Three-dimensional *in vitro* cell biology models of ovarian and endometrial cancer

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Abstract

Objectives: This study aims to establish three-dimensional (3D) cell culture models of human ovarian and endometrial cancers and to compare biological and morphological characteristics of these models with those of two-dimensional (2D) models of the same cell lines and the primary tumours.

Methods: 3D models of ovarian and endometrial cancer cell cultures were established using a Rotary Cell Culture System. Immunohistochemical profiling and differential proteomics were used to characterize biological characteristics of multicellular spheroids (MCS) formed from these cultures. These were compared to characteristics of the same cells established in 2D and of the primary tumours from which the cell lines were derived.

Results: MCSs from 3D cell cultures appeared histologically similar to the primary tumours. Immunohistochemical profiling of multiple markers, including CA125, BCL2 and p53, showed that patterns of protein expression in MCSs resemble those of the primary tumours. Proteomic profiling identified several differentially expressed protein markers between 2D and 3D cultures. These included prohibitin, which was down-regulated in 3D cultures suggesting cells proliferate less compared to 2D cultures; and VDAC1 and annexin 4, which were up-regulated in 3D cultures suggesting greater levels of apoptosis in 3D compared to 2D models.

Conclusion: Establishing 3D models of cancer cell lines is likely to be of value for studying the molecular and biological mechanisms of ovarian/endometrial

tumour progression and for testing novel molecular targets for cancer therapy.

Introduction

Cancer cell lines established from primary tumours are widely accepted as valid model system for studying tumour cell biology. Standard methodology is to culture homogeneous populations of single cells as a monolayer on a plastic surface. However, such models overlook many features of the dynamic three-dimensional (3D) environment in which tumours exist *in vivo*. The micro-environment within a tumour is regulated by interactions between tumour stroma, immune cells and endothelial cells, and through the local support network of the extracellular matrix (1). Complexity of these interactions raises questions about the validity of using standard two-dimensional (2D) cell culture systems, to study tumour biology at a tissue-specific level, and perhaps of cancer *per se*. That is not to say that 2D models of cancer are wholly unrepresentative of tumours *in vivo*. Tumour xenografts of human cancer cell lines grown in nude mice have been shown to be similar in their histology and expression profiles to parental tumours (2). Even so, establishing the same cells in a 3D system may provide an environment that is more reflective of the tumours *in vivo*.

It is perhaps for this reason that use of 3D cell culture methods for studying tumourigenesis has increased over the last few years. Traditionally, *in vitro* 3D models described consist of spontaneously forming cell aggregations, liquid overlay cultures and 'spinner' flasks. More recently, prefabricated collagen scaffolds and microcarrier beads have proved popular for multilayer growth of cells derived from different types of tumour. Currently, the most widely used 3D model systems involve induction of multicellular spheroids (MCS) by preventing cell adhesion to a surface. MCSs have a relatively well-organized spherical symmetry, and show morphological and physiological features that are consistent with primary tumours, including cell–matrix interactions and avascular sites (3). The recently established

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Rotary Cell Culture System (RCCS) developed by NASA is a substantial improvement on the spinner flask method (4–6). The low shear environment in the rotary vessel has an advantage over static and other dynamic tissue culture systems in that it allows cells to form aggregates and to differentiate. This permits cell growth that more closely resembles *in vivo* tissue equivalent (7,8).

Several studies have attempted to draw comparison between the biological characteristics of cell culture models in 2D and 3D. Petersen *et al.* were the first to show that although nonmalignant and malignant breast epithelial cells are phenotypically similar when grown in 2D, they behave very differently when grown as 3D cultures (9). In 3D, nonmalignant breast epithelial cells form organized, growth-arrested acinar structures that have a similar architecture to that of normal human breast tissue. By contrast, cells derived from breast carcinomas form disorganized cellular masses that do not respond to the growth inhibitory cues from the extracellular matrix (10). In another study, Ghosh *et al.* showed that there are significant differences in gene expression profiles of melanoma cells grown in 3D compared to 2D (11).

Studies of ovarian and endometrial cancer cell lines grown in 3D culture are limited and have provided but few insights into differences in cellular and molecular morphology between different culture methods (12–15). Recently, one study of an ovarian cancer cell line suggested that the expression profile of cells grown in 3D is more similar to the expression profile of the cells grown as tumour xenografts than the same cells grown in 2D (16).

The purpose of the current study was to establish a method for 3D culturing of primary, early-passage epithelial cell lines derived from ovarian and endometrial cancers. The aim was to compare biological features between 2D and 3D cultures and the primary tumours from which the cell lines were derived, including morphological characteristics, expression of tumour markers and proteomic profiles.

Materials and methods

Cell lines

Epithelial cancer cell lines were established from primary tumours collected at University College London Hospital. All cells were grown in media 199 and MCDB 105 (Sigma, Gillingham, Dorset, UK) in a 1 : 1 ratio supplemented with 15% foetal calf serum and 200 mM L-glutamine (Invitrogen, Paisley, UK). Either resected pieces of tumour were finely minced or cells from cytobrushings of surfaces of tumours were incubated in complete culture media at 37 °C/5% CO₂. When cells reached confluence they were subcultured according to standard trypsinization methods

using 0.25% trypsin-EDTA (Invitrogen). Cancer cell lines used in this study were OV-TRL12B (established from cytobrushings of a high-grade poorly differentiated, stage II squamous ovarian carcinoma) and EN-TRL 67T (established from a grade 2, stage IIB endometrial adenocarcinoma).

Phenotype analysis of cell lines

Growth rates of the newly established cell lines were assessed. After plating 1×10^5 cells per P60 dish in triplicate, cell numbers were counted and population doublings (PD) calculated using the following formula: $PD = \log (\text{total cell number at each passage}/\text{initial cell number})/\log 2$.

Ability of the established cell lines to grow in a semi-solid medium was determined by plating them in complete medium containing 0.3% Noble Agar (Sigma) on top of a layer of complete medium containing 0.6% Noble Agar. Colonies were fixed with methanol and stained with 1% p-iodonitrotetrazolium violet (Sigma). Colony formation was observed and number of colonies containing more than approximately 50 cells were counted after 4 weeks incubation. Colony-forming efficiency (CFE%) was calculated as the number of colonies counted divided by the number of cells plated. Each assay was performed in triplicate. CFE in a control cell line (TOV112D) was assessed simultaneously.

Invasive ability of the cancer cell lines through MatrigelTM Invasion Chambers (BD Biosciences, Oxford, UK) was assessed. Each assay was performed in triplicates and 30 000 cells were plated per chamber. One per cent bovine serum albumin in serum-free medium was used as a chemo-attractant. Populations that showed evidence of invasion were counted under a light microscope to establish the extent of invasion.

Immunofluorescence and immunohistochemistry analysis

Immunofluorescence analysis was used to study expression of several epithelial, apoptotic and tumour markers in cells from 2D cultures and standard immunohistochemistry was used to analyse expression of the same series of markers in sections of 3D culture spheroids and primary tumour tissues. The following antibodies were used: cytokeratin 7 (CR-UK), BerEp4 (Dako, Ely, UK), CA125 (Dako), FSP (Sigma), all at a dilution of 1 : 1000. Expression of other cancer-associated markers (p53, Mib-1, Bcl-2, ER, PGR) were assessed using standard protocols with appropriate positive controls (CK7: tonsil/breast/oesophagus; BerEp4: appendix/bowel cancer; Ca125: ovarian carcinoma; FSP: skin fibroblasts; p53: colon carcinoma/tonsil; Mib-1: tonsil; Bcl-2: tonsil; ER: breast; PGR: breast) and antibodies from the Clinical Diagnostics

Unit of University College London Hospital and from the Histology Service at Cancer Research UK. As negative controls, the same tissues were used but omitting primary antibodies..

Three-dimensional cultures

Tumour cell lines were cultured in 3D using the RCCS (Synthecon, Houston, TX, USA) following the manufacturer's instructions. Cells in liquid medium were maintained in a dynamic fluid suspension. Aeration was provided through a semipermeable membrane. Cells were grown as 2D cultures in the same culture medium. Ten-millilitre 3D vessels were inoculated with 200 000 cells/ml; cells were grown for 3–4 weeks, or until cell aggregates were visible within the vessel.

Optimizing 3D cultures We investigated effects of a variety of cell carriers on size and stability of cell aggregates grown in 3D. We tested two types of microcarrier beads and an extracellular matrix gel; these were added to the 3D rotation vessel. Hillex® II H112-170 microcarriers (SoloHill Engineering Inc., Ann Arbor, MI, USA), and FACT III microcarriers (SoloHill Engineering Inc.) and extracellular matrix gel from Engelbreth Holm-Swarm mouse sarcoma (Sigma) were used. Cell aggregates were formalin fixed inside the 3D culture vessel, embedded in paraffin wax, sectioned, haematoxylin and eosin stained and analysed for tumour markers as described above.

Fluorescence 2D difference gel electrophoresis

Sample preparation Twenty-four hours prior to cell lysis of 3D cellular aggregates, the complete culture medium was replaced by serum-free medium. Cell suspensions were transferred to 50-ml tubes and were centrifuged to collect the cells. Cell pellets were washed with ice-cold PBS before resuspending in 400 µl 2D lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 0.5% NP40, 10 mM Tris-HCl pH 8.3). For 2D cell cultures, cells were grown until 90% confluent in serum-free medium 24 h prior to washing and cell lysis. Samples were homogenized using a 25 G needle and insoluble material was removed by centrifugation (12 000 g/10 min/4 °C). Protein concentrations were determined by Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) and a bovine serum albumin standard curve.

2D difference gel electrophoresis 2D difference gel electrophoresis (2D-DIGE) analysis was performed in triplicate as described by Gharbi *et al.* (17). Briefly, samples were labelled randomly with NHS-Cy3 or NHS-Cy5 at 4 pmol dye/µg total protein. Equal amounts of protein

from all samples were mixed to create an internal standard, which was labelled with NHS-Cy2 at 4 pmol/µg protein (GE Healthcare, Chalfont St Giles, UK). Cy3- and Cy5-labelled samples and the Cy2-labelled internal standard were mixed appropriately, then reduced with dithiothreitol (DTT) (Sigma) to final concentration of 65 mM and a carrier Ampholine/Pharmalyte mixture (1 : 1; pH 3–10) was added to a final concentration of 2% (v/v). 2D electrophoresis was carried out as previously described (18) using 24-cm Immobiline Dry Strips pH 3–10 NL (GE Healthcare) and 12% homogenous sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels bonded to low-fluorescence glass plates. Gels were scanned between glass plates using a Typhoon™ 9400 variable mode imager and captured using ImageQuant software (both GE Healthcare). Low-resolution scans were initially made to give maximum pixel values within 5–10% for each Cy dye channel and below saturation, prior to high-resolution (100 µm) scans. Images were analysed using DeCyder™ software version 5.0 (GE Healthcare) according to the software guidelines. Only spots displaying an average 2-fold increase or decrease in abundance (from triplicate measurements), matching across all gel images and with *P*-values of ≤ 0.01 , were selected for mass spectrometry-based identification. Gels were poststained with colloidal Coomassie Blue and stained images were matched to the fluorescent images in DeCyder (18). A pick list of spots of interest was then generated from the colloidal Coomassie Blue image and spots were excised using an Ettan spot picker (GE Healthcare).

Protein identification by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) peptide mass fingerprinting In-gel tryptic digestion was performed as previously described (19) and extracted peptides resuspended in 5 µl water plus 0.1% formic acid. Of the sample 0.5 µl was then mixed with matrix (saturated aqueous solution of dihydroxybenzoic acid), spotted onto a stainless steel MALDI target and air dried. MALDI-TOF mass spectrometry was performed using an Ultraflex TOF/TOF instrument (Bruker Daltonics Ltd, Coventry, UK) in the reflectron mode. Prominent peaks in the mass range m/z 700–5000 were used to generate a peptide mass fingerprint, which was searched against the updated NCBI database using MASCOT version 2.0.02 (Matrix Sciences Ltd, London, UK). Identifications were accepted when: (i) ≥ 6 peptide masses matched a particular protein; (ii) sequence coverage was 20%; (iii) the search score was higher than the threshold score at $P = 0.05$; and (iv) the predicted protein mass agreed with the gel-based mass. A standard Western blotting protocol was used to confirm differential expression of protein candidates.

Figure 1. Growth, neoplastic and epithelial characteristics of the cell lines OV-TRL12B and EN-TRL67T grown in two-dimensional (2D) cultures. (a) Growth curves of OV-TRL12B (●) and EN-TRL67T (■) over a period of 60 days in culture. (b) Colony-forming efficiency (CFE%) and (c) invasive ability (cell number) of OV-TRL12B and EN-TRL67T cell lines. OV-TRL12B cells showed a 3% CFE, EN-TRL67T a CFE of 0.5%, and both cell lines also showed evidence of invasive ability.

Results

Establishing tumour cell lines from primary tissues

Studies were performed on two cancer cell lines freshly established from one ovarian and from one endometrial tumour. By using primary, early-passage epithelial cultures, we hoped to ensure that the lines would be as closely related as possible to the primary tumours from which they were derived, since less culture-induced mutations would have occurred. Both cell lines underwent clonal selection to establish homogenous populations of epithelial cells. Growth properties of the cell lines were first established in 2D cultures (Fig. 1). Both the ovarian cancer cell line OV-TRL12B and the endometrial cancer cell line EN-TRL67T showed linear growth over a 60-day period and have now been subcultured continuously for 41 and 28 passages respectively. Population doubling times were 3.6 and 3.4 days respectively.

Cell lines were analysed for their malignant phenotype *in vitro* using anchorage-independent growth and invasion assays. Both cell lines formed colonies in soft agar after 3 weeks and were able to invade through a basement membrane (Fig. 1). These data suggest that EN-TRL67T is a less aggressive cancer cell line than the OV-TRL12B, which is consistent with the clinical phenotype of the primary tumours.

Immunofluorescence analysis was used to confirm the epithelial nature of the two cell lines. Cells were stained for the epithelial-specific markers cytokeratin 7 and BerEp4. Both cell lines expressed BerEp4, while EN-TRL67T also expressed cytokeratin 7.

Establishing tumour cell lines in 3D

We established 3D cultures of OV-TRL12B and EN-TRL67T cells using the RCCS (20–22). This system maintains the

cells in constant rotation, which prevents them from attaching to the walls of the culture vessel; thus, cells grow in-suspension and form 3D multi cellular spheroids (MCSs). MCSs form in 1 week for passage 28 OV-TRL12B cells and within 2 weeks for passage 18 EN-TRL67T cells. MCSs reached a maximum diameter of 4 mm, after which they tended to dissociate.

Both cell lines were able to form MCSs without any material support. Even so, we investigated whether addition of microcarrier beads coated with cationic trimethyl ammonium (HillexII) or gelatin (cationic type 1 porcine collagen Factor III) improved cell growth in 3D. Both coatings have been shown to facilitate attachment and growth of mammalian cells. We also tested whether addition of a gel comprising components of the extracellular matrix provided a support network that would enhance 3D growth. However, none of these support matrices improved the size or stability of MCSs (data not shown).

Cell aggregates from 3D cultures were formalin fixed, paraffin wax embedded, sectioned and examined for their morphological characteristics. These were compared with morphological characteristics of the primary tumours from which the cell lines were derived (Fig. 2). The primary tumour of the cell line EN-TRL67T is an endometrioid adenocarcinoma grade 2 with papillary architecture of the endometrium. Extensive areas of necrosis were present and the tumour cells were pleomorphic with abundant eosinophilic cytoplasm and mitotic activity. 3D MCSs grew in clusters, which resembled a papillary structure. The cells were pleomorphic with enlarged nuclei and prominent nucleoli, and mitotic cells were also observed. Further, there were also foci of apoptosis with cell degeneration and karyorrhexis. Thus, the growth pattern of the cell line in 3D showed close morphological similarity to the primary tumour.

The OV-TRL12B cell line was derived from a poorly differentiated squamous cell carcinoma arising within a

Figure 2. (a–c) Three-dimensional (3D) cultures of the OV-TRL12B cancer cell line (a and b) show cell clusters composed of sheets of cells showing variation in cell size. At higher magnification, nuclear pleomorphism and prominent nucleoli (N) are seen. Histological section of the original tumour (c) shows poorly differentiated squamous carcinoma infiltrating stroma, forming sheets and nests of cells. Tumour cells show similar cytological features with variation in cell size nuclear pleomorphism and prominent nucleoli, as the 3D culture; (d–f) 3D cultures of the EN-TRL 67T cancer cell line (d and f) shows papillary formation of the cell clusters. Tumour cells show nuclear pleomorphism, increased mitotic activity and atypical mitoses (M). Foci of apoptosis (A) and nuclear debris are present. Histological section of the original endometrial adenocarcinoma (f) shows papillary architecture and prominent neutrophil polymorph (P) infiltration of stroma. Tumour cells show similar architectural and morphological features although apoptosis is more prominent in the 3D culture.

mature cystic teratoma of the ovary. Tumour cells infiltrated stroma as nests, sheets and trabeculae. Cells had a variable amount of eosinophilic cytoplasm and pleomorphic nuclei, and showed mitotic activity. There was also focal keratinization and areas of necrosis. Compared to the original tumour the 3D MCS grew in similar sheets and cell clusters. The cells varied in size and had pleomorphic nuclei with prominent nucleoli; foci of apoptotic cells were also present.

Immunohistochemical staining for epithelial markers in cells grown in 3D showed a similar pattern of CK7 and BerEp4 expression as the same cells grown in 2D. The result of staining was judged by taking the percentage of positively stained cells and intensity of stain into account (Fig. 3). We also compared expression of several functional and tumour-associated markers between 2D and 3D cultures and the primary tumours from which both cancer cell lines were derived. These included ovarian tumour marker CA125, apoptotic markers BCL2, proliferation marker and Mib-1, the cell cycle control protein p53; and steroid hormone receptors PGR and ER. In general, levels of expression for these markers were similar between cells from 2D and 3D cultures and in those from the primary tumours. The only notable exception was for Mib-1, which was highly expressed in 2D cultures of EN-TRL67T, but showed weak expression in cells from 3D cultures and the primary tumour (Fig. 3).

Proteomic analysis of tumour cell lines grown in 2D and 3D

We investigated whether there were any differences in proteomic profiles between OV-TRL12B and EN-TRL67T cells grown in 2D and 3D, which may suggest differences in underlying biology associated with these different cell culture approaches. Whole cell protein lysates were analysed using 2D-DIGE (Fig. 4).

For OV-TRL12B, we found 176 protein isoforms that were >2-fold differentially expressed between cells grown in 2D and 3D at a significance level of $P < 0.01$. For EN-TRL67T, we found 78 differentially expressed proteins between the 2D and 3D cultures. Of these, 57 (29%) were common to both the ovarian and endometrial cancer cell lines.

Mass spectrometry was used to identify 22 of the differentially expressed proteins (Table 1). Of these, 20 proteins were either up- or down-regulated in the 3D vs. 2D cultures of both cell lines. These included annexin IV, which is expressed almost exclusively in epithelial cells and plays a role in apoptosis, prohibitin, which is thought to be a negative regulator of cell proliferation and possibly acts as a tumour suppressor in breast cancer development, voltage-dependent anion-selective channel 1 (VDAC1), a plasma membrane protein also involved in apoptosis (23), two members of the peroxiredoxin family (PRDX1 and PRDX6) and mitochondrial superoxide dismutase, which

Figure 3. (a) Immunohistochemical analysis of OV-TRL12B and EN-TRL67T cell lines grown in two- (2D) and three-dimensional (3D) cultures and the corresponding primary tumour staining for the cytokeratin marker CK7, the epithelial antigen BerEp4 and the epithelial tumour marker Ca125. (b) Summary of immunohistochemical staining for several markers. Shading of boxes is graded according to percentage of positive stained cells and staining intensity.

have roles in protecting cells against oxidative stress and effect proliferation during cancer development and progression, vimentin, which is known to be involved in epithelial to mesenchymal cell transition (17), and beta tubulin (TUBB), which may be mutated in small cell lung cancers and is associated with response to treatment with paclitaxel.

For three of these proteins, annexin IV (theoretical pI: 5.84; actual pI: 6.15; theoretical MW: 36 kDa; actual MW: 24 kDa) prohibitin (theoretical pI: 5.57; actual pI: 5.74; theoretical MW: 29.8 kDa; actual MW: 19 kDa) and VDAC1 (theoretical pI: 8.62; actual pI: 8.3; theoretical

MW: 30.8 kDa; actual MW: 25 kDa), Western blotting was used to validate altered expression observed between 2D and 3D cultures (Fig. 4b). These data were consistent with results of 2D-DIGE analysis, and suggested an increase in expression for all three proteins in 3D compared to 2D cultures.

Discussion

Two-dimensional monolayers of cancer cell lines have been the most widely used *in vitro* model for cancer for several years; they are relatively easy to establish and maintain and have long-term viability. However, they lack the 3D

Figure 4. (a) Two-dimensional difference gel electrophoresis (2D-DIGE) analysis of the cancer cell lines OV-TRL12B and EN-TRL67T grown in two- (2D) and three-dimensional (3D) cultures. The further validated proteins are marked and a magnification of these spots is shown. (b) Three proteins identified as being differentially expressed between 2D and 3D cultures of OV-TRL12B and EN-TRL67T cancer cell lines by 2D-DIGE, validated by Western blotting analysis.

structural architecture and are often not as reflective of biological properties of tumours *in vivo*. In principle, establishing cell line models in 3D systems should address some of the limitations of 2D cancer cell line cultures.

In the present study, we have successfully established 3D cultures for two newly derived cancer cell lines, one from an ovarian and one from an endometrial cancer using the RCCS. The RCCS is particularly useful since cultures are easy to set up and maintenance of cell lines is simple. There are very few studies describing 3D cell biology models for these cancer types, and none used the RCCS. One of the earliest and most widely used approaches to 3D *in vitro* modelling of human cancer cells was to embed cells in a collagen-enhanced matrigel. This was used by Park *et al.* to establish a 3D model of an endometrial cancer cell line invading endometrial stroma (16). In a similar study, Kenny *et al.* used a collagen/matrigel support matrix to establish a 3D model of early stages of development of an ovarian cancer metastasis in the omentum (15). Both models provide important information concerning interactions between different cellular types, for endometrial and ovarian cancers. However, one of the disadvantages of collagen/matrigel models is that they are solid systems; as such, they are not readily adaptable to changes in and/or manipulation of environmental stimuli. This prohibits detailed analysis of the 3D architec-

ture of primary tumours in response to changing biological environment (22). In addition, although collagen gels have the ability to mimic various densities of different tissues (soft and connective) depending on the working dilution of collagen, they lack other components present in connective tissue. Matrigel provides basement membrane components but is more of a cell culture supplement rather than an accurate agent mimicking flat basement membranes beneath epithelial cells.

Zietarska *et al.* used a 'hanging droplet' approach to establish 3D spheroids from two ovarian cancer cell lines (24). They showed that gene expression profiles of these spheroids were more similar to expression profiles from xenografts in nude mice compared to gene profiles from 2D cultures. Broadly, this is consistent with the findings of our study. However, the RCCS method used here has the advantage that MCSs can grow for longer periods of time and reach significantly greater 'tumour' volumes than they could by using the hanging droplet approach. MCSs formed by the RCCS can mimic both surface exposure of tumour cells to a manipulated extracellular environments and internal, often hypoxic environment of tumours, probably the cause of areas of necrosis we observed in both primary tumours and the 3D spheroids.

Several other features of MCSs formed by the RCCS suggest that they are good representations of the primary

Table 1. Differentially expressed proteins identified between two (2D) and three-dimensional (3D) cultures of OV-TRL12B and EN-TRL67T cancer cell lines

Protein nomenclature	EN-TRL67T 3D vs. 2D		OV-TRL12B 3D vs. 2D		Known or predicted function
	Ratio	P-value	Ratio	P-value	
Heat shock protein HSPA5	2.2	0.00016	-1	0.52	ATP binding, protein binding, bridging
Heat shock 70 kDa protein 8 isoform 2 variant	-1.2	0.0074	-2.3	6.70×10^{-5}	ATPase activity, coupled/unfolded protein binding/ATP binding/protein folding/response to unfolded protein
Hypothetical protein DKFZp686J13123 TKT	1	0.48	-3.4	2.40×10^{-5}	Calcium ion binding/transferase activity/transketolase activity
Vimentin 50 kDa protein VIM	-2	2.20×10^{-6}	-2.9	0.0001	Protein binding/structural constituent of cytoskeleton/structural molecule activity
Tubulin, beta polypeptide TUBB	-2.1	3.00×10^{-5}	-2.7	0.0002	Structural molecule activity I/GTP binding/unfolded protein binding/GTPase activity/microtubule-based movement/natural killer cell-mediated cytotoxicity/protein polymerization/cytoplasm/tubulin
Isoform alpha-enolase of alpha-enolase ENO1	-1.3	0.044	-4.2	7.50×10^{-7}	Phosphopyruvate hydratase activity/plasminogen activator activity/DNA binding/lyase activity/magnesium ion binding/regulation of transcription, DNA-dependent/transcription/glycolysis/growth control, hypoxia tolerance/allergic responses
Adenosylhomocysteinase AHCY	-1.3	0.0054	-4.7	0.0002	Adenosylhomocysteinase activity/hydrolase activity/one-carbon compound metabolism
Phosphoglycerate kinase 1 PGK1	-1.1	0.18	-2.9	4.90×10^{-5}	ATP binding/phosphoglycerate kinase activity/transferase activity/glycolysis/phosphorylation
Fructose-bisphosphate aldolase A ALDOA	-1.8	0.0017	-3.3	2.00×10^{-5}	Fructose-bisphosphate aldolase activity/lyase activity/fructose metabolism/glycolysis/striated muscle contraction carbohydrate degradation; glycolysis; D-glyceraldehyde 3-phosphate and glyceraldehyde phosphate from D-glucose
L-lactate dehydrogenase B chain LDHB	-1.3	0.0068	-3.2	0.0001	Oxidoreductase activity/L-lactate dehydrogenase activity/tricarboxylic acid cycle intermediate metabolism/anaerobic glycolysis
Voltage-dependent anion-selective channel protein1	6.9	4.30×10^{-5}	14.2	4.00×10^{-5}	Voltage-gated anion channel porin activity/voltage-gated ion-selective channel activity/apoptogenic cytochrome c release channel activity/anion transport/apoptotic program
VDAC1	3.3	0.0003	2.8	2.50×10^{-5}	Voltage-gated anion channel porin activity/voltage-gated ion-selective channel activity/apoptogenic cytochrome c release channel activity/anion transport
Annexin IV ANXA4	3.6	3.90×10^{-5}	1.7	0.024	Calcium ion binding/calcium-dependent phospholipid binding/phospholipase inhibitor activity
Prohibitin PHB	6	2.50×10^{-5}	4.6	3.30×10^{-5}	Transcriptional activator activity/transcriptional repressor activity/negative regulation of transcription/regulation of cell cycle/DNA metabolism/histone deacetylation/negative regulation of cell proliferation
Proteasome activator complex subunit2 PSME2	-2.1	0.0005	-2.2	0.0017	Proteasome activator activity/immune response
PSME1 29 kDa	-1.8	0.0012	-2.9	0.0013	Proteasome activator activity/proteasome activator activity/immune response
Triosephosphate isomerase 1	-1.1	0.38	-2	8.90×10^{-5}	Metabolism/pentose-phosphate shunt/fatty acid biosynthesis/gluconeogenesis/glycolysis
Peroxiredoxin-6 (PRDX6)	-1.7	0.0081	-2.8	0.0002	Peroxidase activity/phospholipase A2 activity/hydrolase activity/oxidoreductase activity/phospholipid catabolism/response to oxidative stress/lipid catabolism
GSTP1	-1.5	0.0026	-2.7	4.10×10^{-5}	Transferase activity/glutathione transferase activity/metabolism/central nervous system development
Peroxiredoxin-1 (PRDX1)	-1.1	0.2	-3.1	0.0001	Oxidoreductase activity/oxidoreductase activity/skeletal development/cell proliferation
PRDX1 19 kDa	41.7	1.60×10^{-6}	5.3	0.0032	Oxidoreductase activity/oxidoreductase activity/skeletal development/cell proliferation
SOD2	3.1	0.0001	2.7	1.20×10^{-5}	Destroys radicals that are normally produced within the cells and that are toxic to biological systems

tumours from which they were derived. Both MCSs from the tumour cell lines showed similarities to avascular tumour nodules and microregions of the solid tumour contained different areas of cells with different growth kinetics (for example, proliferation, quiescence and necrosis). These features are commonly seen in primary cancers.

Proteomic comparisons showed some of the protein changes associated with culturing the same cells under 2D and 3D growth conditions and may suggest which are likely to orchestrate the changes in the biological characteristics of the cells. For example, increased expression of prohibitin we observed in 3D cultures supports the reduced cellular proliferation observed in 3D vs. 2D cultures. Similarly, differences in VDAC1 and annexin 4 expression support our observations that the 3D cultures exhibit more apoptosis. Finally, we also found reduced expression of transketolase and vimentin (and TUBB) when cells were established in 3D; both proteins are frequently down-regulated in solid ovarian tumours (25). These results are consistent with other studies that have compared gene expression changes between solid tumours and the corresponding cell lines (25).

There are several applications of 3D *in vitro* cell line models that could lead to a greater understanding of the underlying biology of a disease, if reliable models that accurately mimic the disease *in vivo* can be developed. The RCCS is an extremely useful tool for further development of 3D *in vitro* models of epithelial ovarian cancers. To date, we have used the RCCS in our laboratory to establish 3D multicellular spheroids of an array of epithelial ovarian cancer cell lines of different histological subtypes (data not shown). This may be particularly significant for a disease such as ovarian cancer, which is still poorly understood. Standard chemotherapeutic regimes are nonspecific and have had little impact on overall mortality; current markers for the early detection of disease (for example, CA125) are not specific enough to detect pre-invasive cancer and there are no existing prognostic ovarian tumour markers that can be used effectively to guide treatment regimes. Tumourigenesis is a process in which interactions of cells within the tumour and their microenvironment determine how aggressive the tumour might be and how it will respond to therapeutic intervention. For example, it is widely accepted that stromal factors can promote angiogenesis, which in turn will promote tumour progression and metastasis. However, very few model systems have been developed that can analyse these cell–cell interactions in such a way that could lead to the discovery of novel genetic and biological targets for developing new therapies. Such interactions could be investigated by extending the RCCS models we have developed to heterotypic cultures of epithelial tumour cells, stromal fibroblasts and

endothelial cells. From this, and with recent technological developments that enable screening of global patterns of DNA and gene and protein expression changes, it is conceivable that identification and experimental validation of novel molecular targets for therapy could be both rapid and effective.

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