REGAINING TAXOL SENSITIVITY IN CHEMORESISTANT OVARIAN CANCER CELLS

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REGAINING TAXOL SENSITIVITY IN CHEMORESISTANT OVARIAN CANCER CELLS

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LIST OF SYMBOLS AND ABBREVIATIONS

ECM	Extracellular Matrix
TR	Taxol-resistant
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
SKOV3-P	SKOV3 Parent cells
SKOV3-T	SKOV3 Taxol-resistant cells
OVCAR3-P	OVCAR3 Parent Cells
OVCAR3-T	OVCAR3 Taxol-resistant cells

SUMMARY

Ovarian cancer is a leading cause of death in women, and despite primary treatment, the majority of patients relapse. As the cancer becomes chemoresistant, prognosis dramatically worsens. Metastasis, or the spread of cancer, is the primary cause of death in more than 90% of ovarian cancers. Taxol, a frontline chemotherapeutic drug, works by stabilizing microtubules, cytoskeletal filaments required for cell division. While it is known that microtubules affect cell adhesion, it remains unclear if chemoresistance alters cell adhesion to the extracellular environment. We tested this by isolating Taxol-resistant cells, which displayed increased microtubule dynamics characterized by more rapid microtubule growth from fluorescently labeled EB3 microtubule protein. Additionally, they displayed faster adhesion rates and decreased adhesion strength, which could help overcome the rate-limiting step of metastasis. Adhesion strength was found to be independent of microtubule polymerization and dependent on up-regulated focal adhesion kinase (FAK) in the Taxol-resistant cells. Upon inhibition of FAK in these cells, microtubule dynamics decreased, signifying that adhesive signaling is up-stream of microtubule dynamics. Hence, by altering cell adhesion to the extracellular environment, Taxol-resistant cells show an increase in down-stream microtubule dynamics. This study consequently used FAK inhibition as a therapeutic target to improve prognosis by regaining drug sensitivity.

CHAPTER 1

INTRODUCTION

Ovarian cancer, a leading cause of death in women, lacks effective, early detection techniques, resulting in more than 75% of the patients being diagnosed at an advanced stage of the disease.[1] While improved surgical cancer removal methods and subsequent adjuvant platinum and taxane chemotherapy have increased progression-free survival, the disease remains largely incurable.[2] Approximately 85% of patients relapse due to the genetic complexity and diversity of ovarian cancer cells; as cancer cells become chemoresistant, only 20% of ovarian cancers respond to the standard cocktail of drugs.[1,3] Additionally, it is known that the majority of cancer-related deaths occur due to metastasis, the spread of cancer. More importantly, the rate-limiting step of metastasis is adhesion at distant sites. The primary site of metastasis is the omentum, a soft tissue present inside the peritoneal cavity. However, the correlation between chemoresistance and metastasis remains largely unknown.

The standard cocktail of drugs commonly consists of Taxol and Carboplatin. Taxol is known to alter microtubule dynamics of cancer cells thus potentially influencing the processes of cellular division, adhesion, and migration. Our lab previously showed that matrix stiffness induces changes in chemoresistance and adhesion.[4] More specifically, we found that metastatic ovarian cancer cells preferentially bind to soft microenvironments and undergo morphological elongation characteristic of epithelial-mesenchymal transition. Additionally, previous studies in our lab have elucidated various mechanisms by which Taxol resistance contributes to metastasis. Taxol-resistant SKOV3 and OVCAR3 ovarian cancer cells display increased microtubule dynamics and weaker

adhesion to the extracellular matrix (ECM), which suggests that they more easily detach from the primary tumor sites compared to parent cancer cells. Staining for the focal adhesion proteins vinculin and paxillin, which are a part of intracellular complexes that serve as points of contact between the cell and the extracellular matrix, demonstrated that Taxol-resistant cells display smaller and fewer focal adhesions; the decrease in size and number of focal adhesions may contribute to the Taxol-resistant cells' weaker adhesion. Moreover, we found Taxol-resistant cells to have increased cell motility and faster adhesion kinetics, helping them overcome the rate-limiting step of metastasis – adhesion at the soft omentum fat pads.

We seek to target and block these characteristics of Taxol-resistant cells in order to not only regain sensitivity to Taxol, but to also prevent the essential steps of metastasis, such as detachment of tumor cells from the primary site and engraftment to distant sites. Previous studies have demonstrated that focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, promotes cell migration and increases focal adhesion turnover by contributing to the processes of actin remodeling and adhesion signaling.[5] Consequently, by specifically targeting FAK, we hope to find novel ways to overcome the poor prognosis of late-stage ovarian cancer.

CHAPTER 2

LITERATURE REVIEW

Ovarian cancer is a leading cause of death in women due to lack of effective, early detection techniques, with more than 75% of the patients presenting with an advanced stage of the disease during initial diagnosis.[1,6] Although improved surgical cancer-debulking methods and subsequent adjuvant platinum and taxane based chemotherapy have increased progression-free survival, only 30% of patients with an advanced stage cancer have an overall survival period of 5 years.[7] This high frequency of relapse despite successful primary treatment is largely observed due to the heterogeneity or genetic complexity of ovarian cancer cells.[8] Moreover, with the majority of cancer-related deaths occurring due to metastasis, or the spread of cancer from its primary tumor site to the metastatic site, there is an increased necessity for understanding the intracellular mechanism by which chemoresistance influences metastasis.

Over the past fifty years, chemotherapeutics have evolved to best prevent cancer growth and metastasis after surgical removal. In the 1960s and early 1970s, alkylating agents like melphalan (Alkeran) and cyclophosphamide were administered singly with numerous prominent side effects. These drugs were replaced in the late 1970s and early 1980s by various combinatorial mixes of doxorubicin, a topoisomerase inhibitor, cyclophosphamide, and cisplatin, a platinum-based therapeutic. Carboplatin, another platinum drug, progressively replaced cisplatin in the late 1980s due to its less toxic nature and fewer side effects.[9] Furthermore, a prominent change occurred in the 1990s with the introduction of taxane drugs such as paclitaxel, which was shown to be increasingly effective against cancer reoccurrence when administered with

carboplatin.[10,11] Recently, the use of carboplatin in conjunction with liposomal doxorubicin has been shown to demonstrate longer progression-free survival in recurrent ovarian cancer compared to carboplatin and paclitaxel chemotherapy.[12] Nevertheless, carboplatin and paclitaxel continue to be more commonly used following surgery.[7,13]

Paclitaxel in normal cancer cells is known to stabilize microtubules and consequently inhibit the regular processes of cellular division, adhesion, and migration. Understanding the variability in the binding of paclitaxel to various tubulin isotypes, after posttranslational modifications and mutations, is a potential primary target for understanding the mechanism of drug resistance. Previous research has demonstrated that higher expression of class III β tubulin plays a role in rendering resistance to paclitaxel because paclitaxel has preferential binding for class I β tubulin.[14] Moreover, it is predicted that paclitaxel induces apoptosis by changing microtubule properties responsible for the release of proteins involved in the onset of apoptosis; activation of pro-apoptotic factors such as p53 and anti-apoptotic factors such as BCL2 are also tethered and/or transported by microtubules.[15,16] However, further research has not demonstrated a direct dependence of paclitaxel on p53-regulated apoptosis, but the presence of functional p53 has been shown to enhance paclitaxel sensitivity in cancer cells.[17] Similarly, research has not been able to directly link paclitaxel-induced apoptosis to the inactivation of BCL2, but there is evidence that overexpression of BCL2 confers inhibition of the downstream effect of paclitaxel on caspase-dependent apoptosis.[18] Moreover, mitotic arrest caused by paclitaxel may cause DNA damage that in turn activates p53 and inactivates BCL2.[19,20] Although p53 has been shown to activate other pro-apoptotic proteins such as BCL2-Antagonist/Killer (BAK), there is still a need to

further investigate the signaling pathways by which paclitaxel influences p53 and BCL2-regulated apoptosis.[21]

Carboplatin is another prominent drug used in the treatment of ovarian cancer, but its therapeutic mechanism differs from that of Paclitaxel. The hydrolyzed analog species of Carboplatin form an adduct with DNA strands through cross-linkage and induces DNA damage that recruits various repair proteins. These proteins consequently mediate an apoptotic response or cause cell cycle arrest.[7] Recent research proposes that the active form of the tumor suppressor protein p14ARF leads to p53 inactivation via E3 ubiquitin ligase MDM2, conferring cells resistance to carboplatin; inhibition of p14ARF prevents p53 aggregation and allows the native form of p53 to be functional as an apoptosis inducer, reestablishing cell sensitivity to carboplatin.[22] Thereafter, p53 perhaps leads to cell cycle arrest or apoptosis via mediating pro-apoptotic proteins such as BAK. Nevertheless, further investigation is still necessary to identify more feasible, potential targets for reestablishing sensitivity in chemoresistant ovarian cancer cells.

While ovarian cancer research has commonly focused on drug-induced alteration of signaling pathways and consequent cell death, a new approach to understanding chemoresistance focuses on finding a connection between chemoresistance and cancer metastasis. For instance, the overexpression of AKT2, an anti-apoptotic protein, has been shown to up-regulate β1-integrin expression.[23] Since the β-subunit of heterodimeric integrin membrane proteins plays an essential role in cellular adhesion to the extracellular matrix, increased expression confers more motility to the cells and increases their metastatic potential.[24–26] Tumorigenesis is marked by several genetic alterations that produce oncogenes with dominant gain of function and tumor suppressor genes with

recessive loss of function. Therefore, it is optimal to find a drug that targets the invasive character of cancer cells and increases drug sensitivity. However, multiple pathways have been identified in increasing the invasiveness of cancer cells. The difficulty lies in identifying which pathway would be the most influential in reestablishing drug sensitivity; in part to the large number of cell cycle regulating proteins, it is also possible that multiple pathways may need to be targeted simultaneously to increase taxane sensitivity.

As mentioned previously, integrins serve as receptors for ECM proteins and transduce biochemical signals that regulate processes such as cell proliferation, cell migration, and cell apoptosis. A critical mediator protein of integrin-based signaling is Focal Adhesion Kinase (FAK), a cytosolic tyrosine kinase co-localized with integrins in focal adhesions.[26] Moreover, although the exact mechanism remains unclear, FAK has been shown to significantly interact with paxillin; while some studies propose paxillin to be a substrate of FAK, others demonstrate it to be simply a protein that anchors FAK to focal adhesion sites.[26] Nevertheless, since FAK has been implicated in the promotion of cell migration and the prevention of cell apoptosis, it may be a promising target in reversing the effects of chemoresistance on both cellular adhesion strength and kinetics.[27,28] This study will further investigate the mechanism by which chemoresistance affects the functionality of FAK by observing how the inhibition of FAK affects cell motility and adhesion in Taxol resistant cells of both the SKOV3 and OVCAR3 lineages. By targeting the adhesion and microtubule dynamics of Taxol resistant cells, the study hopes to increase drug sensitivity and simultaneously reduce the invasive character of cancer cells.

CHAPTER 3

MATERIALS AND METHODS

A. Derivation of Taxol-resistant Cells

Human ovarian carcinoma, SKOV-3 and OVCAR-3, cells were obtained from ATCC and Dr. John McDonald, respectively. Both cell lines were maintained in RPMI 1640 (Corning) containing 10% FBS (Atlanta Biologicals) and 1% penicillin streptomycin (Corning). Cells of both lineages were selected for resistance to Taxol; they were plated at 20% of confluence and exposed to 10 nM Taxol (Enzo) for 48 hours before returning to growth media. After achieving 40% of confluence, cells were re-exposed to 10 nM Taxol for 48 hours before returning to growth media. With the drug dose kept constant, the cells were treated until Taxol could no longer support cell growth, after which the cells were fed with standard growth media. Subsequently, 5 mM Taxol was added once per week since resistant cells require Taxol to maintain normal cell proliferation. Cell viability assays were performed to confirm no significant change in IC_{50} over the course of the study.

B. Cell Viability Assay

Cell viability was examined using a MTT assay. Initially, both SKOV3 and OVCAR3 cells were plated at 20% of confluence before exposing them to varying concentrations of Taxol, 25 μM Carboplatin, or DMSO (<0.1% v/v) as a solvent control. After 72 hours, the cells were incubated in 1mg/mL MTT reagent for four hours in standard growth conditions. Subsequently, the supernatant was replaced with isopropanol acidified with 4nM HCl, which solubilized the reduced formazan product. The absorbance was measured at 620 nm, with 595 nm as a reference value, using a DTX-800

Multimode Detector microwell plate reader (Beckman Coulter). Additionally, the IC_{50} was determined by interpolating within the linear region since the data did not fit the traditional sigmoidal fit commonly associated with cell viability curves.

C. Rhodamine Efflux Assay

Rhodamine 123 was used as a model drug for the drug efflux assay. Studies were carried out in both the parent and Taxol-resistant populations of SKOV3 and OVCAR3 cells. Cells were seeded in a 96-well plate, incubated with 0.5 μg/mL Rhodamine 123 for 60 minutes, and then washed extensively with adhesion buffer. At various time points, the supernatant was removed; the green fluorescence of Rh 123 was excited at 485 nm and measured at an emission of 535 nm. The efflux was calculated as [Effluxed Rh 123/(Effluxed Rh 123 + Rh 123 in cells)]. Efflux rate was determined using $[dC_{external}/dt = kC_{internal}]$ where C is the concentration of Rhodamine 123 and k is the rate constant. A similar procedure was followed for 24-hour efflux, except the cells were incubated in growth media.

D. Actin and Tubulin Stain

Actin and tubulin proteins were visualized using immunofluorescence imaging. Parent and Taxol-resistant cells cultured on glass cover slips were extracted in a buffer containing 80 mM PIPES (pH 6.8), 1 mM $MgCl_2$, 5 mM EDTA, and 0.5% Triton X-100 before fixation with 0.5% glutaraldehyde in PBS. The reaction was subsequently quenched with 1 mg/mL sodium borohydride, permeabilized with Triton X-100, and blocked with FBS. Cells were stained for 1 hour at room temperature with 1:50 Rhodamine Phalloidin rabbit-tubulin followed by 1:1000 goat anti-rabbit Alexa Flour 488

before sealing with Vectashield (Vector Labs) containing DAPI. Cells were visualized using either an inverted Nikon Eclipse Ti microscope.

E. Microtubule Pelleting Assay

The amount of polymerized and depolymerized tubulin in parent and Taxol-resistant SKOV3 and OVCAR3 cells was quantified using a tubulin polymerization assay. Cells grown in the absence of Taxol for 5-7 days were grown to 80% of confluence in a 24-well plate. After lysing the cells with 100 μL of hypotonic lysis buffer (20 mM Tris-Hcl, pH 6.8, 0.5% Nonidet P-40, 1 mM $MgCl_2$, 2 mM EGTA), they were transferred to a 1.5mL microcentrifuge tube. The wells were washed once with 100 μL of hypotonic lysis buffer, and the entire 200 μL volume was vortexed prior to pelleting out the insoluble fraction at room temperature centrifugation at 12,000 rcf for 10 minutes. The remaining cytoskeletal elements were dissolved in aforementioned buffer with addition of 0.5% SDS. The supernatants were transferred to different tube and the pellets were re-suspended in SDS-containing buffer with other insoluble tubulin proteins. Equivalent volumes of samples were loaded for the western blot procedure mentioned below.

F. Western Blot Assay

Parent and Taxol-resistant cells of both SKOV3 and OVCAR3 lineages were lysed using radioimmunoprecipitation buffer, which consisted of a mixture of protease inhibitors. The lysates were separated on either 10% (paxillin/tubulin) or 7.5% (vinculin/FAKp397) polyacrylamide gel before transferring to a PVDF membrane. These membranes were blocked in 5% milk, incubated overnight at 4°C in primary antibodies against tubulin (1:3000; Rockland), paxillin (1:500, BioLegend), vinculin (1:1000,

Invitrogen), or FAKp397 (1:1000, Genetex). The membranes were incubated with 1:1000 HRP-conjugated anti-rabbit IgG (Rockland) and visualized using Novex ECL chemiluminescent substrate (Invitrogen). The total amount of protein was used as a loading control by staining the membranes with Coomassie G250 (BioRad). The protein bands were analyzed using blot analysis tools on ImageJ (NIH) as per the instructions in the user manual.

G. Adhesion Strength Assay

A centrifugal force-based study was used to assess adhesion strength. Both SKOV3 and OVCAR3 cells were allowed to adhere overnight to a collagen matrix in a 96 well plate and labeled with Calcein AM. After replacing the media with adhesion buffer, an initial fluorescence reading was measured. The plate was centrifuged at 29 rcf for five minutes. After washing with adhesion buffer, a final fluorescence reading was taken. In order to calculate the attached fraction, the final fluorescence reading was divided by the initial fluorescence reading. Similar studies were performed with cells pre-incubated with Taxol, Nocodazole (Sigma), or PF-228 (Sigma) for four hours.

H. Focal Adhesion Stain

Focal adhesions in parent and Taxol-resistant cells of both SKOV3 and OVCAR3 lineages were visualized by staining paxillin and vinculin. Cells were also stained for focal adhesion kinase (FAK) to observe its expression. Cells were fixed using 4% formaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 5% horse serum. Subsequently, they were stained with either 1:200 anti-paxillin (Clone Y113), 1:500 anti-vinculin (Invitrogen), or 1:200 anti-FAKp397 (Genetex) diluted in PBS with 1% BSA. After washing, cells were incubated with 1:100 rhodamine phalloidin (Invitrogen) and

anti-rabbit Alexa Fluor 488 (Invitrogen), counterstained with DAPI (AnaSpec), and sealed with Vectashield on collagen I coated coverslips. The coverslips were imaged using an inverted Nikon Microscope at 40x with a CoolSNAP camera (Photometrics), and these images were normalized to the average for that session since light brightness may vary. Paxillin and vinculin images were used to quantify focal adhesions. The method utilized involved overlapping the images with a low pass Gaussian filter prior to the application of a morphological top hat filter. This procedure corrected for differences in basal paxillin and vinculin expression. Pixels that were above two standard deviations of the cell background, as determined based on F-actin fluorescence, were deemed positive. Noise less than 10 pixels (~0.25 μm^2) were ignored, while the remaining segmented focal adhesion for analysis. The focal adhesion density was calculated as the integrated focal adhesion intensity normalized to cell area.

I. Microtubule Tracking Assay using EB3

Parent and Taxol-resistant cells of both SKOV3 and OVCAR3 lineages were transfected with mApple-EB3 (Addgene plasmid #54892), a fluorescently tagged protein, using the TransIT-LT1 transfection reagent (Mirus Bio). The live-cell microtubule dynamics were imaged with a Nikon Eclipse Ti inverted epifluorescent microscope, which was maintained at 37°C and 5% carbon dioxide using an In Vivo Scientific environmental cell chamber and Bioscience Tools CO_2 controller. Imaging was performed using a Nikon CFI Apochromat TIRF 100X oil-immersion lens, while a Photometrics QuantEM CCD camera (Princeton Instruments) was used to minimize exposure time. Videos were capture at 1 Hz for two minutes and quantified using the open source u-track software.

J. Statistics

Statistical analysis was performed using a student's t-test or analysis of variance (ANOVA) followed by post-hoc analysis with Student-Newman-Keuls test; $p < 0.05$ was considered to be significant ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). Pearson correlation coefficients (ρ), ranging from -1 to +1 for perfectly inversely or positively correlated, respectively, were determined in MATLAB. All data are reported as mean ± standard error of the mean from three independent experiments unless otherwise noted.

CHAPTER 4

RESULTS

A. Drug Efflux and Other Pro-survival Adaptions do not Cause Taxol Resistance

I. Establishing Taxol Resistance in SKOV3 and OVCAR3 Ovarian Cancer Cells

In order to derive populations of SKOV3 and OVCAR3 ovarian cancer cells that were resistant to Taxol, parent populations were treated with Taxol and grown over time until Taxol could no longer support cell growth. We confirmed Taxol resistance by carrying out a cell viability assay, in which both the parent and Taxol-resistant cells were treated with Taxol at varying concentrations for 72 hours. The SKOV3 and OVCAR3 parent populations displayed IC_{50} values of 2.3 ± 0.3 nM and 4.1 ± 1.8 nM, respectively, while the Taxol-resistant populations showed IC_{50} values of 22.1 ± 4.3 nM and 45.5 ± 4.9 nM, respectively (Figure 1a). There was approximately a 10-fold increase in the IC_{50} for the Taxol-resistant populations, demonstrating that they show significantly increased viability at Taxol concentrations between 5-10 nM. At concentrations of Taxol larger than 10 nM, the viability of the Taxol-resistant populations decreased.

II. Investigating Drug Efflux as a Mechanism of Chemoresistance

A classical model of chemoresistance is drug efflux, in which the chemotherapeutic is pumped out of the cell at a higher rate than it is taken into the cell. In order to examine if the resistance to Taxol in the Taxol-resistant populations was a consequence of drug efflux, we evaluated the ability of cells to efflux Rhodamine 123. Rhodamine was used as a model drug since both Rhodamine 123 and Taxol use P-glycoprotein as an efflux transporter.[29] Drug efflux was observed over an initial two hours. The SKOV3 parent and Taxol-resistant populations showed rate constants of 0.73

± 0.09 h^{-1} and 0.72 ± 0.11 h^{-1}, respectively, while the OVCAR3 parent and Taxol-resistant populations showed rate constants of 0.70 ± 0.06 h^{-1} and 0.80 ± 0.13 h^{-1}, respectively (Figure 1b). There was no significant difference in the efflux rate constants observed for the parental and Taxol-resistant populations of either the SKOV3 and OVCAR3 cell lines.

III. Testing for Carboplatin Resistance

In order to investigate the possibility of any inherent mechanisms of chemoresistance in both the SKOV3 and OVCAR3 cells, the respective parent and Taxol-resistant populations were treated with 25 μM of Carboplatin and tested for cell viability. As seen in Figure 1c, there was no significant difference in cell viability between the parent and Taxol-resistant populations, which suggests that the resistance established in the Taxol-resistant cells was due to an alternative mechanism.

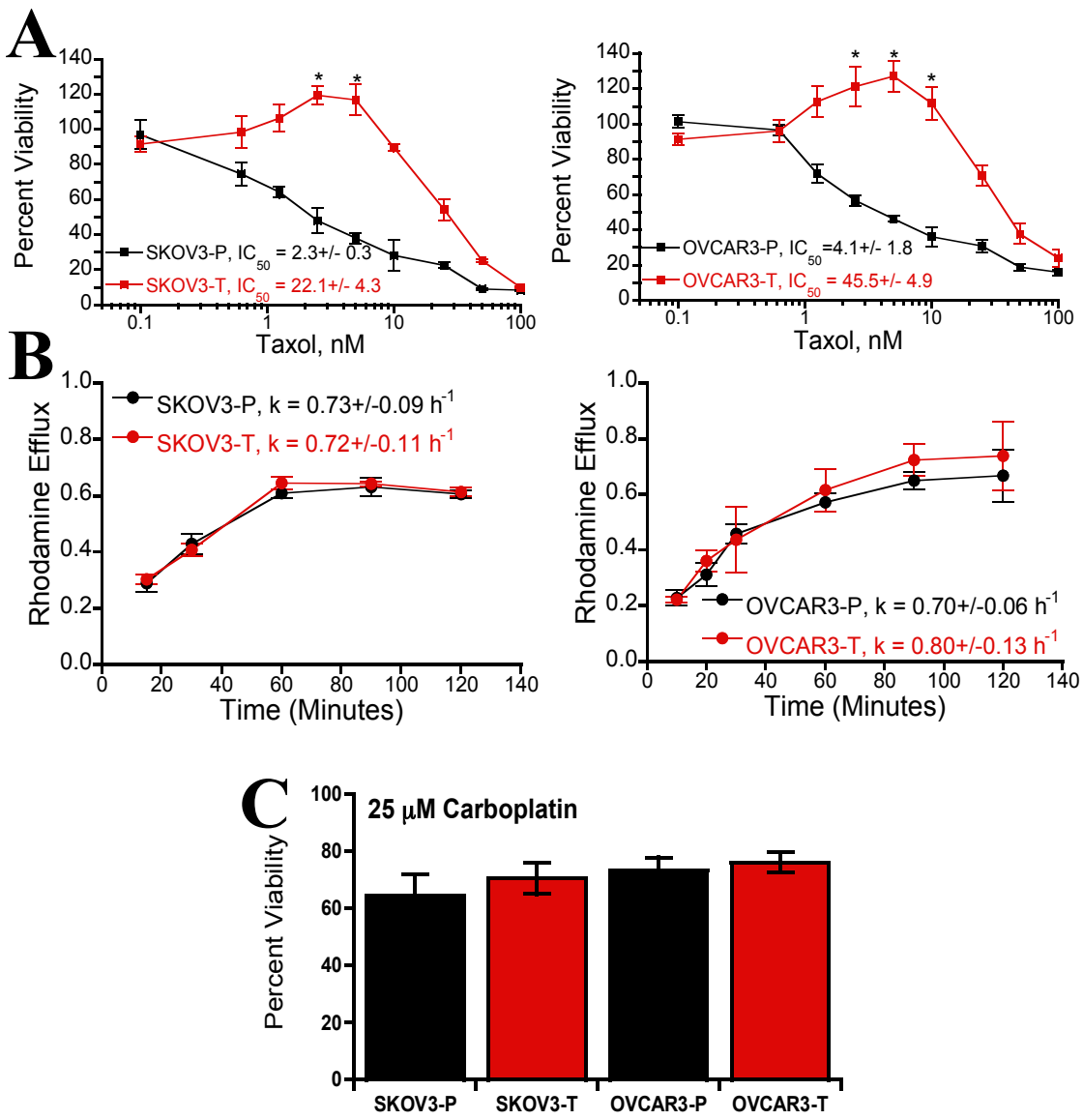

Figure 1. Derivation of Taxol-Resistant Cells and Analysis of Mechanisms of Chemoresistance. (A) Viability of Parent (-P) and Taxol-resistant (-T) populations of SKOV3 and OVCAR3 cells post incubation with varying concentrations of Taxol. Taxol-resistant cells show a 10-fold increase in IC_{50}. (B) Rhodamine Efflux over two hours used to calculate efflux rates (k), which showed no significant difference between the Parent (-P) and Taxol-resistant (-T) populations. (C) Viability of Parent (-P) and Taxol-resistant (-P) populations of SKOV3 and OVCAR3 cells post incubation with Carboplatin. There was no significant difference in viability among the cell populations.

B. Increased Microtubule Dynamics in Taxol-resistant Ovarian Cancer Cells

I. Comparing Actin and Tubulin Expression in Parent and Taxol-resistant Populations

Since Taxol is a microtubule-stabilizing drug, we examined cytoskeleton protein expression in parent and Taxol-resistant populations of SKOV3 and OVCAR3 cells. The Taxol-resistant cells appeared to have more depolymerized microtubules as observed by the less structured tubulin filaments from the immunofluorescent stain (Figure 2a).

II. Comparing Tubulin Polymerization in Parent and Taxol-resistant Populations

We sought to verify the microtubule dynamics seen from the immunofluorescent stain by comparing tubulin polymerization in the parental and Taxol-resistant populations of SKOV3 and OVCAR3 cells. The amount of tubulin was quantified using a microtubule-pelleting assay followed by western blot analysis. In the absence of Taxol, the SKOV3 Taxol-resistant population displayed less polymerized tubulin than its respective parent population (Figure 2b). However, upon treatment with 10 nM Taxol, the level of polymerized tubulin in the Taxol-resistant population increased to that of the parent population. Moreover, treatment with 100 nM Taxol increased tubulin polymerization to a level above that of the parent population. Similar results were obtained for the OVCAR3 cells, which indicates that Taxol-resistant cells have less tubulin polymerization than their parent cells.

III. Comparing Microtubule Growth Rate and Growth Density in Parent and Taxol-resistant Populations

Microtubule dynamics were also characterized by comparing the microtubule growth rates in live parent and Taxol-resistant cells of SKOV3 and OVCAR3 lineages. Both cell populations were transfected with fluorescent end-binding protein (mApple-

EB3), which binds to the growing end of the microtubules. Microtubule growth rates were quantified to be faster in the Taxol-resistant population of SKOV3 cells (Figure 2c). An increased number of microtubule growing plus ends was also observed in the same population (Figure 2d). Similar results were obtained for the OVCAR3 cells, which indicates that Taxol-resistant cells displayed greater dynamics than their parent cells.

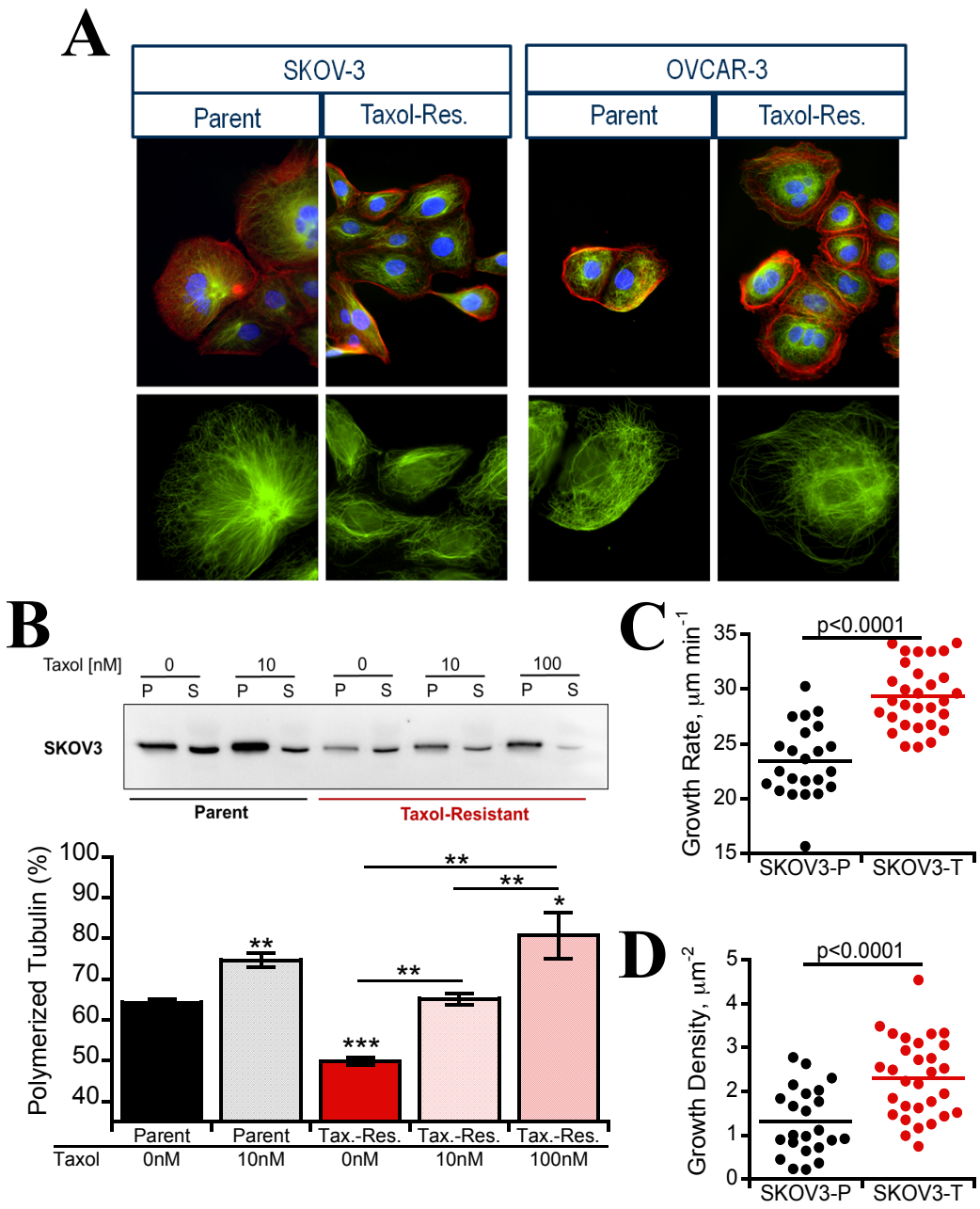

Figure 2. Taxol-resistant cells display increased microtubule dynamics. (A) Actin-Tubulin Stain shows increased tubulin dynamics in Taxol-resistant cells of SKOV3 and OVCAR3 lineages. (B) Polymerized and soluble tubulin was separated using a microtubule pelleting assay and analyzed using Western Blot. Percent of polymerized tubulin was calculated by dividing polymerized tubulin by the sum of soluble and polymerized tubulin. SKOV3 Taxol-resistant cells displayed less polymerized tubulin than the parent cells, which was reversed with low doses of Taxol. (C-D) SKOV3 Taxol-resistant cells show increased microtubule growth rate and density as determined by live-cell microtubule dynamics using EB3 fluorescent tag.

C. Decreased Adhesion Strength in Taxol-resistant Cells due to Smaller and Fewer Focal Adhesion Formation

I. Investigating Adhesion Strength in Parent and Taxol-resistant Populations

Microtubule dynamics have been previously shown to influence adhesion signaling, which plays an essential role in processes such as cell migration, proliferation, and survival.[30] Therefore, we compared the adhesion strength in the parent and Taxol-resistant populations of SKOV3 and OVCAR3 cells using a centrifugal-force base adhesion assay. The cells were allowed to adhere overnight to a collagen-matrix prior to the application of centrifugal force. The adherent fractions of the Taxol-resistant populations were significantly less than that of their parent populations (Figure 3a). These results indicate that adherent fraction is inversely related to the IC_{50} values, suggesting that Taxol resistance confers weak adhesion to the ECM (Figure 3b).

II. Examining Focal Adhesion Formation in Parent and Taxol-resistant Populations

In order to further investigate the weak adhesion observed for the Taxol-resistant cells, we stained the parent and Taxol-resistant populations for focal adhesion protein paxillin. Both the SKOV3 and OVCAR3 parental populations displayed large focal adhesions throughout the cell periphery, while the Taxol-resistant populations featured smaller and fewer focal adhesions (Figure 3c). The focal adhesion density was calculated as the integrated density of segmented focal adhesions relative to the cell area (Figure 3d). The total amount of paxillin was subsequently quantified using western blot analysis, which confirmed a significant decrease in the focal adhesion protein paxillin in both Taxol-resistant populations in comparison to their respective parent populations (Figure 3e). While similar results were obtained for OVCAR3 cell populations when stained for

focal adhesion protein vinculin, neither of the SKOV3 populations displayed vinculin positive focal adhesions from the immunofluorescent stain (data not shown). However, western blot analysis was sensitive enough to measure differences in total vinculin, confirming a reduction of expression in both SKOV3 parent and Taxol-resistant populations. Taken together, these results suggest that decreased adhesion strength in the Taxol-resistant cells correspond to decreased vinculin expression, but not paxillin expression.

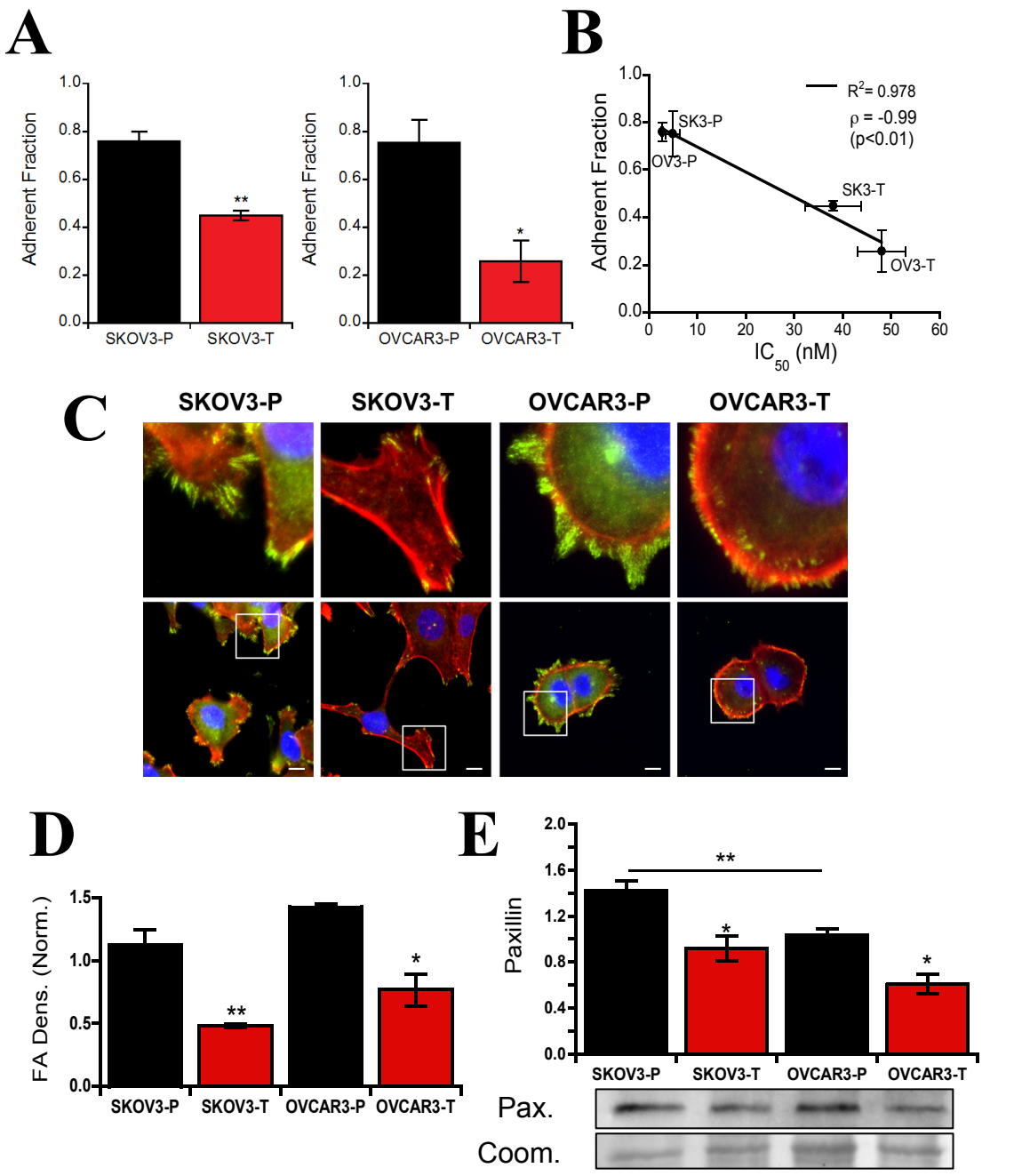

Figure 3. Taxol-resistant cells display weaker adhesion. (A) Centrifugal force-based adhesion strength assay showed that Taxol-resistant populations have a lower adherent fraction than parent populations. (B) The adherent fraction is inversely related to IC_{50} values ($p<0.01$; $\rho = -0.99$). (C) Focal adhesion stain for paxillin revealed that Taxol-resistant cells display smaller and fewer focal adhesions. (D) Focal adhesion density was quantified as the integrated density of segmented focal adhesions relative to cell area. (E) Total paxillin expression was quantified using Western blot and normalized to the total protein expression as determined from Coomassie stain. Taxol-resistant populations express less paxillin than parent populations.

D. Focal Adhesion Kinase Controls Adhesion Strength Which is Upstream of Microtubule Dynamics

I. Examining a Relationship Between Adhesion Strength and Microtubule Dynamics

Taxol-resistant cells of both SKOV3 and OVCAR3 lineages demonstrate increased microtubule dynamics and decreased adhesion strength. In order to determine the correlation between these two observed properties, we first hypothesized that alterations in microtubule dynamics were responsible for the decreased adhesion strength observed in the Taxol-resistant cells. We tested this relationship by treating the parent and Taxol-resistant populations of both SKOV3 and OVCAR3 cells with either Taxol or nocodazole to stabilize or depolymerize microtubules, respectively. We repeated the adhesion strength assay and realized no changes in the adhesive strength of either the parent or Taxol-resistant populations relative to the respective untreated controls (Figure 4a-b). These results demonstrated that changes in adhesion strength were not directly related to microtubule dynamics.

We subsequently hypothesized that the decreased focal adhesion formation was actually altering microtubule dynamics. We then examined the focal adhesion kinase (FAK) signaling pathway since the overexpression of FAK has been demonstrated in variety of invasive cancers.[31] FAK phosphorylation was inhibited with PF228 (Figure 4c), which has been shown to block focal adhesion turnover.[32] After treating both the parent and Taxol-resistant populations with PF228, we repeated the adhesion strength assay. In parent cells, FAK inhibition showed no significant difference in the adherent fraction (Figure 4d). However, in the Taxol-resistant cells, FAK inhibition significantly increased the adherent fraction in comparison to the untreated control in a dose-

dependent manner, reversing the effects of Taxol resistance on adhesion strength for all concentrations greater than 5 μM (Figure 4d). In order to confirm that alterations in adhesive strength were responsible for changes in microtubule dynamics, we repeated the microtubule plus-tip tracking with EB3 post FAK inhibition. It was observed that a significant ($p<0.001$) decrease in growth rate occurred for both the SKOV3 parent and Taxol-resistant cells (Figure 4e). More importantly, the growth rates of the parent and Taxol-resistant populations were comparable through a larger growth rate decrease in the Taxol-resistant cells. These results suggest that microtubule dynamics are down-stream of adhesive signaling and that changes in focal adhesion signaling cause alterations in microtubule dynamics causing an increase in resistance to microtubule-stabilizing drugs such as Taxol.

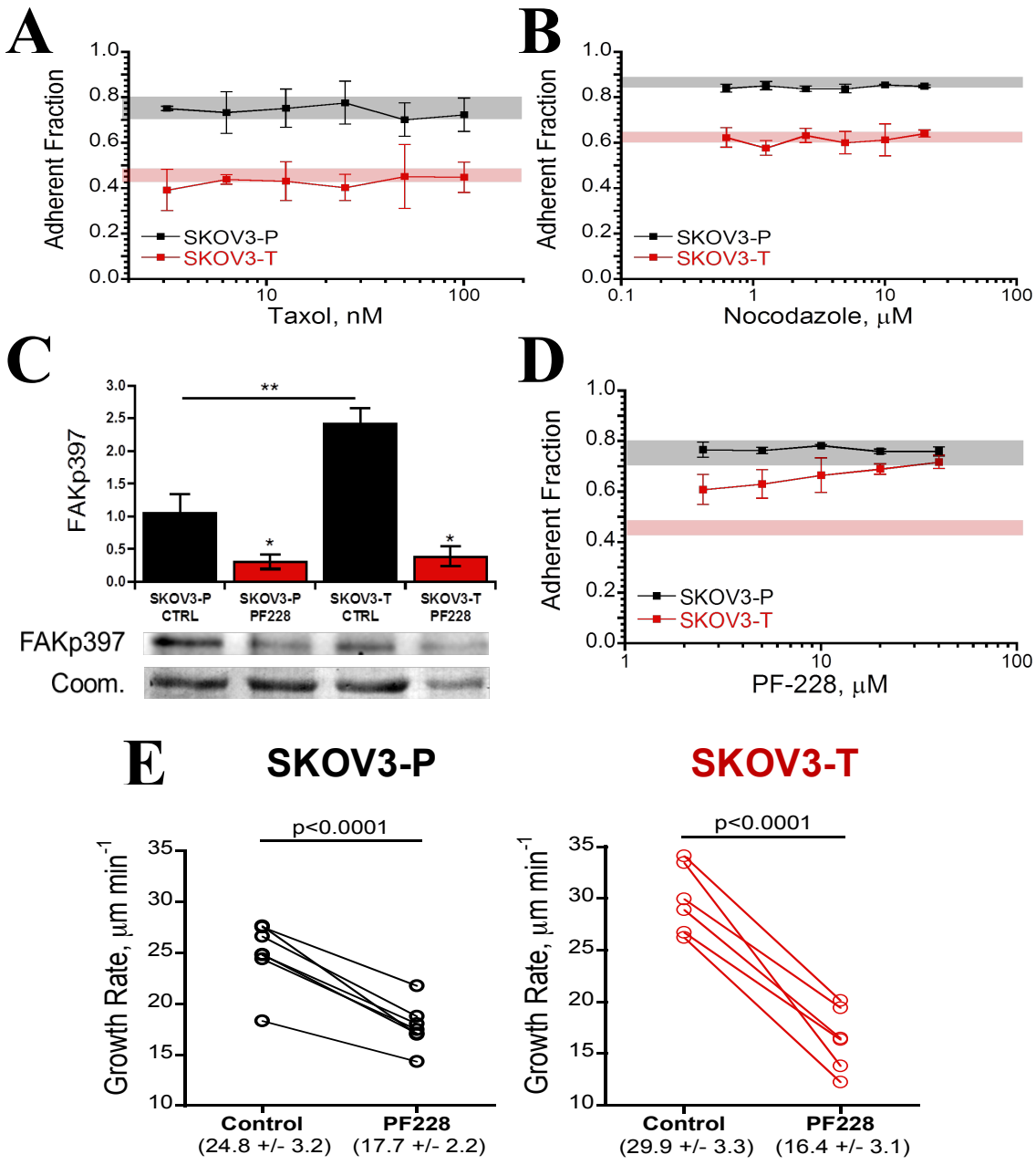

Figure 4. FAK inhibition reverses changes in adhesion and microtubule dynamics. (A-B) SKOV3 parent and Taxol-resistant cells show no change in adherent fraction relative to their untreated controls (shaded regions) after 4 hours of treatment with varying concentrations of Taxol and Nocodazole. (C) FAK phosphorylation in parent and Taxol-resistant cells decreases upon treatment with PF228. (D) SKOV3 Taxol-resistant cells recover adhesive force after 4 hours of treatment with varying concentrations of PF228 until their adherent fraction becomes equivalent to that of the parent cells. (E) Live-cell imaging of microtubules with EB3 fluorescent tag post PF228 treatment. Parent and Taxol-resistant cells show significant decrease in growth rate until the values become statistically equal; however, a greater decrease is observed for Taxol-resistant cells.

CHAPTER 5

DISCUSSION

A major factor attributed to chemoresistance in a variety of cancers is the lack of drug accumulation inside the cell.[33] Previous research has demonstrated that P-glycoprotein (Pgp) mediates this active efflux of chemotherapeutics from cancer cells. However, studies have also shown that not all chemoresistant cancer cells express Pgp; hence, other mechanisms must confer these cells resistance to chemotherapeutics. ABC transporters have also been identified in drug efflux, but inhibition of these proteins poses many clinical limitations.

Taxol is a commonly used chemotherapeutic that targets microtubules and induces apoptosis by initiating mitotic arrest.[34] Since its approval by the FDA in 1992, it has been increasingly used to treat many cancers.[35] However, the development of resistance to the drug prevents the improvement of the overall response and survival of patients. Consequently, it is important to understand the mechanisms of Taxol resistance that arise from factors other than drug efflux and inherent, pro-survival adaptions. After establishing populations of SKOV3 and OVCAR3 Taxol-resistant cells (Figure 1a), we performed a Rhodamine 123 efflux assay to compare the drug accumulation levels in both the parent and Taxol-resistant populations; Rhodamine 123 was used as the model drug since both Rhodamine 123 and Taxol primary used P-glycoprotein as an efflux transporter. There was no significant difference observed in drug efflux between the parent and Taxol-resistant populations (Figure 1b), suggesting that drug efflux was not a dominant determinant of resistance. Furthermore, there was no significant change in cell viability observed between the parent and Taxol-resistant cells post Carboplatin treatment

(Figure 1c). These results led us to deduce that resistance to Taxol in the Taxol-resistant cells was arising due to some alternative mechanism.

Consequently, we stained the parent and Taxol-resistant populations of both SKOV3 and OVCAR3 cells for cytoskeleton proteins and observed that the Taxol-resistant cells appeared more dynamic (Figure 2a). The microtubules are not only growing at a faster rate in Taxol-resistant cells, but there is also a higher density of growing plus-ends (Figure 2c and 2d). Additionally, Taxol-resistant cells displayed less polymerized tubulin in the absence of drug (Figure 2b). However, when treated with low doses of Taxol, the level of polymerized tubulin became comparable to that of parent cells in controlled condition. These results explain the initial peak in viability seen for the Taxol-resistant cells under similar concentrations (Figure 1a). Hence, Taxol acts to stabilize the increased microtubule dynamics in the Taxol-resistant cells and allows these cells to properly divide and proliferate.

Previously, it has been shown that microtubules stimulate the disassembly of focal adhesion through the use of focal adhesion kinase (FAK) and dynamin.[36] Hence, we hypothesized that Taxol resistance was altering cellular adhesion. Using a centrifugal force-based assay, the adhesion strength of Taxol-resistant cells was determined to be lower than the parent cells (Figure 3a). Additionally, adhesion strength correlated strongly with Taxol sensitivity, as characterized by IC_{50} values (Figure 3b). The weaker adhesion observed in Taxol-resistant cells was further explained by the presence of smaller and fewer focal adhesions, as determined by and quantified from an immunofluorescence stain (Figure 3c and 3d). Western blot analysis confirmed that paxillin expression in the Taxol-resistant cells was indeed lower than in the parent cells

(Figure 3e). Thus, these results demonstrated that there is interplay between Taxol resistance and cellular adhesion, but the connection remains unclear.

We further hypothesized that changes in microtubule dynamics were altering adhesion in the Taxol-resistant cells. The adhesion strength assay was repeated for both the parent and Taxol-resistant cells after treatment with either Taxol or Nocodazole, a microtubule-depolymerizing drug.[37] However, there was no significant change in the adherent fraction between the parent or Taxol-resistant cells and their respective untreated controls after treatment with either drug (Figure 4a and 4b), signifying that an alternative microtubule-independent mechanism was conferring Taxol resistance.

We subsequently examined if alterations in focal adhesions were causing changes in microtubule dynamics. In order to target the adhesion dynamics, we inhibited focal adhesion kinase (FAK), which is overexpressed in Taxol-resistant cells.[5] Additionally, FAK silencing has been previously shown to increase sensitivity of ovarian cancer to taxanes.[27]

After confirming that phosphorylation of FAK decreases post treatment with PF228 (Figure 4c), we repeated the adhesion strength assay and observed that the Taxol-resistant cells recover adhesion strength comparable to the parent cells (Figure 4d), which confirms that FAK indeed plays a role in cellular adhesion. Subsequently, we repeated the microtubule plus-tip tracking study with fluorescent EB3 in order to observe any changes in microtubule growth rate post FAK inhibition. As previously shown, Taxol-resistant cells displayed a higher microtubule growth rate than parent cells in the control group. However, after treatment with PF228, Taxol-resistant cells show a greater decrease in microtubule growth rate in comparison to the parent cells, until the rates are

relatively equal (Figure 4e). These results suggest that microtubule dynamics are downstream of adhesion signaling. More importantly, by targeting cellular adhesion in Taxol-resistant cells, we have demonstrated a method for Taxol-resistant cells to regain sensitivity to Taxol.

In addition to overcoming chemoresistance, targeting adhesion dynamics may also reduce the progression of metastasis. The initial step of metastasis, which is detachment of cells from the primary tumor site, is aggravated due to the weaker adhesion observed in Taxol-resistant cells. Hence, by reversing the effects of resistance on adhesion strength via FAK inhibition, we may also be able to inhibit cell migration and invasion. Therefore, FAK not only provides a promising target for increasing chemosensitivity, but may also block metastasis and potentially improve the prognosis of patients with late stage ovarian cancer.

CHAPTER 6

CONCLUSION

Taxol-resistant ovarian cancer populations were confirmed to acquire resistance due to alternative mechanisms other than drug efflux and pro-survival adaptions. In comparison to the respective parent populations, SKOV3 and OVCAR3 Taxol-resistant cells displayed increased microtubule dynamics and weaker adhesion as a result of more depolymerized tubulin and smaller focal adhesions, respectively. Adhesion strength was found to be independent of microtubule polymerization and dependent on up-regulated focal adhesion kinase (FAK) in the Taxol-resistant cells. Furthermore, microtubule dynamics decreased post FAK inhibition, suggesting that microtubule dynamics are downstream of adhesion signaling. Since the inhibition of FAK reverses that changes seen in cell adhesion and microtubule dynamics in Taxol-resistant cells, it may be an effective therapeutic target to improve prognosis and regain drug sensitivity.

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