Bioengineered 3D platform to explore cell–ECM interactions and drug resistance of epithelial ovarian cancer cells

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Abstract

The behaviour of cells cultured within three-dimensional (3D) structures rather than onto two-dimensional (2D) culture plastic more closely reflects their *in vivo* responses. Consequently, 3D culture systems are becoming crucial scientific tools in cancer cell research. We used a novel 3D culture concept to assess cell-matrix interactions implicated in cancerogenesis: a synthetic hydrogel matrix equipped with key biomimetic features, namely incorporated cell integrin-binding motifs (*e.g.* RGD peptides) and the ability of being degraded by cell-secreted proteases (*e.g.* matrix metalloproteases). As a cell model, we chose epithelial ovarian cancer, an aggressive disease typically diagnosed at an advanced stage when chemoresistance occurs. Both cell lines used (OV-MZ-6, SKOV-3) proliferated similarly in 2D, but not in 3D. Spheroid formation was observed exclusively in 3D when cells were embedded within hydrogels. By exploiting the design flexibility of the hydrogel characteristics, we showed that proliferation in 3D was dependent on cell-integrin engagement and the ability of cells to proteolytically remodel their extracellular microenvironment. Higher survival rates after exposure to the anti-cancer drug paclitaxel were observed in cell spheroids grown in hydrogels (40-60%) compared to cell monolayers in 2D (20%). Thus, 2D evaluation of chemosensitivity may not reflect pathophysiological events seen in patients. Because of the design flexibility of their characteristics and their stability in long-term cultures (28 days), these biomimetic hydrogels represent alternative culture systems for the increasing demand in cancer research for more versatile, physiologically relevant and reproducible 3D matrices.

1. Introduction

A growing body of evidence suggests that three-dimensional (3D) models, in contrast to two-dimensional (2D) flat, conventional culture plastic, replicate more accurately the actual microenvironment where cells reside in tissues; and therefore the behaviour of cells cultured in 3D will reflect more closely their *in vivo* responses [1-4]. Consequently, 3D culture models are becoming increasingly crucial research tools, in particular in cancer cell biology [5-7], as they are a bridge between traditional culture on plastic and *in vivo* experiments [8-10].

Currently*, in vitro* 3D culture models for cancer research include multicellular spheroids grown in suspension [11, 12] and cells embedded within naturally derived extracellular matrices (ECM) (*e.g.* reconstituted ECM-protein-based matrices for Matrigel^{TM} [13-17], collagen gels [18, 19] and cell-secreted ECMs [20]). Although these 3D culture systems have profoundly revolutionised fundamental cancer research [2], they have experimental and interactive limitations. Multicellular spheroids grown as independent cell agglomerates in the absence of an ECM do not interact with their extracellular milieu and do not have physical resistance provided by the ECM [21]. The gold standard 3D matrix Matrigel^{TM} and other naturally derived hydrogels have ECM-like biological properties, but their inherent characteristics offer limited design flexibility in fine-tuning different matrix properties (physical and biological) independently [22]. In addition, their relatively poor handling characteristics and batch-to-batch composition differences affect the reproducibility of experimental outcomes and comparative studies [4, 9].

Emerging approaches in biomaterial sciences and regenerative medicine have focused on the development of synthetic hydrogels that mimic the key features of natural extracellular microenvironments due to flexible biochemical and biophysical characteristics [23-26]. As provisional matrices and drug delivery platforms in biomaterial-based regenerative medicine, these biomimetic hydrogels have demonstrated that they can be equipped with specific biological functionalities that influence cellular performance *in vitro* and *in vivo* [27-29]. In the context of cancer biology, cell–ECM interactions are fundamental to cancerogenesis and related signalling events [30-32]. The possibility of re-creating controlled extracellular microenvironments, characterised by cell-integrin binding sites and susceptibility to proteolytic remodelling, are key features of synthetic hydrogels that can be exploited to study cancer cell–ECM interactions. Such synthetic and defined matrices can overcome the limitations of naturally derived matrices. Hence, these attributes make biomimetic hydrogels an exciting alternative to the currently used 3D systems [26, 33, 34].

Specifically, we used synthetic hydrogels that are formed from peptide-functionalised multiarm polyethylene glycol (PEG) macromolecules *via* the factor XIII (FXIII)-catalysed cross-linking mechanism, a reaction occurring during fibrin clot formation in natural wound healing. Desired biological functions can be conferred on these matrices through stable and specific incorporation of peptides (*e.g.* the Arg-Gly-Asp (RGD) integrin-binding motif), and other proteins (*e.g.* growth factors) by means of the same cross-linking reaction and simultaneously with the gel formation [35, 36]. The sensitivity of these matrices to degradation by cell-secreted/activated proteases (*e.g.* matrix metalloproteinases (MMP)), can be precisely controlled by designing

MMP substrates within the hydrogel network [28, 29]. The matrix stiffness can be regulated by changing the polymer dry mass of the hydrogel, without changing their biological and biochemical characteristics [29, 36].

The aim of our study was to provide a proof of concept that this synthetic 3D platform offers a versatile cell culture model to analyse interactions crucial in cancer progression and anti-cancer drug resistance. We have hypothesised that the malleability of the hydrogel characteristics, in contrast to natural gold standard gels (*e.g.* Matrigel^{TM}), may be exploited to dismantle complex cell–ECM interactions of cancerogenesis *in vivo* into more simple and defined questions trackable in an *in vitro* setting. We used two cell lines as cancer cell models – OV-MZ-6 [37] and SKOV-3 [38] – both were derived from peritoneal ascites (fluid that surrounds the cancer in the abdominal cavity) of human epithelial ovarian carcinoma (EOC), an aggressive form of ovarian cancer typically diagnosed at an advanced stage. At this late disease stage, chemotherapy resistance occurs, for reasons as yet unknown [39]. The ability of both cancer cell lines, embedded within these synthetic hydrogels, to grow multicellular spheroids from single cells and their resistance to anti-cancer drugs compared to cell monolayers grown on conventional 2D plastic surfaces [40, 41] were investigated. The influences of extracellular microenvironment characteristics, achieved by systematic and independent alteration of the physical, biological and biochemical properties of these synthetic matrices, on the behaviour of the EOC cells cultured in 3D were determined.

2. Materials and methods

2.1. Abbreviations

CLSM, confocal laser scanning microscopy; EOC, epithelial ovarian carcinoma; FXIII, factor XIII; MMP, matrix metalloproteinase; mab, monoclonal antibody; PFA, paraformaldhyde; PBE, phosphate-buffered EDTA; PEG, polyethylene glycol; RGD, Arg-Gly-Asp; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

2.2. Materials

Cell culture reagents, rhodamine415-conjugated phalloidin, Alexa488-conjugated anti-mouse IgG, DAPI, PCR reagents, CyQuant® and AlamarBlue® assays were from Invitrogen. Amino acids, PFA, porcine gelatin, Brilliant Blue R, BSA, Sigmacote®, paclitaxel and monoclonal antibody (mab) anti-keratin 18 (#KS-B17.2) were from Sigma-Aldrich. Mab anti-keratin 8 (#M20) and anti-vimentin (#V9) were from Abcam. MMP-inhibitor GM6001 [Ilomastat], mab anti-α3-integrin (#P1B5) and anti-β1-integrin (#P5D2) were from Chemicon. Recombinant MMP9 and triton X-100 were from Calbiochem. Bradford™ assay and Brij-35 detergent were from Pierce. Reagents for SEM and TEM were from ProSciTech. RNeasy micro kit was from Qiagen. SYBR® Green PCR master mix was from AB Applied Biosystems. Mab anti-E-cadherin (#HECD-1) was from Zymed Laboratories Inc. MMP9 activity assay was from GE Healthcare.

2.3. Cell lines

The human epithelial ovarian cancer (EOC) cell line OV-MZ-6 was established from malignant ascites and cultured as reported previously [37]. The human epithelial ovarian serous adenocarcinoma cell line SKOV-3 derived from ascites fluid was obtained from American Type Culture Collection (ATCC) and cultured as recommended [38].

2.4. Preparation of PEG-based hydrogels for 3D cell cultures

Polyethylene glycol (PEG)-precursors for factor XIII (FXIII)-catalysed hydrogel formation was engineered as reported previously [35, 36]. Hydrogel networks were formed in Tris-buffered saline buffer (TBS; 50 mM Tris-HCl, 50 mM $CaCl_2$, pH 7.6) by thrombin-activated factor XIII (10.7 U/mL) cross-linking reaction of two precursors having complementary FXIII-substrates: i) PEG-Gln produced with 8-arm PEG (40kDa) end-functionalised with Gln-containing FXIII-substrate (NQEQVSPLER*C*G); ii) PEG-MMP-Lys formed from 8-arm PEG (40 kDa) end-functionalised with the Lys-containing FXIII-substrate and *MMP-sensitive* site for proteolytic degradation of final hydrogels (FKGG*GPQG↓IWGQ*ER*C*G) [29]. To form non-degradable hydrogels, the precursor 8-PEG-MMP-Lys was replaced by 8-PEG-noMMP-Lys in which the MMP-degradable substrate was substituted by a *MMP-insensitive* sequence (FKGG*GDQGIAGF*ER*C*G). Simultaneously, by means of the same reaction, the bio-functional peptide *RGD*, (NQEQVSPL-G*RGDSP*G, 50 μM), was stably incorporated into hydrogel networks. Cross-linking reactions were performed in sterile humidified atmosphere for 35 min at 37°C/5% (v/v) CO_2.

Biomaterial stiffness was tuned using different polymer dry mass percentages (1.5/2.0/2.5% (w/v)) corresponding to different physicochemical properties (elastic shear modulus G'=241±19/637±93/1201±121 Pa) as described previously [42].

Human EOC cells were encapsulated within PEG-based hydrogel precursor solution ($3.5x10^5$ cells/mL). Immediately thereafter and prior to cross-linking, 20 µL of hydrogel precursor solution was sandwiched between two sterile, Sigmacote® pre-treated, hydrophobic glass slides within 1.5 mm spacers. After polymerization at 37°C/5% (v/v) CO_2 for 35 min, hydrogel discs were removed from glass slides and immersed in culture media in 48-well plates. 3D cell cultures were performed over 12 days in 300 µL culture media which was replaced every 3 days. For protease activity studies, the 3D hydrogels were then washed in phosphate-buffered saline (PBS) and further treated for 1 day with serum-free media prior to a fresh 1 day serum-free media collection for analysis.

2.5. Detection of 2D/3D cell proliferation using CyQuant® and AlamarBlue® assays

Three $x10^4$ cells in 100 µL culture media per well ($8.3x10^3$ cells/cm^2) were cultured in 2D in 96-well plates for 1-5 days or in 3D for 1, 4, 12 days, washed in PBS and frozen at -80°C until assayed. Preparation of the 3D samples for CyQuant® assays was performed as described earlier [42]. Briefly, 3D samples were digested with ProteinaseK (0.5 mg/mL) diluted in phosphate-buffered EDTA (PBE, pH 7.1) at 60°C overnight. Subsequently, 3D samples were treated with RNaseA (1.4 units/mL) for 1 hr at room temperature (RT) in cell-lysis buffer supplemented with NaCl (180 mM) and EDTA (1 mM). Measurements of the DNA content by CyQuant® assays were

performed as reported previously [43] and fluorescent signals (excitation 485 nm, emission 520 nm) detected using a plate reader (BMG PolarStar). The provided λ DNA standard (10 ng to 2 μg/mL) was applied to calculate the n-fold change of DNA content per 2D/3D condition.

For AlamarBlue® assays, $3.0x10^4$ cells were cultured as above prior to the addition of the AlamarBlue® reagent (2D - 10% (v/v), 3D - 4% (v/v)) [43] and fluorescent signals (excitation 544nm, emission 590nm) detected as above. For each condition, three different biological assays were performed in triplicate.

2.6. Cell survival post paclitaxel treatment

One day after seeding of 2D cultures on 96-well plates, cells were treated with paclitaxel (0, 0.01, 1, 10, 100 nmol/L) for 3 days. Additionally, 2D monolayers treated for 3 days were cultured for another 3 days in fresh media. 7 or 14 days after 3D culture, spheroids were treated with paclitaxel (10 nmol/L) for a further 7 or 14 days. Cell spheroids grown for 7 days and treated for 7 days were also cultured for another 7 days in fresh media and CyQuant® and AlamarBlue® assays performed as described above. Cell survival was calculated using DNA content/metabolic activity as a percentage of fluorescence in non-treated cells.

2.7. Confocal laser scanning microscopy (CLSM) of 2D/3D cultures

EOC cells were grown to 80% confluent monolayers on sterile coverlips in 24-well plates. Samples were washed in PBS, fixed and permeabilized with 4% (w/v) paraformaldehyde (PFA)/PBS containing 0.2% (w/v) saponin for 30 min at RT. Then,

samples were washed once in glycine buffer (0.1 M glycine in PBS) followed by two washes in PBS and blocked in 1% (w/v) bovine serum albumin (BSA)/PBS for 30 min at RT followed by incubation with primary (keratin 8 (1/100), keratin 18 (1/500), E-cadherin (1/200), vimentin (1/200), α3 (1/500) and β1 integrins (1/500)) and secondary antibodies (Alexa488-conjugated anti-mouse IgG (1/1,000)) in 1% (w/v) BSA/PBS each for 1 hr at RT. The secondary antibody only served as a negative control. F-actin filaments and nuclei were stained with 0.3 U/mL rhodamine415-conjugated phalloidin and 2.5 μg/mL 4'-6-diamidino-2-phenylindole (DAPI) respectively in 1% (w/v) BSA/PBS. Immunofluorescence was visualized and photographed using a confocal microscope (SP5, Leica).

After 12 days of 3D culture, F-actin filaments and nuclei were immunofluorescence stained and imaged by CLSM as described above except that 0.2% (v/v) triton X-100 and 0.8 U/mL rhodamine415-conjugated phalloidin was used. Integrins were stained using mab directed to α3 (1/500) and β1 integrins (1/500) followed by a secondary Alexa488-conjugated anti-mouse IgG (1/1,000). Confocal images of cells spheroids were recorded using a motorized upright on a Leica SP5 CLSM. Typically z-stacks were acquired with a constant slice thickness of 2 μm reconstructing a cross-section profile of approximately 100-150 equidistant XY-scans using the Leica Microsystems LAS AF software (version 1.8.2 build 1465). For each condition, EOC cells grown within 3D hydrogels were visualized at three different positions and repeated three times.

2.8. Software analyses of CLSM images

To quantify the distribution pattern of cell clusters, cluster number and volume, 3D CLSM images were quantitatively analysed using previously described methods [44-46]. Single cell volumes, single cells grown as 3D cultures within PEG-based hydrogels imaged on day 1 by CLSM, were used to determine an appropriate threshold for image data to retain essential information and remove rogue fluorescence signals. A threshold of 1% was shown to retain the structures and give the optimal correlation with the nominal cell volume. One-to-one agreement of cell volumes is not possible, since CLSM introduces a significant aberration in the Z-direction. For image processing and evaluation, 2D raw data was converted to 3D greyscale data for both DAPI and rhodamine phalloidin channels. The data was segmented at 1% and then a filter was applied to remove noise-related clusters from the data (clusters > 1000 voxels). The *total number of clusters* and individual *cluster sizes* (μm^3) for a data set were then calculated, and from this data, *total cluster volume* and *average cluster size* in the image (by dividing total volume with total number of clusters) was measured.

2.9. Time-lapse microscopy of 3D cultures

3D hydrogels were glued onto 24-well plates with 5% (w/v) hydrogel solution and survival and formation of cell spheroids live imaged using a widefield microscope (Leica, AF6000 LX) under sterile humidified atmosphere at 37°C/5% (v/v) CO_2 over 6 days. Images were taken every hour. For each condition, EOC cells grown within 3D hydrogels were visualized at five different positions and repeated three times.

2.10. Scanning electron and transmission electron microscopy (SEM/TEM)

After 12 days of 3D culture, hydrogels were transferred into 24-well plates, washed, fixed (3% (w/v) glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.4, 0.4 M sucrose osmotically adjusted in cacodylate buffer, calcium dichloride) and stored at 4ºC. On analysis, samples were washed (0.1 M sodium cacodylate buffer, twice for 10 min) and post-fixed in Dalton's fixative (1% (w/v) osmium tetroxide in potassium dichromate), and stepwise dehydrated (70% ethanol, twice for 10 min; 90% ethanol, twice for 10 min; 100% ethanol, twice for 15 min; 100% amyl acetate, twice for 15 min). Samples were critical point dried (Denton Vacuum critical point drying apparatus; Moorestown), coated with a thin layer of gold (BIORAD SC500 gold coater; Hercules) and imaged on a FEI Quanta 200 SEM (Hillsboro) operating at 10 kV.

After 12 days of 3D culture, hydrogels were fixed and post fixed as for SEM, with an additional treatment for 30 min in 1% uranyl acetate. Post dehydration in ascending series of ethanol and acetone (50% ethanol, twice for 15 min; 70% ethanol, twice for 15 min; 90% ethanol, twice for 15 min; 90% acetone, twice for 20 min; 100% acetone, twice for 30 min) samples were embedded in Spurr epoxy resin. Ultrathin sections (~ 50nm) were cut (RMC MT7 ultramicrotome), stained with uranyl acetate and lead citrate, then imaged and photographed with a 1200EX TEM (JEOL) operating at 80 kV.

2.11. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (RT-qPCR)

RT-PCR analysis was performed as described previously [47]. Equal amounts of total RNA from cells cultured in 2D monolayers (extracted using Trizol) and as cell spheroids (extracted using an RNeasy micro kit) were treated with DNase 1, reverse transcribed using random hexamers and SuperScript III to prepare first strand cDNA samples. Gene specific primers used are listed in Supplementary Table 1. PCR for 18S served as internal control; PCR without cDNA for each primer as negative control. PCR products were electrophoresed on a 2% (w/v) agarose gel and visualized by ethidium bromide staining using a gel documentation system (Syngene). RT-qPCR was performed as described previously [47] using cDNA samples (1/10) generated above. Gene specific primers used are listed in Supplementary Table 1. RT-qPCR was performed in triplicate using SYBR® Green chemistry on an ABI7300 thermal cycler (Applied Biosystems) according to the manufacturer's instructions. The relative standard curve method was used to quantify gene expression levels; CT values for respective samples were fitted onto a standard curve to obtain respective expression levels. Final gene expression data were reported after normalisation to that of 18S RNA.

2.12. MMP-inhibition, quantitative MMP9 activity assay and zymography

Three $x10^4$ cells in 100 µL culture media per well ($8.3x10^3$ cells/cm^2) were cultured in 2D on 96-well plates for 1-5 days. After 1 day culture, culture media was supplemented with 20 µM MMP-inhibitor GM6001 [Ilomastat] and replaced after 2 days with fresh media with or without 20 µM MMP-inhibitor supplemented for another 2 days. After a total culture period of 5 days, CyQuant® assays were performed as described above. Similar inhibitory studies followed by CyQuant®

assays were performed in 3D over 12 days and culture media containing 20 μM MMP-inhibitor replaced every 3 days. Three different biological assays were performed in triplicate.

To quantify endogenous (pro-form) and total (pro+active) MMP9 levels of cells grown in 2D as monolayers and in 3D as cell spheroids, the biotrak activity assay was performed according to the manufacturer's instruction employing 100 μl of sample. To measure levels of MMP9, serum-free conditioned media was diluted (1/10). MMP9 activity was calculated by subtracting endogenous (pro-MMP9) levels from total (pro+active) levels.

Gelatine zymography using serum-free conditioned media was performed as previously reported [48]. Total protein concentrations were determined using a Bradford™ assay kit according to manufacturer's instructions with BSA standards. Equal amounts of protein (5 ng/mL) and recombinant MMP9 (20 ng/mL) were electrophoresed on 8% (v/v) polyacrylamide gels containing 1 mg/mL porcine gelatine under non-reducing conditions. Gelatinolytic activities were detected at 37 °C for ~20 h and visualized by staining with 0.25% (w/v) Brilliant Blue R.

2.13. Statistical analysis

Statistical analyses were carried out using an ANOVA and Student's t-test with the software 'R' version 2.10.0. Results for all analyses with 'P' value less than 0.05 were considered to indicate statistically significant differences (* - $P<0.05$; ** - $P<0.01$; *** - $P<0.001$).

3. Results

3.1. Cell spheroid formation within biomimetic hydrogels

To replicate the *in vivo* behaviour of the EOC cell lines OV-MZ-6 and SKOV-3, we used synthetic hydrogels displaying key features of the natural ECM (*e.g.* cell-integrin binding sites and degradability by cell-secreted/activated proteases) as a 3D culture system and compared this to conventional monolayer cultures in 2D. EOC cells embedded within hydrogels formed cell spheroids, similar to those found in ascites fluid accumulated in the peritoneal cavity of patients diagnosed with advanced ovarian cancer [40, 49], whereas EOC cells cultured on traditional 2D culture plastic formed typical monolayers (Fig. 1A, B). Both EOC cell types had similar 8-fold increased DNA content when grown in 2D after 5 days. However, OV-MZ-6 (but not SKOV-3) cells grown as spheroids within hydrogels showed a similar proliferation rate to that seen in 2D which was significantly enhanced after 12 days (23-fold increased DNA content; 5-fold SKOV-3; Fig. 1C). Both cell lines expressed a similar pattern of epithelial (keratin 8/18, E-cadherin) and mesenchymal (vimentin) markers detected at both the mRNA and protein level (Fig. S1), thus indicating that these proliferative profiles reflect other more subtle differences than morphological changes. Time-lapse microscopy of live spheroid survival and formation of both cell lines over 6 days revealed that multicellular spheroids were formed from single cells (Supplementary movies A, B). Further investigation of differences between 2D and 3D cultures revealed that mRNA expression of the cell surface receptors *α3/α5/β1* integrins and the protease *MMP9* were significantly increased in 3D compared to 2D (Fig. 1D). Multicellular spheroid formation was also analysed by SEM depicting the

development of lamellipodia and ECM in 3D representative for the *in vivo* behaviour of EOC cells (Fig. 1E-H).

3.2. The effect of matrix stiffness on cell spheroid formation

Matrix stiffness has been shown to influence cell behaviour in 2D [50] and 3D [22, 28, 29]. Exploiting the design flexibility characteristics of our 3D culture platform, we studied the effect of altered matrix stiffness on the response of EOC cell spheroids in 3D. Stiffness of hydrogels was varied as a function of polymer dry mass and decreased from 2.5% (w/v) to 1.5% (w/v) corresponding to the mechanical properties decreasing from 1201±121 Pa to 241±19 Pa. Cell spheroids grown in 3D proliferated less within stiffer (G'=1201±121 Pa) hydrogels. This effect was more pronounced in RGD-functionalised hydrogels (Figure 2A). 3D reconstructions of CLSM images visualized differences in the shape of cell spheroids: irregular and scattered cell spheroids were formed in softer (G'=241±19 Pa) hydrogels, compact and smaller cell spheroids grew in stiffer hydrogels (Fig. 5B). Quantitative cluster analyses showed significantly more smaller/medium clusters (5-10/10-15x10^4 μm^3) in stiff RGD-functionalised hydrogels in contrast to more abundant larger clusters (>20x10^4 μm^3, P=0.07) measured in soft hydrogels (Fig. 2C, D). No significant effect was observed in non-RGD-functionalised hydrogels, only a trend towards smaller clusters (<5x10^4 μm^3, P=0.08) in stiffer hydrogels (Fig. S3A). A medium polymer dry mass of 2.0% (w/v) corresponding to G'=637±93 Pa was chosen for the analysis of the biological and biochemical characteristics of hydrogels.

3.3. The influence of integrin engagement on cell spheroid formation

Since integrin cell adhesion and signalling receptors play crucial roles not only in chemoresistance but also in cell spheroid formation, we investigated this aspect by comparing the behaviour of EOC cells cultured within hydrogels with and without the incorporated cell-integrin binding sequence RGD [35, 36, 49, 51, 52]. Non-RGD-functionalised hydrogels led to a 13-fold change of DNA content of OV-MZ-6 cells spheroids after 12 days. Upon RGD-functionalisation (50 μM) of hydrogels cell spheroids proliferated up to 23-fold (Fig. 3A). The DNA content of SKOV-3 cell spheroids also increased 50% when grown within RGD-functionalised hydrogels (Fig. S3B). 3D reconstructions of CLSM images confirmed differences in cell growth with enhanced spheroid formation in RGD-functionalised hydrogels compared to hydrogels without RGD peptides (Fig. 3B). Immunofluorescent staining of α3/β1 integrins illustrated a higher expression in cell spheroids grown in RGD-functionalised hydrogels than in 2D (Fig. 3C). Quantitative cluster analyses demonstrated significantly enhanced cluster number upon RGD-incorporation compared to hydrogels without RGD peptides, but no significant difference in cluster volume and size (Fig. 3D, E).

3.4. Importance of cell-mediated matrix remodelling in 3D

The main feature of our 3D system is the possibility of modulating its characteristics flexibly and independently. Hence, we varied the biochemical properties of hydrogels, (*i.e.* their sensitivity to cell-secreted MMPs [28, 29, 36]) to assess the cell proteolytic machinery and to study its implication in ECM remodelling and consequent cancer progression [36, 48, 53, 54]. The proliferation of EOC cells within MMP-sensitive

hydrogels was 50% enhanced compared to MMP-insensitive hydrogels. Similarly, the growth of cell spheroids within MMP-sensitive hydrogels was significantly reduced by inhibition of MMP activity through a broad-spectrum MMP-inhibitor (GM6001, 20 μM) added to the culture media (Fig. 4A). Control experiments using the same MMP-inhibitor in 2D when cells were grown as monolayers showed no effect on proliferation (Fig. S3C). 3D reconstructions of CLSM images depicted the influence of MMP-sensitivity of hydrogels and MMP-inhibition on cell spheroid formation (Fig. 4B). Quantitative cluster analyses revealed that MMP-sensitivity had a significant effect on total number but not volume of clusters whereas MMP-inhibition led to both smaller ($<5x10^4$ μm^3) and fewer clusters (Fig. 4C). Without MMP-inhibition of MMP-sensitive hydrogels, significantly more medium/large clusters (<10-$15/>20x10^4$ μm^3) were formed. A significant number of larger clusters ($>20x10^4$ μm^3) were formed in MMP-insensitive hydrogels compared to MMP-sensitive hydrogels (Fig. 4C), confirmed by histograms including density plots (Fig. 4D). SEM analyses showed a more ordered or structured growth of cell spheroids in MMP-sensitive hydrogels and that MMP-inhibition led to smaller colonies and cell debris (Fig. S3F).

Total MMP9 production in conditioned media of cell spheroids in 3D, measured by a quantitative activity assay, was decreased independent from RGD-incorporation within hydrogels compared to cell monolayers in 2D. Endogenous (pro-)MMP9 levels did not change upon 3D culture (Fig. S3D). Zymography confirmed pro-MMP9 in conditioned media of cell spheroids grown within hydrogels independent from their MMP-sensitivity. The pro-MMP9 level was reduced after MMP-inhibition (Fig. S3E).

3.5. Chemoresistance of cell spheroids within biomimetic hydrogels

Due to the long-term stability of hydrogels (28 days; Fig. S2), we sought to examine the chemoresistance of EOC cells grown as spheroids towards clinically used anti-cancer drugs like paclitaxel. Paclitaxel is a microtubule-stabilizing agent mediating cell cycle arrest and apoptosis [55] that is used for treatment of advanced ovarian carcinomas. Treatment of monolayers of OV-MZ-6 cells (and SKOV-3 cells - data not shown) in 2D with paclitaxel (0.01-100 nmol/L) for 3 days inhibited cell survival and proliferation dramatically (Fig. 5A). A concentration, at which 20% survival of cell monolayers (10 nmol/L) was reached, was used to treat cell spheroids grown in 3D. Cell survival and proliferation, as measured by DNA content or metabolic assays (data not shown) in spheroids grown for 7/14 days prior to 7/14 days paclitaxel treatment, was significantly reduced by 40-60% (Fig. 5B). 3D reconstructions of CLSM images depicted reduced cell spheroid formation upon treatment with paclitaxel (Fig. S4A). Quantitative cluster analyses of CLSM images revealed formation of significantly larger clusters (>$20x10^4$ μm^3) in non-treated samples, compared to paclitaxel-treated conditions, in which clusters remained mostly small (<$10x10^4$ μm3; Fig. 5C, left panel). Total cluster numbers increased significantly upon treatment for 7 days (Fig. 5C, right panel). Histograms including density plots illustrated more and smaller clusters after treatment compared to non-treated clusters (Fig. S4B). Further studies on the effects of paclitaxel treatment of 3D cultures revealed that *α3/α5/β1* integrin and *MMP9* expression was significantly increased after treatment compared to non-treated conditions (Fig. 5D).

Ultrastructural differences of cell spheroids upon paclitaxel treatment were detected by TEM. No major morphological changes were observed in 3D compared to 2D cultures of OV-MZ-6 cells [37]. OV-MZ-6 cells within spheroids were partially

separated by intercellular spaces bridged by cytoplasmic interdigitations and hemidesmosomes. The cytoplasm was rich in mitochondria and ribosomes, whereas rough endoplasmatic reticulum and fat droplets were rarely observed. The nuclei were moderately irregular in shape and encompassed over 50% of the cell organelle. Spheroids of OV-MZ-6 cells (and SKOV-3 cells - data not shown) within hydrogels showed compact cell organisation with large lysosomes and were mostly mitotic, only a few were apoptotic. Upon paclitaxel treatment, mainly necrotic areas and smaller cell spheroids were found (Fig. 5E).

4. Discussion

4.1. Third dimension in cell cultures

Matrix-based 3D *in vitro* culture models are increasingly becoming essential tools in cancer research as they allow cell responses that more closely mimic events occurring *in vivo* during cancer formation and progression. They provide a pathophysiological context that more accurately replicates solid cancer microenvironments compared to monolayer cultures in 2D [1, 2, 9, 10]. In this context, we first examined the behaviour of two EOC cell types in 2D on conventional culture plastic, and in 3D within novel synthetic biomaterials. These matrices can be functionalised to include key features of naturally derived ECMs (*e.g.* the interaction with cells *via* integrin binding sites and the ability of proteolytically remodelling by cell-mediated processes). Similar to reported studies on epithelial cancer cells in 3D [15, 17] and *in vivo* observations of ovarian cancer growth [49, 56], single EOC cells embedded within hydrogels grew as multicellular spheroids. In contrast, in 2D, on traditional plastic surfaces, they formed cell monolayers (Fig. 1A, B).

Intriguingly, both EOC cell lines displayed similar growth profiles in 2D, but in 3D they differed significantly. OV-MZ-6 cells grown in 3D as spheroids proliferated more than spheroids of SKOV-3 cells (Fig. 1C). Hence, 2D cultures approached confluence within the monitored time frame over 5 days and decreased proliferation, while slower growing cell spheroids continued to grow for 12 days (Fig. 1C) up to 28 days (Fig. S2). Due to dramatic differences in cell organization resulting from culture conditions in 2D and 3D, with 3D being more close to that observed *in vivo*, it is

questionable whether plastic-based cultures may be selective enough to study cell behaviours.

4.2. Physiological aspects of 3D cell cultures

Despite the complex variables involved in the transition from 2D to 3D, the additional dimensionality of 3D cultures compared to 2D cultures is a crucial feature leading to differences in cell responses. This extra dimensionality not only influences the spatial organization of cell surface receptors engaged in interactions with surrounding cells (*e.g.* the development of lamellipodia (Fig. 1E, F)) and ECM [8, 52], but also induces the physical constraint experienced by cells in 3D [22, 28, 29]. These spatial and physical aspects affect the signal transduction from the outside to the inside of cells, and ultimately have a profound influence on gene expression and cell behaviour [8]. Interestingly, these cell responses have been shown to occur similarly *in vivo* and in 3D rather than in 2D [40, 57, 58]. Within 3D matrix adhesions enhanced cell function compared to 2D (*e.g.* the involvement of α3/α5/β1 integrins and MMP9) was detected (Fig. 1D). Elevated integrin expression in EOC cell spheroids was also reported earlier and underlines the physiological relevance of 3D models [49, 51].

The more physiological 3D microenvironment caused not only formation of cell spheroids (diameter >50 μm), mimicking the peritoneal ascites where EOC cells exist as individual cells or aggregate to multicellular spheroids (diameter 50-750 μm) [49], but also an increase in growth rates of both EOC cell types (Fig. 1C; S2). The presence of keratins 8/18 supports their epithelial nature (Fig. S1). 'Partial or incomplete epithelial-mesenchymal transition (EMT)' is a common feature in EOC displaying a dysregulated adhesion phenotype characterized by loss of epithelial (E)-

cadherin [59]. The EOC cells used here already have a mesenchymal phenotype as noted by vimentin expression (Fig. S1). This expression concurs with reports that serous ovarian carcinomas express vimentin *in vivo*, and is a characteristic of a limited spectrum of these carcinoma types [37].

4.3. Tunable 3D models for cell–ECM interactions

The transition from 2D to 3D cultures represents a landmark step in cancer research towards replicating cell events occurring *in vivo*. Various 3D studies have shown that specific cell–matrix interactions influencing cancer cell behaviour and cancer development [4, 52, 60, 61]. Despite hallmark outcomes obtained using current gold standard 3D models, there is a need for 3D systems that are more reliable, reproducible and possess controllable characteristics [2, 4, 9, 25]. In our study, we used a synthetic hydrogel-based 3D platform that offers high design flexibility of natural matrix microenvironments. We focused on replicating three key features of the ECM implicated in interactions between cancer cells and their microenvironment: i) matrix stiffness, ii) display of cell-integrin binding sites, and iii) ability to undergo cell-mediated proteolytic degradation. To our knowledge, this is the first study which explores systematically the influence of these key features of extracellular microenvironments on epithelial cancer cells cultured in 3D. In contrast to naturally derived matrices [17, 22, 58] and (semi)-synthetic biomaterials [52, 62], each of these hydrogel characteristics can be manipulated independently.

Contrary to studies using ‘overlay 3D cultures’ [22, 31, 51] or ‘3D on top assays’ [17], we used 3D cultures where cells were embedded within an anisotropic biomimetic hydrogel formed from liquid precursor solutions containing

homogenously dispersed single EOC cells. Consequently, cells are exposed in all directions to similar biomechanical and biochemical stimuli. Since functions of matrix-embedded cells are dependent on surrounding microenvironments, we sought firstly to modulate the biomaterial stiffness [35, 36, 42]. Unlike cells grown within a 3D matrix or in tissues, cells cultured on plastic are attached to essentially rigid materials. Similarly, the stiffness of hydrogels altered 3D adhesions, shape and proliferation. With increasing material stiffness cell spheroid formation and size were reduced. 3D reconstructions visualized irregular and scattered cell spheroids formed in softer hydrogels; compact and dense cell spheroids in stiffer hydrogels (Fig. 2). Our results underpin published data showing morphological asymmetry and growth differences. Increased matrix concentrations inhibited cell spheroid growth and raised cell density due to enhanced resistance posed by the matrix and higher density of MMP-substrates recognized by cells, rather than being caused by limitations of nutrients or waste removal diffusion [21, 50]. Interestingly, t~~T~~hese data are different from another~~that of one~~ study that showed larger~~increased~~ cell spheroid formation with increased matrix stiffness [22]. These differences could be ~~However, one should be aware that comparisons between some 3D studies is difficult~~ due to the ~~use of~~ different 3D culture techniques employed (*e.g.* type of matrices, overlay or on top vs. matrix-embedded cell cultures)~~, cell lines and experimental approaches (e.g. culture time, biomaterial stiffness)~~. Additionally, in contrast to the biomimetic PEG-based hydrogels described here, with the use of naturally derived matrices due to their inherent characteristics, it is often challenging to investigate the influence of the biological and mechanical characteristics independently and separately. In general, c~~C~~ellular sensing towards biomaterial stiffness was reported to be consistent with a

role for signalling like tyrosine phosphorylation, but molecular mechanisms involved remain unknown [50].

4.4. Specific integrin activation

Cell-integrin engagement and interactions with ECM molecules in 3D are essential in regulating cancer development and growth [8]. We explored the involvement of integrins in cell spheroid formation in 3D by specific functionalization of hydrogels with the prototypic cell-adhesion motif RGD. This cell integrin-binding has proven ability to impact on function of various cancer cells [52], including EOC cells [63]. Proliferation of EOC cells was significantly enhanced in RGD-functionalised hydrogels (Fig. 3A). In contrast to other cell types (*e.g.* fibroblasts [36, 42] and oral squamous carcinoma cells [52]), EOC cells proliferated and formed significantly more medium-sized clusters within non-RGD-functionalised hydrogels (Fig. 3D). This suggests that EOC cells are producing their own ECM (Fig. 1G, H) (*e.g.* vitronectin [64], fibronectin [56], collagen type I (data not shown)) and/or rely on cell–cell interactions for survival and proliferation of multicellular spheroids. Formation of cell-secreted ECM within spheroids reduces anti-cancer drug penetrance and enhances integrin-mediated pro-survival signalling [56].

Taken together these results not only strengthen the hypothesis that integrin activation is crucial in EOC progression [65, 66], but also open up new avenues to explore complex aspects of cancer cell–ECM interactions using this versatile 3D platform. Different cell integrin-binding domains singularly or in combination and/or various sequences of target biomolecules can be tested using a similar approach [67]. By integrating multiple cell types to generate pathologically more relevant cell–cell

interactions, the impact of cancer-associated stroma on spheroid formation and chemoresistance can be dissected. A 3D omental model developed by Kenny et al. [31] involves different cell types, ovarian cancer cells, mesothelial cells, and omental fibroblasts, implicated in the early steps of ovarian cancer metastasis by replicating a pathophysiologically cancer microenvironment. Such 3D organotypic models can then be used to examine expression and activity of cancer-associated proteases and their therapeutic inhibition [32, 54].

The biological mechanisms by which cell spheroids are formed and sustained as well as their integrin expression is poorly understood [51]. Inhibition of β1 integrin and MMP9 suppressed spheroid–ECM interactions in EOC cells [68, 69]. Hereby, interactions between α5β1 integrin and fibronectin mediate formation [49], whereas α2β1 integrin plays a role in disaggregation and invasion of EOC cell spheroids [51]. Expression of integrins and cancer-associated proteases differ in 3D compared to 2D [8] confirmed by our own data demonstrating significantly increased α3/α5/β1 integrins and MMP9 expression in 3D cultures compared to 2D cultures (Fig. 1D). The altered α3/β1 integrin levels are not in line with other studies because spheroids were derived from different EOC cell lines, grown on top of agarose-coated wells and/or due to another detection method being used [51]. The impact of altered stiffness of hydrogels was even more pronounced upon RGD-incorporation stimulating integrin-dependent growth (Fig. 3). Integrins act as mechanoregulators on cell fate upon changed matrix rigidity by promoting Rho and ERK activation [22].

4.5. Cell proteolytic machinery in 3D

Since the biodegradability of biomaterials influences survival and migration of cell spheroids, hydrogels sensitive to specific and localized cell-mediated proteolytic degradation by MMPs were generated [48, 53, 54]. Indeed, proliferation of cell spheroids within MMP-sensitive hydrogels was significantly enhanced compared to MMP-insensitive hydrogels and blocked by a broad-spectrum MMP-inhibitor (GM6001) leading to smaller spheroids (Fig. 4A-C, S3F). Cell growth in MMP-insensitive hydrogels was higher than in MMP-inhibited conditions. In MMP-insensitive matrices fewer cell spheroids were formed than in MMP-sensitive hydrogels (Fig. 4A). However, the fewer cell spheroids in MMP-insensitive hydrogels grew bigger than in non-inhibited MMP-sensitive matrices (Fig. 4B, C). Since MMP-inhibition is most likely not affecting or altering the cell proliferative behaviour (Fig. 4C, S3F), these differences might be caused by unspecific and slower degradation of MMP-insensitive hydrogels as previously observed with other cell types [28, 29]. Inconsistent with other reports, only pro-MMP9 was detected in cell spheroids, even less than in cell monolayers (Fig. 4D; S3C), due to the embedded nature of our 3D cultures whereas cell spheroids grown on top of agarose-coated assays secreted active MMP9 [51]. In 3D omental co-cultures MMP9 was expressed by mesothelial cells and fibroblasts and by ovarian cancer cells once they bound to the 3D co-culture [54]. To further investigate the involvement of MMPs, we incubated cell monolayers with the MMP-inhibitor. MMP-inhibition of 2D cultures had no effect on cell proliferation (Fig. S3C) indicating a role of MMPs in cell spheroid formation in 3D. This result is supported by a report blocking cell spheroid invasion to mesothelial monolayers using the same MMP-inhibitor [68]. These and our studies suggest that 3D models need to be carefully designed to mimic specific *in vivo* situations. Collectively, our data demonstrate the possibility of investigating separately different aspects of cell–matrix

interactions that are important in cancer growth and progression by exploiting the design flexibility of the matrix characteristics offered by these biomimetic hydrogels.

4.6. Chemoresistance of 3D cell cultures

Typically, flat surfaces, compared to tissues or 3D systems, force cells to adapt to abnormal microenvironmental conditions, which can then lead to altered cell responses [8, 70]. Consequently, this may result in 2D being less selective than 3D cultures in dissecting behavioural differences among cell types and in their sensitivity towards chemotherapeutics as reported in our 3D study. In fact, early multicellular models described influences of cancer micro-regions on the responsiveness to cytotoxic agents [11, 16]. Landmark 3D cancer studies are in line with this observation and demonstrated that phenotypical differences between normal and malignant epithelial breast cells were observed exclusively in 3D [2, 15, 30]. EOC cell spheroids revealed an enhanced resistance to chemotherapeutics, including paclitaxel and cisplatin [41, 47, 71].

Here, we report that paclitaxel treatment of EOC cells in 2D cultures inhibited cell survival and proliferation significantly (Fig. 5A, S4C). Cell survival and proliferation in 3D cultures after paclitaxel treatment was reduced by 40 or 60% in conditions where cell spheroids were grown for 1/2 weeks prior to 1/2 weeks treatment, respectively, while the same treatment led to 80% reduced cell viability of cell monolayers. Cells grown as spheroids develop multicellular-dependent chemoresistance (Fig. 5B, C) as observed *in vivo* [49, 56]. For EOC cells, it was demonstrated that paclitaxel triggered apoptosis in 2D accompanied with decreased

Bcl-XL expression while it did not induce cell death in 3D [41]. Bcl-2 expression was related to paclitaxel-induced apoptosis of 2D cultures but not in 3D [71].

The phenomenon of cell spheroids displaying elevated chemoresistance to anti-cancer reagents has been attributed to several mechanisms, including a decreased penetrance of anti-cancer drugs, increased pro-survival signalling, and/or upregulation of genes conferring drug resistance. In fibroblast-derived 3D matrices differential responses of epithelial cancer cells grown as multicellular spheroids to cytotoxic drugs were described. Paclitaxel treatment led to elevated integrin expression, as shown in our 3D cultures (Fig. 5D), and respective signalling [20] Therefore, 3D systems can be used as *in vitro* drug evaluation tools [20, 62, 72].

5. Conclusions

We provide evidence that this 3D hydrogel system offers well-defined and reproducible extracellular microenvironments for studying pathophysiological processes in cancerogenesis. Both EOC cell lines embedded within hydrogels grew as multicellular spheroids from single cells, mimicking ovarian cancer metastases *in vivo* where cells are shed from the primary cancer and aggregate as spheroids within the ascites in the peritoneal cavity. Changes in extracellular microenvironment characteristics achieved by the systematic and independent alteration of the stiffness, biological and biochemical properties of these synthetic matrices tangibly and specifically influenced the behaviour of EOC cells cultured in 3D. Additionally, these multicellular spheroids have shown higher resistance to anti-cancer drugs than cell monolayers grown in 2D. Thus, these design features render this 3D model an exceptionally versatile cell culture tool. Because of their controlled and reproducible properties, their ability to replicate natural microenvironments, and their stability in long-term cultures, these bioengineered 3D matrices are suited to study specific cell–matrix interactions implicated in cancer development as well as to screen anti-cancer drugs and chemoresistance.

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Figure legends

Figure 1. Differences in cell organisation and proliferation on conventional 2D plastic and in physiologically relevant 3D cultures.

A. Schematic illustration (modified from [7]) of EOC cells grown in 2D as monolayers (left) on traditional plastic surfaces and in 3D as spheroids embedded within hydrogels (right). **B.** Both EOC cell types – OV-MZ-6, SKOV-3 – formed in 2D (left) typical monolayers shown by phase contrast (top panel) and confocal (bottom panel; cell actin filaments stained with rhodamine phalloidin, nuclei with DAPI) microscopy images. In 3D, both EOC cell types embedded within hydrogels grew as spheroids imaged by phase contrast (top panel) and confocal (bottom panel, 3D reconstruction) microscopy. Scale bars, 75 μm. **C.** EOC cells proliferated similar over 5 days when cultured in 2D as monolayers. Grown in 3D as spheroids, OV-MZ-6 cells proliferated significantly more than SKOV-3 cells over 12 days; mean ± SEM, 4 experiments. **D.** Quantitative analysis of mRNA levels normalised to *18S* expression revealed significantly increased levels of *α3/α5/β1* integrins and *MMP9* in 3D compared to 2D (OV-MZ-6). **E.-H.** SEM analyses depicted multicellular spheroid formation of EOC cells (OV-MZ-6) within hydrogels. **F.** Cells within a spheroid were connected via the development of lamellipodia. **G./H.** The growth and anchorage of multicellular spheroids within hydrogels was supported by cell-secreted ECM. Scale bars, 20μm (F, G), 10μm (E, H). Statistical significance, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 2. Formation and proliferation of cell spheroids in 3D as a function of matrix stiffness~~polymer dry mass~~.

A. Proliferation of OV-MZ-6 cell spheroids within hydrogels was decreased with increasing biomaterial stiffness altered by variation of the mechanical properties. This effect was even more pronounced when hydrogels were RGD-functionalised; mean ± SEM, 3 experiments. **B.** 3D reconstructions of CLSM images illustrated differences in shape and cell spheroid formation upon altered hydrogel stiffness and RGD-functionalisation: formation of irregular and scattered cell spheroids was exclusively observed in softest (G'=241±19 Pa) hydrogels (lower panel). Scale bars, 100 μm. **C.** Quantitative cluster analyses showed more small/medium clusters in stiff (G'=1201±121 Pa) RGD-functionalised hydrogels and a trend towards larger clusters (P=0.07) in softer hydrogels. **D.** Histograms (grey columns) and density plots (black line) revealed total distribution of clusters; 3 experiments. Fewer and smaller clusters were formed in softer RGD-functionalised hydrogels. Statistical significance, ** P<0.01.

Figure 3. Incorporation of cell integrin-binding sites within hydrogels influences formation and proliferation of cell spheroids.

A. OV-MZ-6 cell spheroids proliferated significantly more within RGD-functionalised (50 μM) hydrogels (filled symbols) compared non-RGD-functionalised conditions (open symbols) over 12 days; mean ± SEM, 4 experiments. **B.** 3D reconstructions of CLSM images depicted differences in formation of cell spheroids upon RGD-functionalisation of hydrogels: more and larger cell spheroids were formed upon RGD-incorporation. Scale bars, 100 μm. **C.** Cell morphology between 2D and 3D cultures imaged by immunofluorescent staining by CLSM confirmed changed expression of α3/β1 integrins in monolayers (left panel; scale bars, 20 μm) and spheroids (right panel; scale bars, 100 μm). **D.** Quantitative cluster analyses

revealed significant formation of medium clusters in non-RGD-functionalised hydrogels (top panel). Total number of clusters was significantly enhanced upon RGD-incorporation within hydrogels compared to non-RGD-functionalised conditions. No difference in total cluster volume upon RGD-incorporation was detected (lower panel). **E.** Histograms (grey columns) and density plots (black line) illustrated total distribution of clusters; 3 experiments. Fewer clusters were formed in non-RGD-functionalised hydrogels. Statistical significance, ** $P<0.01$, *** $P<0.001$.

Figure 4. Formation and proliferation of cell spheroids in 3D as a function of matrix biochemical characteristics.

A. Proliferation of OV-MZ-6 cell spheroids within MMP-insensitive hydrogels was significantly reduced compared to MMP-sensitive hydrogels over 12 days. MMP-inhibition (GM6001, 20 μM in culture media) led to significantly decreased proliferation of cell spheroids within MMP-sensitive hydrogels; mean ± SEM, 3 experiments. **B.** 3D reconstructions of CLSM images depicted influence of MMP-sensitivity of hydrogels and MMP-inhibition on cell spheroid formation: more and larger cell spheroids were formed upon MMP-sensitivity; after MMP-inhibition fewer and smaller cell spheroids were observed. Scale bars, 100 μm. **C.** Quantitative cluster analyses revealed significant formation of smaller clusters upon MMP-inhibition and medium/large clusters in non-inhibited MMP-sensitive hydrogels. Significant larger clusters were formed in MMP-insensitive hydrogels compared to MMP-sensitive hydrogels (top panel). MMP-sensitivity and MMP-inhibition significantly influenced total number and volume of clusters: fewer clusters were formed in MMP-insensitive hydrogels and after MMP-inhibition, smaller clusters detected in MMP-inhibition of MMP-sensitive hydrogels (lower panel). **D.** Histograms (grey columns) and density

plots (black line) illustrated total distribution of clusters; 3 experiments. MMP-sensitivity of hydrogels led to more clusters compared to MMP-insensitive hydrogels; MMP-inhibition caused less and smaller clusters. Statistical significance, ** $P<0.01$, *** $P<0.001$.

Figure 5. Paclitaxel treatment of EOC cells grown in 2D as monolayers and in 3D as spheroids.

A. Survival of OV-MZ-6 cell monolayers in 2D as a function of paclitaxel concentration (0.01-100 nmol/L) showed a dramatic decline from 10nM; mean ± SEM, 4 experiments. **B.** Survival of OV-MZ-6 cells spheroids within hydrogels was significantly decreased after paclitaxel (10 nmol/L) treatment; however, to a lesser extent compared to cell monolayers at the same concentration; mean ± SEM, 3 experiments. **C.** Quantitative cluster analyses revealed significantly larger clusters in non-treated conditions; in paclitaxel-treated samples clusters remained mostly small. Upon paclitaxel treatment a significantly increased number and decreased volume of clusters was detected; mean ± SEM, 3 experiments. **D.** Quantitative mRNA analysis revealed significantly increased levels of *α3/α5/β1* integrins and *MMP9* upon normalisation to *18S* levels in cell spheroids in 3D upon exposure to paclitaxel. **E.** TEM analyses showed morphological differences of cell spheroids after treatment: without treatment defined cell structures and cell–cell contacts within spheroids were visible (N-nucleus, ER-endoplasmatic reticulum, AC-apoptotic cell, TJ-tight junctions), after treatment necrotic areas and smaller cell spheroids were detected (NEC-necrotic cell). Scale bars, overview 1 μm, zoom 0.2 μm. Statistical significance, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Supplementary figure 1. Characterisation of EOC cells grown in 2D as monolayers and in 3D as spheroids by morphological markers.

A. Expression of epithelial markers (keratin 8/18, E-cadherin) and mesenchymal markers (vimentin) were detected via RT-PCR, with 18S as loading control, in both OV-MZ-6 (top panel) and SKOV-3 (bottom panel) EOC cell types grown in 2D as monolayers and in 3D as spheroids. **B.** Immunocytochemistry confirmed the expression of epithelial and mesenchymal markers in both OV-MZ-6 (top panel) and SKOV-3 (bottom panel) EOC cell lines, sustained in adhesive culture over the course of this study, depicted as overlay staining; no primary antibody served as control (cell actin filaments stained with rhodamine phalloidin, nuclei with DAPI, morphological marker with respective primary and Alexa488-conjugated secondary antibodies). Scale bars, 50 μm.

Supplementary figure 2. Long-term culture and proliferation of cell spheroids in 3D grown within biomimetic hydrogels.

A. EOC cells were grown as spheroids within hydrogels up to 28 days and visualized by phase contrast microscopy. Scale bars, 100 μm. **B.** 3D reconstructions of CLSM images depicted shape and growth of cell spheroids embedded within hydrogels. Whereas OV-MZ-6 cells grew slightly less at 21 to 28 days in 3D culture, SKOV-3 cells formed large (diameter >300 μm) spheroids. Scale bars, 100 μm. **C.** When grown as spheroids, OV-MZ-6 cells reached their maximum proliferation after 12 days, whereas SKOV-3 proliferated less but continuously up to 28 days; mean ± SEM, 4 experiments.

Supplementary figure 3. Influence of RGD-incorporation and MMP-sensitivity of biomimetic hydrogels on formation of cell spheroids.

A. Quantitative cluster analyses revealed no difference upon altered polymer dry mass of hydrogels without RGD peptides, but a trend towards smaller clusters (P=0.08) in stiff (G'=1201±121 Pa) hydrogels. **B.** Proliferation of SKOV-3 cell spheroids was increased within RGD-functionalised (50 μM) hydrogels (filled symbols) compared non-RGD-functionalised conditions (open symbols) over 12 days; mean ± SEM, 4 experiments. 3D reconstructions of CLSM images depicted differences in formation of SKOV-3 cell spheroids upon RGD-functionalisation of hydrogels: more and larger cell spheroids were formed upon RGD-incorporation. Scale bars, 100 μm. **C.** MMP-inhibition of OV-MZ-6 cell monolayers grown in 2D had no effect on their proliferation; mean ± SEM, 3 experiments. **D**. Quantitative measurement of endogenous (pro) and total (pro+active) MMP9 production in conditioned media of OV-MZ-6 cell spheroids grown within MMP-sensitive hydrogels showed a reduction of total MMP9 independent from RGD-incorporation compared to cell monolayers. Endogenous MMP9 levels did not change upon 3D culture. Upon 2D culture minor amounts of active MMP9 were produced. **E.** Zymography confirmed pro-MMP9 in conditioned media of OV-MZ-6 cell spheroids within hydrogels independent from MMP-sensitivity. Pro-MMP9 level was reduced after MMP-inhibition (GM6001, 20 μM in culture media). **F.** SEM analyses confirmed changes of OV-MZ-6 cell spheroid formation upon MMP-sensitivity of hydrogels and MMP-inhibition. Scale bars, overview 500 μm (inserted panel), zoom 10 μm.

Supplementary figure 4. Effects of paclitaxel treatment on EOC cells grown as spheroids within biomimetic hydogels.

A. 3D reconstructions of CLSM images depicted less cell spheroid formation within hydrogels after treatment up to 28 days. Scale bars, 100 μm. **B.** Histograms (grey columns) and density plots (black line) illustrated total distribution of clusters; 3 experiments. Paclitaxel treatment led to more and smaller clusters compared to non-treated conditions.

Supplementary figure 5. Live cell imaging of survival and formation of cell spheroids in 3D cultures.

Time-lapse microscopy of live cell spheroid survival and formation of OV-MZ-6 (**A, movie**) and SKOV-3 (**B, movie**) cells cultured within hydrogels over 6 days revealed that multicellular spheroids were formed from single cells. Scale bars, 100 μm.

Tables

Supplementary table 1. Primers used for RT-qPCR and PCR conditions.

Annealing temperature used for all primers was 60°C.

Primers	Sequence (5'-3')	Cycles
α3 integrin forward	TACGTGCGAGGCAATGACCTA	40
α3 integrin reverse	TTTGGGGGTGCAGGATGAAGCT	
α5 integrin forward	CATTTCCGAGTCTGGGCCAA	40
α5 integrin reverse	TGGAGGCTTGAGCTGAGCTT	
β1 integrin forward	AGGTGGTTTCGATGCCATCAT	40
β1 integrin reverse	AAGTGAAACCCGGCATCTGTG	
MMP9 forward	TCGTGGTTCCAACTCGGTTT	40
MMP9 reverse	GCGGCCCTCGAAGATGA	
keratin 8 forward	CTGGGATGCAGAACATGAGTATTC	35
keratin 8 reverse	GTAGCTGAGGCCGGGGCTTGT	
keratin 18 forward	GAGACGTACAGTCCAGTCCTTGG	35
keratin 18 reverse	CCACCTCCCTCAGGCTGTT	
E-cadherin forward	GCCCATTTCCTAAAAACCTGG	35
E-cadherin reverse	TTGGATGACACAGCGTGAGAG	
Vimentin forward	TGGAAGAGAACTTTGCCGTTG	35
Vimentin reverse	AAGGTGACGAGCCATTTCCTC	
18S forward	GATCCATTGGAGGGCAAGTCT	35
18S reverse	CCAAGATCCAACTACGAGCTTTTT	

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