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Differential Effects of Integrin αv Knockdown and Cilengitide on Sensitization of Triple-Negative Breast Cancer and Melanoma Cells to Microtubule Poisons [S]

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ABSTRACT

Low survival rates of patients with metastatic triple-negative breast cancer (TNBC) and melanoma, in which current therapies are ineffective, emphasize the need for new therapeutic approaches. Integrin β1 appears to be a promising target when combined with chemotherapy, but recent data have shown that its inactivation increases metastatic potential owing to the compensatory upregulation of other integrin subunits. Consequently, we analyzed the potential of integrin subunits αv, α3, or α4 as targets for improved therapy in seven TNBC and melanoma cell lines. Experiments performed in an integrin αvβ1-negative melanoma cell line, MDA-MB-435S, showed that knockdown of integrin subunit αv increased sensitivity to microtubule poisons vincristine or paclitaxel and decreased migration and invasion. In the MDA-MB-435S cell line, we also identified a phenomenon in which change in the expression of one integrin subunit changes the expression of other integrins, leading to an unpredictable influence on sensitivity to anticancer drugs and cell migration, referred to as the integrin switching effect. In a panel of six TNBCs and melanoma cell lines, the contribution of integrins αv versus integrins αvβ3/β5 was assessed by the combined action of αv-specific small interfering RNA or αvβ3/β5 inhibitor cilengitide with paclitaxel. Our results suggest that, for TNBC, knockdown of integrin αv in combination with paclitaxel presents a better therapeutic option than a combination of cilengitide with paclitaxel; however, in melanoma, neither of these combinations is advisable because a decreased sensitivity to paclitaxel was observed.

Introduction

Integrins are a family of noncovalently associated heterodimeric adhesive receptors composed of one α and one β subunit; integrins play roles in mediating cell-extracellular matrix and cell-cell interactions. Ligation of integrins with extracellular matrix ligands induces a variety of intracellular signals and regulates several cellular responses, including migration, differentiation, proliferation, and survival (Guo and Giancotti, 2004). Integrins control a diverse array of cellular functions crucial to the initiation, progression, and metastasis of solid tumors and generally are overexpressed in aggressive breast cancer and melanoma (Desgrosellier and Cheresh, 2010).

The integrin αv subunit is known to associate with five different β subunits (β1, β3, β5, β6, and β8) to form integrin heterodimers. Among these, integrins αvβ3 and αvβ5 have been extensively studied. In normal epithelial cells, the expression of integrin αvβ3 is low, whereas high levels have been shown in some tumors, for example, breast carcinoma (Parvani et al., 2015) and melanoma (Nip et al., 1992; Danen et al., 1994). Expression of integrin αvβ3 is increased in brain metastases compared with primary tumors in breast adenocarcinoma and malignant melanoma (Vogetseder et al., 2013). It has a pivotal role in human melanoma growth (Mitjans et al., 2000) and initiates the transition from the benign radial growth phase to the malignant vertical growth phase (Albelda et al., 1990). Bianchi-Smiraglia et al. (2013) demonstrated a critical role for integrin αvβ5 in the tumorigenic potential of triple-negative breast cancer (TNBC) cells. The importance of integrin αvβ5 was also demonstrated in melanoma, showing

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ABBREVIATIONS: Ad5, adenovirus type 5; ANOVA, analysis of variance; cDDP, cisplatin; cilengitide (SML1594), cyclo(L-arginylglycyl-L-a-aspartyl-D-phenylalanyl-N-methyl-L-valyl), 2,2,2-trifluoroacetate; FAK, focal adhesion kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PE, phycoerythrin; PF-573228 or PF-228, 6-[4-(3-methanesulfonyl-benzylamino)-5-trifluoromethyl-pyrimidin-2-ylamino]-3,4-dihydro-1H-quinolin-2-one; PTX, paclitaxel; siRNA, small interfering RNA; TNBC, triple-negative breast cancer; VCR, vincristine.

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its involvement in the highly aggressive phenotype of melanoma cells expressing neuropilin 1 (Ruffini et al., 2015). Preclinical studies have revealed that α3β1 promotes protumorigenic gene expression and function in breast cancer cells (Subbaram and Dipersio, 2011). Primary melanomas express low levels of α3β1, whereas metastatic melanomas express high levels of this integrin (Melchiori et al., 1995). Downregulation of cell-surface α4 integrin and the consequent loss of α4β1 plasma fibronectin receptors in ERBB-2–overexpressing breast cancer cells are likely to be of functional significance to their ability to invade basement membranes and metastasize (Ignatoski et al., 2000). In experimental models of melanoma metastasis, incubation of melanoma cells with antibodies directed against α4β1 can significantly reduce the frequency of lung metastases in mice pretreated with proinflammatory cytokines (Kuphal et al., 2005).

Metastatic melanoma and TNBC of patients who have become refractory to immune-based or targeted therapies are still treated by chemotherapy. Several studies indicate that β1 integrins mediate drug resistance and stimulate metastasis in many different tumor types, including TNBC (Aoudjit and Vuori, 2001; Lahlou and Muller, 2011) and melanoma (Aoudjit and Vuori, 2001). It would be favorable for TNBC and melanoma treatment to find a way to enhance the efficacy of chemotherapy and concomitantly reduce metastasis. Many studies have used RNA interference to analyze whether integrins might be a target for combined cancer therapy strategies, and most have targeted integrin subunit β1 (Aoudjit and Vuori, 2012); however, few reports analyzing integrin β1 knockdown identified integrin switching, a phenomenon in which a change of expression of one integrin subunit can change the expression of other integrins and observed increased metastasis (Parvani et al., 2013; Truong et al., 2014). These data raised concerns with respect to the use of β1 integrins as drug targets to sensitize tumors to radiotherapy or chemotherapy.

Results presented in this article evaluate, in TNBC and melanoma cell lines, the use of small interfering RNAs (siRNAs) specific for integrin α subunits (αv, α3, and α4) to enhance the efficacy of chemotherapy and reduce migration and invasion. Integrin αv knockdown, in combination with vincristine (VCR) or paclitaxel (PTX), but not with cisplatin (cDDP), showed beneficial effects in TNBC but not in melanoma cell lines. The integrin αv knockdown also reduced cell migration and invasion. The therapeutic effect of combined action of the αvβ3/β5 inhibitor cilengitide and PTX in TNBC and melanoma cell lines did not entirely reflect the results of integrin αv knockdown. Since this combination showed both beneficial and detrimental effects in TNBC and melanoma, it cannot be recommended for therapy.

Materials and Methods

Cell Culture. The TNBC mesenchymal stem-like (MDA-MB-231 and MDA-MB-436) and basal-like MDA-MB-468 cell lines, as well as human melanoma MDA-MB-435S (a spindle-shaped variant of the parental MDA-MB-435), RPMI-7951, human melanoma cell line MeWo, and A375 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C with 5% CO_2 in a humidified atmosphere.

Drugs and Chemicals. cDDP (Sigma, St. Louis, MO) was dissolved in water and stored at −20°C; VCR (Sigma) and PTX (Sigma) were dissolved in phosphate-buffered saline (PBS) and dimethylsulfoxide, respectively, and stored at −20°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Millipore, Billerica, MA) was dissolved in PBS and stored at 4°C. Cilengitide (SML1594, Cyclo(L-arginylglycyl-L-a-aspartyl-D-phenylalanyl-N-methyl-L-valyl), 2,2,2-trifluoroacetate; Sigma-Aldrich, Darmstadt, Germany), a cyclized Arg-Gly-Glu(RGD)-containing pentapeptide that selectively blocks activation of αvβ3 and αvβ5 integrins, dissolved in water, and stored at −20°C; PF-228 (PF-573228, 6-[4-(3-methanesulfonyl-benzylamino)-5-trifluoromethyl-pyrimidin-2-ylamino]-3,4-dihydro-1H-quinolin-2-one; Sigma-Aldrich), a focal adhesion kinase (FAK) inhibitor, was dissolved in dimethylsulfoxide and stored at −20°C.

siRNA, Transfection, and Determination of Cell Survival. For silencing of integrin subunits β3, β5, αv, α3, and α4, the predesigned human integrin-specific siRNA sequences (Silencer Select Predesigned siRNA; Ambion, Austin, TX; siRNA ID no. s7581 (si(β3)), s7591 (si(β5)), s7568 (si(αv)), s7569 (si(αv)), s7543 (si(α3)), s7544 (si(α4)) and control nonspecific siRNA (si(-)) (Silencer Select Predesigned siRNA negative control no. 1 siRNA; Ambion) were used. The transfections of siRNAs were performed using LipofectAMINE RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. For all experiments 50 nM β3-, β5-, αv-, α3-, and α4-specific siRNA was used, except for knocking down β3 in MDA-MB-435S and MDA-MB-231, where 120 nM β3-specific siRNA was applied. The success of silencing was assessed using flow cytometry 48 hours after siRNA transfection (i.e., in the moment when cells were treated with anticancer drugs for assessment of sensitivity).

The sensitivity of cells to anticancer drugs was determined using an MTT assay as described in (Stojanović et al., 2016). Briefly, 24 hours after siRNA transfection, cells were seeded in 96-well tissue culture plates ($0.4–2 \times 10^4$ cells/well, depending on the cell line). Twenty-four hours later (i.e., 48 hours upon siRNA transfection), cells were treated with different concentrations of anticancer drugs. Seventy-two hours later, the absorbance of MTT-formazan product was measured using a microplate reader (Awareness Technology Inc., Palm City, FL) at 600 nm. The only exceptions in this protocol were MDA-MB-231 cells, which were seeded into 96-well plates, 48 hours later transfected with siRNA, and 48 hours later exposed to anticancer drugs for assessment of cell survival. Absorbance data used for analysis were obtained by subtracting the signal in a blank well.

Determination of Integrin αvβ3, αvβ5, αv, α3, and α4 Levels by Flow Cytometry. Flow cytometry was used to analyze the expression of αvβ3, αvβ5, αv, α3β1, and α4 in cells. Briefly, adherent cells were grown in tissue culture dishes, detached by Versene (Invitrogen), and washed twice with PBS. Membrane fluorescence staining for αvβ3, αvβ5, αv, α3β1, and α4 was performed (1 hour, 4°C) with monoclonal antibodies directed against αvβ3, αvβ5, α3β1, and α4 (Chemicon, Temecula, CA) and αv (Calbiochem, Darmstadt, Germany). Binding of the unlabeled primary antibodies was revealed by incubation (30 minutes, 4°C) of phycoerythrin (PE)-conjugated anti-mouse (DAKO, Carpenteria, CA) or fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (BD Pharmingen, San Jose, CA). Isotype control samples were incubated with mouse IgG1 (Sigma-Aldrich), followed by PE- or FITC-conjugated anti-mouse antibody.

Determination of Cell Migration and Invasion. For monitoring cell migration and invasion, cells transfected with control siRNA and integrin-specific siRNA (48 hours after transfection) were serum starved (24 hours), seeded (number depending on cell type) in migration or Matrigel-coated Invasion Transwell Cell Culture Inserts (pore size, 8 μm) (Corning, Corning, NY), and left to migrate or invade for 22 hours toward 10% FBS in Dulbecco's modified Eagle's medium as a chemoattractant. The number of cells migrated/invaded to the underside of the filter was determined using NIH ImageJ as previously described (Christmann et al., 2017).

Confocal Microscopy of Actin, Paxillin, and Phospho-Paxillin. For confocal microscopy analysis, MDA-MB-435S cells were seeded on coverslips and transfected with control siRNA or integrin

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αv- or α4-specific siRNA. Forty-eight hours after transfection, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% TritonX-100, and incubated with primary rabbit polyclonal anti-paxillin antibody or rabbit monoclonal anti-phospho Paxillin antibody [Y113] (Abcam, Cambridge, MA) for 1 hour, washed, and incubated with donkey Alexa-Fluor 555-conjugated-anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 hour. F-actin fibers were stained with phalloidin-FITC (Sigma-Aldrich), nuclei were counterstained with TO-PRO-3 iodide (Invitrogen) and slides mounted in Dako Fluorescent Mounting Media (DAKO). Analysis was performed using Leica TSC SP2 (Lasertechnik GmbH, Jena, Germany) microscope, and obtained images were processed with ImageJ.

Western Blot Analysis. Cells grown in six-well plates were lysed with 96°C heated 1× Laemmli buffer, scraped off the plate, boiled for 3 minutes at 96°C, and sonicated. Proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane (Amersham, Braunschweig, Germany), blocked in 5% nonfat dry milk, and membranes were incubated with rabbit monoclonal antibody against phosphorylated focal adhesion kinase Y397, pFAK(Y397), or total FAK (FAK) (Abcam, Cambridge, UK), followed by incubation with horseradish peroxidase-coupled secondary antibody (GE Healthcare, Chicago, IL, Invitrogen). Detection was done using a chemiluminescence reagent (GE Healthcare).

Statistical Analysis. Each experiment was repeated at least three times, and GraphPad Prism software v5.0 was used to analyze the data. All data from MTT experiments were analyzed by related-measure two-way analysis of variance (ANOVA) with Bonferroni posttest. Data obtained from migration, invasion, and immunofluorescence were analyzed by related measure one-way ANOVA with Dunnett's multiple comparison: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Integrin Expression Patterns in TNBC and Melanoma Cell Lines. To investigate the role of knockdown of αv, α3, and α4 integrins in TNBC and melanoma cells in sensitivity to different anticancer drugs, migration, and invasion, the expression of integrins αv, αvβ3, αvβ5, α3β1, and α4 was analyzed in TNBC and melanoma cell lines. Cell lines used were three TNBC cell lines (MDA-MB-231, MDA-MB-468, and MDA-MB-436), three melanoma cell lines (RPMI-7951, MeWo, and A375), and cell line MDA-MB-435S, which has been used for years as a TNBC cell line, but recent data clearly showed that these cells originate from melanoma (Korch et al., 2018). The debate regarding the authenticity of this cell line started when this cell line was shown to be identical to M14 melanoma cell line (Rae et al., 2007). It has just been resolved by authentication testing of M14 from 1975 (before the establishment of MDA-MB-435S) with comparison, with donor serum and lymphoblastoid cell line ML14, that M14 is the authentic cell line and MDA-MB-435S is a misidentified derivative (Korch et al., 2018).

Cell-surface expression of integrins in live cells of all seven cell lines was measured by flow cytometry (Fig. 1). Integrin αv was highly expressed in all cells except in MDA-MB-436 cells, which express a moderate amount of this subunit. The αv subunit forms a complete integrin complex through heterodimerization with one of five β subunit binding partners: β1, β3, β5, β6, and β8. We were particularly interested in the expression of integrins αvβ3 and αvβ5 and less interested in αvβ6 and αvβ8. Namely, integrin αvβ6 is weakly expressed in MDA-MB-435S, MDA-MB-231, and MDA-MB-468 but absent in most melanoma cells, similarly to integrin αvβ8 (Goodman et al., 2012). Flow cytometry analysis showed that integrin αvβ3 was highly expressed in MDA-MB-435S and A375 cells, moderately expressed in MDA-MB-231, MDA-MB-468, and RPMI-7951 cells, whereas the expression was absent in MDA-MB-436 and MeWo cells. On the other hand, integrin αvβ5 was highly expressed in all cell lines except for MDA-MB-468 and MDA-MB-436, which express low amounts of this heterodimer. The most evenly expressed integrin in TNBC and melanoma cells was integrin α3β1, and the least represented integrin heterodimer in our cell panel was integrin α4β1 (α4 subunit forms heterodimer with β1 or β7 (Raab-Westphal et al., 2017)) with high expression in melanoma cell lines MDA-MB-435S, RPMI-7951, and A375 and very low expression in TNBC cell line MDA-MB-436. Expression of integrin subunits αv and α4 and integrin heterodimers αvβ3, αvβ5, and α3β1 in all cell lines is summarized in Supplemental Table S1.

Integrin Subunit β3 or β5 Knockdown Alters Expression of Both αv Integrin Heterodimers αvβ3 and αvβ5: Observation of the Integrin-Switching Effect in MDA-MB-435S Cells. We started our investigation with the melanoma cell line MDA-MB-435S because it has been previously shown that silencing of integrin αv in this cell line enhanced radiosensitivity (Cao et al., 2006). Another reason for using this cell line is that it expresses high levels of integrins αvβ3, αvβ5, α3β1 and α4β1 but does not express integrin αvβ1 (Wong et al., 1998; Palmieri et al., 2002; Taherian et al., 2011). Therefore, by inclusion of this cell line, we aimed to differentiate between the contribution of integrins αvβ3 and αvβ5 in drug sensitivity, migration, and invasion and to analyze the function of these integrins independent of integrin αvβ1 interference. Our goal was to determine which of the integrin heterodimers might be used as candidates for increasing sensitivity to anticancer drugs and inhibition of migration and invasion. Therefore, we decided to knockdown integrin subunits β3 or β5 to decrease the expression of integrin heterodimers αvβ3 or αvβ5, respectively, and knockdown integrin subunits αv, α3, or α4, with the aim of decreasing the expression of all αv integrin heterodimers, integrin α3β1, or α4β1, respectively. The cell-surface expression of integrin heterodimers αvβ3, αvβ5, and α3β1 and the amount of integrin subunits αv and α4 were measured 48 hours after siRNA transfection using flow cytometry and compared with the expression of corresponding molecules in cells transfected with control siRNA. The control siRNA had minimal effect on the expression of all integrin subunits and heterodimers compared with nontransfected MDA-MB-435S cells (data not shown). Upon transfection with integrin subunit β3-specific siRNA (Fig. 2, first row) integrin αvβ3 expression decreased to 71% of the value of control siRNA. Concomitantly, we observed significant upregulation of integrin αvβ5 (i.e., 59% higher level compared with control siRNA). Silencing of integrin subunit β3 decreased the total amount of integrin subunit αv very slightly. A similar balance effect was observed when MDA-MB-435S cells were transfected with integrin subunit β5-specific siRNA (Fig. 2, second row), which resulted in significant decrease of integrin αvβ5 expression (44% of the value for control siRNA) and simultaneous increase of integrin αvβ3 expression by 27%. Silencing of integrin subunit β5 slightly decreased the total amount of integrin αv. To reduce simultaneously the expression of integrins αvβ3 and αvβ5, cells were transfected with integrin

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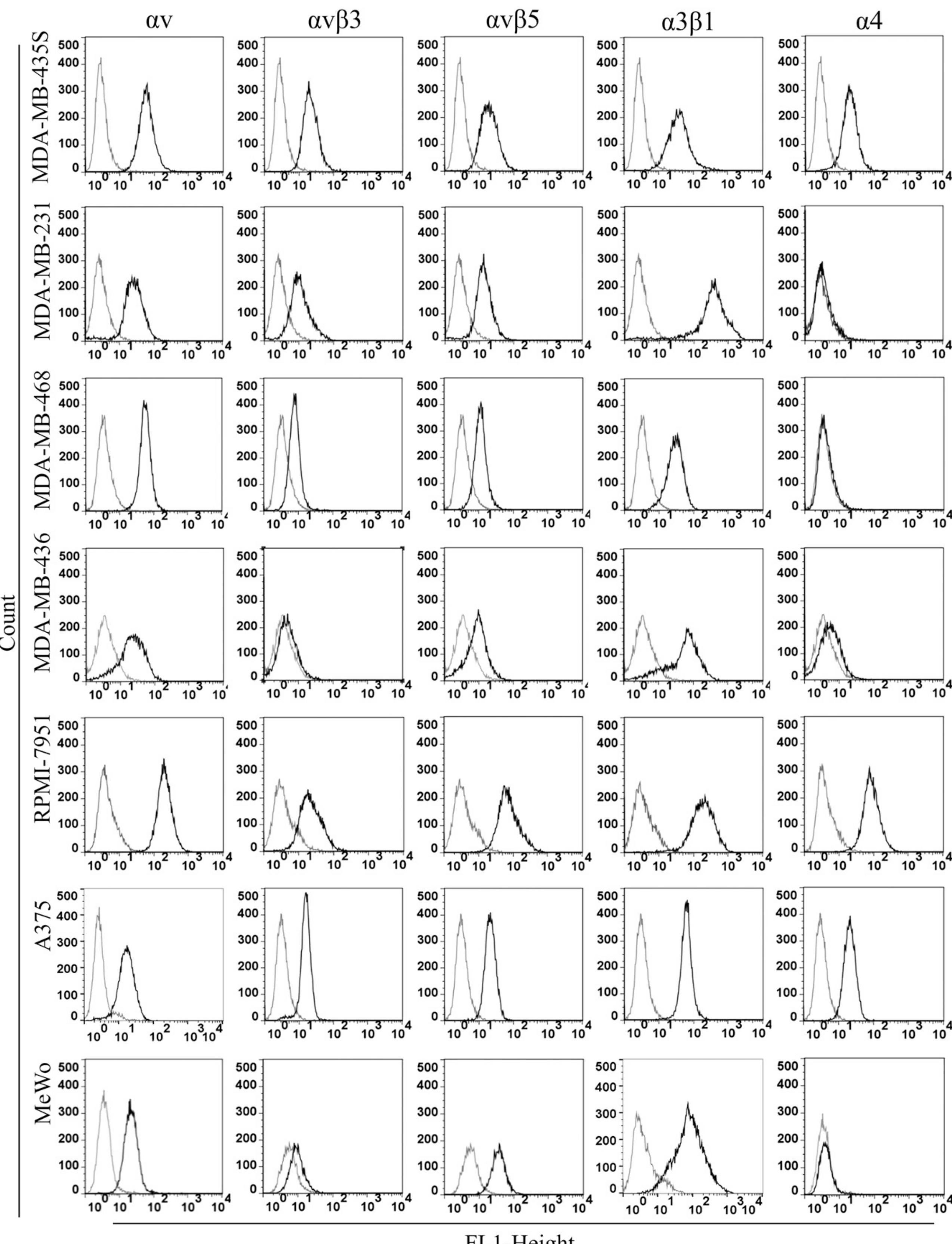

Fig. 1. Surface expression of integrin subunit αv, integrins αvβ3, αvβ5, α3β1, and integrin subunit α4 in melanoma cell lines MDA-MB-435S, RPMI-7951, A375, MeWo, and TNBC cell lines MDA-MB-231, MDA-MB-468, and MDA-MB-436. Cells were detached by Versene and analyzed by flow cytometry using antibodies against integrin subunit αv, α4, or integrins αvβ3 and αvβ5 (black histogram), and isotype-matched antibody as a negative control (gray histogram), followed by rabbit FITC-conjugated-antimouse antibody. The representative data of three independent experiments yielding similar results are shown.

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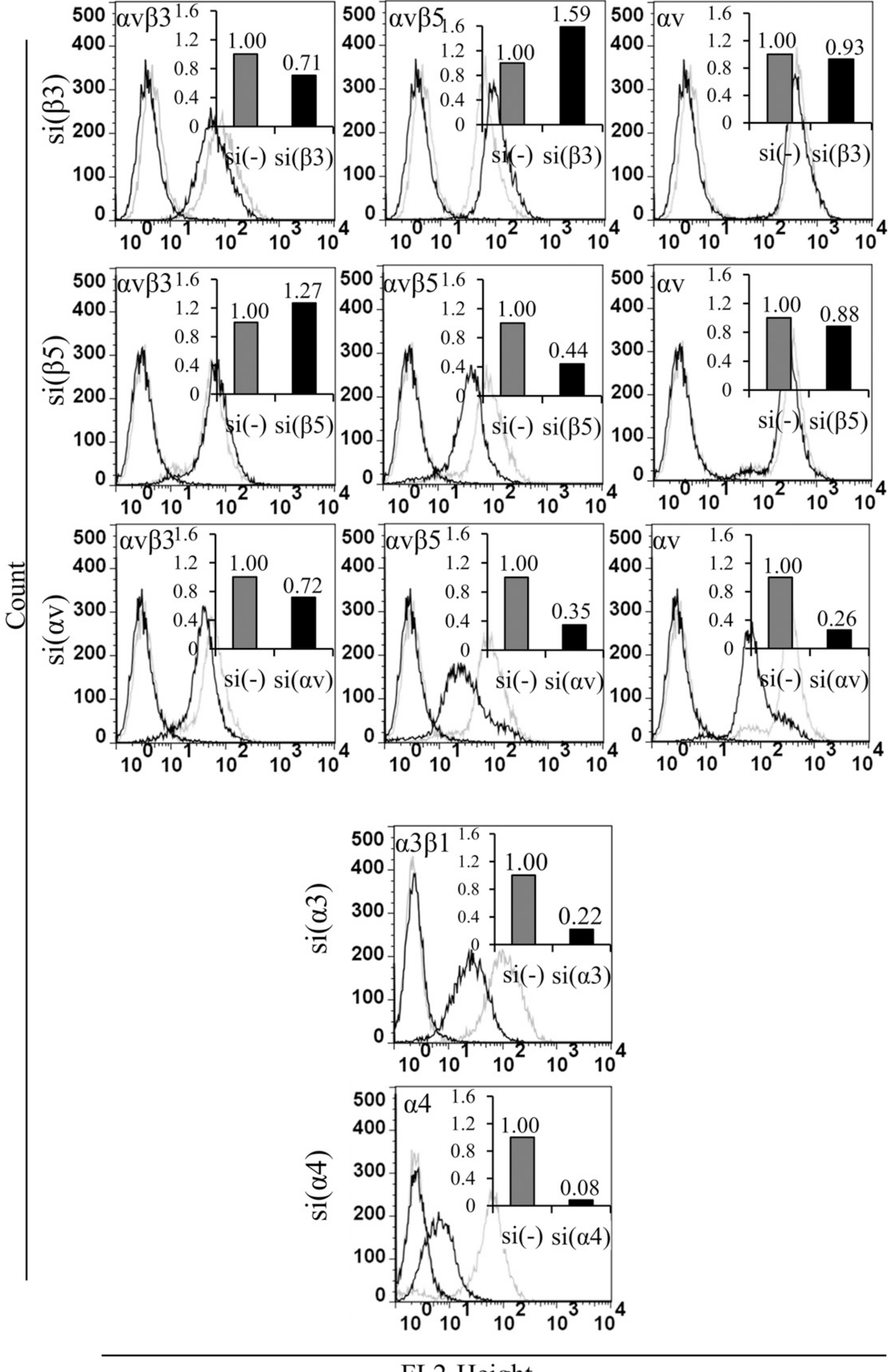

Fig. 2. Integrin subunit β3, β5, αv, α3, or α4 knockdown in MDA-MB-435S cells reduces the expression of integrins αvβ3, αvβ5, integrin subunit αv, integrin α3β1, or integrin subunit α4, respectively. Surface expression of integrins αvβ3 and αvβ5, integrin subunit αv, integrin α3β1, or integrin subunit α4 in MDA-MB-435S cells was analyzed by indirect flow cytometry 48 hours after transfection with integrin subunit β3-, β5-, αv-, α3-, or α4-specific siRNA (black histogram) and compared with cells transfected with control siRNA (gray histogram). Representative data of three independent experiments yielding similar results are shown. Mean fluorescence intensities (MFI) relative to cells transfected with control siRNA for corresponding histograms are shown in upper right corners.

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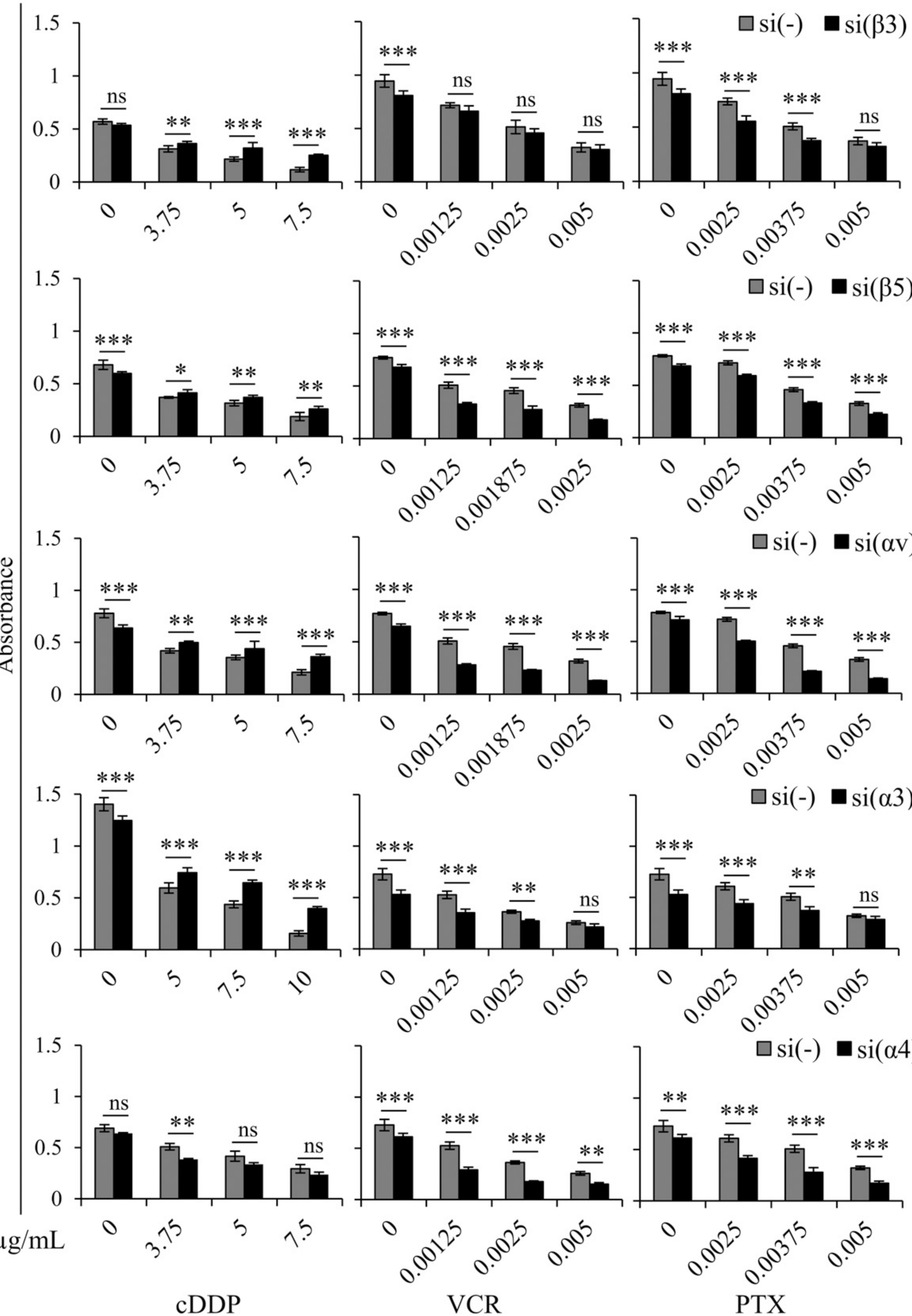

Fig. 3. Modulation of MDA-MB-435S cell sensitivity to cDDP, VCR, and PTX upon integrin subunit $\beta3$, $\beta5$, αv, $\alpha3$, or $\alpha4$ knockdown. MDA-MB-435S cells were transfected with control or integrin subunit $\beta3$-, $\beta5$-, αv-, $\alpha3$-, or $\alpha4$-specific siRNA. Twenty-four hours after transfection cells were seeded in 96-well plates, treated the next day with cDDP, VCR, or PTX, and cytotoxicity was measured by MTT 72 hours later. Average absorbance data ± S.D. shown are representative of at least three independent experiments yielding similar results. Data were analyzed by two-way ANOVA with Bonferroni post-test. ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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TABLE 1
Effect of different integrin subunits knockdown on sensitivity of integrin $\alpha v\beta 1$-negative melanoma cell line MDA-MB-435S cells to cisplatin (cDDP), vincristine (VCR), and paclitaxel (PTX)

Integrin Subunit	Antitumor Drug		
	cDDP	VCR	PTX
$\beta 3$	R	R	R
$\beta 5$	R	S	NI
αv	R	S	S
$\alpha 3$	R	R	R
$\alpha 4$	NI	S	S

NI, no interaction between treatments in combination; R, resistance (decreased sensitivity to combination); S, sensitization (increased sensitivity to combination).

subunit αv-specific siRNA (Fig. 2, third row). We observed decreased expression of αv (26% of the value for control siRNA) and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, down to 72% and 35% of the value for control siRNA, respectively. MDA-MB-435S cells were also transfected with integrin subunit $\alpha 3$- (Fig. 2, fourth row) or $\alpha 4$-specific siRNA (Fig. 2A, fifth row), which led to significant downregulation of integrin $\alpha 3\beta 1$ (22% of the value for control siRNA) or $\alpha 4\beta 1$ (8% of the value for control siRNA). We hypothesize that in the MDA-MB-435S cell line, expressing principally two αv integrin heterodimers ($\alpha v\beta 3$ and $\alpha v\beta 5$), knockdown of either integrin subunit $\beta 3$ or $\beta 5$ releases integrin subunit αv. This subunit likely heterodimerizes with free $\beta 5$ or $\beta 3$ subunits in the cell, increasing $\alpha v\beta 5$ or $\alpha v\beta 3$, respectively. To test this hypothesis, we measured the adenovirus type 5 (Ad5)–mediated transgene expression and the amount of adenoviral DNA upon transduction. Briefly, we used the property of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ to have an analogous role in Ad5 attachment (binding) to the cell surface and internalization (entry) into the cell. When the $\alpha v\beta 3/\alpha v\beta 5$ ratio is disturbed, the amount of attached or internalized Ad5 remains the same, but integrin $\alpha v\beta 5$ has a crucial role in Ad5 release from endosome (i.e., the amount of integrin $\alpha v\beta 5$ that modulates Ad5 release can be measured as transgene expression) (Majhen et al., 2009). Therefore, the $\alpha v\beta 3/\beta 5$ balance effect in MDA-MB-435S cells was indeed confirmed in this way since the Ad5 transgene expression was increased upon integrin $\beta 3$-specific siRNA transfection (2.36-fold owing to the integrin $\alpha v\beta 5$ upregulation) or decreased upon integrin $\beta 5$-specific siRNA transfection (0.42-fold owing to the integrin $\alpha v\beta 5$ downregulation) (Supplemental Fig. S1A). Concurrently the amount of attached (Supplemental Fig. S1B) and internalized (Supplemental Fig. S1C) Ad5 DNA did not change significantly.

Beneficial Effect of Integrin Subunit $\beta 5$, αv, or $\alpha 4$ Knockdown in Combination with VCR and PTX in Melanoma Cell Line MDA-MB-435S. To test whether silencing of integrins could be used to increase the sensitivity of MDA-MB-435S cells to chemotherapy, sensitivity was measured by MTT assay (Fig. 3). The control siRNA had minimal effect on the survival of MDA-MB-435S cells upon treatment with all selected anticancer drugs (data not shown). Silencing of integrin subunits decreased the survival of MDA-MB-435S cells; therefore, in Fig. 3, we present absorbance data from MTT tests for each combination of integrin subunit knockdown and anticancer drug. The various effects of combinations are summarized in Table 1. We determined whether integrin knockdown increases sensitivity (marked as S), decreases sensitivity (marked as R), or showed no interference (marked as NI), which can also be considered a beneficial effect since additive effect results in overall lower cell survival. Our results show that silencing of integrin subunits αv, $\beta 3$, $\beta 5$, or $\alpha 3$ decreased the sensitivity of MDA-MB-435S cells to cDDP, whereas silencing of integrin subunit $\alpha 4$ showed no interaction with cDDP sensitivity (Fig. 3, left column; Table 1). On the other hand, silencing of integrin subunits αv, $\beta 5$, or $\alpha 4$ increased sensitivity of MDA-MB-435S cells to VCR and PTX, whereas silencing of integrin subunits $\beta 3$ or $\alpha 3$ decreased sensitivity to VCR and PTX (Fig. 3, middle and right column; Table 1). We conclude that knockdown of integrin subunits αv, $\beta 5$, or $\alpha 4$ could be used for sensitization of MDA-MB-435S cells to VCR and PTX.

Activation of apoptosis is the main mode of cell death induced by chemotherapeutic agents. By binding to β tubulin, paclitaxel and its derivatives stabilize cytoskeletal microtubules, which leads to cell-cycle arrest via G2/M phase block. Prolonged arrest of cell division eventually activates a checkpoint and induces programmed cell death (Jordan and Wilson, 2004). To test whether knockdown of integrin subunit αv increases the fraction of apoptotic cells in MDA-MB-435S upon PTX treatment, we determined the apoptosis rate by Annexin V/PI staining. Cells transfected with the integrin subunit αv siRNA demonstrated a significantly greater number of cells in apoptosis 48 hours after PTX treatment in comparison with cells transfected with control siRNA (Supplemental Fig. S2A). We conclude that knockdown of integrin subunit αv increases sensitivity of MDA-MB-435S cells to PTX through the mechanism that increases apoptosis. Integrin αv knockdown had no effect on MDA-MB-435S cell-cycle progression. Nevertheless, the percentage of sub-G1 population after PTX treatment was higher in cells transfected with integrin subunit αv siRNA compared with control siRNA (Supplemental Fig. S2B).

Integrin Subunit αv or $\alpha 4$ Knockdown in MDA-MB-435S Cells Differently Influence Migration, Organization of Actin Network, and Focal Adhesions. We next compared the ability of MDA-MB-435S cells transfected with control siRNA or integrin subunits αv, $\beta 3$, $\beta 5$, or $\alpha 4$ siRNA to migrate using FBS as the chemoattractant (Fig. 4A). Migration of the MDA-MB-435S cells transfected with control siRNA resembled the parental MDA-MB-435S cells (data not shown). Migration of MDA-MB-435S cells transfected with integrin subunit αv-specific siRNA was dramatically reduced. To investigate specifically the role of integrins $\alpha v\beta 3$ or $\alpha v\beta 5$ in the migration of MDA-MB-435S cells, we determined cell migration upon integrin subunit $\beta 3$ or $\beta 5$ silencing. Silencing of neither integrin subunits $\beta 3$ or $\beta 5$ significantly influenced MDA-MB-435S cell migration. In light of previously shown integrin balance (switching) effect (Fig. 2), we conclude that both integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are equally important for cell migration; consequently, their altered ratio does not significantly change the overall migration. Interestingly, silencing $\alpha 4\beta 1$, an integrin that generally has a promigratory role (Wang et al., 2005), significantly increased cell migration (Fig. 4A). We hypothesize that this could be a consequence of released integrin subunit $\beta 1$ upon integrin subunit $\alpha 4$-specific siRNA transfection, but we can only speculate which $\beta 1$ heterodimers could be formed. Our hypothesis is somewhat supported by the fact that transfection of MDA-MB-435S cells with integrin subunit $\alpha 3$-specific siRNA results in significantly

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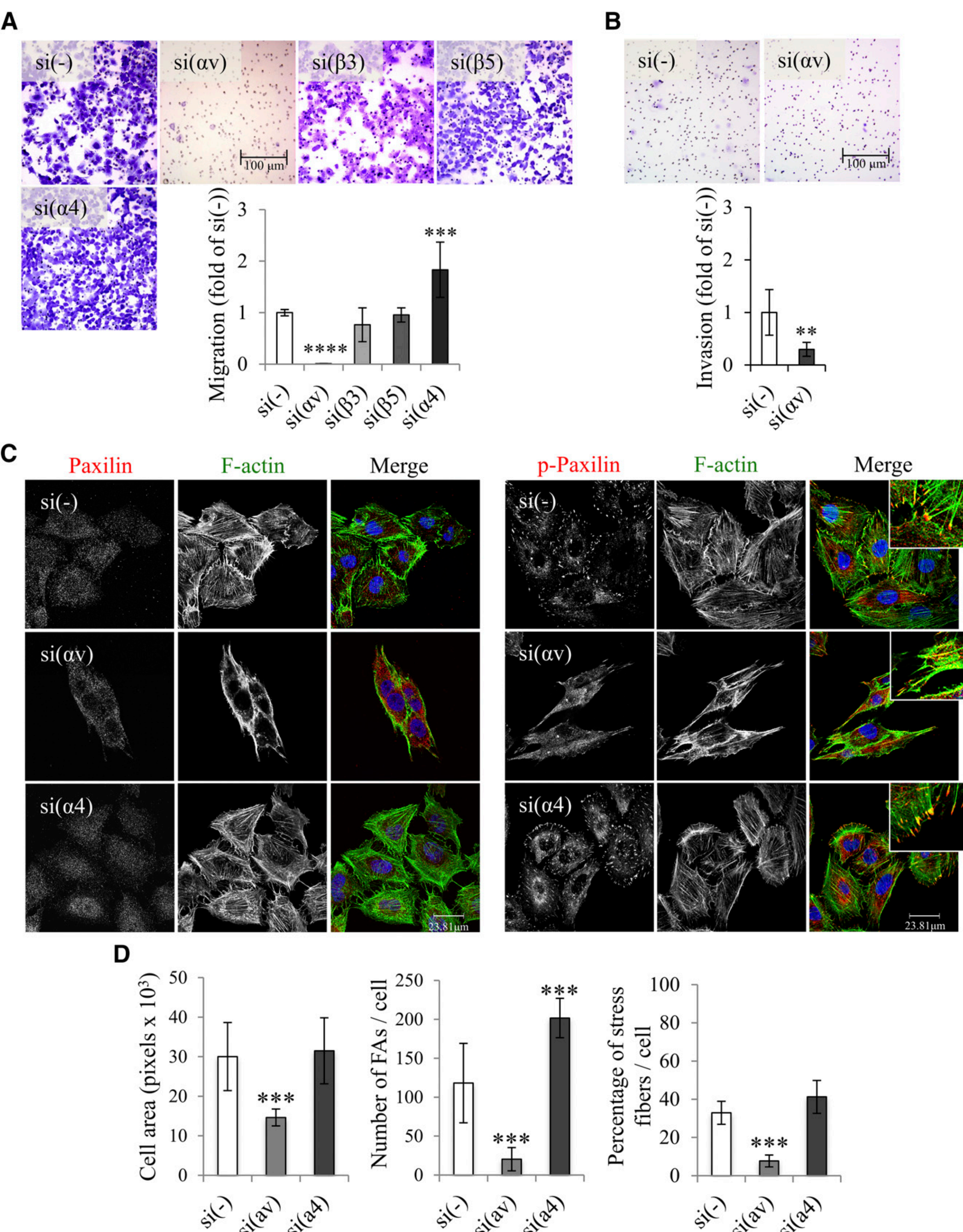

Fig. 4. Alteration of migration, invasion, actin network organization, and focal adhesions upon integrin subunit αv or α4 knockdown in MDA-MB-435S cells. (A) In MDA-MB-435S cells, integrin αv knockdown decreases and integrin α4 knockdown increases migration compared with cells transfected with control siRNA, whereas no change was observed upon integrin subunit β3 or β5 knockdown. Forty-eight hours after transfection with control, integrin subunit αv-, β3-, β5-, or α4-specific siRNA, cells were serum starved for 24 hours and then seeded in Transwell cell culture inserts and left to migrate for 22 hours toward serum. Cells on the underside of the inserts were stained with crystal violet, photographed, and counted. Averages of five microscope fields of three independently performed experiments ± S.D. are shown compared with control cells transfected with control siRNA set as 1. Data were analyzed by one-way ANOVA with Dunnett's multiple Comparison. ***$P < 0.001$; ****$P < 0.0001$. (B) Decreased invasion of integrin subunit αv-specific

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increased migration compared with cells transfected with control siRNA (Supplemental Fig. S3). Since integrin subunit α3 silencing does not have any effect on sensitivity to VCR and PTX, we postulate that increased sensitivity to microtubule poisons in cells transfected with integrin subunit α4-specific siRNA is more likely the consequence of integrin α4β1 downregulation than formation of some integrin β1-containing integrin heterodimer in the cell upon α4-specific siRNA transfection.

To evaluate in vitro invasiveness through Matrigel, we performed invasion assay upon integrin αv knockdown (Fig. 4B), which mimics the three-step hypothesis of invasion-adhesion, proteolytic dissolution of the extracellular matrix, and migration (Albini, 1998), and we observed significantly decreased invasion.

Next, we monitored whether knockdown of integrin subunits αv and α4 influences actin remodeling and focal adhesions. MDA-MB-435S cells transfected with control siRNA resembled the parental MDA-MB-435S cells (data not shown), with cells well spread, prominent actin stress fibers, and cortical actin apparent. We observed punctuate paxillin staining in MDA-MB-435S cells (data not shown), cells transfected with control, integrin subunit αv-, or α4-specific siRNA (Fig. 4C, left panel). Tyrosine phosphorylation of paxillin is important for focal adhesion formation and for function of paxillin as a docking molecule in focal adhesions (Nakamura et al., 2000). The phospho-paxillin (Y113) staining in MDA-MB-435S cells transfected with control siRNA showed typical discrete short clusters of focal adhesion complex staining enriched at both the central region and the periphery of cells, which was coincident with the F-actin at focal adhesion sites (Fig. 4C, right panel, first row; enlarged focal adhesion sites are presented). Upon silencing integrin subunit αv, MDA-MB-435S cells were less well spread and appeared smaller; paxillin staining therefore seemed intense (Fig. 4C, left panel, second row); however, we verified that upon integrin αv knockdown, the size of the cells was similar to that of MDA-MB-435S cells transfected with control siRNA because their FSC/SSC ratio in flow cytometry analysis was similar (data not shown). Upon integrin αv knockdown, MDA-MB-435S cells were showing disorganization of actin with loss of stress fibers and significant loss of phospho-paxillin (Y113) staining, indicating focal adhesion number reduction (Fig. 4C, right panel, second row; 4D). An increase in phospho-paxillin staining was observed in MDA-MB-435S cells after integrin subunit α4-specific siRNA transfection (Fig. 4C, right panel, third row; 4D), especially at the leading edge of migrating cells, which is in line with increased migration (Fig. 4A).

Integrin Subunit αv Knockdown Differently Affects Sensitivity of TNBC and Melanoma Cell Lines to Microtubule Poisons. Given the results obtained in the MDA-MB-435S cell line (Figs. 2–4; Supplemental Fig. S3), we decided not to proceed with integrin α3 or α4 silencing because we observed either decreased sensitivity to anticancer drugs or increased migration, which would not benefit cancer treatment. We decided to analyze the influence of integrin αv knockdown (Fig. 5A) on sensitivity to cytotoxic activity of cDDP, VCR, and PTX (Fig. 5B) in three TNBC and three melanoma cell lines. We verified in the MDA-MB-435S cell line the absence of integrin αv siRNA off-target effects by comparison with another integrin αv-specific siRNA and obtained similar results by flow cytometry, as well as MTT, using PTX (data not shown). The control siRNA had minimal effect on the survival of cells upon treatment with all selected anticancer drugs (data not shown). The various effects of the integrin subunit αv knockdown and anticancer drug combinations are summarized in Table 2, with increased sensitivity marked as S, decreased sensitivity marked as R, and NI showing no interference. Our results show that knockdown of integrin αv achieved by αv-specific siRNA transfection in all cell lines was successful (Fig. 5A) and resulted in beneficial effects in all three TNBC cell lines in combination with VCR and PTX (Fig. 5B; Table 2). Conversely, in three additional melanoma cell lines, we observed beneficial effects (similar to effects observed in MDA-MB-435S) in one (RPMI-7951); in the other two (A375 and MeWo) cell lines, we observed a clear detrimental effect (i.e., decreased sensitivity to all three drugs, cDDP, VCR, and PTX) (Fig. 5B; Table 2). Regarding cDDP, in two TNBC (MDA-MB-468 and MDA-MB-436) and two melanoma (A375 and MeWo) cell lines, we observed decreased sensitivity, which, if we take into account that similar results were obtained in the MDA-MB-435S melanoma cell line (Fig. 3; Table 1), suggests against the combination of integrin subunit αv knockdown and cDDP for improved therapy. In conclusion, knockdown of integrin subunit αv could be used for sensitization to VCR and PTX of TNBC, but not melanoma.

To analyze the involvement of integrin heterodimers αvβ3 or αvβ5, in sensitization to VCR or PTX observed upon integrin αv knockdown, we performed silencing of integrin subunits β3 or β5 in MDA-MB-231 and RPMI-7951 cell lines and measured cell survival. Data are presented in Supplemental Fig. S4, and the various effects of combinations are summarized in Supplemental Table S2. Surprisingly, in MDA-MB-231, integrin β5 knockdown did not show interactions with sensitivity to VCR or PTX, whereas integrin β3 knockdown decreased cell sensitivity to both drugs. On the contrary, in melanoma cell line RPMI-7951, knockdown of either

siRNA transfected MDA-MB-435S cells compared with cells transfected with control siRNA. Forty-eight hours after transfection with control or integrin subunit αv-specific siRNA, cells were serum starved for 24 hours and then seeded in Matrigel-coated Invasion Transwell cell culture inserts and left to invade for 22 hours toward serum. Cells on the underside of the inserts were fixed, stained with crystal violet, photographed, and counted. Averages of five microscope fields of three independently performed experiments ± S.D. are shown compared with control cells transfected with control siRNA set as 1. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison. $^{**}P < 0.01$. (C) Integrin subunit αv or α4 knockdown influences actin network organization and focal adhesions in MDA-MB-435S cells. Cells were seeded onto coverslips 1 day before transfection with control (first row), integrin subunit αv- (second row), or α4-specific (third row) siRNA. Forty-eight hours after transfection, cells were fixed, permeabilized, and stained for anti-paxillin or anti-phospho-paxillin [Y117] antibody, followed by Alexa-Fluor 555-conjugated antibody (red). F-actin staining (green) was performed in all samples, and nuclei were stained with TO-PRO-3 iodide (blue). Analysis was performed using TCS SP Leica. (D) MDA-MB-435S cells transfected with integrin subunit αv-specific siRNA are smaller, have fewer focal adhesions per cell, and have fewer stress fibers compared with cells transfected with control siRNA. Data represent measurements of >20 cells and are plotted as mean ± S.D. ($n = 3$). Data were analyzed by one-way ANOVA with Dunnett's multiple comparison. $^{***}P < 0.001$.

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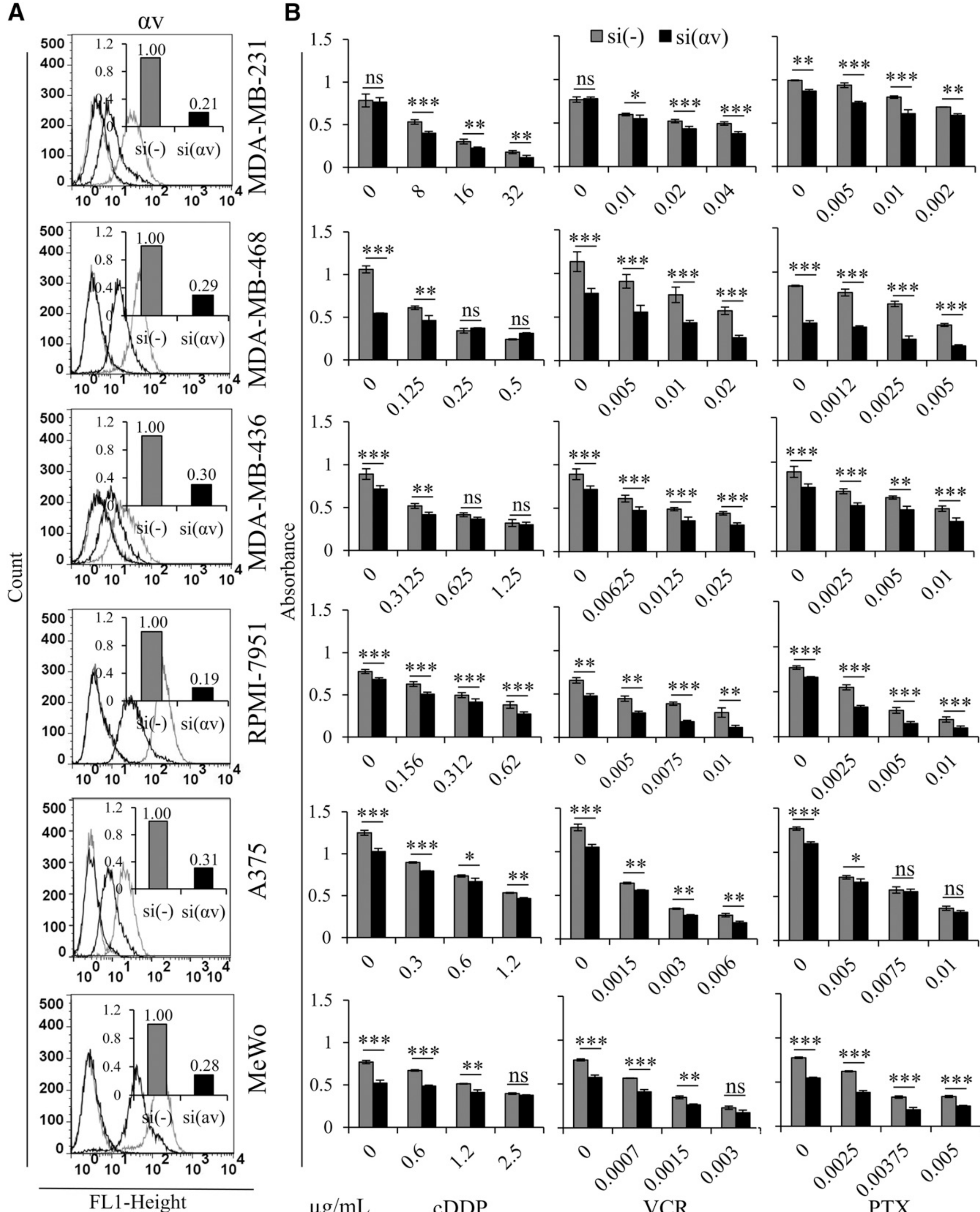

Fig. 5. Integrin subunit αv knockdown in TNBC (MDA-MB-231, MDA-MB-468, and MDA-MB-436) and melanoma (RPMI-7951, A375, and MeWo) cells reduces expression of αv integrins and modulates sensitivity to cDDP, VCR, and PTX. (A) Surface expression of integrin subunit αv in TNBC and melanoma cells after transfection with integrin subunit αv-specific siRNA compared with cells transfected with control siRNA. Forty-eight hours after transfection, cells were analyzed by flow cytometry using integrin subunit αv-specific antibody followed by FITC-conjugated antibody. Gray histogram represents control siRNA, and black histogram represent integrin subunit αv-specific siRNA. Mean fluorescence intensities (MFI) relative to cells transfected with control siRNA for each histogram are shown in upper right corners. Representative data of three independent experiments yielding similar results are shown. (B) Integrin subunit αv knockdown modulates the sensitivity of TNBC and melanoma cells to cDDP, VCR, and PTX. Cells were transfected with control or integrin αv-specific siRNA. Twenty-four hours after transfection, cells were seeded in 96-well plates, treated the next day with

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TABLE 2
Effect of integrin subunit αv knockdown on sensitivity of triple-negative breast cancer (TNBC) and melanoma cell lines to cisplatin (cDDP), vincristine (VCR), and paclitaxel (PTX)

Cell Line	Anticancer Drug		
	cDDP	VCR	PTX
MDA-MB-231	S	S	S
MDA-MB-468	R	NI	NI
MDA-MB-436	R	NI	NI
RPMI-7951	NI	NI	S
A375	R	R	R
MeWo	R	R	R

NI, no interaction between treatments in combination; R, resistance (decreased sensitivity to combination); S, sensitization (increased sensitivity to combination).

integrin $\beta3$ or $\beta5$ led to increased sensitivity to VCR or PTX (Supplemental Fig. S4A; Supplemental Table S2). It should be noted that both cell lines express integrin αv$\beta1$, and it is likely that this integrin also plays a role in sensitivity to VCR or PTX upon integrin αv knockdown. Because of the observed decreased sensitivity of MDA-MB-231 cells to VCR or PTX upon integrin $\beta3$ knockdown, we also checked whether knockdown of integrin subunits $\beta3$ or $\beta5$ causes the balance effect we observed in MDA-MB-435S cells. We did not observe this effect (Supplemental Fig. S4B); however, this result does not exclude the possibility of other integrin-switching effects.

Knockdown of Integrin αv Significantly Decreases Migration of TNBC and Melanoma Cell Lines: Key Role of Integrin αv$\beta5$. Integrins αv have a well established role in the migration and invasion of tumor cells (Desgrosellier and Cheresh, 2010). Thus, we examined the in vitro migration of TNBC MDA-MB-231 and MDA-MB-468 and melanoma cell line RPMI-7951 upon integrin αv knockdown, achieved by αv-specific siRNA transfection, using Transwell inserts and FBS as a chemoattractant (Fig. 6A). Migration of MDA-MB-231 and MDA-MB-468 cells upon integrin αv knockdown was reduced to 40% or 35% of the migration capacity of parental cells transfected with control siRNA, respectively, whereas in melanoma cell line RPMI-7951, integrin αv knockdown dramatically reduced cell migration to the extent that it was difficult to find cells that migrated to the bottom side of the Transwell insert membrane. To investigate specifically which integrin heterodimer, αv$\beta3$ or αv$\beta5$, is more important for inhibition of migration, we measured cell migration upon integrin $\beta3$ or $\beta5$ knockdown that led to decreased expression of integrin αv$\beta3$ or αv$\beta5$. Cell migration was not altered upon knockdown of integrin $\beta3$ in MDA-MB-231, MDA-MB-468, or RPMI-7951 cell lines. It was the decreased expression of integrin αv$\beta5$, obtained by integrin $\beta5$ knockdown, that significantly inhibited cell migration in all three cell lines; although in MDA-MB-231 and RPMI-7951, it was not as successful as the knockdown of all integrin αv heterodimers, as observed in MDA-MB-468 cells.

To evaluate in vitro invasiveness of representative TNBC and melanoma cell lines through Matrigel, we also performed Transwell insert invasion assays (Fig. 6B). In MDA-MB-231 cells, we observed decreased invasion, in values similar to the migration inhibition, down to 40% of control, whereas in RPMI-7951 cells, the invasion was even more dramatically inhibited, down to 15% of the control. The effects of integrin subunit knockdown on migration and invasion are summarized in Table 3.

Targeted Inactivation of Integrin Heterodimers αv$\beta3$ and αv$\beta5$ Using Cilengitide Combined with PTX Does Not Mimic the Combination of Integrin αv Knockdown and PTX. An integrin/anticancer drug combination, shown to be the most promising in our experiments, was integrin αv knockdown/VCR or PTX in TNBC cell lines because of the beneficial effect of the combination, which simultaneously decreases migration and invasion. To analyze further the importance of integrins αv$\beta3$ and αv$\beta5$ in response to PTX, we assessed the combination therapy of cilengitide (a potent and selective inhibitor of integrins αv$\beta3$ and αv$\beta5$) and PTX. Cilengitide enabled us, unlike integrin αv knockdown, to analyze the role of inhibition of integrins αv$\beta3$ and αv$\beta5$ signaling without affecting the expression of other integrins αv.

To measure the combined effect of cilengitide and PTX, first we assessed the cytotoxic activity of different cilengitide concentrations in our panel of seven cell lines and found a dose-dependent effect of cilengitide on cell survival (Fig. 7). The highest level of sensitivity was observed in the melanoma cell line MeWo; TNBC cell line MDA-MB-468 was the least sensitive cell line. MDA-MB-468 cells were highly resistant to cilengitide treatment, which was not expected. Namely, they express a comparable amount of integrin αv$\beta5$ as MDA-MB-436 but do express integrin αv$\beta3$, which is not the case in MDA-MB-436 (Fig. 1; Supplemental Table S1). Nevertheless, MDA-MB-436 showed a cytotoxic effect with a 20-fold lower concentration of cilengitide. All other cell lines were similarly sensitive to cilengitide. In Fig. 7, we present the data for each combination of cilengitide and PTX, and the various effects of combinations are summarized in Table 4. Cilengitide showed a beneficial effect in all cell lines except the TNBC cell line MDA-MB-468 and melanoma cell line MeWo, in which detrimental effect was observed. More specifically, cilengitide increased the sensitivity of TNBC MDA-MB-231 and melanoma RPMI-7951 and A375 cell lines while demonstrating an additive effect with PTX in TNBC MDA-MB-436 and melanoma cell line MDA-MB-435S. In conclusion, comparison of the combination of integrin αv knockdown/PTX (Table 2) versus cilengitide/PTX (Table 4) shows discrepancy in the TNBC cell line MDA-MB-468 and melanoma cell line A375.

Inhibition of FAK Phosphorylation at Tyrosine 397 Decreases Sensitivity to PTX in Six of Seven TNBC and Melanoma Cell Lines. Focal adhesion kinase is involved in integrin-induced signal transduction pathways. It plays a central role in tumor progression and metastasis and represents the link to growth factor receptors. In FAK, several sites of tyrosine phosphorylation have been identified. Tyrosine 397, the major autophosphorylation site in FAK, is essential for most FAK functions (Zhao and Guan, 2011). In a variety of human cancers, increased expression and/or activation of FAK has been found. Therefore, small molecular inhibitors of FAK kinase activity might be used in treatment of metastatic cancer

cDDP, VCR, or PTX, and cytotoxicity was measured by MTT 72 hours later. Average absorbance data ± S.D. shown are representative of at least three independent experiments yielding similar results. Data were analyzed by two-way ANOVA with Bonferroni post-test. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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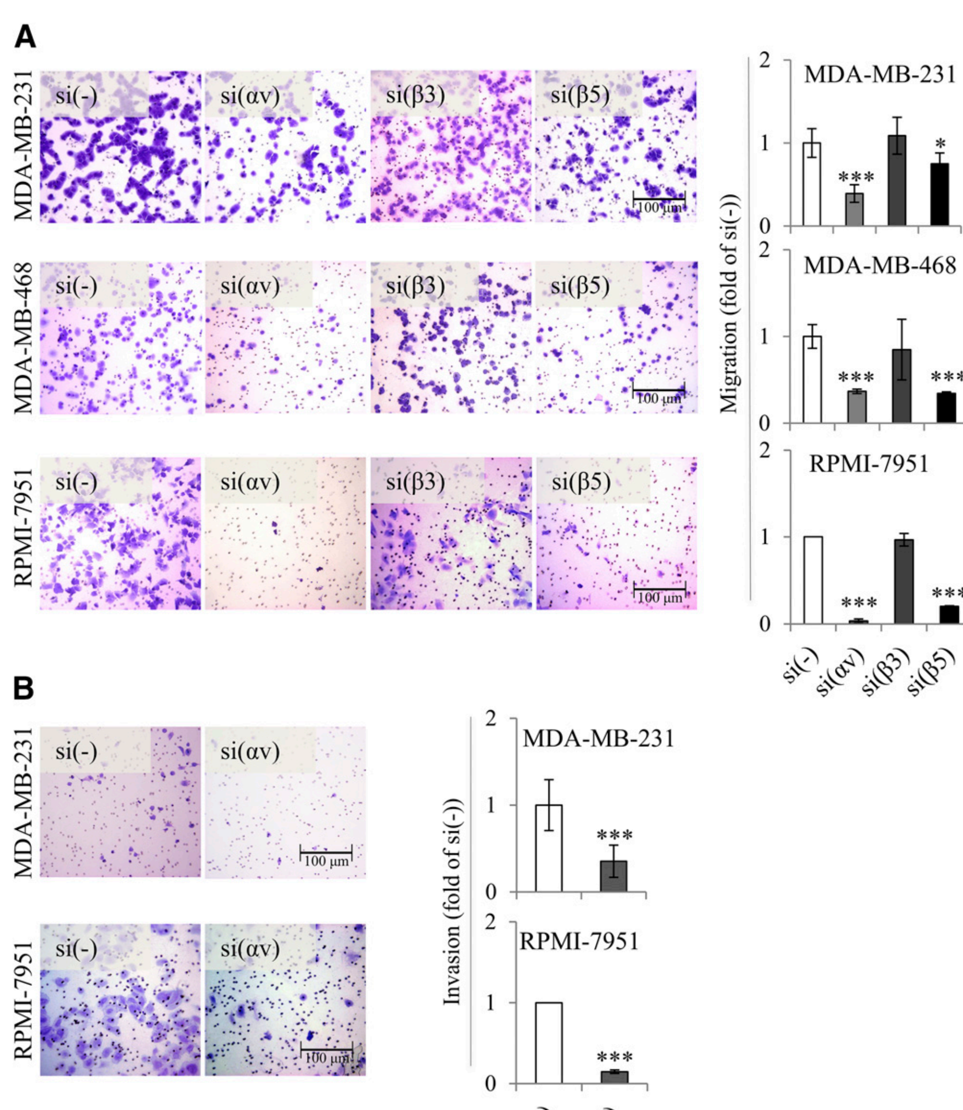

Fig. 6. Decreased migration and invasion of TNBC and melanoma cells upon integrin subunit αv knockdown. (A) Migration of MDA-MB-231, MDA-MB-468, and RPMI-7951 cells transfected with integrin αv-, β3-, or β5-specific siRNA compared with cells transfected with control siRNA. Cell migration was determined in cells upon transfection with control, integrin subunit αv-, β3- or β5-specific siRNA. Forty-eight hours after transfection, cells were serum starved for 24 hours, then seeded on Transwell cell culture inserts and left to migrate toward serum. After 22 hours, cells on the underside of the inserts were fixed, stained with crystal violet, photographed, and counted. Representative photographs are shown. Averages of five microscope fields from at least two independent experiments ± S.D. are shown relative to the migration of cells transfected with control siRNA set as 1. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison. $^{*}P < 0.05$; $^{***}P < 0.001$. (B) Invasion of TNBC cells MDA-MB-231 and melanoma cells RPMI-7951 upon integrin subunit αv knockdown. Cell invasion was determined in cells upon transfection with control or integrin subunit αv-specific siRNA. Forty-eight hours after transfection, cells were serum starved for 24 hours and cell invasion was measured in Transwell cell culture inserts coated with Matrigel. After 22 hours, cells on the underside of the filters were fixed, stained with crystal violet, photographed, and counted. Representative photographs are shown, and average number of five microscopic fields of invaded cells ± S.D. from at least two independent experiments is shown relative to invasion of cells transfected with control siRNA set as 1. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison. $^{***}P < 0.001$.

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(Sulzmaier et al., 2014). We hypothesized that integrin αv knockdown or cilengitide-mediated inhibition of pFAK(Y397) phosphorylation might be a crucial event in increased sensitivity to PTX. Therefore, we analyzed the phosphorylation of FAK(Y397) versus total amount of FAK using Western blot in all cell lines upon silencing integrin αv or exposure to cilengitide. Decreased phosphorylation of FAK(Y397) upon knockdown of integrin αv (Fig. 8A) was found in all three TNBC cell lines and melanoma cell line MDA-MB-435S, but not in the other three melanoma cell lines RPMI-7951, A375, and MeWo. No correlation was found to increased sensitivity to PTX because in cell line MDA-MB-436, no change in sensitivity after integrin αv knockdown was seen (Fig. 5B; Table 2), even though integrin αv-specific siRNA transfection strongly reduced pFAK(Y397) level down to 20% of the control value. The level of pFAK(Y397) upon cilengitide exposure was also reduced in all three TNBC cell lines and in all melanoma cell lines except MeWo (Fig. 8B). No correlation was found to

TABLE 3
Effect of integrin subunit αv, β3, or β5 knockdown on migration and invasion of TNBC and melanoma cell lines

Integrin Subunit-Specific siRNA	MDA-MB-435S		MDA-MB-231		MDA-MB-468		RPMI-7951	
	Migration	Invasion	Migration	Invasion	Migration	Invasion	Migration	Invasion
si(αv)	↓	↓	↓	↓	↓	na	↓	↓
si(β3)	=	na	=	na	=	na	=	na
si(β5)	=	na	↓	na	↓	na	↓	na

↓, decreased migration/invasion; =, no change in migration/invasion; na, not analyzed.

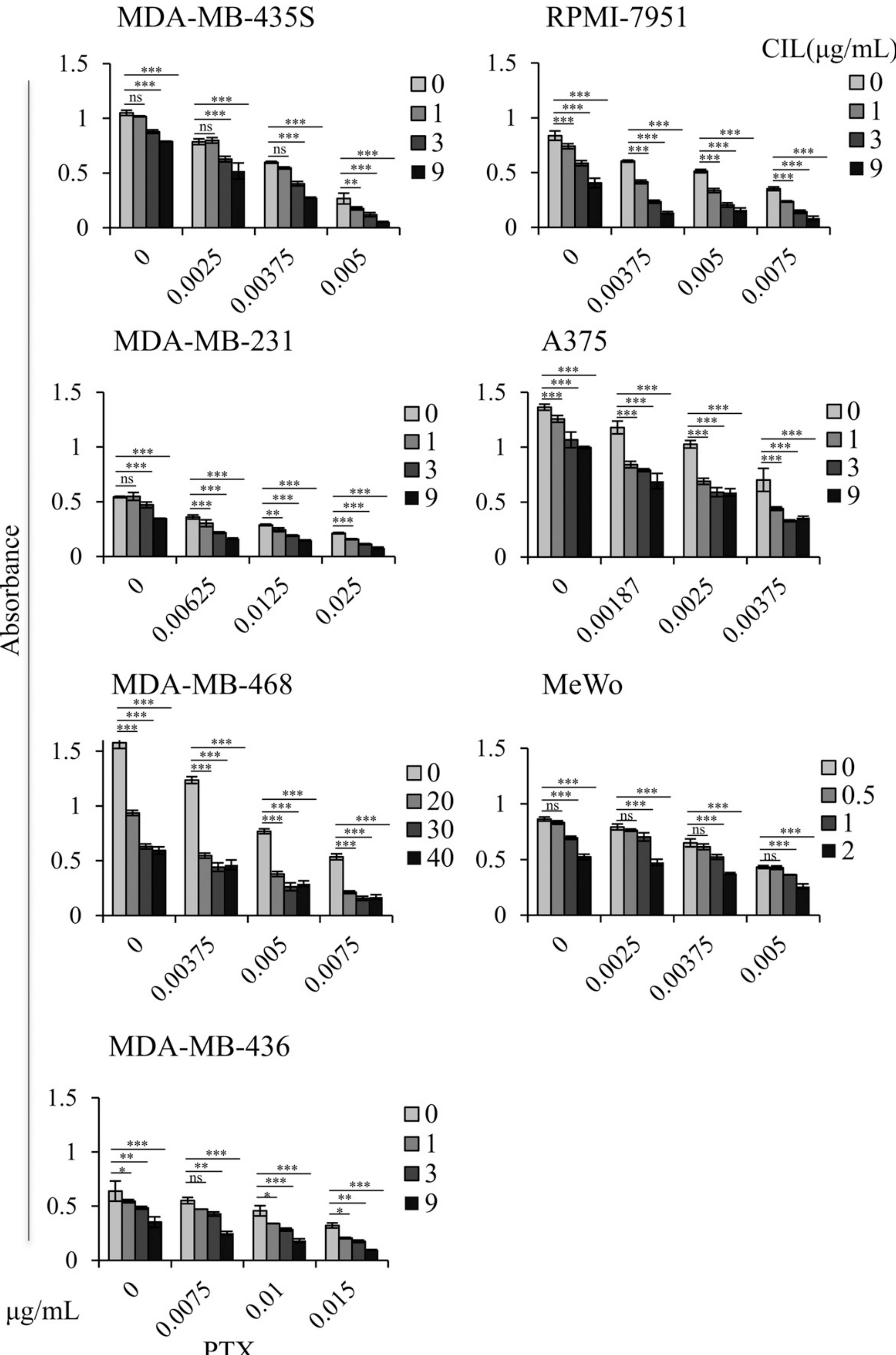

Fig. 7. Sensitivity of TNBC (MDA-MB-231, MDA-MB-468, and MDA-MB-436) and melanoma (MDA-MB-435S, RPMI-7951, A375, and MeWo) cells to PTX upon cilengitide exposure. Cells were seeded in 96-well plates and 24 hours later treated with cilengitide and PTX. Survival was measured by MTT assay 72 hours later. Absorbance data presented are representative of at least three independent experiments with similar results ± S.D. Data were analyzed by two-way ANOVA with Bonferroni post-test. ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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TABLE 4
Effect of αvβ3/β5 inhibitor cilengitide on the sensitivity of triple-negative breast cancer and melanoma cell lines to paclitaxel

Cell Line	Cilengitide
MDA-MB-435S	NI
MDA-MB-231	S
MDA-MB-468	R
MDA-MB-436	NI
RPMI-7951	S
A375	S
MeWo	R

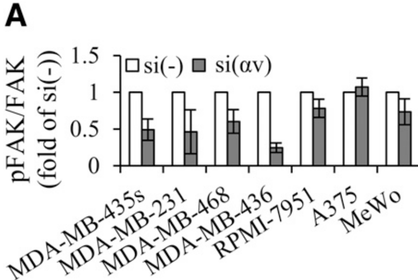
NI, no interaction between treatments in combination; R, resistance (decreased sensitivity to combination); S, sensitization (increased sensitivity to combination).

increased sensitivity to PTX since in cell line MDA-MB-468, demonstrating reduced pFAK(Y397) upon cilengitide exposure, decreased sensitivity to PTX was observed (Fig. 7; Table 4). Interestingly, in melanoma cell lines RPMI-7951 and A375, we did not observe a reduction of pFAK(Y397) levels on integrin αv knockdown, but we observed reduction upon cilengitide exposure. We hypothesize that this discrepancy might be the consequence of integrin-switching effects.

Finally, we investigated whether targeted inhibition of pFAK(Y397) could increase the sensitivity of TNBC and melanoma cells to PTX. We first analyzed the reduction of pFAK(Y397) upon exposure to inhibitor PF-228 using Western blot (Supplemental Fig. S5). Then, the combined effect of pFAK(Y397) inhibitor PF-228 and PTX was analyzed in all seven cell lines. In Fig. 9, we present data for each combination of PF-228 and PTX; the various effects of combinations are summarized in Table 5. Surprisingly, PF-228 showed a detrimental effect in all cell lines except in melanoma cell line A375, in which no interaction between pFAK(Y397) inhibition and PTX was observed. We conclude that pFAK(Y397) is downstream from integrins αv or only integrins αvβ3 and αvβ5 in TNBC and melanoma cell lines (Fig. 8), but it was not implicated in integrin-mediated response to PTX. The discrepancy between change of sensitivity to PTX upon integrin αv knockdown or cilengitide exposure and pFAK(Y397) inhibitor might be explained by the fact that FAK is a cytoplasmic tyrosine kinase that plays critical roles in integrin, but also in other cell-surface receptor signaling pathways (Kleinschmidt and Schlaepfer, 2017). Therefore, the use of pFAK(Y397) inhibitors in combination with PTX is not recommended for therapy of TNBC and melanoma.

Discussion

Integrins may represent candidate targets for combination treatments that can increase sensitivity to anticancer drugs and inhibit metastasis (Dickreuter and Cordes, 2017); however, change of expression of one integrin can lead to change in expression of other integrins in an event called integrin switching, which can have both mechanistic and therapeutic implications. Data showing that the specific inactivation of β1 integrin elicits metastatic progression owing to compensatory upregulation of β3 integrin (Parvani et al., 2013; Truong et al., 2014) raised concerns with respect to the use of β1 integrins as drug targets to sensitize tumors to radiotherapy or chemotherapy. Therefore, we analyzed the potential of different integrin α subunit knockdowns (αv, α3, or α4) to increase the sensitivity of TNBC and melanoma cell lines to different



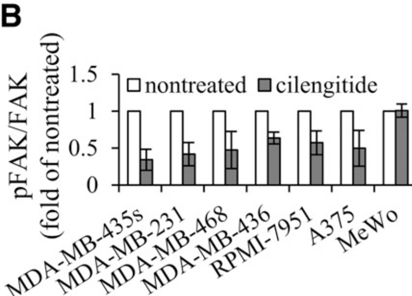


Fig. 8. Levels of pFAK(Y397) in TNBC (MDA-MB-231, MDA-MB-468, and MDA-MB-436) and melanoma (MDA-MB-435S, RPMI-7951, A375, and MeWo) cells upon integrin subunit αv knockdown (A) or cilengitide exposure (B). Cells were seeded in six-well plates, transfected with αv-specific siRNA (A), or exposed to cilengitide (IC70) (B). Forty-eight hours upon transfection (A) or 1 hour upon cilengitide exposure (B), whole-cell extracts were prepared and analyzed by Western blot. The level of pFAK(Y397) was normalized against total FAK and presented as relative to the expression in cells transfected with control siRNA or untreated cells. The results presented are average of at least three independent experiments ± S.D.

anticancer drugs (cDDP, VCR, and PTX), as well as its potential to inhibit migration and invasion.

We started our investigation in melanoma cell line MDA-MB-435S (Korch et al., 2018), in which we observed integrin switching between integrin heterodimers αvβ3 and αvβ5. In WM-266-4 melanoma cells, the number of αvβ3 and αvβ5 heterodimers is regulated at the level of the β3 and β5 genes, respectively, but the activity of the αv gene dictates the number of αvβ1 heterodimers (Koistinen and Heino, 2002). Since MDA-MB-435S cells lack integrin αvβ1 (Wong et al., 1998), the observed balance is in line with the proposed regulation model.

Knockdown of integrin subunits αv, β5, or α4 increased the sensitivity of MDA-MB-435S cells to VCR and PTX, whereas knockdown of integrin subunits α3 or β3 produced the opposite effect. The knockdown of αvβ3 using β3-specific siRNA, which simultaneously increases the expression of integrin αvβ5, resulted in decreased sensitivity to VCR or PTX. This finding indicates that the signaling pathway triggered by integrin αvβ5 protects MDA-MB-435S cells and can be augmented by increased expression of integrin αvβ5, which is somewhat different from our previously published results on the absence of dose-response effect in conferring resistance to cDDP upon de novo expression of integrin αvβ3 in head and neck cell lines (Brozovic et al., 2008; Stojanović et al., 2016). Silencing of integrin subunits αv, β3, β5, or α3 decreased the sensitivity of MDA-MB-435S cells to cDDP, whereas silencing of the integrin subunit α4 did not have a significant effect. We do not know whether there is a common mechanism between increased sensitivity to VCR and PTX and decreased sensitivity to cDDP. According to our results in melanoma cell line MDA-MB-435S, however, it seems that these two events have separate signaling

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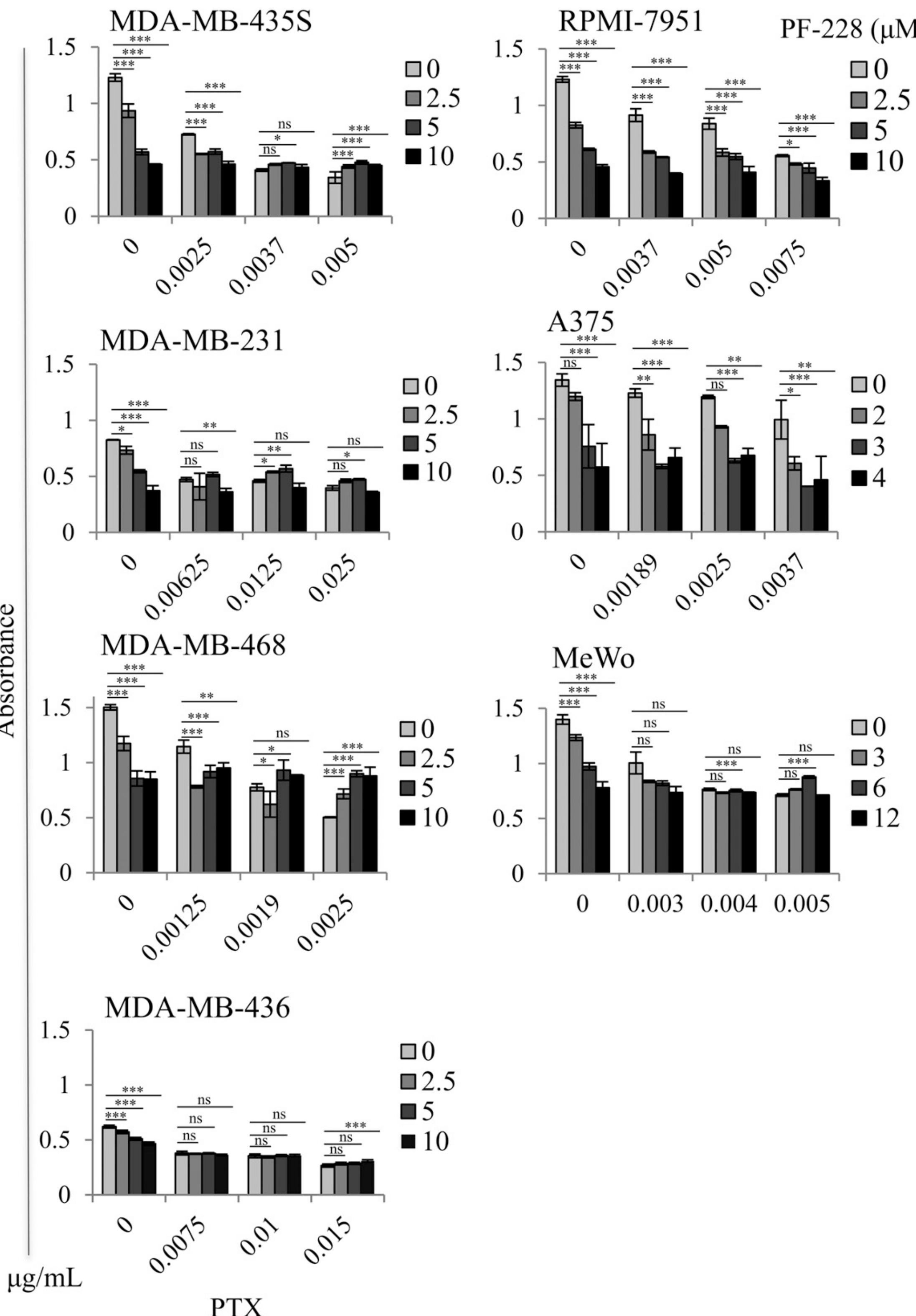

Fig. 9. Sensitivity of TNBC (MDA-MB-231, MDA-MB-468, and MDA-MB-436) and melanoma (MDA-MB-435S, RPMI-7951, A375, and MeWo) cells to PTX upon inhibition of pFAK(Y397) using PF-228. Cells were seeded in 96-well plates and 24 hours later treated with PF-228 and PTX. Survival was measured by MTT assay 72 hours later. Absorbance data presented are representative of three independent experiments with similar results ± S.D. Data were analyzed by two-way ANOVA with Bonferroni post-test. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

pathways. The concomitant sensitivity to cDDP in cells that develop resistance to PTX has been frequently described in the literature (Stordal and Davey, 2009).

Integrins have an important role as migration- or invasion-promoting receptors (Ganguly et al., 2013). The knockdown of integrin αv in the MDA-MB-435S cell line resulted in

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TABLE 5
Effect of pFAK(Y397) inhibitor on sensitivity of triple-negative breast cancer and melanoma cell lines to paclitaxel

Cell Line	Inhibitor PF-228
MDA-MB-435S	R
MDA-MB-231	R
MDA-MB-468	R
MDA-MB-436	R
RPMI-7951	R
A375	NI
MeWo	R

NI, no interaction between treatments in combination; R, resistance (decreased sensitivity to combination).

dramatically decreased cell migration and invasion. Interestingly, knockdown of integrin subunits $\beta 3$ or $\beta 5$ did not influence migration at all, very likely because of the balance effect between integrin heterodimers $\alpha v\beta 3$ and $\alpha v\beta 5$. Our results are in line with animal studies showing that integrin $\alpha v\beta 3$ is associated directly with the metastatic potential of MDA-MB-435S cells (Felding-Habermann et al., 2001; Harms et al., 2004). Integrin $\alpha 4\beta 1$ generally has a promigratory role (Jordan and Wilson, 2004); however, integrin subunit $\alpha 4$ siRNA transfection in MDA-MB-435S cells unexpectedly increased cell migration. We hypothesize that this could be due to integrin switching effect ((i.e., release of integrin subunit $\beta 1$ upon integrin subunit $\alpha 4$-specific siRNA transfection, but we can only speculate which of the $\beta 1$ heterodimers could have formed.

A key antitumor action of the microtubule poisons is kinetic stabilization of spindle microtubule dynamics, leading to mitotic arrest and subsequent cell death; however, they may induce apoptotic cell death independent of cell-cycle arrest (Jordan and Kamath, 2007). The first potential mechanism proposed to account for the increased sensitivity to VCR and PTX is decreased expression of drug transporters that efflux (pump out) drugs from cells (Jang et al., 2001); however, we found no difference in the ability of MDA-MB-435S cells to efflux calcein AM, a process dependent on the activity of both multidrug resistance protein and P-glycoprotein, upon integrin αv knockdown (data not shown). Since tubulin mutations, the second potential cause of modulation of sensitivity to microtubule drugs, are not expected upon integrin αv knockdown, we presume that a third potential mechanism (i.e., modulation of apoptosis is very likely involved). Indeed, we showed in melanoma cell line MDA-MB-435S an increased number of apoptotic cells upon the combination of integrin αv knockdown and PTX, which might be a consequence of modulation of apoptotic proteins or changes in the expression of drug target (i.e., microtubules). Deschesnes et al. (2007) showed that, regardless of the drug that depolymerizes microtubules, a similar sequence of molecular and cellular alterations occurs that culminates with a cell death program reminiscent of anoikis. In addition, their study supports that remodeling of focal adhesion structures is closely linked to the changes in response to microtubule-depolymerizing agents, in line with our results showing that integrin αv knockdown significantly reduces the number of focal adhesions in MDA-MB-435S cells.

We have shown that, in TNBC cell lines, integrin αv knockdown in combination with VCR or PTX resulted in beneficial effect, whereas in melanoma cell lines, either beneficial (MDA-MB-435S and RPMI-7951) or detrimental (A375 and MeWo) effect was observed. In two TNBC and two melanoma cell lines, in which a beneficial effect of integrin αv knockdown, in combination with microtubule poisons was observed, we detected decreased migration and invasion. We have also shown the key role of integrin $\alpha v\beta 5$ in migration, except in melanoma cell line MDA-MB-435S, in which both integrins, $\alpha v\beta 3$ and $\alpha v\beta 5$, are implicated. Our results are in line with data obtained in cell line MDA-MB-231, in which integrin αv knockdown inhibited migration and invasion in vitro and metastasis in vivo (Li et al., 2015) and with the report showing that depletion of integrin $\beta 5$ in MDA-MB-231 cells markedly reduced tumor growth and angiogenesis, whereas re-expression of integrin $\beta 5$ rescued this phenotype (Bianchi-Smiraglia et al., 2013). This is also in accordance with published results showing that MDA-MB-231-derived $\beta 3$ knockdown cells exhibited no changes in migration speed (Costa et al., 2013). It has been shown, however, that integrin $\beta 3$ knockdown in MDA-MB-231–derived tumor-bearing mice inhibits metastasis (Parvani et al., 2015). Integrin $\alpha v\beta 3$ in TNBC contributes to the spontaneous metastasis of breast tumors to bone (Sloan et al., 2006) and is a pivotal integrin for the growth of human melanomas (Mitjans et al., 2000). In conclusion, αv siRNA-mediated knockdown of both integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ might be beneficial for therapy.

Cilengitide-driven inactivation of integrin heterodimers $\alpha v\beta 3$ and $\alpha v\beta 5$ showed a beneficial effect in combination with PTX in five of seven TNBC and melanoma cell lines, although in a different set of cells compared with integrin αv knockdown. The extreme example is melanoma cell line A375, in which decreased sensitivity to PTX was observed upon integrin αv knockdown, whereas cilengitide exposure led to increased sensitivity to PTX. These discrepancies might be explained by the fact that integrin αv knockdown affects all integrin αv heterodimers, whereas cilengitide inhibits signaling from integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ only. The additive effect of cilengitide in integrin $\alpha v\beta 1$-negative MDA-MB-435S cells versus the sensitizing effect observed upon integrin αv knockdown might be explained by the 16-fold higher IC_{50} for $\alpha v\beta 5$ (79 nM) (Nisato et al., 2003), shown to be responsible for sensitivity to PTX, than for $\alpha v\beta 3$ (4.1 nM) (Reardon and Cheresh, 2011). Further, integrin switching might occur and change cell response to anticancer drugs. Although it seems less likely that integrin inhibition induces integrin switching, this possibility has been confirmed in TNBC cell line MDA-MB-231, in which two different monoclonal antibodies against integrin $\alpha 3\beta 1$ increased attachment of MDA-MB-231 cells to collagen (Lichtner et al., 1998). Interestingly, a phase 1 study of cilengitide and PTX in patients with advanced solid tumors (a third of patients had breast cancer) has shown antitumor activity and clinical benefit in six of the 12 evaluable patients (Haddad et al., 2017).

Although decreased levels of pFAK(Y397) upon integrin αv knockdown or cilengitide were found in most used cell lines, we did not find correlation with modulation of sensitivity to PTX. Moreover, direct inhibition of pFAK(Y397) by PF-228 consistently decreased sensitivity to PTX in all cell lines except A375, in which combination of PF-228 and PTX showed an additive effect on cell viability, indicating that pFAK(Y397) is not a suitable target for therapy. Opposite results were obtained in ovarian cancer cells by Kang et al. (2013), who showed that pFAK(Y397) inhibition by VERSUS-6063 sensitizes taxane-resistance ovarian cancer cells to PTX and by Halder

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et al. (2005), who showed that FAK knockdown promotes an in vitro efficacy of docetaxel in both taxane-sensitive and taxane-resistant cell lines.

In conclusion, our results suggest that, for TNBC, the knockdown of integrin αv in combination with PTX presents a better therapeutic option than cilengitide and decreases cell migration and invasion at the same time. Contrarily, in melanoma, neither of these combinations is advisable because decreased sensitivity to PTX was observed. Further study is needed to reveal the mechanism behind this phenomenon.

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Authorship Contributions

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