote apoptotic procedures to suppress cell growth in different kinds of cancers (Sun et al., 2009). In addition to inhibiting mitochondrial activities, doxycycline suppresses transcription of matrix metalloproteinases (MMPs), MMP-2 and MMP-9 (Wang et al., 2015). MMPs degrade matrix to induce cell migration and thus promote tumor cell spread and metastasis (Chen et al., 2005). Doxycycline suppresses lipopolysaccharide (LPS)-activated phosphorylation of p38 mitogen-activated protein kinase (MAPK) and nuclear translocation of nuclear factor kappa B (NF- $\kappa$ B) (Santa-Cecilia et al., 2016). Furthermore, it inhibits focal adhesion kinase (FAK) phosphorylation and its transcription (Sun et al., 2009; Wang et al., 2015). FAK modulates normal cells proliferation, survival, and motility as well as in tumor cells (Hanks et al., 2003). FAK is a central signaling component triggered by numerous stimuli *via* growth factor receptors and integrins. On the other hand, doxycycline further reduces the transcription of the microglial activation marker ionized calcium binding adaptor molecule 1 (IBA-1), the production of reactive oxygen species (ROS), and nitrogen monoxide (NO) (Santa-Cecilia et al., 2016). Doxycycline also reduces proinflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 1 beta (IL-1 $\beta$ ) (Di Caprio et al., 2015). Doxycycline down-regulates vascular endothelial growth factor A (*VEGF-A*) expression which may also be connected to cancer proliferation and inflammation (Veron et al., 2012). Knockdown of VEGF disrupts integrin  $\alpha\beta3$  activity *via* decreasing VEGFR2 signaling. In addition, the integrin  $\alpha\beta3$  is a critical regulator of programmed death-ligand 1 (*PD-L1*) expression (Vannini et al., 2019). The immune checkpoint programmed cell death-1/PD-ligand 1 (PD-1/PD-L1) is associated with pro-inflammatory cytokine production and cancer growth (Huang et al., 2019). However, the link between *PD-L1* suppression *via* modulation of

integrin  $\alpha\beta3$  and doxycycline-induced anti-proliferation of cancer cells is uncertain. Regardless, doxycycline is shown to suppress gastric cancer cell growth and the potential formation of colonies and spheroid (Pandian et al., 2020). Furthermore, a clinical trial study has shown that doxycycline can reduce cancer stem cells (CSCs) in breast cancer patients *in vivo* (Scatena et al., 2018). It is merit to investigate whether doxycycline can be used to treat breast cancer.

Integrin  $\alpha\beta3$  is one of 24  $\alpha\beta$ -paired integrins that play vital roles in cell adhesion, migration, and interaction with extracellular matrix (ECM) proteins (Mezu-Ndubuisi and Maheshwari, 2021). The integrin  $\alpha$  subunit pairs with  $\beta1$ ,  $\beta3$ ,  $\beta5$ ,  $\beta6$ , and  $\beta8$  to form heterodimers (Luo et al., 2007). Although studies indicate that overexpressed integrin  $\alpha\beta3$ s are observed in normal cells, they may function differently from the matter on cancer cells. Integrin  $\alpha\beta3$  is recognized by several binding ligands such as fibrillin, fibrinogen, fibronectin, osteopontin, tenascin, thrombospondin vitronectin, and von Willebrand factor (VWF), however, integrin  $\alpha\beta5$  only preferentially binds to vitronectin (Weis and Cheresch, 2011). The overexpression of integrin  $\alpha\beta3$  is observed in highly-growth endothelial cells and cancer cells (Cheng et al., 2021). The integrin extracellular domain controls ligand-binding specificity. On the other hand, the cytoplasmic short tail regulates intracellular signaling pathways by recruiting and activating specific kinases and signaling intermediates. Consequently, coordination between domains controls the adhesion (Altei et al., 2020), morphology (Lange et al., 2016), migration (Sales et al., 2019), invasion (Hood and Cheresch, 2002), proliferation (Moreno-Layseca and Streuli, 2014), and survival (Desgrosellier and Cheresch, 2010) in cancer cells. These signals manipulate the host microenvironment to deliver abundant blood vessel and stromal resources to maintain proliferation and metastasis of cancer cells (Luo et al., 2007). Targeting integrin  $\alpha\beta3$  has been interesting research for cancer prevention and targeting theragnostic therapy (Slack et al., 2022). In addition to thyroid hormone, dihydrotestosterone (DHT) and estrogen can also bind to integrin  $\alpha\beta3$  and thus activate biological activities (Meng et al., 2011; Ho et al., 2020). Conversely, other molecules such as resveratrol bind to the integrin  $\alpha\beta3$  receptor to activate anti-proliferation in different types of cancer cells (Lin et al., 2006). Additionally, thyroid hormone analogue, tetraiodothyroacetic acid (tetrac), and its nano-derivative (nano-diamino-tetrac, NDAT) not only bind to integrin  $\alpha\beta3$  competently with thyroid hormones but also induce anti-proliferation against cancer cells (Yang et al., 2021). The previous study reveals that doxycycline down-regulates integrin  $\alpha\beta3$  downstream FAK signaling in leukemia cells (Wang et al., 2015). Accordingly, those observations encourage us to investigate that the activity of doxycycline may be controlled by an integrin  $\alpha\beta3$ -dependent signal transduction pathway.

Early studies demonstrate that doxycycline is highly lipophilic, hence it can penetrate the cell membrane and enter cells to inhibit the synthesis of 30S ribosomal subunits in bacteria (Chopra and Roberts, 2001). In the present study, we investigated the role of doxycycline-induced anti-proliferation in both estrogen receptor (ER)-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cell lines. In contrast to the antibiotic activity of doxycycline, it inhibits the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and regulates genes that regulate cell proliferation by interacting with cell surface integrin  $\alpha\beta 3$  in ER-negative breast cancer cells. On the other hand, thyroxine ( $T_4$ )-induced cancer proliferation is also moderated by integrin  $\alpha\beta 3$ , which regulates ERK1/2 activation and gene expressions. Additionally, the ERK1/2 activation is associated with the *PD-L1* expression. In contrast to  $T_4$ , the *PD-L1* gene expression is suppressed by doxycycline. The doxycycline-induced signal transductions *via* integrin  $\alpha\beta 3$  play pivotal modulators in several gene expressions and anti-proliferation in breast cancer cells.

## 2 MATERIALS AND METHODS

### 2.1 Materials

All reagents were obtained commercially and used without further purification. Thyroxine ( $T_4$ ; Cat. No. T2376) and doxycycline (Cat. No. D9891) were purchased from Sigma-Aldrich (St. Louis, MO, United States). The integrin  $\alpha\beta 3$  inhibitor (HSDVHK-NH<sub>2</sub>) was purchased from Calbiochem (San Diego, CA, United States). The ERK1/2 inhibitor (PD98059; Cat. No. #9900) was purchased from Cell Signaling Technology (Danvers, MA, United States).

### 2.2 Cell Lines

Human breast cancer ER-positive MCF-7 cells and ER-negative MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, United States). Cell lines were tested and authenticated with BCRC (isoenzyme analysis, *Mycoplasma*, cytogenetics, tumorigenesis, receptor expression testing). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies Corp., Carlsbad, CA, United States). Media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell cultures were maintained in the incubator with a 5% CO<sub>2</sub> supply at 37°C. Before the study, cells were placed in a 0.25% hormone-depleted serum-supplemented medium for 48 h.

### 2.3 Cell Viability Assay

MCF-7 and MDA-MB cells were plated at a density of  $4 \times 10^3$  cells/well in 96-well plates. Cell viability was determined by using the alamarBlue<sup>®</sup> Assay Kit (Thermo Fisher Scientific Inc.) at 72 h after treatment. At the time of detection, the medium was removed, and cells were incubated with alamarBlue<sup>®</sup> reagent for 2 h at 37°C according to the manufacturer's instruction. Plates were then analyzed by using a microplate reader (VERSAmax<sup>™</sup> Tunable Microplate Reader, Molecular Devices) at a wavelength of 570 and 600 nm as reference.

### 2.4 Real-Time Quantitative Reverse Transcription PCR

Total RNA was extracted with genomic DNA removed by Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). 1  $\mu$ g of DNase I-treated total RNA was reverse-transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies Corp.) into cDNA to use as the template for real-time PCR reaction and analysis. The real-time PCR reactions were conducted using QuantiNova<sup>™</sup> SYBR<sup>®</sup> Green PCR Kit (QIAGEN, Hilden, Germany) on CFX Connect<sup>™</sup> Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The reaction was conducted according to the manufacturer's instructions that an initial denaturation was at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 5 s and combined annealing/extension at 60°C for 10 s. The primer sequences were shown as follows (Table 1): *Integrin  $\alpha v$* , *Integrin  $\beta 3$* , *Homo sapiens cyclin D1 (CCND1)*, *c-Myc*, Vascular endothelial growth factor A (*VEGF-A*), *Homo sapiens Caspase 2 (CASP2)*, matrix metalloproteinase-9 (*MMP-9*), *Homo sapiens BCL2-antagonist of cell death (Bad)* and *Homo sapiens programmed death-ligand 1 (PD-L1)*.

### 2.5 Western Blotting Analysis

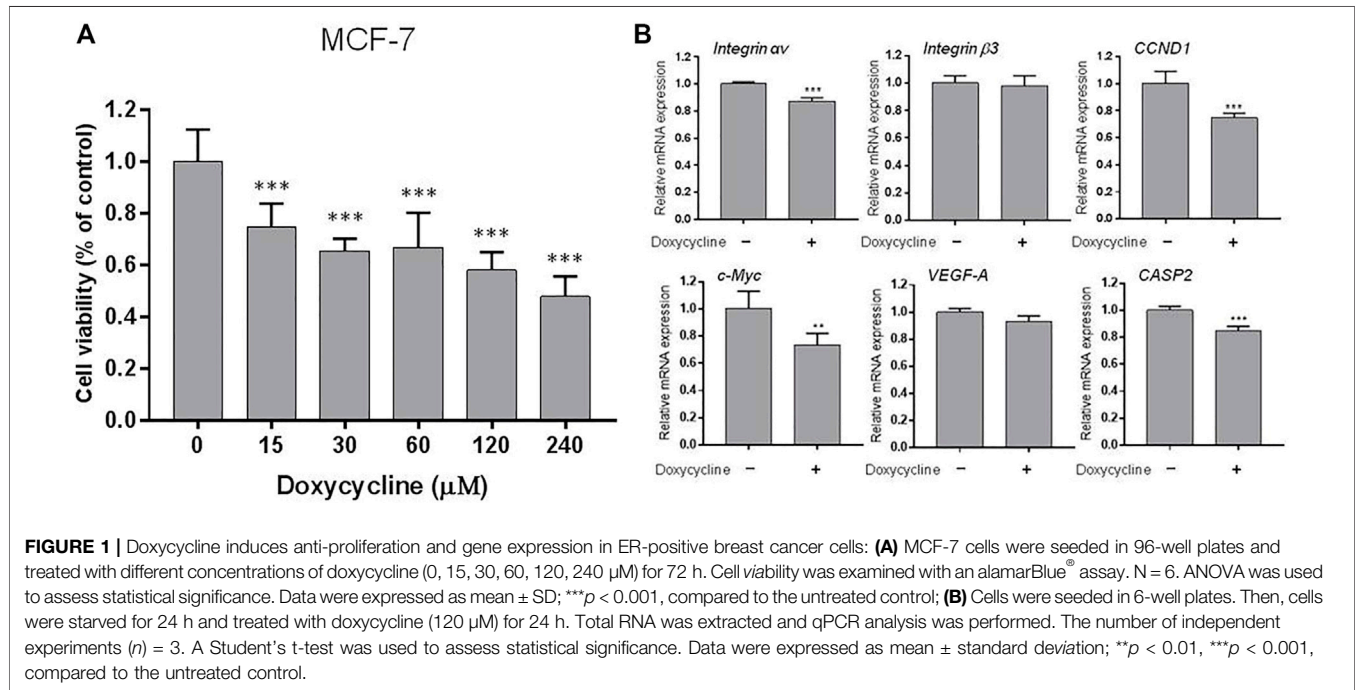
To examine the effects of doxycycline on the signaling pathways, Western blot analyses were conducted to quantify protein expression levels of pFAK, pERK1/2, and PD-L1 in MDA-MB-231 cells. For western blot analyses, cells were lysed and extracted protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). A 15- $\mu$ g quantity of protein was loaded in each well with 5x sample buffer and the samples were separated by electrophoresis at 100 V for 2 h. The separated proteins were transferred from the polyacrylamide gel to Millipore Immobilon-PSQ Transfer PVDF membranes (Millipore, Billerica, MA, United States) with the Mini Trans-Blot<sup>®</sup> Cell (Bio-Rad Laboratories, Inc.). Membranes then were incubated with NaCl/Tris blocking buffer containing 2% BSA (bovine serum albumin). Membranes were incubated with primary antibodies to phosphorylated (phospho)-ERK, phospho-FAK, and their corresponding proteins (Cell Signaling Technology, Inc, Beverly, MA, United States), PD-L1, and GAPDH (GeneTex International Corp, Hsinchu City, Taiwan) overnight at 4°C. The proteins were detected with HRP-conjugated secondary antibodies and Immobilon<sup>™</sup> Western HRP Substrate Luminol Reagent (Millipore). Western blots were visualized and recorded with the Amersham Imager 600 (GE Healthcare Life Sciences, Pittsburgh, PA, United States). The densitometric analysis of Western blot was conducted by ImageJ 1.47 software (National Institute of Health, United States) according to the software instruction.

### 2.6 Statistical Analysis

All of the collected data for immunoblot, nucleotide densities, and cell densities were analyzed by IBM<sup>®</sup> SPSS<sup>®</sup> Statistics software version 19.0 (SPSS Inc, Chicago, IL, United States). One-way analysis of variance (ANOVA) with Duncan's post-hoc test was conducted for multiple groups' comparison and Student's *t*-test

**TABLE 1** | Primer sequences for the qPCR.

	Forward	Reverse
<i>Integrin <math>\alpha v</math></i>	5'-GATTCCAAACTGGGAGAC-3'	5'-AAGGCCACTGAAGATGGAGC-3'
<i>Integrin <math>\beta 3</math></i>	5'-CGAGTGCCTCTGGTCAAT-3'	5'-AGAAGTCGTCACAC-3'
<i>CCND1</i>	5'-CAAGGCCTGAACCTGAGGAG-3'	5'-GATCACTCTGGAGAGGAAGCG-3'
<i>c-Myc</i>	5'-TTCGGGTAGTGGAACCAG-3'	5'-CAGCAGCTCGAATTTCTCC-3'
<i>VEGF-A</i>	5'-TACCTCCACCATGCCAAGTG-3'	5'-GATGATTCTGCCCTCCCTCCTT-3'
<i>CASP2</i>	5'-GCATGTACTCCCACCGTTGA-3'	5'-GACAGGCGGAGCTTCTTGTA-3'
<i>MMP-9</i>	5' TGTACCGCTATGGTTACACTCG-3'	5' GGCAGGGACAGTTGCTTCT-3'
<i>Bad</i>	5'-CTTTAAGAAGGGACTTCTCGCC-3'	5'-CTTTAAGAAGGGACTTCTCGCC-3'
<i>PD-L1</i>	5'-GTTGAAGGACCAGCTCTCCC-3'	5'-ACCCTGCATCCTGCAATTT-3'



was also conducted. The  $p$ -values < 0.05 (\* or #), 0.01 (\*\* or ##) and 0.001 (\*\*\*) or ###) as the threshold for significance.

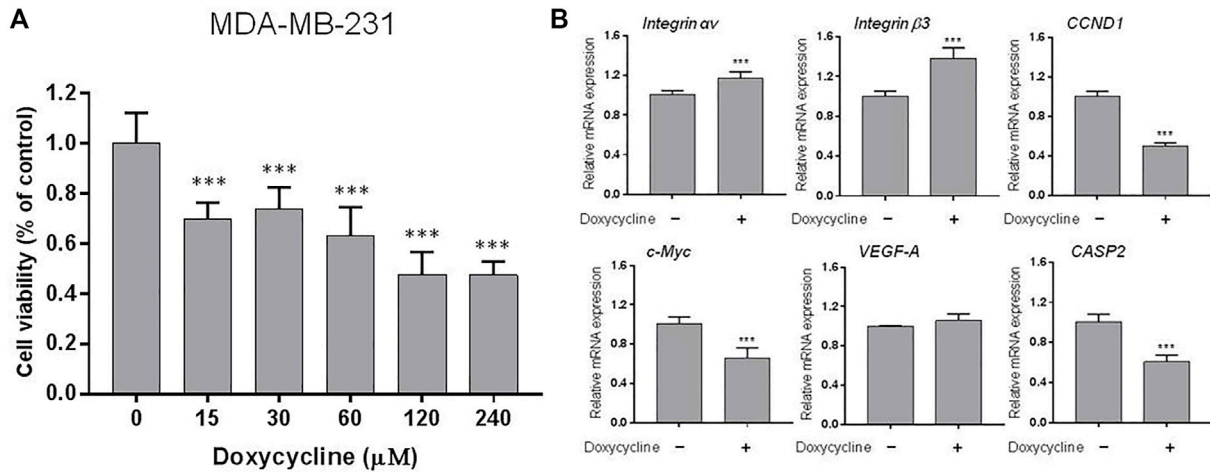
### 3 RESULTS

#### 3.1 Doxycycline Induces Anti-Proliferation in Breast Cancer Cells

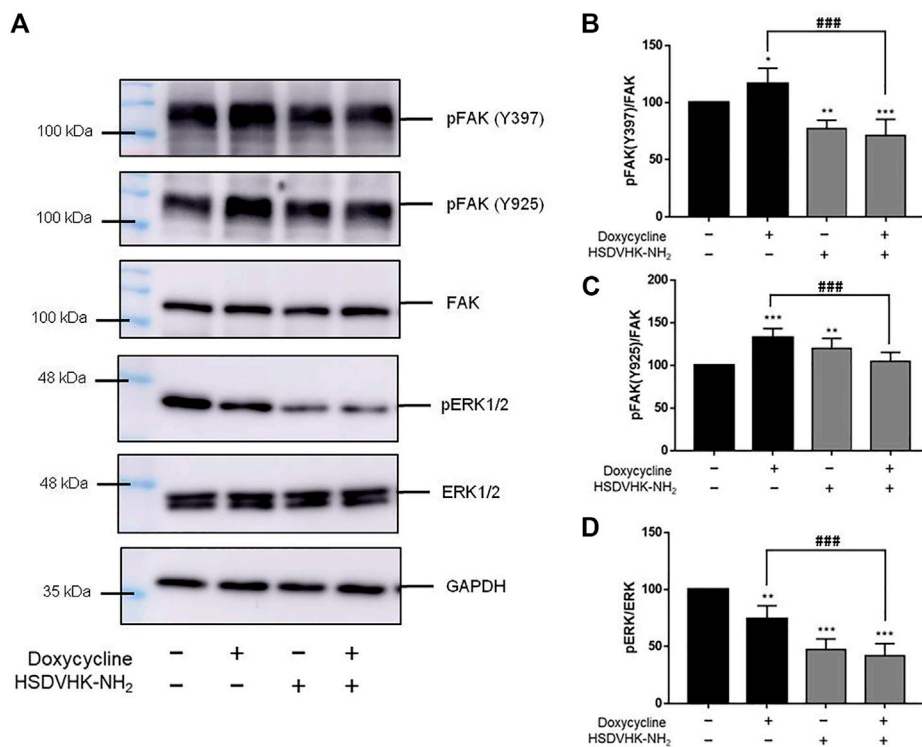
To investigate the effects of doxycycline-induced anti-proliferation in different types of breast cancer cells, ER-positive MCF-7 cells and ER-negative MDA-MB-231 cells were seeded in 96 well trays. Cells were fed with serum-free medium for 48 h and then re-fed with 10% FBS medium containing various concentrations of doxycycline. The anti-proliferative effect was detected by Alamar Blue Assay kit at the end of treatment. Results indicated that doxycycline inhibited cell proliferation in a concentration-dependent manner in both MCF-7 and MDA-MB-231 cells (**Figures 1A, 2A**).

Because doxycycline suppressed cell growth in both ER-positive and negative breast cancer cells, we investigated whether doxycycline could stimulate the expression of pro-apoptotic genes and inhibit proliferative genes. MCF-7 cells were harvested and total cellular RNA was extracted after 24 h treatment with doxycycline (120  $\mu\text{M}$ ). The qPCR was conducted for *integrin  $\alpha v$* ,  *$\beta 3$* , *CCND1*, *c-Myc*, *VEGF-A*, *CASP2*. Doxycycline suppressed *integrin  $\alpha v$*  significantly but not *integrin  $\beta 3$*  in MCF-7 cells (**Figure 1B**). It also inhibited proliferative genes such as *CCND1* and *c-Myc*. In addition, doxycycline had no significant effect on the angiogenic gene *VEGF-A*. Interestingly, doxycycline also inhibited pro-apoptotic gene expression (*CASP2*).

Similar studies were performed in triple negative breast cancer MDA-MB-231 cell line. MDA-MB-231 cells were harvested and total RNA was extracted after treatment with doxycycline (60  $\mu\text{M}$ ) for 24 h. The qPCR was conducted for *integrin  $\alpha v$* , *integrin  $\beta 3$* , *CCND1*, *c-Myc*, *VEGF-A*, *CASP2* (**Figure 2B**). Doxycycline treatment inhibited the expression of *CCND1* and *c-Myc* in MDA-MB-231 cells. Instead, doxycycline treatment did



**FIGURE 2** | Doxycycline induces anti-proliferation and gene expression in ER-negative breast cancer cells: **(A)** MDA-MB-231 cells were seeded in 96-well plates and treated with different concentrations of doxycycline (0, 15, 30, 60, 120, 240  $\mu\text{M}$ ) for 72 h. Cell viability was examined with an alamarBlue<sup>®</sup> assay.  $N = 6$ . ANOVA was used to assess statistical significance. Data were expressed as mean  $\pm$  SD;  $***p < 0.001$ , compared to the untreated control; **(B)** Cells were seeded in 6-well plates. Then, cells were starved for 24 h and treated with doxycycline (60  $\mu\text{M}$ ) for 24 h. Total RNA was extracted and qPCR analysis was performed. The number of independent experiments ( $n$ ) = 3. A Student's  $t$ -test was used to assess statistical significance. Data are expressed as mean  $\pm$  standard deviation;  $***p < 0.001$ , compared to the untreated control.



**FIGURE 3** | Blockage of integrin  $\alpha\beta3$  activity inhibits doxycycline-induced signal transduction in breast cancer MDA-MB-231 cells. **(A)** Cells were pre-treated with HSDVHK-NH<sub>2</sub> (10  $\mu\text{M}$ ) for 24 h, then treated with doxycycline (60  $\mu\text{M}$ ) for 24 h. Cells were harvested and protein was extracted for Western blotting analysis of pFAK (Y397), pFAK (Y925), FAK, pERK1/2, and total ERK1/2. The HSDVHK-NH<sub>2</sub> compound reduced doxycycline-induced phosphorylation of Y397 **(B)**, Y925 **(C)**, and pERK1/2 **(D)**. Numbers of independent studies ( $n$ ) = 3. ANOVA was used to assess statistical significance.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , compared to the untreated control.  $###p < 0.001$ , compared to doxycycline alone.

not suppress the expression of *integrin  $\alpha\text{v}$ ,  $\beta3$* , and *VEGF-A* in MDA-MB-231 cells. On the other hand, doxycycline increased the expression of *integrin  $\alpha\text{v}$*  and *integrin  $\beta3$*  (Figure 2B). Moreover, doxycycline-induced gene expressions of *integrin  $\alpha\beta3$*  displayed different patterns between MCF-7 and MDA-MB-231 cells. These results indicated that doxycycline might promote different pathways to induce anti-cancer growth in ER-positive and ER-negative breast cancer cells. The antiproliferative effect demonstrated that the cell viability of ER-negative breast cancer MDA-MB-231 cells was lower than that of doxycycline-treated MCF-7 cells (Figures 1A, 2A).

### 3.2 Integrin $\alpha\beta3$ Downstream ERK1/2 Plays an Important Role in Doxycycline-Induced Biological Activities in ER-Negative Breast Cancer Cells

In our previous studies, thyroid hormone binds to the receptor on integrin  $\alpha\beta3$  to activate signal transduction and regulate gene expression of *integrin  $\alpha\beta3$*  (Lin et al., 2013; Hsieh et al., 2017; Abadi et al., 2021) and promote cancer cell proliferation (Lin et al., 2016b; Ho et al., 2020). Our recent findings show that doxycycline affects both gene expression of *integrin  $\alpha\text{v}$*  and  $\beta3$  in MDA-MB-231 cells (Figure 2B). Therefore, we investigated the role of integrin  $\alpha\beta3$  on doxycycline-induced signal transduction in MDA-MB-231 cells by a specific integrin  $\alpha\beta3$  inhibitor, HSDVHK-NH<sub>2</sub> (10  $\mu\text{M}$ ) for 24 h, followed by doxycycline (60  $\mu\text{M}$ ) for 24 h. Proteins were extracted and Western blotting analyses were conducted to examine FAK, phosphoFAK, and phosphoERK1/2 (Figure 3). Doxycycline induced the gene expression of *integrin  $\alpha\beta3$* , which then activated downstream FAK phosphorylation. The effects of doxycycline on FAK phosphorylation and *integrin  $\alpha\beta3$*  expression were consistent in MDA-MB-231 cells. Doxycycline treatment significantly increased the phosphorylation of Tyr397/925 (Figures 3B,C). Notably, HSDVHK-NH<sub>2</sub> decreased Tyr397 phosphorylation, whereas doxycycline did not affect pFAK after HSDVHK-NH<sub>2</sub> pretreatment. However, the doxycycline-induced pFAK (Y397) (Figure 3B) and pFAK (Y925) (Figure 3C) were significantly reduced by the integrin  $\alpha\beta3$  inhibitor (HSDVHK-NH<sub>2</sub>). These results implied that doxycycline interacted with integrin  $\alpha\beta3$  and activated FAK phosphorylation. Although the active binding site might be different from that of HSDVHK-NH<sub>2</sub>, the activation of FAK could still be attenuated by HSDVHK-NH<sub>2</sub>. On the other hand, doxycycline inhibited the further downstream of integrin  $\alpha\beta3$ , the ERK1/2 phosphorylation, and the specific mechanism of action relevant to pERK1/2 inhibition remained unclear. Regardless, HSDVHK-NH<sub>2</sub> significantly reduced the inhibitory effect of doxycycline on ERK1/2 phosphorylation (Figure 3D).

### 3.3 Doxycycline Modulates Different Genes Expression in ER-Negative Breast Cancer Cells

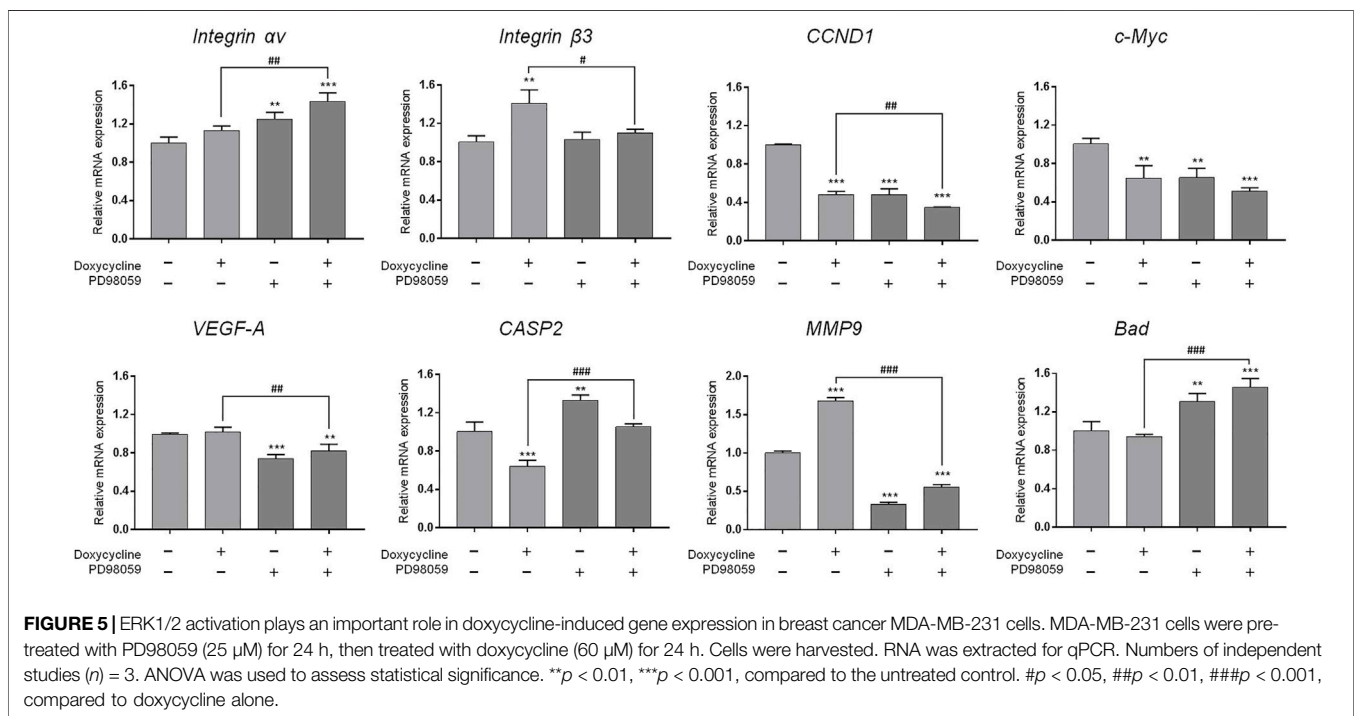
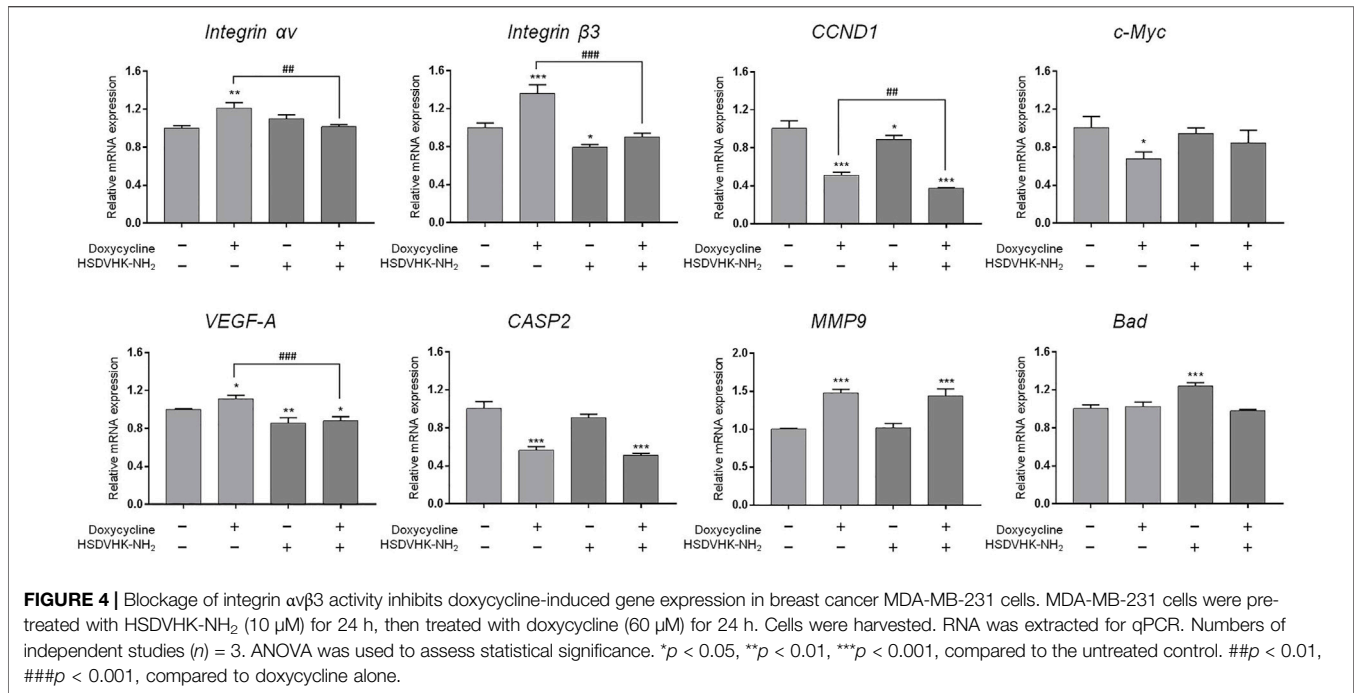
We further investigated the effect of integrin  $\alpha\beta3$  on doxycycline-induced gene expression in breast cancer MDA-

MB-231 cells. Cells were pre-treated with a specific integrin  $\alpha\beta3$  inhibitor, HSDVHK-NH<sub>2</sub> (10  $\mu\text{M}$ ) for 24 h, followed by 60  $\mu\text{M}$  doxycycline treatment for 24 h. Cells were harvested, and total RNA was extracted. The qPCR was conducted for *integrin  $\alpha\text{v}$* , *integrin  $\beta3$* , *CCND1*, *c-Myc*, *VEGF-A*, *CASP2*, *MMP9*, and *Bad*. As shown in Figure 4, doxycycline inhibited a cell-cycle related gene (*CCND1*) and the oncogene (*c-Myc*) in MDA-MB-231 cells, whereas *integrin  $\alpha\text{v}$* , *integrin  $\beta3$* , *VEGF-A*, *MMP-9*, and *Bad* were not suppressed. The effects of doxycycline-induced gene expression were disturbed in the presence of HSDVHK-NH<sub>2</sub>. Although doxycycline significantly increased the expression of integrin  $\alpha\text{v}$  and  $\beta3$  mRNA, the increased effect of gene expression could be reversed by HSDVHK-NH<sub>2</sub>. Results presented in Figures 3, 4 suggested that doxycycline might modulate signal transduction by regulating integrin  $\alpha\beta3$  in MDA-MB-231 cells. It would be interesting to investigate whether doxycycline-induced biological activity is also affected by blocking the integrin  $\alpha\beta3$  binding site.

Since our findings (Figure 3) and others had shown that doxycycline inhibited ERK1/2 activation, we further investigated the role of the ERK1/2-dependent signal transduction pathway in doxycycline-induced biological activities in breast cancer MDA-MB-231 cells. MDA-MB-231 cells were treated with 60  $\mu\text{M}$  doxycycline in the presence or absence of an ERK1/2 inhibitor, PD98059 (25  $\mu\text{M}$ ) for 24 h. Cells were harvested and total RNA was extracted for qPCR (Figure 5). The results are similar to Figure 4. Doxycycline inhibited *CCND1* and *c-Myc* in MDA-MB-231 cells, whereas *integrin  $\alpha\text{v}$* , *integrin  $\beta3$* , *VEGF-A*, *MMP-9*, and *Bad* were not suppressed. These results were consistent with pre-treatment of HSDVHK-NH<sub>2</sub>. In addition, the gene expressions of *integrin  $\beta3$* , the proliferative genes (*CCND1*), the angiogenic gene (*VEGF-A*), and the migration-related gene (*MMP9*) were further inhibited by PD98059 significantly. However, the pro-apoptotic gene (*CASP2* and *Bad*) was significantly activated by PD98059. These results indicate that activated ERK1/2 is involved in doxycycline-induced gene expressions.

### 3.4 Doxycycline Inhibits PD-L1 Expression in ER-Negative Breast Cancer Cells

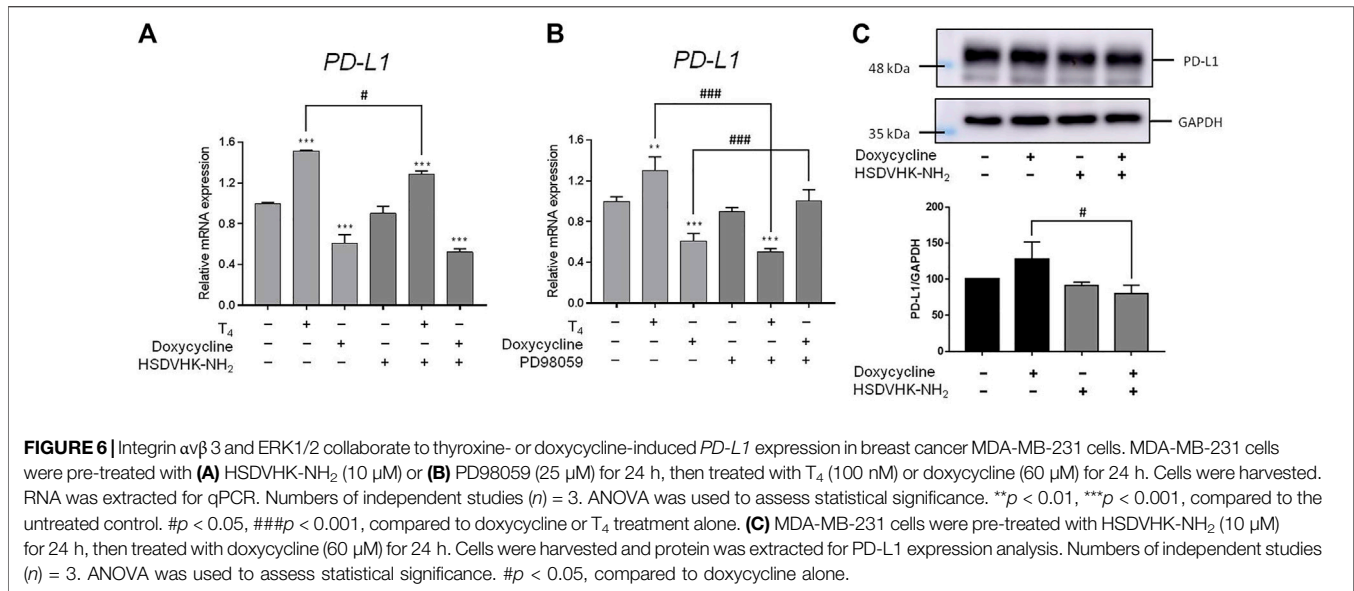
Activation of ERK1/2 is also involved in *PD-L1* gene expression in cancer cells (Lin et al., 2016a). Since doxycycline inhibited ERK1/2 activation, we further investigated the role of integrin  $\alpha\beta3$  on the inhibitory effect of *PD-L1* expression induced by doxycycline in MDA-MB-231 cells (Figure 6). Thyroxine (T<sub>4</sub>) was used as a positive control for integrin  $\alpha\beta3$  binding (Lin et al., 2016b; Davis et al., 2018; Lin et al., 2018; Davis et al., 2020; Davis et al., 2021). T<sub>4</sub> increased *PD-L1* expression, and the inducible expression of *PD-L1* could be reversed by an integrin  $\alpha\beta3$  inhibitor (HSDVHK-NH<sub>2</sub>) (Figure 6A). Conversely, doxycycline suppressed *PD-L1* expression, and the levels of *PD-L1* mRNA were not significantly altered by the integrin  $\alpha\beta3$  inhibitor. Of note, doxycycline increased PD-L1 protein accumulation in MDA-MB cells (Figure 6C). The effect was significantly inhibited by HSDVHK-NH<sub>2</sub> (Figure 6C). These results suggested that integrin  $\alpha\beta3$  may play different



regulations on *PD-L1* expression and its protein accumulation. To determine the role of ERK1/2 in doxycycline-induced *PD-L1* expression, MDA-MB-231 cells were pre-treated with an ERK1/2 inhibitor (PD98059) before treatment with doxycycline or thyroxine. T<sub>4</sub>-induced *PD-L1* expression was significantly reduced in the presence of PD98059 (Figure 6B). On the other hand, suppressive effect of doxycycline on *PD-L1*

expression was interrupted by PD98059, therefore, the suppressive expression of *PD-L1* might be up-regulated by negative feedback. These results indicate that regulations of *PD-L1* expression by T<sub>4</sub> and doxycycline were ERK1/2-dependent.

In summary, both doxycycline and thyroxine interacted with integrin  $\alpha\beta 3$  and regulated ERK1/2 activation consequently



modulating proliferation in ER-negative breast cancer MDA-MB-231 cells. Eventually, doxycycline inhibited ERK1/2 activation, a downstream of integrin  $\alpha\beta3$ , regulated gene expression and suppressed cancer cell growth sequentially in ER-negative MDA-MB-231 cells.

## 4 DISCUSSION

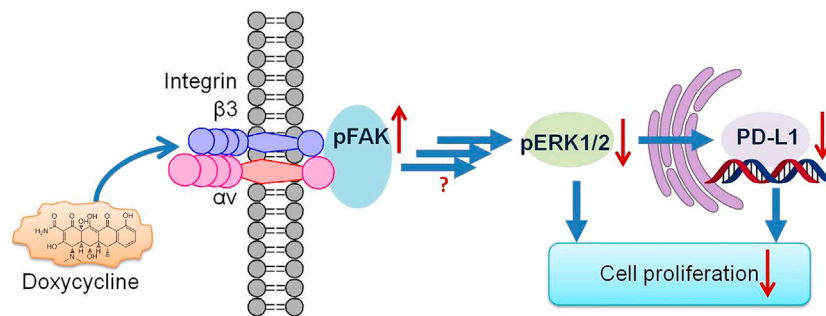
Several studies have shown that doxycycline can induce apoptosis in a range of cancer cells (Mouratidis et al., 2007; Son et al., 2009; Dijk et al., 2020; Alsaadi et al., 2021), indicating that the drug can be applied as an anti-cancer drug for cancer patients in clinical practice. In addition, doxycycline can reduce cancer stem cell (CSC) progression through apoptosis (Yang et al., 2015; Matsumoto et al., 2017), and it has been tested in the clinical trial study to reduce cancer stem cells (CSCs) in breast cancer patients *in vivo* (Scatena et al., 2018). Evidence has supported that breast cancers originate from breast cancer stem cells (BCSCs) (Zhang et al., 2017). The study also indicates that doxycycline inhibits the vitality and growth of BCSCs by reducing effective mammosphere formation, migration, and infiltration. Biosynthesis in mitochondria plays a vital role in clonal proliferation and survival independent of CSC scaffolds. Therefore, it makes mitochondria to be an important target for new treatment methods. Doxycycline impairs mitochondrial function and protects hypoxia-induced apoptosis in glioma cells (Luger et al., 2018). It also shows that doxycycline targets mitochondria (De Luca et al., 2015; Dijk et al., 2020). Besides, doxycycline inhibits NF- $\kappa$ B activation and reduces plasma lysophosphatidate concentrations to attenuate breast cancer-related inflammation (Tang et al., 2017). In our study, doxycycline was shown to inhibit the proliferation of ER-positive and negative breast cancer cells in a concentration-dependent manner (Figures 1, 2). Studies by Le Zhang et al. indicate that doxycycline suppressed the transcription of autophagy markers protein 1 light chain 3-B (LC-3B) I and LC-3BII (Zhang et al., 2017). Doxycycline treatment effectively inhibits epithelial-to-

mesenchymal transition (EMT) and cancer rod-like features in breast cancer cells. In addition, doxycycline has been shown to significantly down-regulate transcription of the stem cell factors such as octamer-binding transcription factor 4 (Oct4), sex determining region Y-Box transcription Factor 2 (Sox2), Nanog, and CD44 (Zhang et al., 2017). Those observations suggested that autophagy inhibited by doxycycline may be in part due to the effects on expression of stem cell markers, EMT, and proliferation (Zhang et al., 2017).

Doxycycline is verified to block cancer cell progression by different mechanisms (Sagar et al., 2010; Hadjimichael et al., 2020; Yi et al., 2021). The previous studies have shown that doxycycline suppresses MMP expression and FAK phosphorylation to inhibit leukemic cell migration (Wang et al., 2015). Accordingly, doxycycline inhibits migration and metastasis in cancer cells that may be related to the activities of integrins (Sun et al., 2009). However, the study of integrin  $\alpha\beta3$  regulating breast cancer growth through doxycycline is unclear. In the present study, our preliminary gene screen results indicated that doxycycline down-regulated expression of *integrin  $\alpha v$*  and the proliferative genes (*CCND1* and *c-Myc*) and the pro-apoptotic gene (*CASPS2*) in MCF-7 cells (Figure 1B). Interestingly, doxycycline treatment did not suppress the expression of *integrin  $\alpha v$*  in MDA-MB-231 cells (Figure 2B). Alternatively, doxycycline increased expression of *integrin  $\alpha v$*  and *integrin  $\beta3$* , but inhibited the expression of *CCND1*, *c-Myc* and *CASPS2* in MDA-MB-231 cells (Figure 2B).

The integrin  $\alpha\beta3$  is overexpressed in endothelial cells and interrupting ligand binding to block integrin  $\alpha\beta3$  function may produce anti-angiogenic effects (Weis and Cheresh, 2011). Expression of integrin  $\alpha\beta3$  on different cell types contributes to cell growth and mobility. Although integrin  $\alpha\beta3$  may also express on normal cells, the tertiary conformation of integrin  $\alpha\beta3$  on cancer cells attract potential antagonists to interrupt multiple aspects of cancer progression. Our results indicate that doxycycline inhibits expression of *integrin  $\alpha v$*  in MCF-7 cells but





**FIGURE 7** | The signaling pathways of doxycycline-induced anti-proliferation in human breast cancer MDA-MB-231 cells.

not in MDA-MB-231 cells (**Figures 1B, 2B**). Interestingly, our results showed that the half-maximal inhibitory concentration ( $IC_{50}$ ) of MDA-MB-231 cells was between 60 and 120  $\mu\text{M}$  (**Figure 2A**), whereas the  $IC_{50}$  of MCF-7 cells was as high as 120–240  $\mu\text{M}$  (**Figure 1A**). These results imply that MDA-MB-231 cells are more sensitive to doxycycline than MCF-7 cells. A previous study showed that integrin  $\alpha\beta 3$  was overexpressed in MDA-MB-231 cells compared to MCF-7 cells (Godugu et al., 2021). The higher  $\alpha\beta 3$  expression was found in MDA-MB-231 (21%) and the lower expression of  $\alpha\beta 3$  was found in the MCF-7 breast cancer cell line (9%) by using flow cytometry. Accordingly, we support that doxycycline-induced antiproliferation may be related to integrin  $\alpha\beta 3$  activity in ER-negative breast cancer MDA-MB-231 cells. On the other hand, anti- $\beta 1$ -integrin antibodies showed inhibitory effects on leukemic cell migration induced by doxycycline (Wang et al., 2015) suggesting that doxycycline may inhibit cancer migration *via* integrin functional inhibition. As shown in **Figure 3**, doxycycline significantly activates both Tyr397/925 phosphorylation of FAK, but inhibits the ERK1/2 phosphorylation. The results are partially consistent with the previous study showing that doxycycline highly inhibits the levels of ERK/MAPK proteins and their regulated transcriptional activity (Pandian et al., 2020). Furthermore, we investigate whether the FAK and ERK1/2 phosphorylation are suppressed in the presence of an integrin  $\alpha\beta 3$  inhibitor (HSDVHK-NH<sub>2</sub>). Our findings demonstrate that both FAK and ERK1/2 activities induced by doxycycline are significantly inhibited by HSDVHK-NH<sub>2</sub>. In addition, the combination with an integrin  $\alpha\beta 3$  inhibitor enhances the inhibition of ERK1/2 phosphorylation compared to doxycycline alone (**Figure 3D**). Previously, we have investigated that thyroxine (T<sub>4</sub>) can induce the ERK1/2 phosphorylation and PD-L1 protein accumulation (Lin et al., 2016a). Compared with our previous study, the results suggest that both thyroxine and doxycycline can interact with integrin  $\alpha\beta 3$  to regulate ERK1/2 signal transduction pathways. The former activates ERK1/2 and the latter suppresses ERK1/2 activation. Besides, the effects of doxycycline-induced gene expression are also disturbed in the presence of HSDVHK-NH<sub>2</sub> (**Figure 4**). We further investigated whether ERK1/2 inhibition affects doxycycline-induced gene expressions. Effects of doxycycline-induced gene expression are disturbed in the

presence of a MEK inhibitor (PD98059) in MDA-MB-231 cells (**Figure 5**). These results also provide evidence that doxycycline interacts with integrin  $\alpha\beta 3$  to regulate various gene expressions *via* ERK1/2 signaling and suppress cancer growth in ER-negative breast cancer cells.

In our previous study, the activation of *PD-L1* expression is up-regulated by ERK1/2-dependent phosphorylation (Lin et al., 2016a). Thyroxine (T<sub>4</sub>) acts on integrin  $\alpha\beta 3$  to activate specific signal transduction. T<sub>4</sub> induces ERK1/2 activation and cell proliferation *via* binding to cell surface integrin  $\alpha\beta 3$  (Lin et al., 2016b; Davis et al., 2018; Lin et al., 2018; Davis et al., 2020; Davis et al., 2021). Blockage of integrin  $\alpha\beta 3$  signaling and ERK1/2 pathway (**Figures 6A,B**) reduces the biological activities of thyroxine by an integrin  $\alpha\beta 3$  inhibitor (HSDVHK-NH<sub>2</sub>) or a MEK inhibitor (PD98059). Those results confirm our previous studies that thyroxine *via* integrin  $\alpha\beta 3$  activates the ERK1/2-dependent signal transduction pathway to modulate proliferation in cancer cells (Lin et al., 2013; Davis et al., 2014; Davis et al., 2015; Lin et al., 2016a; Lin et al., 2016b). In contrast with T<sub>4</sub>, doxycycline down-regulates the gene expression of *PD-L1*, and the suppressive effect is partially removed by a MEK blocker, PD98059 (**Figure 6B**). Those results illustrate that doxycycline may modulate the *PD-L1* expression *via* the ERK1/2 pathway.

In conclusion, doxycycline is a widely used antibiotic for a long time, therefore, it is safer than other novel drugs and has well-established pharmacokinetics. It is merit to investigate for other applications such as anticancer. The summary of doxycycline-induced antiproliferative effect on breast cancer cells is illustrated in **Figure 7**. Our findings indicated that doxycycline exhibits dose-response regarding anti-proliferation in ER-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells. Results show that ER-negative cells are more sensitive to the anticancer activities of  $\alpha\beta 3$  integrin and doxycycline. Doxycycline modulates different expression patterns of genes in those types of breast cancer cells. Furthermore, doxycycline inhibits ERK1/2 phosphorylation, a downstream of integrin  $\alpha\beta 3$ , and suppresses *PD-L1* expression. Blockade of *PD-1/PD-L1* should prevent cancer cells from escaping immune defenses, thereby enhancing doxycycline-induced antitumor activity, ultimately resulting in a synergistic therapeutic effect. Collectively, these findings suggest that doxycycline interacts with integrin  $\alpha\beta 3$  to downregulate ERK1/2 phosphorylation and downstream *PD-L1*

signaling and gene expression, thereby inhibiting cancer cell growth.

supervision: H-YL, KW, and JW-P, and PD. All authors have read and agreed to the published version of the manuscript.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

Conception and design, Y-FC, Y-NY, KW, H-YL, and PD; Development of methodology, H-RC, T-YH, H-YC, and Z-LL; Acquisition of data: H-RC, T-YH, H-YC, and Z-LL; Analysis and interpretation of data: Y-FC, H-RC, Y-CY, H-YL, and PD; Writing, and review of the manuscript: Y-FC, S-HW, Y-CY, H-YL, and PD; Technical support and advice: AH, H-YL, and KW; Study

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