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Construction and characteristics of an E-cadherin-related three-dimensional suspension growth model of ovarian cancer

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Ovarian cancer is the deadliest of all gynecologic malignancies. Metastatic ovarian cancer cells exist mainly in the form of multi-cellular spheroids (MCSs) in the ascites of patients with advanced ovarian cancer. We hypothesized that E-cadherin, as an important cell-adhesion molecule, might play an important role in the formation and survival of MCSs. Therefore, we established a three-dimensional suspension culture model of ovarian cancer cells that express high levels of E-cadherin to investigate their growth, proliferation, and resistance to chemotherapeutic drugs by CCK-8 assays. Compared to the cell suspension masses formed by cells with low or absent E-cadherin expression, the MCSs of high E-cadherin SKOV-3 cells had larger volumes, tighter cellular connections, and longer survival times. Although the suspension cell masses of all three cell lines were proliferatively stagnant, possibly due to cell cycle arrest at G1/S, cell mortality at 72 h after cisplatin treatment was significantly decreased in the high E-cadherin SKOV-3 cells compared to SKOV-3 cells without E-cadherin expression and to OVCAR-3 cells with low E-cadherin expression. We conclude, therefore, E-cadherin plays a vital role in MCS formation, maintenance, and drug resistance in ovarian cancer and could be a potential target for late-stage ovarian cancer treatment.

varian cancer is one of the most common cancers in women and is the deadliest of all malignant gynecological tumors^{1,2}. Due to the absence of symptoms in early ovarian cancer, most patients are diagnosed at a late stage with extensive abdominal metastasis³. In late-stage cancer, the development of refractory ascites will not only aggravate the patient's pain, but also provide a suitable environment for the survival and transfer of the metastatic cancer cells leading to the poor prognosis of advanced ovarian cancer^{4,5}. Ovarian cancer cells exist in the ovary as single cells or as a spherical multi-cell aggregated mass known as a multicell spheroid (MCS) in ascites. Increasing evidence has shown that the formation of MCSs is necessary for ovarian cancer cells to survive and metastasize after shedding from original tumor lesions⁶. Kristy found that suspended ovarian cancer cell masses cultured in an appropriate media could survive more than 10 days and expand in volume, but suspended normal ovarian cells could survive only up to 2 days7. Suspension MCSs behave in a similar manner to tumor cell masses in vivo. This property overcomes the limitations of using adherent monolayer cells in research because such monolayers have different biological behaviors, including differences in drug resistance, and are poor representations of *in vivo* tumor cells⁸⁻¹¹. Therefore, it is of great clinical relevance to establish a stable suspension MCS model of ovarian cancer cells because this will enable us to properly study the characteristics of tumor cells in the ascites of late-stage ovarian cancer, especially in terms of resistance to chemotherapy drugs.

MCSs allow for the anchorage-independent growth of tumor cells, and the maintenance and function of MCSs in suspension depends to large extent on intracellular adhesion molecules¹². Kin suggested that members of the cadherin family play an important role in the formation of MCS suspensions¹³. Shane demonstrated that tight junctions among HT29 colon tumor cells in MCS suspensions desensitized the cells to cytotoxic drugs and that disruption of E-cadherin–mediated cell-cell adhesion could restore the sensitivity to chemotherapeutics¹⁴. E-cadherin, as an intercellular adhesion molecule, was initially believed to be a tumor suppressor^{15–19}. However, recent research has revealed that E-cadherin plays a more complicated role than just inhibiting the metastasis of



Figure 1 | Differences of E-cadherin expression and morphology among three ovarian cancer cell lines. SK-H, SKOV-3 cells with high expression of E-cadherin; OV-L, OVCAR-3 cells with low expression of E-cadherin; SK-N, SKOV-3 cells with no expression of E-cadherin. The scale bar is 200 μ m. The gels have been cropped to show the specific bands.

tumor cells²⁰. In breast cancer, the down-regulation or loss of Ecadherin indicates tumor aggressiveness and poor prognosis, but the expression of E-cadherin is necessary for the adhesion and aggregation of cells in MCS suspensions²¹. It is noteworthy that Ecadherin might play different roles in ovarian cancer compared to other types of cancers. For instance, in normal ovarian surface epithelium (OSE), E-cadherin over-expression is found only in the OSE located in the deep clefts, invaginations, and inclusion cysts that are prone to cancerization^{22,23}. OSE exhibits remarkable phenotypic plasticity that displays both epithelial and mesenchymal characteristics and undergoes mesenchymal to epithelial transition (MET) with elevated expression of E-cadherin and other epithelial markers during transformation^{24,25}. Stable expression of E-cadherin was also found in advanced ovarian cancer and its metastases^{22,26}. The E-cadherin expression level is significantly higher in ovarian cancer cells than in normal ovarian epithelial cells, and it activates the PI3K/AKT and MEK/ERK signaling pathways by mediating cell-cell adhesion²⁷⁻³⁰. This promotes the growth and proliferation of ovarian cancer cells indicating a possibly unique and critical function of E-cadherin in ovarian cancer cells in MCS suspensions. Nevertheless, the roles and mechanisms of E-cadherin in ovarian cancer cells remain uncertain³¹.

In this study, we compared the growth and proliferation in threedimensional suspension cultures of three types of ovarian cancer cells with different levels of E-cadherin expression. We established an MCS model using cells with high E-cadherin expression, and this allowed us to analyze the role of E-cadherin in the formation, maintenance, and drug-resistance of ovarian cancer MCSs.

Results

Differences in E-cadherin expression level and cell morphology among three kinds of ovarian cancer cells. Both western blot analysis (Figure 1A and 1B) and immunofluorescence experiments (Figure 1D) confirmed that the E-cadherin expression level was high in SK-H cells (SKOV-3 cells expressing high levels of E-cadherin), low in OV-L cells (OVCAR-3 cells expressing low levels of E-cadherin), and absent in SK-N cells (SKOV-3 cells expressing no E-cadherin). Figure 1C shows the differences in cell morphology among the three cell lines with different levels of E-cadherin expression. SK-H cells were larger, more spherical, and grew in aggregations of cells. OV-L cells were small, polygonal, and evenly distributed. SK-N cells were also small but were spindle-shaped. This suggests that E-cadherin might play a role in the maintenance of cell morphology and growth distribution.

Establishment of the suspension MCS model of ovarian cancer cells. Table 1 shows the growth characteristics of the three ovarian cancer cell lines with different E-cadherin expression levels. Both the suspension SK-N cell mass (SK-N-M) and OV-L cell mass (OV-L-M) showed small volumes, short lifetimes, and loose intercellular connections with unstable and incomplete sphere formation. SK-H cells formed complete spheres with larger volumes, had tighter intercellular connections, and had longer lifetimes of almost a month. Because the suspension SK-H cell mass (SK-H-M) showed the best suspension effect, this was chosen to establish the suspension MCS growth model to mimic and study the biological characteristics of ovarian cancer cells that float, survive, and metastasize in ascites.

Based on the formation, maintenance, and dissociation of the SK-H-M, we divided the entire development process into the following five stages (Figure 2 and Figure 3):

A. Small cell mass stage (Day 1). Individual ovarian cancer cells in suspension approach each other and begin to aggregate. Some scattered small cell masses can be seen.

Tuble 1 Characteristics of suspended cent masses of three ovariant cancer cent lines with different reversion account of the pression					
Cell lines	Mean lifetime	Maximum size (mm)	Morphology	Cell-cell connection	
SK-H-M	Longest, 30 days	4.38×2.86	Standard spheroid	Tight	
SK-N-M OV-L-M	Shortest, 13 days Medium, 22 days	4.49 × 2.31 3.53 × 1.96	Irregular Irregular	Loose Loose	

- Table 1 | Characteristics of suspended cell masses of three ovarian cancer cell lines with different levels of E-cadherin expression
- B. Medium cell mass stage (Day 1–2). The number of small cell masses increases and they begin to gather into medium-sized cell masses.
- C. MCS formation stage (Day 2–3). The cell masses further aggregate into large and intact MCSs with tight intercellular connections.
- D. MCS maintenance stage (Day 3–30). The MCSs are maintained with a stable morphology for a relatively long time.
- E. MCS dispersion stage (Day 30–32). Intercellular connections gradually become weaker and the MCSs disperse into small cell masses or individual cells. (Figure 3).

E-cadherin promotes the formation of suspended ovarian cancer MCSs. To further investigate the function of E-cadherin in suspended ovarian cancer MCSs, we used the calcium chelator EDTA to conduct a separation experiment on the SK-H-M at 72 h after placing the cells in suspension (stage D in the scheme shown above). Trypsin-treated cells were used as the control group. EDTA chelates calcium ions and thus blocks the function of E-cadherin, which is calcium-dependent. As shown in Figure 4, ten minutes after the addition of 4 mmol/L EDTA the intercellular connections between the suspended MCS became loose and the large cell mass fell apart into smaller masses. For the next 20 minutes the cell masses gradually became smaller. The EDTA-treated cells were finally digested for 10 minutes with trypsin and this resulted in the small cell masses dissociating into individual cells or into mini cell masses containing only a few cells (Fig. 4A-4C). In the control group, the MCSs digested with trypsin for 10 minutes remained as large cell masses (Fig. 4D), and this demonstrated a far weaker effect of trypsin compared to EDTA. After 30 minutes of trypsin digestion, the intercellular connections became loose but the cell mass remained large (Fig. 4E). EDTA was then added to these cell masses and after 30 minutes the cell masses had dispersed into small cell masses or individual cells (Fig. 4F).

Suspended ovarian cancer cells remain in a proliferationquiescent state due to G1/S arrest. To study the growth and proliferation state of suspended ovarian cancer cells and the possible relationship of this state with E-cadherin, we cultured the cells with different levels of E-cadherin expression in suspension or as adherent monolayers. We used the CCK-8 kit to detect cell proliferation activity at 24 h, 48 h, and 72 h and also studied their cell cycles by fluorescence-activated cell sorting (FACS) flow cytometry. The results showed that after 72 h of culture the proliferation activities of all three suspended cell masses were significantly lower than their respective controls grown as adherent cells (P < 0.05). The optical density readings of SK-H-M at three different time points showed no obvious difference (P > 0.05) nor did the readings of SK-N-M or OV-L-M cells (P > 0.05). In addition, no significant difference in proliferation activity was observed among these three cell lines in suspension at any of the three time points (P > 0.05 for all). Thus we inferred that ovarian cancer cells in suspension exist in a proliferation-quiescent state and that this is not correlated with the E-cadherin expression level. (Figure 5).

From the cell cycle analysis, an obvious G1/S arrest was observed in suspended cells of all the three cell lines compared to the cells in adhesive growth despite different expression levels of E-cadherin. In suspended cells of all three ovarian cancer cell lines, the percentage of cells in G1 phase increased significantly and the percentage of cells in S phase decreased significantly in contrast to their respective adhesive phenotypes (P < 0.05 for all). However, there were no obvious differences in the proportions of cells at different stages of the cell cycles among the three cells lines studied, in either suspension or adhesion and regardless of the expression level of E-cadherin (P > 0.05 for both) (Figure 6).

Positive correlation between drug resistance and E-cadherin expression level in suspended ovarian cancer cells. To study the drug resistance of suspended cells and its correlation with E-cadherin expression, we treated suspended and adherent cultures of the



Figure 2 | Staging of multi-cellular spheroid (MCS) formation in three ovarian cancer cell lines with different E-cadherin expression levels. Stage A) Small cell mass stage. Stage B) Medium cell mass stage. Stage C) MCS formation stage. Stage D) MCS maintenance stage. Stage E) MCS dispersion stage. The scale bar is 2.0 mm.



Figure 3 | Changes in the characteristics of the MCS dispersion process in SKOV-3 cells with high E-cadherin expression. The scale bar is 500 μ m.

three cell lines with cisplatin (5 mg/mL), a common chemotherapeutic drug. CCK8 assays were conducted to determine the cell survival at 24 h, 48 h, and 72 h after cisplatin treatment. As shown in Table 2, the mortality rates of the suspended cells were all significantly lower than the respective adherent cells for all three cell lines after 72 h of cisplatin treatment (P < 0.05) indicating a higher drug resistance to cisplatin in suspended cells than in adherent cells. In addition, the mortality of SK-H cells was significantly lower than SK-N cells and OV-L cells in both the suspended and adherent cultures respectively after 72 h of cisplatin treatment (P < 0.05 for both). This result suggests that E-cadherin plays an important role in drug resistance in both suspended cell masses and adherent ovarian cancer cells (Figure 7).

Discussion

The metastasis of ovarian cancer cells occurs primarily in the abdominal cavity and appears as regional infiltration, and this is different from the systemic metastasis through the vasculature seen in other advanced tumors. It has also been demonstrated that the invasion and metastasis of ovarian cancer cells in the abdominal cavity is usually accompanied by the generation of massive refractory ascites that assist cancer cell migration during peritoneal metastasis, especially in late-stage and high-grade serous carcinoma³².

In order to study the biological characteristics of ovarian cancer cells in ascites during metastasis, we established a three-dimensional suspension MCS model to mimic the state of ovarian cancer cells in ascites and investigated behaviors such as growth, proliferation, and drug resistance. We found that SKOV-3 cells with high E-cadherin expression had tighter cell-cell contacts, intact MCS formation, and longer lifetimes compared to ovarian cancer cells with low or absent expression of E-cadherin. Upon blocking the function of E-cadherin



Figure 4 | MCS separation test in SKOV-3 cells with high E-cadherin expression. Seventy-two hours after SK-H suspension culture – when the MCSs were stable – the medium of the experimental group was changed to serum-free medium containing the following additives. A) The SK-H-M treated with 4 mmol/L EDTA for 10 minutes. B) The SK-H-M treated with 4 mmol/L EDTA continuously for 30 minutes. C) The SK-H-M treated with trypsin digestion for 10 minutes without EDTA after the EDTA treatment in (B). D) The SK-H-M digested with trypsin without EDTA for 10 minutes. E) The SK-H-M digested with trypsin for 30 minutes. F) The SK-H-M treated with EDTA for 30 minutes after treatment with trypsin in (E).

with the calcium chelator EDTA, the suspended MCSs gradually dissociated and lost volume as the cells separated and escaped from the MCS. This result is consistent with previous studies in which Hayward found that LIM1863 colon cancer cells could only form as single-cell suspensions instead of MCSs in a low-calcium culture medium indicating that calcium-dependent cell adhesion was indispensable for MCS formation³³. It has also been shown that E-cadherin can mediate the non-anchorage dependent growth of HSC-3 oral squamous carcinoma cells and Ewing sarcoma cells^{34,35}. Moreover, the E-cadherin expression level in MCSs of Ewing sarcoma cells is significantly increased. A number of studies have confirmed that in addition to the expression of E-cadherin on the cell surface, the expression of downstream signaling proteins P27 and P130 might also participate in the formation of MCSs^{36,37}.

We propose that high expression of E-cadherin might be a key factor in the formation and maintenance of suspensions of ovarian cancer MCSs. However, it has been reported that the expression level of E-cadherin in ovarian cancer cells in ascites and metastatic sites is lower than that of cells in the primary tumor sites³⁸ and that the cells with low expression levels of E-cadherin are more invasive and more prone to spread intra-abdominally³². We speculate that E-cadherin expression is likely to be a dynamic process during abdominal ovarian cancer development that includes the formation, maintenance, dissociation, and secondary colonization of cell masses in the ascites. We propose that E-cadherin plays an indispensable role in the MCS formation and maintenance phases during the above process.

In the cisplatin sensitivity test, the cells in suspension showed significantly reduced mortality compared to the corresponding adherent cells after 72 h of cisplatin treatment regardless of whether the E-cadherin expression was high, low, or absent. These data suggest that suspension cultures significantly reduce the sensitivity of cells to cisplatin, which is in line with the results from Kang³⁵ who demonstrated that Ewing sarcoma cells in a three-dimensional state exhibited significantly greater carboplatin resistance than cells in a monolayer state. Previous research showed that adherent monolayer cells had completely different biological characteristics compared to MCSs. In particular, it was shown that paclitaxel can induce G2-M phase arrest and cell apoptosis in adherent monolayer ovarian cancer cells but not in ovarian cancer MCSs^{8,9}.



Figure 5 | Cell proliferation assays of suspended or adherent cultures of ovarian cancer cells with different levels of E-cadherin expression. *: After 72 hours of culture, the proliferation activities of the three suspended masses were significantly lower than their respective adherent cells (P < 0.05). **A**: The optical density value of SK-H-M at the three different time points showed no obvious difference (P > 0.05). Neither did that of SK-N-M or OV-L-M cells (P > 0.05 for both). #: There was no significant difference in proliferation activity at 24 h among the three cell lines (P > 0.05). @: There was no significant difference in proliferation activity at 48 h among the three cell lines (P > 0.05). \$: There was no significant difference in proliferation activity at 72 h among the three cell lines (P > 0.05).



Figure 6 | Cell cycle analysis of suspended or adherent ovarian cancer cells with different levels of E-cadherin expression.

Table 2 Mortalities of suspended or adherent cells with different levels of E-cadherin expression after cisplatin treatment for 72 h					
Cell line Growth state	SK-H	OV-L	SK-N		
Suspension Adherence	$\begin{array}{c} 9.5\% \pm 0.1\% \\ 47.5\% \pm 2.1\% \end{array}$	$\begin{array}{c} 38.5\% \pm 9.2\% \\ 58.0\% \pm 5.3\% \end{array}$	$\begin{array}{c} 34.0\% \pm 1.4\% \\ 60.3\% \pm 2.1\% \end{array}$		

In the present study, the MCSs with high E-cadherin expression had significantly lower mortality after 72 h of cisplatin treatment compared to the MCSs with absent or low expression of E-cadherin, and this suggests the important role of E-cadherin in the chemoresistance of MCSs in suspension. In vitro studies have shown that anti-E-cadherin antibodies can restore the sensitivity of suspended MCSs of HT29 colon cancer cells to the chemotherapy drugs paclitaxel, fluorouracil, and etoposide14. E-cadherin promotes the formation and maintenance of suspension MCSs by mediating the intercellular adhesion among adjacent cells. This enables the MCSs to survive in the ascites by resisting the toxic effects of chemotherapy drugs, which might serve as a prerequisite for ovarian cancer cells to diffuse or relapse into the abdominal cavity. Gary suggested that the formation of suspended MCSs could decrease the drug sensitivity of the tumor cells by preventing the drug from penetrating into the MCS core³⁹. Thus, E-cadherin supports the structure of the MCSs with tight cellular junctions that can prevent the diffusion and infiltration of chemotherapeutic drugs into the MCS. This can be a potential drug-resistance mechanism for suspended MCSs. Notably, E-cadherin also played a role in drug resistance in adherent cells through an unknown mechanism. In suspended cells, this function of E-cadherin might be further strengthened by inducing the formation of MCSs. In addition to the role of E-cadherin, Ncadherin-mediated apoptosis evasion mechanisms⁴⁰ and related drug-resistance gene changes are both likely to contribute to drug resistance in suspended MCSs⁴¹.

Cell proliferation was found to be relatively static at different time points in suspensions of all three cell lines with different levels of Ecadherin expression. This situation is similar to hepatocellular carcinoma cell line BEL7402 whose growth and proliferation were blocked after cell aggregation in a 'synoikis-like' manner resulting in anoikis evasion and fluorouracil resistance⁴². We hypothesize that the relatively quiescent state of MCS proliferation is closely related to the increase in drug resistance. Furthermore, although there are significant differences in the ability of MCSs to form among the three cell lines, no significant differences exist in proliferation among these cells. This finding suggests that E-cadherin might enhance cell adhesion and aggregation rather than promote cell proliferation via certain mechanisms⁴³.

It is well known that tumor cells at different phases of the cell cycle have different sensitivities to chemotherapy drugs, i.e., more active proliferation leads to higher drug sensitivity. Green and his colleagues found that expression of cyclin-dependent kinase inhibitor p27 decreased the sensitivity to cyclophosphamide in HT29 colon cancer cells⁴⁴. Therefore, the stability and lack of proliferation of the suspension cell mass might be essential for its drug resistance. In our research, FACS analysis with propidium iodide staining showed a significant increase in the proportion of cells in the G1-S phase in the suspended cells compared with adhesive monolayer cells. This G1-S arrest phenomenon could account for the quiescent phenotype of cells by blocking DNA replication at the restriction checkpoint. Although some researchers reported that the expression of p130 and retinoblastoma protein (p130/Rb) contributed to the regulation of the cell cycles of MCSs⁴⁵, the mechanism behind the cell cycle arrest of suspended cultures remains elusive and needs further research. The role of E-cadherin in this process is still unclear. We



Figure 7 | Drug resistance to cisplatin in suspended or adherent ovarian cancer cells with different levels of E-cadherin expression. A: After 72 h of cisplatin treatment, the mortality rates of of all three types of suspended cells were significantly lower than their respective adherent cells (P < 0.05). #: After 72 h of cisplatin treatment, the mortality of suspended SK-H-M cells was significantly lower than SK-N-M cells and OV-L-M cells (P < 0.05). *: After 72 h of cisplatin treatment, the mortality of adherent SK-H-A cells was significantly lower than SK-N-A cells and OV-L-A cells (P < 0.05).

propose that under the regulation of relevant gene expression, suspended ovarian cancer cell masses would pass through G1 phase and enter the S phase of the cell cycle when the surrounding environment becomes appropriate for invasion and re-colonization at a new site to complete the process of peritoneal dissemination. However, the mechanism behind such a process and its relationship with drug resistance remains ambiguous and complicated and further research is required.

In summary, E-cadherin plays a vital role in MCS formation, maintenance, and drug resistance in ovarian cancer and this might account for one of the mechanisms of anoikis resistance, survival, migration, and re-colonization of advanced ovarian cancer cells in the abdomen. Therefore, E-cadherin could be a potential target for preventing metastasis of ovarian cancer cells. Down-regulation of Ecadherin expression might inhibit the formation and maintenance of MCSs and restore the sensitivity of ovarian cancer cells to chemotherapeutic drugs. Such research has the potential to increase chemotherapeutic efficiency and prolong survival in late-stage ovarian cancer patients.

Methods

Cell culture and immunofluorescence staining. SK-H and OV-L cells were purchased from the Cancer Center Lab, Chinese Academy of Medical Sciences. SK-N cells were purchased from the Cell Bank, Chinese Academy of Science. SK-H and SK-N cells were cultured in RPMI-1640 (Gibco, UK) and OV-L cells were cultured in DMEM-F12 (Hyclone, China). All cell lines were maintained in an incubator at 37°C with a relative humidity of 90% and 5% CO₂. Cells were passaged when confluence reached 90%. Immunofluorescence staining was performed as described earlier²⁹.

Three-dimensional suspension culture of ovarian cancer cells. To mimic the suspension condition of ovarian cancer cells in the ascetic fluid, poly 2-hydroxylethyl methacrylate (Poly-HEMA) gel was coated on the bottoms of the wells of a 24-well culture plate to prevent cell adherent growth^{46,47}. A stock solution of Poly-HEMA gel with a final concentration of 120 mg/mL was prepared by mixing 4.8 g Poly-HEMA gel with 40 mL 95% ethanol with stirring at 55°C-65°C until the gel was totally dissolved. The working solution was made by diluting the stock solution with 95% ethanol to 12 mg/mL. A total of 500 µL of the working solution was added to each well of the plate. The plate was air-dried in a laminar flow cabinet and washed with PBS three times before use. A total of 5×10^4 cells per well of each type of ovarian cancer cell were cultured both in the treated plates (the suspension cell group) and in untreated plates as controls (the adhesion cell group). Cell morphology was monitored daily and the subsequent experiments were carried out accordingly.

Calcium depletion experiment. Stable MCSs had formed in the SK-H suspension culture by 72 hours, at which time the medium of the experiment group was changed to serum-free medium with 4 mmol/L EDTA for 10–30 minutes followed by trypsin digestion for 10 minutes without EDTA. For the control group, the MCSs were first digested with trypsin without EDTA for 10–30 minutes and then were treated with

EDTA for 30 minutes. The separation processes of the MCSs in both groups were observed and compared.

Cell proliferation assay. SK-H, SK-N, and OV-L cells were inoculated at 5 \times 10⁴ cells per well in Poly-HEMA gel-coated 96 well plates. Adherent monolayer cells were used as the control group, and three replications were performed for each group. Cell proliferation was measured with the CCK8 cell proliferation assay kit (Bestbio, China) at 24 h, 48 h, and 72 h after inoculation.

Cell cycle analysis. The three kinds of ovarian cancer cells under adherent or suspended culturing were harvested by trypsinization and the calcium-chelating function of EGTA (for suspended cells only) at 72 h after incubation. After washing with pre-cooled PBS, cells were centrifuged at 400 × *g* for 5 min at 4°C and were fixed with 70% pre-cooled ethanol at 4°C overnight. Cells were then centrifuged for 5 min at 1200 rpm, washed with PBS again and incubated for 30 min in PBS containing 10 mg/mL of RNase A at 37°C. After filtering through 400-mesh filter traps, the cells were stained with 5 mg/mL of propidium iodide in darkness for 30 min. The stained cells were measured on a FACSCanto II (BD Biosciences, San Jose, CA). The data were analyzed for cell cycle stage using the ModFit software.

Drug resistance assay. After 60 h of cell culture as described above, the medium of each well was changed to serum-free medium for a 12 h starvation. This was followed by the addition of 5 mg/mL cisplatin to the experimental group and an equivalent volume of pure medium to the control group. Three replications were performed for each group. Cell survival was determined using a CCK8 cell proliferation assay kit (Bestbio, China), and cell mortality was calculated to indicate the drug resistance of the cells.

Western blot analysis. Cells were washed in cold PBS twice before lysis with a mixture of lysis buffer and PMSF (Life, USA) in a 100:1 ratio. The lysate was centrifuged and the supernatant was collected for measuring the total protein concentration with a Bradford protein concentration test kit (Beyotime, China). Five times the volume of loading buffer (Beyotime) was mixed with the lysate and heated for 10 minutes at 99°C. An 8% SDS-PAGE gel was used for electrophoresis with 25 μ g protein in each group. The proteins were transferred to PVDF membranes and the membranes were blocked with 5% skim milk powder for 2 hours followed by overnight incubation with mouse-anti-human E-cadherin monoclonal antibody (1:3000 dilution, BD, USA) or β -actin (1:5000, Abcam Ltd., Cambridge, UK) as the internal control. The membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5000, Pierce, USA). Blots were developed using 1:1 ECL developing solution and visualized with a MiniBIS Pro gel imaging system (DNR, Israel). Quantitative analysis was done with Image J software.

Statistical analysis. All experiments were repeated at least three times. SPSS 17.0 was used for statistical analysis. The expression level of E-cadherin protein and cell proliferation data were tested by one-way ANOVA. Survival rate data were analyzed by Chi-squared test, and P < 0.05 was considered statistically significant. Data were plotted and graphed using GraphPad Prism.

- Naora, H. & Montell, D. J. Ovarian cancer metastasis: integrating insights from disparate model organisms. *Nat. Rev. Cancer* 5, 355–366 (2005).
- Pisani, P., Parkin, D. M., Bray, F. & Ferlay, J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int. J. Cancer* 83, 18–29 (1999).
- Ayantunde, A. A. & Parsons, S. L. Pattern and prognostic factors in patients with malignant ascites: a retrospective study. *Ann. Oncol.* 18, 945–949 (2007).
- 4. Casey, R. C. *et al.* Beta 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids. *Am. J. Pathol.* **159**, 2071–2080 (2001).
- Bast, R. C. Jr, Hennessy, B. & Mills, G. B. The biology of ovarian cancer: new opportunities for translation. *Nat. Rev. Cancer* 9, 415–428 (2009).
- Shield, K., Ackland, M. L., Ahmed, N. & Rice, G. E. Multicellular spheroids in ovarian cancer metastases: Biology and pathology. *Gynecol. Oncol.* 113, 143–148 (2009).
- Shield, K. *et al.* Alpha2beta1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis. *J Carcinog* 6, 11 (2007).
- Paulus, W., Huettner, C. & Tonn, J. C. Collagens, integrins and the mesenchymal drift in glioblastomas: a comparison of biopsy specimens, spheroid and early monolayer cultures. *Int. J. Cancer* 58, 841–846 (1994).
- Frankel, A., Buckman, R. & Kerbel, R. S. Abrogation of taxol-induced G2-M arrest and apoptosis in human ovarian cancer cells grown as multicellular tumor spheroids. *Cancer Res.* 57, 2388–2393 (1997).
- Kim, J. B., Stein, R. & O'Hare, M. J. Three-dimensional in vitro tissue culture models of breast cancer-- a review. *Breast Cancer Res. Treat.* 85, 281–291 (2004).
- Shaw, K. R., Wrobel, C. N. & Brugge, J. S. Use of three-dimensional basement membrane cultures to model oncogene-induced changes in mammary epithelial morphogenesis. J Mammary Gland Biol Neoplasia 9, 297–310 (2004).
- Waleh, N. S. et al. Selective down-regulation of integrin receptors in spheroids of squamous cell carcinoma. *Cancer Res.* 54, 838–843 (1994).
- Kim, Y. J. et al. Modulating the strength of cadherin adhesion: evidence for a novel adhesion complex. J. Cell. Sci. 118, 3883–3894 (2005).

- Green, S. K., Francia, G., Isidoro, C. & Kerbel, R. S. Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy in vitro. *Mol. Cancer Ther.* 3, 149–159 (2004).
- Vleminckx, K., Vakaet, L. Jr, Mareel, M., Fiers, W. & van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 66, 107–119 (1991).
- Cavallaro, U. & Christofori, G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat. Rev. Cancer* 4, 118–132 (2004).
- Mittari, E., Charalabopoulos, A., Batistatou, A. & Charalabopoulos, K. The role of E-cadherin/catenin complex in laryngeal cancer. *Exp. Oncol.* 27, 257–261 (2005).
- Birchmeier, W. E-cadherin as a tumor (invasion) suppressor gene. *Bioessays* 17, 97–99 (1995).
- 19. Risinger, J. I., Berchuck, A., Kohler, M. F. & Boyd, J. Mutations of the E-cadherin gene in human gynecologic cancers. *Nat. Genet.* 7, 98–102 (1994).
- Berx, G. & Van Roy, F. The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. *Breast Cancer Res.* 3, 289–293 (2001).
- Ivascu, A. & Kubbies, M. Diversity of cell-mediated adhesions in breast cancer spheroids. *Int. J. Oncol.* 31, 1403–1413 (2007).
- Sundfeldt, K. *et al.* E-cadherin expression in human epithelial ovarian cancer and normal ovary. *Int. J. Cancer* 74, 275–280 (1997).
- 23. Maines-Bandiera, S. L. & Auersperg, N. Increased E-cadherin expression in ovarian surface epithelium: an early step in metaplasia and dysplasia. *Int. J. Gynecol. Pathol.* **16**, 250–255 (1997).
- Hudson, L. G., Zeineldin, R. & Stack, M. S. Phenotypic plasticity of neoplastic ovarian epithelium: unique cadherin profiles in tumor progression. *Clin. Exp. Metastasis* 25, 643–655 (2008).
- Wong, A. S. *et al.* Constitutive and conditional cadherin expression in cultured human ovarian surface epithelium: influence of family history of ovarian cancer. *Int. J. Cancer* 81, 180–188 (1999).
- Sundfeldt, K. Cell-cell adhesion in the normal ovary and ovarian tumors of epithelial origin; an exception to the rule. *Mol. Cell. Endocrinol.* 202, 89–96 (2003).
- Darai, E. *et al.* Expression of cadherins in benign, borderline, and malignant ovarian epithelial tumors: a clinicopathologic study of 60 cases. *Hum. Pathol.* 28, 922–928 (1997).
- Reddy, P. et al. Formation of E-cadherin-mediated cell-cell adhesion activates AKT and mitogen activated protein kinase via phosphatidylinositol 3 kinase and ligand-independent activation of epidermal growth factor receptor in ovarian cancer cells. Mol. Endocrinol. 19, 2564–2578 (2005).
- Dong, L. L. *et al.* E-cadherin promotes proliferation of human ovarian cancer cells in vitro via activating MEK/ERK pathway. *Acta Pharmacol. Sin.* 33, 817–822 (2012).
- De Santis, G., Miotti, S., Mazzi, M., Canevari, S. & Tomassetti, A. E-cadherin directly contributes to PI3K/AKT activation by engaging the PI3K-p85 regulatory subunit to adherens junctions of ovarian carcinoma cells. *Oncogene* 28, 1206–1217 (2009).
- Lau, M. T., Klausen, C. & Leung, P. C. E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via beta-catenin-Egr1-mediated PTEN expression. Oncogene 30, 2753–2766 (2011).
- 32. Lengyel, E. Ovarian cancer development and metastasis. Am. J. Pathol. 177, 1053–1064 (2010).
- Hayward, I. P. & Whitehead, R. H. Patterns of growth and differentiation in the colon carcinoma cell line LIM 1863. *Int. J. Cancer* 50, 752–759 (1992).
- Kantak, S. S. & Kramer, R. H. E-cadherin regulates anchorage-independent growth and survival in oral squamous cell carcinoma cells. *J. Biol. Chem.* 273, 16953–16961 (1998).
- Kang, H. G. *et al.* E-cadherin cell-cell adhesion in ewing tumor cells mediates suppression of anoikis through activation of the ErbB4 tyrosine kinase. *Cancer Res.* 67, 3094–3105 (2007).
- Shimazui, T. *et al.* Role of complex cadherins in cell-cell adhesion evaluated by spheroid formation in renal cell carcinoma cell lines. *Oncol. Rep.* 11, 357–360 (2004).
- Shimoyama, Y. *et al.* Cadherin dysfunction in a human cancer cell line: possible involvement of loss of alpha-catenin expression in reduced cell-cell adhesiveness. *Cancer Res.* 52, 5770–5774 (1992).
- Veatch, A. L., Carson, L. F. & Ramakrishnan, S. Differential expression of the cellcell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells. *Int. J. Cancer* 58, 393–399 (1994).
- 39. West, G. W., Weichselbaum, R. & Little, J. B. Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumor resistance to adjuvant chemotherapy. *Cancer Res.* 40, 3665–3668 (1980).
- Peluso, J. J., Pappalardo, A. & Trolice, M. P. N-cadherin-mediated cell contact inhibits granulosa cell apoptosis in a progesterone-independent manner. *Endocrinology* 137, 1196–1203 (1996).
- Kerbel, R. S. *et al.* Multicellular resistance: a new paradigm to explain aspects of acquired drug resistance of solid tumors. *Cold Spring Harb. Symp. Quant. Biol.* 59, 661–672 (1994).
- 42. Zhang, Z. *et al.* Acquisition of anoikis resistance reveals a synoikis-like survival style in BEL7402 hepatoma cells. *Cancer Lett.* **267**, 106–115 (2008).
- St, C. B. *et al.* E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). *J. Cell Biol.* 142, 557–571 (1998).

- 44. Green, S. K., Karlsson, M. C., Ravetch, J. V. & Kerbel, R. S. Disruption of cell-cell adhesion enhances antibody-dependent cellular cytotoxicity: implications for antibody-based therapeutics of cancer. Cancer Res. 62, 6891-6900 (2002).
- 45. Carduner, L. et al. Cell cycle arrest or survival signaling through alphav integrins, activation of PKC and ERK1/2 lead to anoikis resistance of ovarian cancer spheroids. Exp. Cell Res. 320, 329-342 (2014).
- 46. Diaz-Montero, C. M. & McIntyre, B. W. Acquisition of anoikis resistance in human osteosarcoma cells does not alter sensitivity to chemotherapeutic agents. BMC Cancer 5, 39 (2005).
- 47. Hong, J. H. et al. Iron promotes the survival and neurite extension of serumstarved PC12 cells in the presence of NGF by enhancing cell attachment. Mol. Cells 15, 10-19 (2003).

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Author contributions

L.L. designed the experiment. S.X., Y.N.Y. and L.L.D. carried out the experiments. L.L. and S.X. interpreted the results, analyzed the data, prepared the figures and tables, and wrote, reviewed, and revised the manuscript. W.L.Q. and L.Y. provided technical or material support. X.W.W. supervised the study. All authors reviewed the manuscript.

Additional information

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