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Commonly used Platinum based chemotherapy drug-cisplatin has been shown to modulate the single cell mechanics of ovarian cancer cells via actin remodeling but the molecular mechanism is not well understood. This work reveals the correlation between single cell stiffness and cisplatin IC_{50} of ovarian cancer cells ranging from highly cisplatin-resistant to cisplatin-sensitive cell lines. By combining cell mechanics approach with actin structural organization and drug sensitivity, our findings reveal for the first time, a direct role of Rho mediated actin remodeling in cisplatin resistance of ovarian cancer cells.

The role of Rho GTPase in Cell Stiffness and Cisplatin Resistance in Ovarian Cancer Cells

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Changes in cell stiffness (Young's modulus, *E*), as measured via Atomic Force Microscopy (AFM), is a newly recognized characteristic of cancer cells and may play a role in platinum drug resistance of ovarian cancers. We previously showed that, compared to their syngeneic cisplatin-sensitive counterpart, cisplatin-resistant ovarian cancer cells are stiffer, and this cell stiffness was dependent on actin polymerization and presence of stress fibers. Here, we measured the correlation between Young's modulus (via AFM measurements on live, non-apoptotic cells in physiological buffer) and cisplatin-sensitivity (IC_{50} as determined via the XTT cell viability assay) in a panel of nine ovarian cancer cell lines representing a range of cisplatin sensitivities. We found that cisplatin-sensitive cells had a lower Young's modulus, compared to cisplatin-resistant cells and resistant cells had a cytoskeleton composed of long actin stress fibers. As Rho GTPase mediates stress fiber formation, we examined the role of Rho GTPase in cell stiffness and platinum resistance. Rho inhibition decreased cell stiffness in cisplatin-resistant CP70 cells and increased their cisplatin sensitivity while Rho activation increased cell stiffness in cisplatin-sensitive A2780 cells and decreased their cisplatin sensitivity. Based on changes in cell stiffness, IC_{50} and cellular actin stress fiber organization in CP70 and A2780 cells, our findings reveal a direct role of Rho mediated actin remodeling mechanism in cisplatin resistance of ovarian cancer cells. These findings suggest the potential applicability of cell mechanical phenotyping as a model for determining sensitivity of ovarian cancer cells that could have major implications in ovarian cancer diagnosis and personalized medicine.

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Commonly used platinum based chemotherapy drug-cisplatin has been shown to modulate the single cell mechanics of ovarian cancer cells via actin remodeling but the molecular mechanism is not well understood. This work reveals the correlation between single cell stiffness and cisplatin IC_{50} of ovarian cancer cells ranging from highly cisplatin-resistant to cisplatin-sensitive cell lines. By combining cell mechanics approach with actin structural organization and drug sensitivity, our findings reveal for the first time, a direct role of Rho mediated actin remodeling in cisplatin resistance of ovarian cancer cells.

Introduction Nano-mechanical changes at the single cell level[1] can reveal significant insights into cancer cell invasiveness[2], metastatic potential[3] as well as drug sensitivity e.g. sensitivity of ovarian cancer cells to cisplatin, a commonly used platinum based chemotherapeutic agent[4]. Recently, we showed using Atomic Force Microscopy (AFM)-based analysis that cisplatin sensitive ovarian cancer cells have reduced cell stiffness (Young's modulus *E*) compared to cisplatin resistant cells at the single cell level[4]. The variations in cell stiffness of cisplatin sensitive and resistant ovarian cancer cells were mainly attributed to the differences in the actin cytoskeleton. The cisplatin resistant ovarian cancer cells OVCAR5-CisR and SKOV3-CisR[6] showed more robust actin cytoskeleton and stress fibers compared to cisplatin sensitive ovarian cancer cells-OVCAR5 and SKOV3[6].

Rho is a member of a family of small G-proteins with GTPase activity. It has been shown to mediate actin polymerization[5]. Notably, Rho GTPases have a well-documented role in activating signaling pathways required for actin assembly and organization[6]. The effect of cisplatin on actin cytoskeleton of ovarian cancer cells, stimulated a more detailed probing of nanomechanical effects of cisplatin on actin cytoskeleton in order to understand the mechanism by which actin polymerization and de-polymerization affects cisplatin sensitivity of ovarian cancer cells. In the current study, we specifically probed actin cytoskeletal regulator, Rho, that may be a key player in modulating the cisplatin sensitivity of ovarian cancer cells through actin-dependent pathways.

We first examined the correlation between cell stiffness of single isolated cells (as measured via AFM) and their cisplatin sensitivity (IC_{50}, as determined via the XTT cell viability assay), in an extended panel of nine established human ovarian cancer cell lines. We found that lower cisplatin sensitivity correlates with greater cell stiffness in the ovarian cancer cell lines studied. Further, we probed the role of actin cytoskeleton, in particular, the Rho signaling pathway to investigate the observed correlation between cell stiffness and IC_{50}. The role of actin cytoskeleton was determined by activation and inhibition of the actin Rho pathways in A2780 and CP70 cells respectively. The cell stiffness and IC_{50} of ovarian cancer cells were measured in the presence of either Rho activator or inhibitor in combination with cisplatin treatments. Activation of Rho GTPase increased cell stiffness and decreased cisplatin sensitivity in A2780 cells. However, inhibition of Rho GTPase decreased cell stiffness and increased cisplatin sensitivity in CP70 cells. Furthermore, we probed the structural basis of the observed changes in cell stiffness by visualizing the actin structural changes via modulation of Rho signaling. Our results reveal a direct role of Rho mediated actin remodeling in cisplatin resistance of ovarian cancer cells. The findings suggest single cell mechanics play an important role in platinum sensitivity and may serve as a marker for platinum drug sensitivity of ovarian cancers.

Materials and methods

Cell Culture

SKOV3 (American Type Culture Collection; Manassas, VA, USA) and OVCAR5 (gift from Dr. T. Lane; Department of Obstetrics and Gynecology, University of California, Los Angeles, CA, USA) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). A2780 (NCI) and CP70 (kind gifts from Dr. B. Karlan; Cedars Sinai, Los Angeles, CA, USA), and PE01 and PE06 (Dr. S.P. Langdon; Edinburgh Cancer Research Center, University of Edinburgh, Edinburgh, UK) were grown in Roswell Park Memorial Institute (RPMI) medium containing 0.25 IU/mL insulin, 1% minimum essential medium (MEM) non-essential amino acids and 10% FBS. SKOV3-CisR and OVCAR5-CisR[7] were grown in DMEM and 10% FBS in the presence

of cisplatin. RMG-1 cells (Dr. G. Konecny, Jonsson Cance Center, UCLA) were grown in Ham's F-12 medium supplemented with 10% FBS and 1% L-glutamine. All growth media were supplemented with 1% penicillin and 1% streptomycin. All cells were incubated in a humidified 5% CO_2 atmosphere at 37°C.

Cisplatin Sensitivity Assays

Cisplatin sensitivity was determined via the XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] cell viability assay. Cells seeded in 96-well plates were incubated overnight, followed by treatment with increasing concentrations of cisplatin for 72 hours at 37° C. XTT (1 mg/mL) and PMS (phenazine methosulphate; 1 mg/mL) were added, and the metabolism of XTT was measured at 450 nm on an absorbance microplate reader (ELx800, Bio-Tek Instruments; Winooski, VT, USA).

For Rho activation cell viability assays, cells were pretreated with Rho Activator II (Cytoskeleton, Inc.; Denver, CO, USA) for 24 hrs, followed by cisplatin treatment for 72 hr. For Rho inhibition cell viability assays, cells were pretreated with Rho Inhibitor I (Cytoskeleton, Inc.) for 4 hrs, washed twice with growth medium, and then treated with cisplatin for 72 hrs.

AFM measurements

Cells were plated on tissue culture dishes and the following day the cells were left untreated or were treated with Rho inhibitor I for 4 hrs. Cells were then stained with Annexin V as an indicator for apoptosis. Positive-staining cells were excluded from AFM analysis. All measurements were conducted using a Catalyst atomic force microscope (Bruker Instruments, Santa Barbara, California) with a combined inverted optical/confocal microscope (Zeiss Corporation; Thornwood, New York, USA). This combination permits lateral positioning of the AFM tip over the cell center with submicron precision. Mechanical measurements were obtained at 37°C in contact mode using sharpened silicon nitride cantilevers with experimentally determined spring constants of 0.02 N/m and a tip radius of 20nm. Force displacement curves (at 1nN trigger force and cell indentation depths within 500-800 nm range) were recorded at a pulling rate of 1 Hz for determination of Young's modulus. Conversion of force-displacement curves to force indentation curves allows the determination of cell elasticity or **"**stiffness**"** (Young's modulus, E). The measurement outcomes include averaged E value. AFM measurements were obtained in at least 60 cells in three different experiments. The AFM tip was always precisely positioned (within micron range) on top of the nucleus using a motorized stage and inverted optical view of the combined confocal-AFM microscope. Lack of any change in measured stiffness, upon subsequent measurements over the same cellular region, suggest no AFM tip induced damage to the cells during indentations.

Annexin V Staining

Prior to AFM measurements cells were stained with Annexin V as a measure of apoptosis. Staining was performed according to the manufacture's recommendations for the Annexin V-FLUOS staining kit (Roche Diagnostics Corporation; Indianapolis, IN, USA). Briefly, cells were washed with buffer and stained with Annexin V-FLUOS. AFM measurements were taken 15 minutes after staining and completed within 2 hrs. Cells that stained positive with Annexin were excluded from the AFM measurements.

F-Actin Staining

Cells seeded on glass coverslips were incubated overnight and stained the following day, or treated the following day and stained the next day. To stain, cells were fixed in

4% formaldehyde, permeabilized in 0.2% Triton-X100, blocked in 2% BSA, and incubated with ATTO 647N-phalloidin at a 1:7500 dilution. Cells were imaged on a Nikon Eclipse 90i using a 100X oil immersion objective.

Statistical analyses:
All statistical analyses were performed using GraphPad Prism, Version 4.00c for Macintosh, GraphPad Software, www.graphpad.com. A nonlinear regression curve fit (one phase exponential decay) was used to analyze cisplatin dose response experiments. A 2-tailed Pearson's correlation was used to correlate the IC_{50} for cisplatin with the Young's modulus, E. Two-tailed unpaired t tests were used to calculate the significance of differences in cell viability between the single agent and dual agent treatments. For all t tests, NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

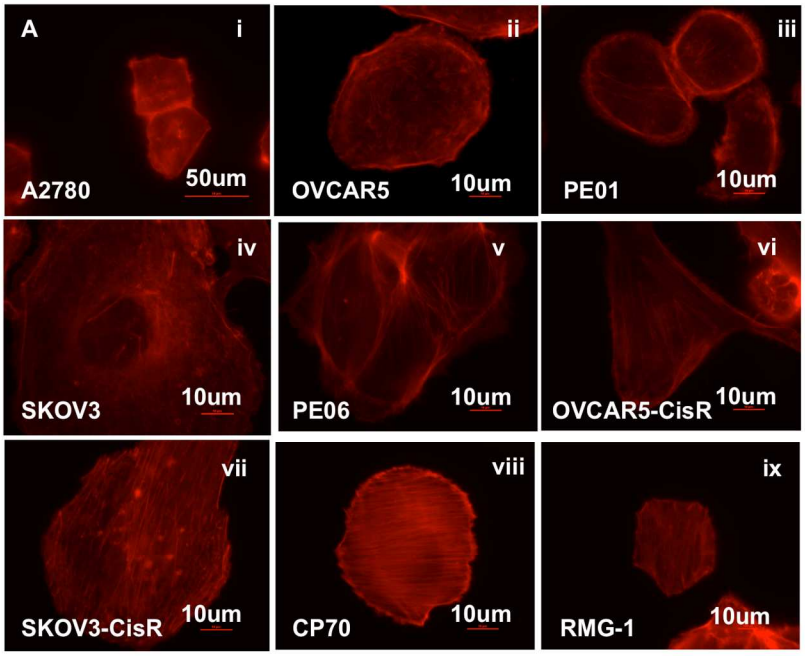
Results

Lower cisplatin sensitivity correlates with greater cell stiffness in ovarian cancer cell lines

Using two pairs of isogenic cisplatin-sensitive and cisplatin-resistant human ovarian cancer cells, we previously demonstrated an increase in cell stiffness in cisplatin-resistant cells compared to cisplatin-sensitive cells[4]. Here, we determined whether cell stiffness correlates with cisplatin sensitivity using a panel of nine human ovarian cancer cell lines (A2780, CP70, OVCAR5, OVCAR5-CisR, PE01, PE06, RMG-1, SKOV3 and SKOV3-CisR). Cisplatin resistance in CP70, OVCAR5-CisR and SKOV3-CisR were developed *in vitro* via the long-term exposure of the parental cells (A2780, OVCAR5 and SKOV3, respectively) to cisplatin[7]. PE01 cells were isolated from a patient's ovarian tumor before the development of platinum-resistance, whereas PE06 cells were isolated after the development of resistance[8]. A2780, OVCAR5, PE01, RMG-1 and SKOV3 cells were cisplatin-naïve. The cisplatin sensitivity of each cell line was determined by measuring the effect of increasing concentrations of cisplatin on cell viability using the XTT assay. The IC_{50}s measured for the cell lines within the panel fell into two main groups, those that were cisplatin-sensitive with a lower IC_{50} (A2780, OVCAR5, PE01, SKOV3; Fig. 1A, i-iv), and those that were cisplatin-resistant with a higher IC_{50} (OVCAR5-CisR, SKOV3-CisR, CP70, RMG-1, PEO6; Fig. 1A, v-ix). Cisplatin sensitivities varied from an IC_{50} of 0.8-20.2 μg/ml, with A2780 cells being the most sensitive and RMG-1 cells being the most resistant to cisplatin (Fig. 1B left axis, black line).

Cell stiffness in each of these cell line was measured via Atomic Force Microscopy (AFM). The average cell stiffness of cell lines were obtained based on the Young's modulus of single isolated cells measured over the nuclear region. Force versus separation curves were recorded over a total of at least 50 cells (4 curves per cell) for each cell line from two or more biological replicates and used to calculate Young's modulus, E (KPa). To avoid measuring unhealthy or apoptotic cells, the cells that stained positive for Annexin V (an indicator of early apoptosis) were excluded from AFM analysis. In all cases, the cisplatin-resistant version of each pair of cell lines had a higher Young's modulus (stiffer cells) as compared to the cisplatin-sensitive cell line (softer cells) (Fig. 1B, right axis). For example, SKOV3 cells had a Young's modulus of 0.4 $\pm$ 0.1 KPa, while SKOV3-CisR cells had a Young's modulus of 1.4 $\pm$ 0.2 KPa ($p < 0.05$). The highest difference in cell stiffness was seen in the A2780 and CP70 cells (A2780 = 0.4 $\pm$ 0.01 KPa; CP70 = 1.5 $\pm$ 0.2 KPa; $p < 0.05$). Moreover, PE06 cells, isolated from a

patient's tumor after the development of platinum-resistance, were found to be stiffer than PE01 cells, isolated before the development of platinum-resistance (PE01 = 0.8 ± 0.3 KPa; PE06 = 1.02 ± 0.3 KPa; $p < 0.05$).



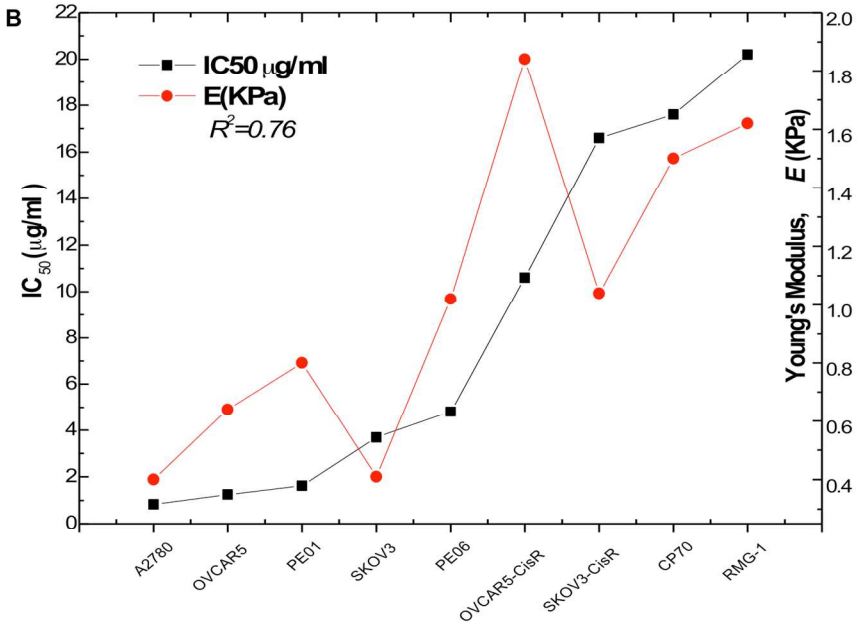


Fig.1 Actin Stress Fibers and Cell Stiffness Increases with Cisplatin Resistance (A) Cellular actin cytoskeleton and (B) cisplatin IC_{50} (left y-axis) measured by XTT cell viability assay and Young's modulus, E (right y-axis) measured using AFM, for a panel of cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines.

Interestingly, A2780 cells showed both the lowest IC_{50} (0.83 μg/ml) and the lowest Young's modulus (0.4 ± 0.01 KPa). In contrast, RMG-1 cells showed the highest IC_{50} (20.2 μg/ml), as well as the highest Young's modulus (1.62 ± 0.1 KPa). A Pearson product-moment correlation coefficient was computed to assess the relationship between the cell stiffness of ovarian cancer cells and their cisplatin IC_{50}. We found a positive correlation between the two variables, ovarian cancer cell stiffness and IC_{50} R^2 = 0.76, p = 0.002 (Fig. 1B). Increases in cell stiffness were correlated with increases in IC_{50} for cisplatin. Hence, based on these measurements, we hypothesized that cell stiffness correlates with cisplatin resistance.

Cisplatin-resistant cells are characterized by a dense cytoskeleton of actin stress fibers

To determine whether actin stress fibers are a distinguishing feature of platinum-resistant cells, we examined F-actin cytoskeletal organization in all of the nine human ovarian cancer cell lines studied with varying cell stiffness and IC_{50}. Fig. 1A (i to iv) shows F-actin staining in cisplatin-sensitive cells (A2780, OVCAR5, PE01, and SKOV3), as compared to their isogenic cisplatin-resistant counterpart (CP70, OVCAR5-CisR, PE06, and SKOV3-CisR, respectively; Fig. 1A (v to viii). Consistent with previous findings[4], the cytoskeletons of the cisplatin-resistant cells were composed of long actin fibers not seen in the cisplatin-sensitive cells. A2780 cells showed almost a complete lack of actin stress fibers, which was in sharp contrast to the isogenic CP70 counterpart, where the stress fibers could be seen extending along the entire length of the cells as shown in representative cells (Fig. 1A). Similarly, SKOV3 cells possessed less actin stress fibers compared to SKOV3-CisR cells with abundant radially elongated actin stress fibers. Moreover, actin stress fibers were also present in RMG-1 cells, a cell line inherently resistant to cisplatin.

Activation of Rho GTPase increases cell stiffness and decreases cisplatin sensitivity

As actin polymerization is required for cell stiffness, and Rho GTPase regulates the actin cytoskeleton[6, 9], we next examined whether Rho plays a role in regulating cell stiffness in human ovarian cancer cells. We first tested whether Rho activation increases cell stiffness in cisplatin-sensitive cells. Rho Activator II was used to constitutively activate the Rho isoforms RhoA, Rac1 and Cdc42 via the blockage of their intrinsic and GAP stimulated GTPase activity[10]. The efficacy of Rho Activator II was tested on actin organization, cell viability and changes in cell stiffness of treated cells. To demonstrate efficacy of Rho Activator II, cisplatin-sensitive A2780 cells were incubated with 2 or 4 μg/ml of the Rho Activator II for 24 hrs, or left untreated and used for cell viability and AFM measurements or stained with ATTO 647N-phalloidin to visualize actin cytoskeleton. Untreated A2780 cells were seen to have a rounded morphology with an actin cytoskeleton consisting of mainly short actin fibers with only a few long fibers visible (Fig. 2A, i). In contrast, A2780 cells treated with Rho Activator II were more spread out on the surface, and the actin cytoskeleton was reorganized into long F-actin filaments that spanned the cell membrane (Fig. 2A, ii (2 μg/ml) and iii (4 μg/ml)). A similar effect was also observed in OVCAR5 cells (Fig. S2).

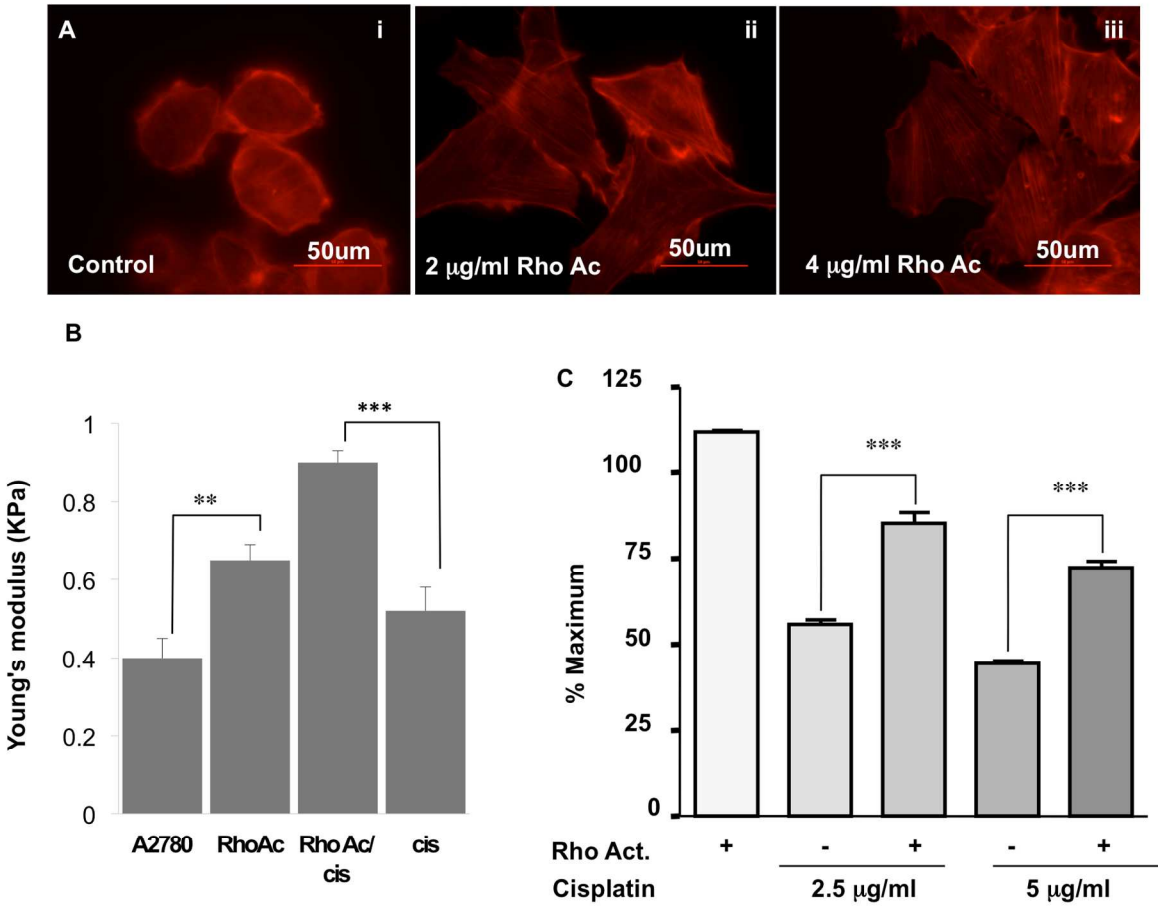


Fig. 2 Rho activation increases actin fibers and cell stiffness and decreases cisplatin sensitivity in A2780 cells (A) Actin cytoskeleton after treatment of A2780 cells with increasing concentrations (2 and 4μg/ml respectively) of Rho Activator II (Rho Ac) for 4 hrs (B) Cell stiffness after treatment with or without Rho Activator II and/or cisplatin for 72 hrs. *** p < 0.05 (C) Cell viability after treatment with or without Rho Activator II (Rho Ac) and/or cisplatin for 72 hrs. *** p < 0.001.

The cell stiffness measurements obtained using AFM showed that Rho activator II increased cell stiffness in A2780 cells in a dose-dependent manner. Cells treated with two μg/ml Rho Activator II for 24 hrs showed an increase in cell stiffness, from 0.4 $\pm$ 0.1 KPa (control) to 0.65 $\pm$ 0.04 KPa ($p<0.05$). A further increase in cell stiffness was seen upon 4μg/ml treatment (0.74 $\pm$ 0.02 KPa; $p<0.05$).

Since cell stiffness positively correlated with cisplatin resistance, we next asked whether Rho mediates this resistance. A2780 cells were pre-treated with 4 μg/ml Rho Activator II

for 24 hrs, after which 2.5 or 5μg/ml cisplatin was added for 72 hrs. Cisplatin-only treated cells received cisplatin on day 2 and were treated for 72 hrs, after which the effect on cell stiffness and cell viability was determined. Interestingly, Rho Activator II alone increased cell stiffness in A2780 cells (0.65 ± 0.04 KPa) compared to untreated cells (0.4 ± 0.05; Fig. 2B). Additionally, cells treated with the combination of Rho Activator II and 2.5μg/ml cisplatin showed greater cell stiffness (cisplatin + Rho Activator II= 0.9 ± 0.03 KPa) versus cells treated with cisplatin alone (cisplatin= 0.52 + 0.06; $p < 0.05$). Meanwhile, Rho Activator II alone increased cell viability beyond that in untreated cells (Rho Activator II = 112%; Fig. 2C). Furthermore, cells treated with the combination of Rho Activator II and 2.5μg/ml cisplatin had a higher level of cell viability versus cells treated with cisplatin alone (cisplatin = 56.0%; cisplatin + Rho Activator II = 85.4%; $p < 0.001$). A similar increase occurred at 5μg/ml cisplatin (cisplatin = 44.5%; cisplatin + Rho Activator II = 72.3%; $p < 0.001$).

Inhibition of Rho GTPase decreases cell stiffness and increases cisplatin sensitivity

To corroborate the above results, the effect of Rho inhibition on cell stiffness was examined. Rho inhibition was achieved via ADP-ribosylation of the effector domain of the Rho GTPase using Rho Inhibitor I, C3 Transferase covalently linked to a cell-penetrating moiety to allow rapid and efficient transport through the plasma membrane[11]. Similar to Rho activation, the efficacy of Rho Inhibitor I was tested based on actin organization, cell viability and cell stiffness measurements. Cisplatin-resistant CP70 cells were treated with 2 or 4 μg/ml of Rho Inhibitor I for 4 hours, after which cells were used for AFM measurements and cell viability assays. Cellular actin was imaged via staining with ATTO 647N-phalloidin. Fig. 3A (i) shows untreated CP70 cells with long fibers of actin stretching across the width of the cell. Treatment with Rho Inhibitor I completely obliterated these fibers (Fig. 3A ii and iii). We also observed similar effect in actin organization of OVCAR5_CisR cells treated with Rho inhibitor (Fig. S2).

To test the effect of Rho inhibition on cell stiffness, CP70 cells were treated with 2 or 4μg/ml of Rho Inhibitor I for 4 hours. A statistically significant decrease in the Young's modulus was observed in cells treated with 2.5μg/ml of Rho Inhibitor I (control = 1.7 ± 0.1 KPa; 2.5 μg/ml Rho Inhibitor I = 1.3 ± 0.09 KPa; $p < 0.001$). Four μg/ml Rho inhibitor treated cells showed further decrease in cell stiffness (4 μg/ml Rho Inhibitor I = 1.15 ± 0.06 KPa; $p < 0.001$). These results support our Rho activation data.

To determine whether Rho mediates cisplatin sensitivity, we again tested the effect of Rho Inhibitor I on CP70 cell stiffness and cell viability in the presence of cisplatin. Cisplatin-resistant CP70 cells were pretreated with 2 μg/ml Rho Inhibitor I for 4 hours, after which the medium was removed and the cells washed twice before the addition of specified amounts of cisplatin. Rho Inhibitor alone decreased cell stiffness in CP70 cells (1.3 ± 0.09 KPa) compared to untreated cells (1.7 ± 0.05; Fig. 3B). Additionally, cells treated with the combination of Rho Inhibitor and 2.5μg/ml cisplatin showed lower cell stiffness (cisplatin + Rho Inhibitor = 0.7 ± 0.1 KPa) versus cells treated with cisplatin alone (cisplatin= 1.67 ± 0.08; $p < 0.005$).

Correspondingly, Rho Inhibitor I treatment alone decreased cell viability. Treatment with 10 μg/ml cisplatin alone also decreased cell viability, and the addition of Rho Inhibitor I decreased viability further (measured relative to Rho Inh treatment; $p < 0.001$). The results (Fig. 3C) are in agreement with those above using Rho Activator II.

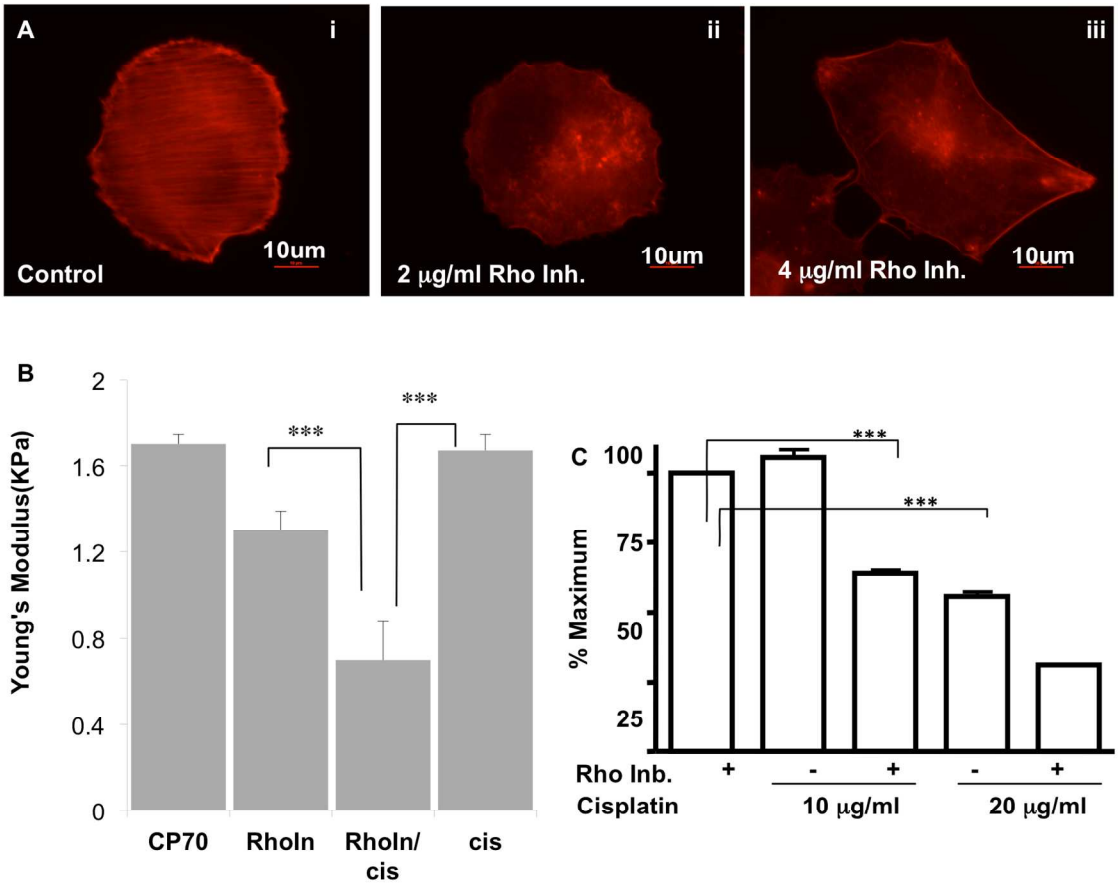


Fig. 3 Rho Inhibition Decreases Cell Stiffness and Increases Cisplatin Sensitivity in CP70 cells (A) Actin cytoskeleton after treatment with Rho Inhibitor I for 4 hrs. (B) Cell stiffness after treatment with 2.5μg/ml Rho Inhibitor I (C) Cell viability after treatment with or without 2.5μg/ml Rho Inhibitor I (Rho Inh) and/or cisplatin for 72 hrs. *** p < 0.001.

Discussion

There is an emerging role of cellular nanomechanics in understanding cancer progression, therapeutic efficacy and development of drug resistance at the cellular and sub-cellular level. In particular, the development of novel cell nanomechanical approaches to determine platinum -drug sensitivity of ovarian cancers could have major implications in personalized ovarian cancer management, currently limited by lack of reliable biomarkers of therapeutic efficacy and resistance to platinum-therapy[12]. AFM[13] has been used to study various types of cellular events on cultured cell lines[14]. AFM based single cell measurements can provide quantitative information[15] regarding cell stiffness (Young's modulus) of cancer cells[16]. Our previous observations revealed distinct nanomechanical profiles of human ovarian cancer cell lines varying in cisplatin sensitivities, as measured by AFM. Cisplatin resistant ovarian cancer cells showed greater cell stiffness compared to the cisplatin sensitive cells[4]. This increase in cell stiffness was reversed by treatment of cells with the actin-depolymerizing agent-

cytochalasin D. Furthermore, STED[17] super resolution confocal microscopy showed that cisplatin resistant ovarian cancer cells possess more robust and organized actin cytoskeleton organization compared to cisplatin sensitive cells[4].

In the current study we tested the correlation between observed changes in cell stiffness in a range of ovarian cancer cell lines with varying cisplatin sensitivities (Fig. 1), which included untreated A2780, CP70, OVCAR5, SKOV3, RMG-1, SKOV3CisR and OVCAR5CisR cells. Additionally, we used adenocarcinoma panel model cell lines (PE01 and PE06), obtained from same patient before and after development of resistance to cisplatin. Our results highlight a positive correlation between the chemosensitivity profiles of the nine human ovarian cancer cell lines studied and their single cell nanomechanics. Actin organization plays a major role in determining the mechanical properties of cells[18]. Thus the actin cytoskeleton of cells with varying cisplatin sensitivities was probed for structural insights into the mechanisms leading to the observed differences in cell stiffness. We found that cell lines with a higher cisplatin resistance also show greater cell stiffness (Fig. 1) and increased actin cytoskeleton organization (also see supplemental Fig. S1).

Furthermore, we explored the cisplatin induced mechanical phenotype in ovarian cancer cells to identify the molecular mechanisms that enable cancer cells to modulate their mechanical responses via remodeling of actin cytoskeleton. Rho proteins have been shown to have key roles as intermediaries between extracellular signals and internal actin organization[9]. In humans, ~20 Rho GTPases exist including Rho, Rac and Cdc42 [19].The small GTPase Rho are involved in cancer progression, including proliferation, evasion of apoptosis, invasion, and metastasis [20]. Rho acts as a molecular switch in which the GTP-bound form is “active” and the GDP-bound form is “inactive.” Once activated, Rho GTPases bind to a variety of effectors, including some actin-binding proteins. In the specific context of cell mechanics, Rho A, can directly or indirectly affect the local polymerization and de-polymerization of filamentous (F)-actin and actin stress fibres[9]. Therefore, we analyzed the effect of Rho Activator II on ovarian cancer cell lines that constitutively activates the Rho isoforms RhoA, Rac1 and Cdc42. Rho Activator II robustly increases the level of GTP bound RhoA in cells within 2-4 h[10]. Moreover, the targeted action of Rho Activator II is an attractive tool for the study of Rho GTPase signaling than indirect activators (e.g. LPA) that concomitantly activate other signaling pathways (e.g. Ras, PI3K and PLC). In comparison, Rho inhibition was achieved via ADP-ribosylation of effector domain of Rho GTPase. Rho Inhibitor C3 Transferase can freely diffuse intra-cellularly to inactive RhoA, RhoB, and RhoC, but not related GTPases such as Cdc42 or Rac1[11]. The optimized conditions and concentrations of both the Rho Activator II and Rho Inhibitor were then used in the cisplatin combination experiments.

The influence of cisplatin in presence of either Rho activation or Rho inhibition was analyzed in cisplatin sensitive and resistant cells lines respectively. Our results demonstrate that Rho inhibition decreased cell stiffness in cisplatin-resistant CP70 cells and increased their cisplatin sensitivity (Fig. 3); while Rho activation increased cell stiffness and decreased cisplatin sensitivity in cisplatin sensitive A2780 cells (Fig. 2). The observed changes in cell stiffness and cisplatin IC_{50} in the presence of Rho activator or inhibitor suggest that the stiffness increase with cisplatin-resistant cells is at least largely due to dynamic changes in the actin cytoskeleton. It should be noted however, that Rho GTPases[21] regulate many other additional signal transduction pathways in addition to those linked to the actin cytoskeleton such as cell polarity, gene transcription and microtubule dynamics[22]. A more detailed understanding of the actin signaling

molecular pathways explicitly involved in development of platinum resistance in ovarian cancers is further needed. Nevertheless, our findings reveal for the first time, a direct role of Rho mediated actin remodeling in cisplatin resistance of ovarian cancer cells.

Conclusions

The findings suggest the potential applications of cell mechanical phenotyping as a model for determining sensitivity of ovarian cancer cells to commonly used chemotherapy drugs, such as cisplatin. Our study highlights the relevance of cell mechanics based approaches for determining new targets for therapy for platinum resistant phenotype in ovarian cancer. In future, measurements of single cell mechanics of live ovarian cancer cells isolated from patient tumors could transform biomarkers used for personalized ovarian cancer drug sensitivity and management of platinum based chemotherapy.

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Electronic Supplementary Information (ESI) available: See supplemental Figure S1

References

1. S. E. Cross, Y. S. Jin, J. Rao and J. K. Gimzewski, *Nature nanotechnology*, 2007, **2**, 780-783.
2. S. Suresh, *Acta biomaterialia*, 2007, **3**, 413-438.
3. T. Watanabe, H. Kuramochi, A. Takahashi, K. Imai, N. Katsuta, T. Nakayama, H. Fujiki and M. Suganuma, *Journal of cancer research and clinical oncology*, 2012.
4. S. Sharma, C. Santiskulvong, L. A. Bentolila, J. Rao, O. Dorigo and J. K. Gimzewski, *Nanomedicine : nanotechnology, biology, and medicine*, 2011.
5. A. B. Jaffe and A. Hall, *Annu Rev Cell Dev Biol*, 2005, **21**, 247-269.
6. A. Hall and C. D. Nobes, *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 2000, **355**, 965-970.

7. C. Santiskulvong, G. E. Konecny, M. Fekete, K. Y. Chen, A. Karam, D. Mulholland, C. Eng, H. Wu, M. Song and O. Dorigo, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 2011, **17**, 2373-2384.

8. P. Mullen, D. A. Cameron, M. Hasmann, J. F. Smyth and S. P. Langdon, *Molecular cancer therapeutics*, 2007, **6**, 93-100.

9. S. T. Sit and E. Manser, *Journal of cell science*, 2011, **124**, 679-683.

10. G. Flatau, E. Lemichez, M. Gauthier, P. Chardin, S. Paris, C. Fiorentini and P. Boquet, *Nature*, 1997, **387**, 729-733.

11. M. J. Kim, S. Kim, Y. Kim, E. J. Jin and J. K. Sonn, *Biochem Biophys Res Commun*, 2012, **418**, 500-505.

12. Z. Su, W. S. Graybill and Y. Zhu, *Clinica chimica acta; international journal of clinical chemistry*, 2013, **415**, 341-345.

13. G. Binnig, C. F. Quate and C. Gerber, *Phys Rev Lett*, 1986, **56**, 930-933.

14. S. Sharma and G. J.K, in *Life at the nanoscale: Atomic Force Microscopy of live cells*, ed. Y. Dufrene, Pan Stanford Publishing, 2011, ch. 20, pp. 421-436.

15. S. Sharma, K. Das, J. Woo and J. K. Gimzewski, *Journal of the Royal Society, Interface / the Royal Society*, 2014, **11**, 20131150.

16. F. T. Arce, B. Meckes, S. M. Camp, J. G. Garcia, S. M. Dudek and R. Lal, *Nanomedicine : nanotechnology, biology, and medicine*, 2013, **9**, 875-884.

17. S. W. Hell and J. Wichmann, *Opt Lett*, 1994, **19**, 780-782.

18. C. Rotsch and M. Radmacher, *Biophys J*, 2000, **78**, 520-535.

19. S. J. Heasman and A. J. Ridley, *Nature reviews. Molecular cell biology*, 2008, **9**, 690-701.

20. F. M. Vega and A. J. Ridley, *FEBS Lett*, 2008, **582**, 2093-2101.

21. C. Gest, P. Mirshahi, H. Li, L. L. Pritchard, U. Joimel, E. Blot, J. Chidiac, B. Poletto, J. P. Vannier, R. Varin, M. Mirshahi, L. Cazin, E. Pujade-Lauraine, J. Soria and C. Soria, *Cancer letters*, 2012, **317**, 207-217.

22. S. Li, B. P. Chen, N. Azuma, Y. L. Hu, S. Z. Wu, B. E. Sumpio, J. Y. Shyy and S. Chien, *The Journal of clinical investigation*, 1999, **103**, 1141-1150.