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Spontaneous spheroid budding from monolayers: a potential contribution to ovarian cancer dissemination

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Summary

Ovarian cancer is the most lethal gynaecologic cancer, in large part because of its early dissemination and rapid development of chemotherapy resistance. Spheroids are clusters of tumor cells found in the peritoneal fluid of patients that are thought to promote this dissemination. Current models suggest that spheroids form by aggregation of single tumor cells shed from the primary tumor. Here, we demonstrate that spheroids can also form by budding directly as adherent clusters from a monolayer. Formation of budded spheroids correlated with expression of vimentin and lack of cortical E-cadherin. We also found that compared to cells grown in monolayers, cells grown as spheroids acquired progressive resistance to the chemotherapy drugs Paclitaxel and Cisplatin. This resistance could be completely reversed by dissociating the spheroids. Our observations highlight a previously unappreciated mode of spheroid formation that might have implications for tumor dissemination and chemotherapy resistance in patients, and suggest that this resistance might be reversed by spheroid dissociation.

Key words: EMT, Ovarian cancer, Platinum, Taxol

Introduction

Ovarian cancer is the fifth leading cause of cancer death in women (Jemal et al., 2010). The incidence of ovarian cancer is lower than many other tumor types, but its mortality is higher – over 50% – and this has not appreciably changed in the past eight decades, although a small, recent trend towards improvement has been seen (Jemal et al., 2010).

Ovarian cancer differs from almost all other solid tumors in that its primary mode of dissemination is directly across the peritoneal space, rather than through the vasculature (Shield et al., 2009). Individual tumor cells can be shed into the peritoneum, and a current model proposes that their aggregation into spheroids – free-floating multicellular clusters – facilitates their transit and implantation (Ahmed et al., 2007; Shield et al., 2009). Spheroids have been observed in the ascites of ovarian cancer patients and are believed to make a significant contribution to intraperitoneal spread (Allen et al., 1987; Burleson et al., 2006; Burleson et al., 2004).

Most of our understanding of spheroid cell biology has come from *in vitro* culture of cancer cell lines. Spheroids can be produced from many cancer cell lines under conditions that favor cell-cell over cell-matrix adhesion, such as growth in serum-free media, or the “hanging drop” method (Kelm et al., 2003; Lund-Johansen et al., 1989). Not all cancer cell lines form spheroids, and the features that allow spheroid growth are not well characterized.

A major property of cultured spheroids, including spheroids from ovarian cancer cell lines, is resistance of spheroid cells to cytotoxic drugs compared to the same cells grown as monolayers (Frankel et al., 1997; Green et al., 2004; Green et al., 1999). This drug resistance is proposed to arise from one or more factors, including creation of a physical barrier to drug penetration; induction of genes or signalling pathways that enhance survival, such as drug efflux pumps; and selection for a subpopulation of drug-resistant cells, which could include cancer stem cells (Desoize and Jardillier, 2000; Hirschhaeuser et al., 2010; Kerbel, 1994–1995).

We observed a novel means of spheroid formation for four ovarian cancer cell lines cultured in standard media. When the cells were grown as adherent monolayers, spheroids arose directly from the monolayer in dense regions and areas of vertical cell growth. Spheroid budding correlated with the absence of cortical E-cadherin and the presence of vimentin filaments throughout the cells of the monolayer, suggesting that the ability of a cell line to form spheroids was a property of the cell line rather than a biological change only at the site of budding. Serial passage of ovarian cancer cells between spheroid and monolayer culture induced progressive resistance to the chemotherapy drugs Paclitaxel and Cisplatin, and dissociating the spheroids back into monolayers could reverse this drug resistance. Our observations suggest that these cancer cell lines have acquired the ability to form spheroids by budding from an adherent monolayer, that a similar process might occur *in vivo*, and that dissociating spheroids could improve ovarian cancer cell sensitivity to chemotherapy.

Results

A novel means of spheroid formation: budding from a monolayer

When we cultured A2780 ovarian cancer cells as a monolayer in standard culture media supplemented with 10% FBS, we

observed formation of multilayered colonies similar to those typically associated with loss of contact inhibition. Upon continued culture, these colonies expanded both vertically and horizontally. Frequently, rounded spherical clusters of cells arose from these colonies and appeared to be attached to the monolayer by a narrow stalk. These cell clusters eventually (within 2–3 days) detached from the monolayer, producing floating spheroids in the media that were morphologically identical to spheroids of this cell line formed by aggregation in commercially-available serum-free spheroid media (Fig. 1A–C). The spheroids were of relatively uniform size, (30 to 100 μm), similar to the size of spheroids formed by aggregation. We called this novel type of spheroid production "budding" to distinguish it from aggregation or clonal expansion from a single cell, both of which are established mechanisms of spheroid formation.

To assess the morphology of these budded spheroids and their relationship to the underlying monolayer, we stained them with the vital DNA dye DRAQ5 and imaged them using a water-immersion lens with confocal sectioning. Imaging of living cells was necessary because fixation caused the spheroids to break off from the monolayer. This live-cell imaging of spheroids in various stages of budding revealed a variety of spheroid morphologies, including large mounds and round balls attached to the monolayer at a single point (Fig. 1D,E).

We tested the viability of budded spheroids by several methods. DNA staining with DRAQ5 showed occasional cells with condensed nuclei consistent with apoptosis, at the same frequency as in the parental monolayer. When we trypsinized spheroids and stained with Trypan blue dye, a majority of cells (~90%) excluded the dye. Finally, spheroids could be transferred to a new culture dish coated with 2% Matrigel and spread as a monolayer without obvious cell death.

We also tested growth factor requirements of budded spheroids, to determine whether the budding process depended on the presence of serum or added growth factors. Commercial spheroid media is typically supplemented with a cocktail of growth factors, some of which are proprietary, instead of serum. We tested spheroid budding by replacing serum-containing media with spheroid base media lacking these added growth factors.

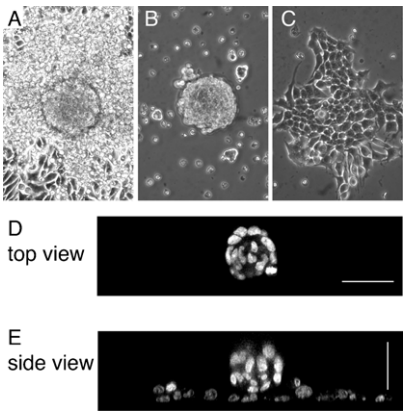

Fig. 1. Ovarian cancer spheroids can bud from a monolayer. A2780 cells were grown as a monolayer in media containing serum. (**A–C**) Phase contrast images of the spheroid budding process. (A) Representative spheroid budding from the monolayer at a site of vertical expansion. (B) Representative spheroid that had detached from the monolayer and was floating freely in the culture media. (C) Representative spheroid plated in the presence of serum, which had re-adhered to the culture dish and formed a new monolayer. (**D**,**E**) Fluorescence images of a budding spheroid stained with DRAQ5 to visualize cell nuclei and imaged while alive. The same spheroid is shown from the top, or X–Y view (D), and the side, or Y-Z view, which was digitally reconstructed from confocal image stacks (E). Scale bars: 30 μm.

Remarkably, A2780 spheroids could bud from a monolayer, adhere to a new dish to form a new monolayer, and produce new budded spheroids, in a continuous cycle for over five weeks, in base media without any growth factors except for a 2% solution of growth factor-reduced Matrigel used to adhere them to the dish. This suggests that the ability to form budded spheroids does not require supplemental serum or growth factors added to the spheroid media.

Spheroid budding was not unique to A2780 cells. We found that four out of six ovarian cancer cell lines produced budded spheroids: A2780, SKOV3, HEY, and OVCA420 (Fig. 2). For most of these, spheroids detached from the monolayer spontaneously and were found floating in the culture media. Neither of the cell lines that failed to form spheroids (BG1 or OVCA433) formed multilayered colonies, suggesting that vertical growth of the monolayer might be a prerequisite for spheroid formation.

Spheroid budding correlates with epithelial to mesenchymal transition (EMT) markers

Spheroid budding could be due to a biological change in a subset of cells within the monolayer, or to a global property of the cell line. To distinguish between these possibilities, we performed a microarray experiment to compare gene expression in A2780 cells grown as monolayers or budded spheroids. This showed no major differences in gene expression between monolayers and spheroids (data not shown), suggesting that altered gene expression is unlikely to be responsible for local changes within a subset of cells that form a budded spheroid.

We next asked whether ovarian cancer cell lines capable of forming budded spheroids showed any properties that differed from cell lines that failed to form budded spheroids. We focused on proteins associated with the epithelial to mesenchymal transition (EMT), a process that promotes cancer progression and loss of traditional epithelial polarity (see Discussion) (Ahmed et al., 2010; Godde et al., 2010).

We performed immunofluorescence for E-cadherin, which is typically found at sites of cell-cell contact in epithelial monolayers, and vimentin, which is typically expressed in mesenchymal cell types. Of six ovarian cancer cell lines assayed, three showed a lack of E-cadherin at cell-cell borders and the presence of vimentin filaments throughout the cytoplasm, a typical pattern for cells that have undergone an EMT (Fig. 2). These three cell lines (A2780, SKOV3, and HEY) were also capable of forming budded spheroids. A fourth cell line, OVCA420, showed lack of cortical E-cadherin and lack of vimentin; OVCA420 cells formed budded spheroids at a much lower frequency. In contrast, two cell lines showed strong expression of E-cadherin at cell-cell borders and either no vimentin (BG-1) or a low level of patchy vimentin expression (OVCA433). Neither of these cell lines formed budded spheroids. Thus, lack of cortical E-cadherin and the presence of vimentin filaments correlated strongly with the ability of an ovarian cancer cell line to form budded spheroids.

Serial passage of ovarian cancer cells between monolayer and spheroid culture increases resistance to cytotoxic drugs

Ovarian cancer cell line spheroids formed by the liquid overlay method were reported to show resistance to Paclitaxel but

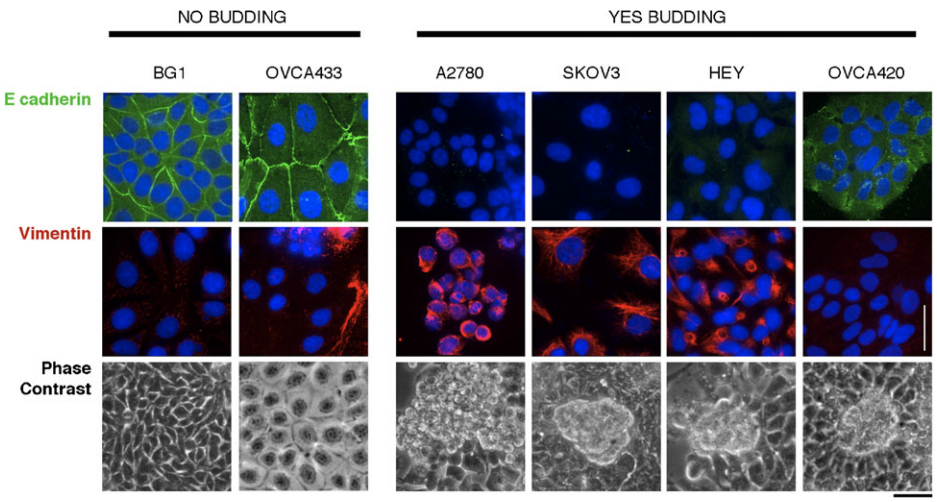

Fig. 2. Ability to form budded spheroids correlates with lack of cortical E-cadherin and expression of vimentin throughout the monolayer. Cell lines were grown as monolayers and immunofluorescence for E-cadherin and vimentin performed. Top panel, E-cadherin (green) merged with DNA (blue) at 60× magnification. Middle panel, vimentin (red) merged with DNA (blue) at 60× magnification. Cell lines that failed to form budded spheroids from the monolayer showed strong cortical E-cadherin signal and lack of vimentin (BG-1) or constant cortical E-cadherin and intermittent vimentin (OVCA433). Cell lines that formed budded spheroids (A2780, SKOV3, HEY) showed absence of cortical E-cadherin and presence of vimentin filaments throughout the monolayer. OVCA420 cells lacked both proteins, and formed rare budded spheroids. Bottom panel, phase contrast images of budded spheroids from these cell lines at 20× magnification. Scale bars: top and middle panels, 20 μm; bottom panel, 50 μm.

remained sensitive to Cisplatin (Frankel et al., 1997; Kobayashi et al., 1993). We tested the drug sensitivity of A2780 and HEY spheroids as compared to monolayers, to determine if spheroid formation in the absence of serum conferred drug resistance. We were able to compare spheroids to monolayers in identical media conditions by first seeding monolayers in media containing serum to promote attachment to the dish, and then replacing the media with spheroid media. Likewise, spheroids formed by budding in serum-containing media were transferred to spheroid media prior to drug treatments.

We tested sensitivity to Paclitaxel and Cisplatin using cells grown in serum free media for at least 24 hours prior to drug treatment. Cells were exposed to either drug for 24 hours, and then trypsinized, stained with Trypan blue, and counted to determine cell viability. As expected, cells grown in monolayers were sensitive to both drugs, with approximately 30% of cells viable compared to vehicle-treated samples (Fig. 3).

Interestingly, the first generation of A2780 and HEY cells grown as spheroids were also sensitive to Paclitaxel and Cisplatin, with approximately 30% of cells viable compared to vehicle-treated controls (Fig. 3). This was seen both for budded spheroids and for spheroids formed by aggregation. Thus, initial spheroid growth did *not* confer drug resistance to either of these cell lines. This was true despite significantly slower cell growth within spheroids as compared to monolayers (the doubling time for monolayer cells in spheroid media was ~24 hours, versus a doubling time for spheroid cells in the same media of ~48 hours). This result implies that neither the spheroid microenvironment nor the slower proliferation rate it conferred was sufficient to produce resistance to these drugs.

In a separate study, we found that serial passage of the breast cancer cell line MCF-7 as spheroids caused a wave of epigenetic changes associated with EMT, which included acquisition of drug resistance (Guttilla et al., 2012). These changes increased with each successive generation of spheroid passage. To test whether serial passage of ovarian cancer cell spheroids could promote drug resistance, we cultured A2780 and HEY cells for several generations of spheroid growth. Rather than trypsinizing spheroids to create the subsequent generation, we allowed them to dissociate spontaneously by transferring them to a culture dish with media containing FBS, in which they spread as a monolayer. Once the monolayer had adhered, we trypsinized the cells, transferred them to serum-free media, and allowed them to form spheroids by aggregation. We repeated this for three cycles, to produce first, second and third generation monolayers and spheroids. This method of serial passaging might approximate an *in vivo* situation in which spheroids must undergo at least partial spontaneous dissociation to implant on serosal surfaces. In all cases, drug testing was done on samples processed in parallel that had not previously been exposed to any drugs.

Compared to first generation spheroids, spheroids from the second and third generation showed increasing drug resistance to both Paclitaxel and Cisplatin. For A2780 cells treated with Paclitaxel, cell viability increased from 37% in first generation spheroids, to 87% in second generation spheroids, to 91% in third generation spheroids (Fig. 3). For A2780 cells treated with Cisplatin, viability increased from 32% in first generation spheroids, to 84% for second generation spheroids, to 91% in third generation spheroids.

HEY cells showed a similar progressive increase in drug resistance. For HEY cells treated with Paclitaxel, viability increased from 37% for first generation spheroids, to 78% for second generation spheroids, to 90% for third generation spheroids. For HEY cells treated with Cisplatin, viability increased from 29% for first generation spheroids, to 82% for second generation spheroids, to 86% for third generation spheroids.

These data suggest that serial spheroid culture induces progressive drug resistance with each cycle alternating between monolayer and spheroid culture. Interestingly, whereas previous reports of multicellular drug resistance showed an association with spheroid compaction, with these two ovarian cancer cell

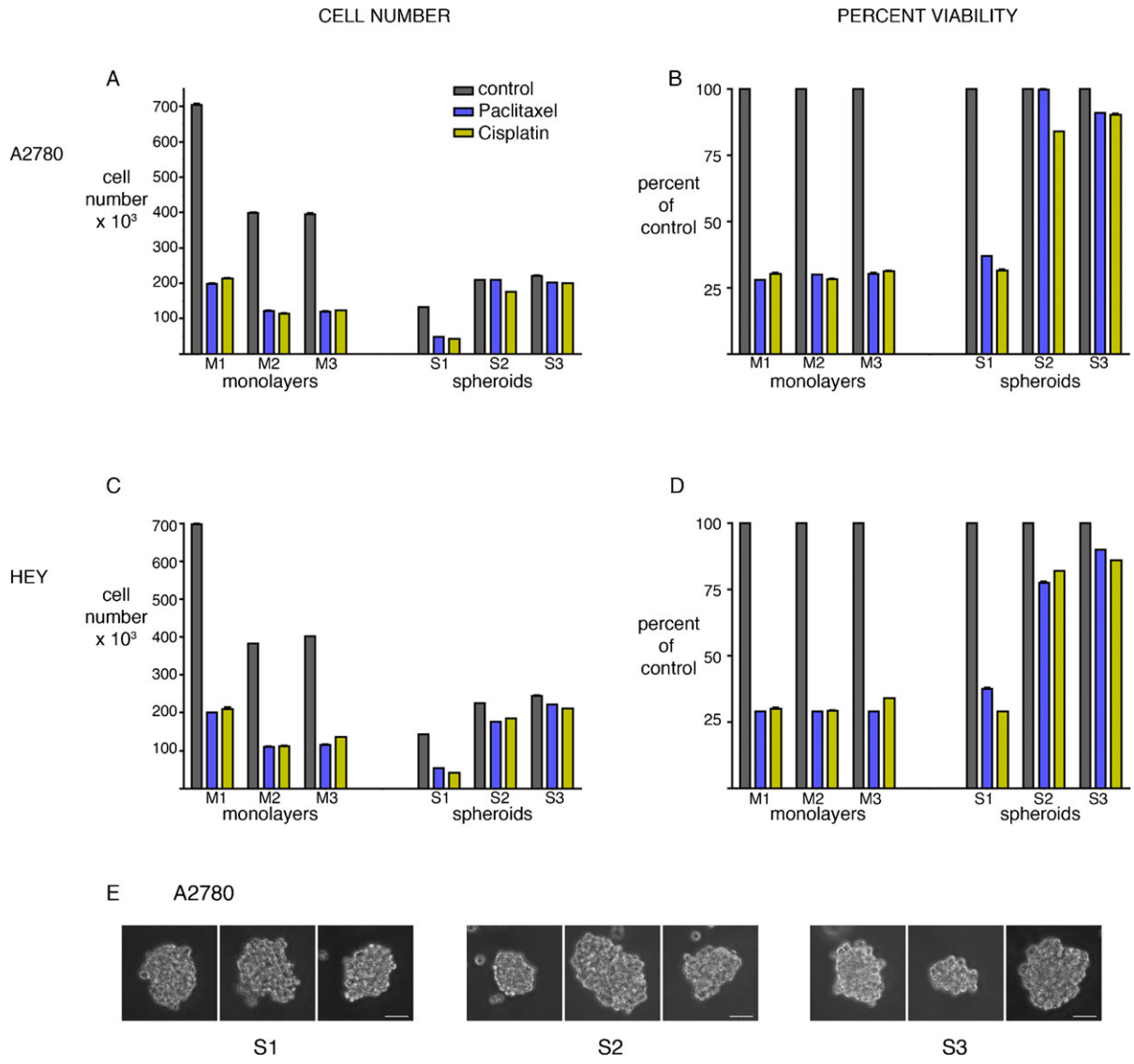

Fig. 3. Spheroid culture induces progressive, reversible resistance to Paclitaxel and Cisplatin that is not due to an increase in spheroid size. A2780 or HEY cells were propagated serially by alternating between monolayer and spheroid culture to produce three generations of monolayers (designated M1, 2, and 3) and three generations of spheroids (designated S1, 2, and 3). Each generation was exposed to the indicated drugs at 7 μM in serum-free media for 24 hours, followed by Trypan blue staining and counting of viable cells. All cells were grown in serum-free media for 24 hours before drug treatment, and no cells had been exposed to drug prior to testing their sensitivity. (**A**,**B**) Cell number and percent viability of A2780 cells treated with vehicle (grey bars), Paclitaxel (blue bars) or Cisplatin (green bars). (**C**,**D**) Cell number and percent viability of HEY cells treated with vehicle (grey bars), Paclitaxel (blue bars) or Cisplatin (green bars). The same data are presented as cell number (A,C) and as percent viability (percent of control, B,D). Error bars are present for each set and show the mean and SEM of three independent repeats. The monolayers are grouped together and spheroids are grouped together, but they were generated in the order M1→S1→M2→S2→M3→S3. (**E**) A change in spheroid size or compaction was not responsible for the progressive drug resistance seen for either cell line. Representative A2780 cell first, second, and third generation spheroids (S1, S2, S3) are shown; similar results were seen for HEY cells. Scale bars: 50 μm.

lines, subsequent generations of spheroids showed similar or slightly reduced compaction, similar to MCF-7 cells grown serially as spheroids (Guttilla et al., 2012; Kobayashi et al., 1993; St. Croix et al., 1996).

Dissociation of ovarian cancer spheroids restores sensitivity to cytotoxic drugs

Spheroid drug resistance could be due to reversible or irreversible mechanisms. Since our method of serial spheroid passaging alternated between spheroid and monolayer culture, we were able to test the drug sensitivity of the intervening monolayer for each spheroid generation. As with serial spheroid generations, this was done in parallel samples that had not previously been treated with any drugs. Media conditions and initial cell numbers were kept constant.

Remarkably, dissociation of spheroids into monolayers completely restored drug sensitivity for every generation. A2780 monolayers treated with Paclitaxel showed 28%, 31%, and 30% viability for first, second, and third generation monolayers, respectively, and these monolayers treated with Cisplatin showed 30%, 29%, and 31% viability, respectively. For HEY cells, monolayers treated with Paclitaxel showed 29% viability for all three generations, and monolayers treated with Cisplatin showed 30%, 29%, and 34% viability for first, second

and third generation monolayers. Thus, the drug resistance induced by serial passaging between monolayer and spheroid culture was completely reversible, despite the generation of resistance during successive spheroid generations.

Discussion

Implications of spheroid budding from a monolayer

We describe here a novel means of spheroid formation – budding from a monolayer of adherent cells – that required minimal growth factor support and could continue for many cycles of budding and dissociation. Our observation raises the possibility that this means of spheroid formation could contribute to ovarian cancer dissemination in patients. Spheroids could form by budding from the primary ovarian tumor, and these budded spheroids could dissociate and float across the peritoneum. This could represent an additional means of spheroid formation for cancers that can form spheroids by aggregation of single tumor cells, and it could represent an alternate mechanism of spheroid formation for tumor cells that lack the ability to form aggregated spheroids.

Budding was associated with vertical growth, continued cell-cell interactions, and eventual release of the budded spheroid from the monolayer. Since cell lines capable of budding lacked obvious cortical E-cadherin, cellular cohesion within the bud is likely to be mediated by other cadherin- or integrin-based cell-cell interactions such as through N-cadherin.

At this point, we do not know the detailed mechanism of the budding process. Because it correlated with a lack of cortical E-cadherin and with the presence of abundant vimentin, and both of these findings were uniform across monolayers, we believe the ability to form budded spheroids could represent a global property of the cancer cell line rather than a biological change limited to distinct sites within the monolayer. If this hypothesis is correct, it implies that the actual site of budding is likely to be random within the monolayer.

The final step in budding is release of the spheroid from the monolayer. This might be due to selective cell death, although we were unable to observe an increase in dying cells near the spheroid base. Alternatively, spheroids could detach from the underlying monolayer through microenvironmental signals as they enhance their attachments within the spheroid. Studies of the biology of spheroid budding will be useful for understanding its relationship to loss of contact inhibition and the creation of a spheroid microenvironment.

Connection between spheroid budding and EMT

EMT is a phenomenon typically associated with epithelial cells, for which the normal physiologic state is one of abundant cortical E-cadherin expression and lack of vimentin. In contrast, normal ovarian surface epithelium is mesodermally-derived and expresses high levels of vimentin without cortical E-cadherin (Ahmed et al., 2007; Auersperg et al., 2001). The relatively unique ability of the ovarian surface epithelium to undergo EMT-like behavioural changes such as cell migration in response to ovulation is thought to represent a homeostatic mechanism for maintaining a continuous, intact epithelial layer (Ahmed et al., 2010).

During tumorigenesis, ovarian cancer cells can adopt an aberrant mullerian differentiation pattern, in which they gain expression of cortical E-cadherin and lose expression of vimentin (Ahmed et al., 2010; Auersperg et al., 2001; Hudson et al., 2008). At later stages of the disease that are associated with greater dissemination, ovarian cancers lose this pattern and again become cortical E-cadherin negative and vimentin positive (Ahmed et al., 2010; Auersperg et al., 2001; Hudson et al., 2008). Ovarian cancers may thus show a high degree of phenotypic plasticity, as described for normal ovarian surface epithelium, and the concept of EMT in this tumor type may be more complex than in others (Ahmed et al., 2007). We hypothesize that a lack of cortical E-cadherin and the presence of vimentin alone is insufficient for spheroid budding, because immortalized ovarian surface epithelium (IOSE) cells with this pattern did not form budded spheroids (data not shown). However, this EMT-like pattern in malignant ovarian cancer cells correlated with the ability to form budded spheroids, suggesting it might contribute to the budding process or be a marker for the ability to form budded spheroids.

Reversible drug resistance in ovarian cancer spheroids

Despite their slower growth, spheroids formed by budding or aggregation initially showed sensitivity to cytotoxic drugs that was equivalent to monolayers. This was followed by progressive acquisition of drug resistance during several cycles of spheroid passaging. This acquired drug resistance was not due to prior drug exposure, because each generation of spheroids had not been exposed to any drugs prior to testing their drug sensitivity. Therefore, it must have been induced by the sequential culture protocol.

The mechanism of progressive drug resistance upon serial spheroid passaging remains unexplained. Breast cancer cells cultured serially as spheroids underwent an EMT with reduced expression of E-cadherin and gain of vimentin expression (Guttilla et al., 2012). However, the ovarian cancer cells we tested express abundant vimentin and minimal E-cadherin at baseline, consistent with these EMT-associated properties prior to the acquisition of drug resistance. Thus, a traditional EMT cannot fully account for the acquired drug resistance. It is possible that these cancer cell lines exist in a state of partial EMT that is enhanced upon further cycles of spheroid culture.

The resistance of A2780 cells to Paclitaxel upon multicellular spheroid growth was demonstrated by Kerbel's group 15 years ago (Frankel et al., 1997). Interestingly, that study also tested Cisplatin treatment and found that resistance upon spheroid culture did not extend to this agent (Frankel et al., 1997). Our finding of Cisplatin resistance could be due to the method of spheroid growth (serum free growth with added growth factors instead of liquid overlay in the presence of serum). Further, our study did not demonstrate drug resistance with the first spheroid generation, while Frankel et al. observed resistance in *de novo* spheroids. Finally, our finding of resistance to two drugs with distinct mechanisms of action (microtubule stabilization versus DNA crosslinking) suggests the resistance is unlikely to be specific to the drug mechanism, but might arise from alterations in drug transport or the cell death machinery.

What additional differences between first, second, and third generation spheroids could confer drug resistance? Genetic changes are unlikely, because these would not be rapidly acquired, reversible, or reproducible in two different cell lines. We also do not think the reduced growth rate of spheroids is responsible, because this reduced growth rate was seen in the first generation of spheroids, which were not drug resistant. Spheroid size and compaction also did not change appreciably from one spheroid generation to the next (Fig. 3E), arguing against a

purely physical change being responsible for progressive drug resistance. A further experiment to test whether spheroid size could account for differing drug sensitivity by size-selecting spheroids through a 40 μm filter did not show greater drug resistance in larger spheroids (data not shown).

Epigenetic changes are a possible explanation for increasing drug resistance upon serial spheroid culture, but these would have to be rapidly reversible or be counteracted by monolayer growth. Altered signalling pathways that block cell death might be engaged upon loss of integrin contacts with the extracellular matrix and restored upon reestablishment of these contacts. However, this would not explain the progressive increase in drug resistance from one spheroid generation to the next. The explanation for the progressive drug resistance might therefore include a combination of epigenetic and signalling changes, possibly with some EMT characteristics. Studies to test the mechanism of drug resistance are ongoing.

Regardless of the drug resistance mechanism, our study demonstrates that drug sensitivity can be restored to ovarian cancer cells, even after multiple rounds of spheroid formation, by simply dissociating the cells. This is true for two independent ovarian cancer cell lines and for two drugs with different mechanisms of action. This is consistent with findings by other groups that show dissociation of spheroids from other cancer types reverses drug resistance, and suggests that strategies to dissociate spheroids *in vivo* could be effective in increasing the efficacy of cytotoxic drugs (Frankel et al., 1997; Green et al., 2004; Green et al., 2002; Kobayashi et al., 1993). Ovarian cancer spheroids might be especially amenable to dissociation since they are confined to the peritoneal space.

Strategies to dissociate ovarian cancer spheroids *in vivo* could include calcium chelators that disrupt cell-cell contacts. Remarkably, intraperitoneal chelators such as EGTA have been well tolerated in animals at reasonably high doses (Llobet et al., 1991; Llobet et al., 1990). To date, we are not aware of the use of antiadhesive agents as a means to chemosensitize tumors in human trials, although anti-N-cadherin antibodies were given as a single agent (without concomitant cytotoxic drugs) and were well tolerated (Perotti et al., 2009). For ovarian tumors, many of which do not express E-cadherin, these therapies would need to target other cadherins and possibly integrin family members. The intraperitoneal dissemination of ovarian cancer makes this tumor a particularly attractive candidate for testing anti-adhesive approaches.

Materials and Methods

Cell lines

A2780 cells were purchased from European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK) and grown in RPMI 1640 with 2mM Glutamine and 10% fetal bovine serum (FBS, not heat inactivated). OVC433 and OVCA420 cells (gift from Dr. Laurie Hudson, University of New Mexico Health Sciences Center, Albuquerque, NM) were grown in MEME plus 0.5% penicillin/streptomycin, 1% Glutamine, 1% Sodium Pyruvate, and 10% FBS. BG1 cells (gift from Matthew Burow, Tulane University School of Medicine, New Orleans, LA) were grown in DMEM plus 10% FBS. SKOV3 cells (gift of Zou Changpeng, University of CT Health Center, Farmington, CT) were grown in DMEM-F12 plus 10% FBS. HEY cells (gift from Dr. Robert Bast, MD Anderson Cancer Center, Houston, Texas) were grown in DMEM-F12 plus 10% FBS. All cells were kept at 37°C in a 5% CO_2 incubator.

Spheroid culture

Spheroid formation

Aggregated spheroids were formed by culturing cells in serum-free media on low adhesion tissue culture plates in one of two commercial media preparations: Mammocult Mammosphere media supplemented with a proprietary cocktail plus heparin and hydrocortisone (Stem Cell Technologies, Vancouver, BC, Canada), or Mammary Epithelium Spheroid Media supplemented with hydrocortisone, bovine pituitary extract, epidermal growth factor (rhEGF), insulin, and antibiotics (Lonza Walkersville Inc., Walkersville, MD).

Budding spheroids were formed by culturing cells in their regular growth media on adherent plates and allowing spheroids to form spontaneously. HEY cells were cultured as a monolayer in their regular growth media followed by growth in Mammocult media (Lonza) for budding.

Spheroid dissociation

We tested several methods of dissociating spheroids: trypsinization, calcium chelation with EGTA, mechanical trituration, and allowing spheroids to dissociate spontaneously by plating them in serum-containing media or plating them in spheroid media on plates pre-coated with 2% Matrigel (growth factor reduced, phenol red free; BD Biosciences, Bedford, MA). Spontaneous dissociation was associated with the least cell death, and resulting monolayers showed equal ability as standard monolayers to produce new budding spheroids.

Immunofluorescence

Cells were grown on glass coverslips to near-confluence. E-cadherin was detected after fixing cells in 4% formaldehyde, using a rat anti-E-cadherin antibody from Invitrogen (Camarillo, CA) (dilution 1:80) and Oregon-green goat anti-rat secondary antibody from Molecular Probes (Eugene, OR). Vimentin was detected after fixing cells in ice-cold methanol for 5 minutes, using mouse anti-Vimentin antibody Ab-2 (Clone V9) from Thermo Scientific (Fremont, CA) (dilution 1:100) and Alexa-568 anti-mouse secondary antibody (Molecular Probes). DNA was stained with Hoechst stain at 10 μg/ml. Coverslips were mounted using 0.5% p-phenylenediamine in 20 mM Tris 8.8 and 90% glycerol.

Microscopy

Budded spheroids could only be imaged live, as fixation caused these spheroids to detach from the monolayer. Phase contrast imaging was done using a 4× or 20× objective on a Nikon inverted microscope (TE2000-U, Nikon Instruments, Melville, NY) equipped with an ORCA AG CCD camera (Hamamatsu Photonics, Bridgewater, NJ) and controlled by MetaMorph software (Molecular Devices Corp, Sunnyvale, CA).

Fluorescence imaging of budded spheroids was done on cells grown on glass coverslips. Cell nuclei were stained with DRAQ5 (Biostatus, Leicestershire, UK) at 5 μM for 20 minutes prior to imaging. Cells were imaged live using a 40× 1.2 NA c-apochromat water immersion lens on a Carl Zeiss, Inc. LSM510 laser scanning confocal microscope equipped with a MetaDetector spectral detector (Thornwood, NY). Optical sections were collected at 0.5 μm steps.

Immunofluorescence was imaged using a 60× objective on a Nikon TE2000-U microscope with a spinning disk confocal head (Perkin Elmer; Wellesley, MA) controlled by MetaMorph software.

Drug treatments

Paclitaxel (Taxol, Sigma Aldrich, St Louis, MO) was reconstituted as a 10 mM stock solution in DMSO. Cis-Diammineplatinum (II) dichloride (Cisplatin, Sigma Aldrich) was reconstituted as a 5 mM stock solution in phosphate buffered saline (PBS).

Spheroids and monolayers were plated at 1×10^3 cells/well in 24 well low adhesion plates in regular growth media containing serum (monolayers) or Lonza spheroid media (spheroids). Monolayer 2 was produced from the spontaneous dissociation of Spheroid 1 onto a culture dish in media containing FBS; Spheroid 2 was produced from the natural budding of Monolayer 2 or the aggregation of Monolayer 2 cells; Monolayer 3 was produced from the natural dissociation of Spheroid 2; and Spheroid 3 was produced from the natural budding or aggregation of Monolayer 3.

To eliminate effects of serum on drug bioavailability and cell proliferation, media for all spheroids and monolayers was exchanged for Lonza spheroid media after 24 hours of growth, and cells were cultured for an additional 24 hours in this media before drug treatment. Monolayers or spheroids were treated with Paclitaxel or Cisplatin at a final concentration of 7 μM for 24 hours. These treatments were done on samples processed in parallel, so no drug treatment was done on cells that had previously been exposed to any drug. Viability was determined by trypsinizing cells, staining with Trypan blue, counting, and calculating total numbers of viable and dead cells, in triplicate, in at least two independent experiments for each condition. Drug sensitivity for budded spheroids and spheroids formed by aggregation in serum free media were found to be the same; thus, most drug experiments were done using aggregated spheroids since we could generate greater numbers of these more quickly. Graphs of drug resistance were made using Prism (GraphPad Software, La Jolla, CA).

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Competing Interests

The authors declare that there are no competing interests.

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