Culturing substrates influence the morphological, mechanical and biochemical features of lung adenocarcinoma cells cultured in 2D or 3D

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1. Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide (Ferlay et al., 2010), and it has one of the highest predicted incidence rates (Malvezzi et al., 2015). Despite the large research and economic investments, the survival rate of lung cancer patients remains poor due to the development of drug resistances (Tsverkova and Goss, 2012). Consequently, the development of highly effective treatments against lung cancer remains a vital area of cancer research. In the last years, however, around 95% of new anticancer drugs against lung cancer eventually failed in clinical trial (Arrowsmith and Miller, 2013; Hakanson et al., 2014; Kola and Landis, 2004; Landry et al., 2013). One of the major reasons for failure, accounting for around 60% of failed clinical trials, is the lack of efficacy in humans despite robust indications of activity in preclinical models (Arrowsmith, 2011; Hay et al., 2014; Kamb, 2005). Although the reasons for this poor clinical translation are multiple, it has been suggested that major contributors to such high failure rate may be attributed to the preclinical testing models used, which can indeed generate false-positive results in the evaluation of drug efficacy (Arrowsmith and Miller, 2013; Cook et al., 2014; Kamb, 2005; Smalley et al., 2006; Unger et al., 2014).

In conjunction to \textit{in vitro} two-dimensional (2D) cell cultures, genetically engineered mice, ectopic/orthotopic xenografts, and primary human tumourgrafts implanted into immunodeficient mice are the most common preclinical models used in drug development. An important feature of all tissues is that cells grow in three dimensions (Abbott, 2003; Smalley et al., 2006). Solid tumours are not an exception, and they are now recognized as complex tissues able to constantly instruct
and react to chemical and mechanical signals to/from neighbouring cells and the microenvironment (Egeblad et al., 2010). In vitro 2D cell models represent therefore simplified microenvironments mimicking the tumour tissue (Gillett et al., 2013). They indeed impose limitations in reproducing the cellular behaviour (e.g., division, proliferation, migration, differentiation) of cancer cells (Discher et al., 2005), as well as in reproducing the physical barriers hindering drug permeation into the tumour tissue (Poondu et al., 2002). It is believed that such limitations negatively impact upon the relevance of the efficacy data on novel chemotherapeutic agents generated during the preclinical studies (Landry et al., 2013; Rehföldt et al., 2007), and are also responsible for the poor in vitro-in vivo correlation of drug activities (Poondu et al., 2002). On the other hand, animal models, although indispensable in the discovery and development process of new cancer drugs, have various limitations as preclinical cancer models (Aparicio et al., 2015; Mak et al., 2014; Rangarajan and Weinberg, 2003; Ruggeri et al., 2014). Various systematic reviews describe in fact the limitations of animal research in the efficacy assessments of new drug candidates (Aparicio et al., 2015; Hackam, 2007; Hartung, 2008; Mak et al., 2014; Olson et al., 2000; Pount et al., 2004; van der Worp et al., 2010). Thus, alternative methods of producing pertinent representations of human solid tumours must be implemented in the preclinical phase to reliably test the response of lung cancer cells to specific drugs. Considering this and in agreement with the “3Rs (Reduction, Refinement and Replacement) principle” in animal research, alternative three-dimensional (3D) in vitro models and test platforms have been developed for application in lung cancer drug discovery (Nya et al., 2011; Vinci et al., 2012).

3D culture models can more accurately reflect the complex interactions of cancer cells with the surrounding microenvironment, not only on a biochemical and biomechanical level but also on the level of gene and protein expression (Friedrich et al., 2009). The pioneering work by Mina Bissell’s group was central in providing the early experimental evidence that moving cell cultures into 3D systems can make a difference in cancer research (Weaver et al., 1997). Thus, 3D cell models have permeated into such research field (Hutmacher, 2010), producing a clear shift in preclinical studies towards 3D alternative systems. Since then, in vitro 3D culture models have been developed from various human tumour cells (Yamada and Cukierman, 2007). Furthermore, the continuous progress in tissue engineering (Hakanson et al., 2014), including the development of various 3D scaffolds, hydrogels and bioreactor systems, has also contributed to the improved the diversity, fidelity and capacity of such models (Achilli et al., 2012; Friedrich et al., 2007; Stratmann et al., 2014). They have become a prevailing alternative to animal experimentation (Kimlin et al., 2013) in the study of: (i) the micro-environmental regulation of tumour cell physiology (Kural and Billiar, 2013); (ii) therapeutic problems associated with metabolic and proliferative gradients in the tumour tissue (Rodriguez-Enriquez et al., 2008); and (iii) cell-cell and cell–matrix interactions triggering radio-/chemo-resistance of tumours (Friedrich et al., 2007; Shield et al., 2009). For the latter, 3D co-culture tumour models have been developed. These include heterotypic cellular components (e.g. cancer cells and fibroblasts) and play a critical role in recreating the tumour microenvironment in vitro. The tumour micro-environment greatly impacts the therapeutic efficiency of chemotherapeutic drugs. Co-culture 3D tumour models represent therefore one of the most promising in vitro systems for predictive testing of compound efficacy in oncology (Hirschhaeuser et al., 2010). Accordingly, numerous studies have shown that 3D cell models offer the promise of improving clinical translation of drugs by allowing a more accurate examination of drug sensitivity and resistance (Gudugu et al., 2013; Mehta et al., 2012), whilst reducing the need (or number) of animal studies. As a result, 3D cell cultures can allow for improved in vitro-to-in vivo correlations than conventional 2D cell models (Fitzgerald et al., 2015; Hartung, 2017; Weiswald et al., 2013).

Nevertheless, to fully understand how efficacy data originating from 3D cell models could be beneficial to the drug discovery pipeline, it is of prime interest for the cancer research community to characterize such models. The crucial point is to consistently relate the 3D model of choice to specific pathological conditions (Friedl et al., 2012). Realistically, each in vitro model reflects a particular aspect of the in vivo condition and the ways that cells adapt to that context. While it is highly intuitive that 2D cell cultures do not recapitulate the real in vivo settings as well as 3D models, it is not so intuitive to actually define which in vitro 3D system is as close as possible to a precise in vivo environment. This is due to the current lack of scientific literature describing the morphological, mechanical and biochemical features of cancer cells cultured in specific 3D microenvironments.

Considering this, in our study we characterized various in vitro models of Non-Small-Cell Lung Cancer (NSCLC), the most prevalent form of tumour originating from lung epithelial cells. We examined live or fixed lung adenocarcinoma (A549) cells cultured in 2D on glass or in 3D within two different substrates: Matrigel™ and PuraMatrix™. A549 cells were chosen as (i) a physiologically relevant in vitro model of NSCLC (Caino et al., 2012) and as (ii) established cell line for anticancer drugs discovery and screening (Gazdar et al., 2010). Matrigel™ and PuraMatrix™ were selected as well-accepted hydrogel scaffolds for the creation of defined 3D microenvironments (Worthington et al., 2015). Generally, scaffolds used for forming 3D in vitro models are grouped into two broad categories (Horváth et al., 2016). The first includes biologically-derived/natural materials or hydrogels such as Matrigel™. The second category corresponds to synthetically-derived matrices (e.g. PuraMatrix™). Hence, we decided to focus our investigation to one substrate for each of the two categories of scaffolds just described. Matrigel™ is also widely established in cancer research as cell culture substrate (Albini and Noonan, 2010). As compared to 2D glass substrates, Matrigel™ and PuraMatrix™ have very low storage moduli, corresponding to a soft gel (Georges and Jannine, 2005). The storage modulus (elastic contribution) value reported in the literature for Matrigel™ is 80 Pa as measured by rheometry, while PuraMatrix™ is less stiff, with a storage modulus value of 5 Pa (Allen et al., 2011).

In our study, we carefully examined the extent to which, shifting from 2D to 3D cell cultures, lung adenocarcinoma cells changed their shape, cytoskeleton organization and mechanical properties (quantified by AFM as cell stiffness), as well as their biochemical signature: expression of Epithelial-to-Mesenchymal Transition (EMT) markers and invadopodia, cytokine secretion, and activation of Erk1/2 and PI3K/Akt signalling pathways. Finally, we analysed potential differences in the response to an anticancer drug used in clinical settings (docetaxel). Therefore, to our knowledge, our study provides one of the first in-depth investigations of the physiological features of 3D in vitro models that relate to cancer development, progression and treatment efficacy in human patients, stimulating the adoption of these models in drug discovery as alternatives to animal experimentation.

2. Materials and methods

2.1. Chemicals and materials

Chemicals and solvents were purchased from commercial sources (Sigma-Aldrich, Fisher Scientific, Invitrogen and Calbiochem) and used as received, unless otherwise specified. Glass-bottom petri-dishes were from TED-Pella (TED PELLA, Inc., USA) and other cell-culture plastic products were purchased from Nunc (Thermo Fisher, Fisher Scientific, Ireland).

2.2. Cell culture

Human alveolar adenocarcinoma cells (A549 cell line) were obtained from the American Tissue Culture Collection (ATCC, LGC Standards, Middlesex, UK) and maintained in Hams F12K media supplemented with 2 nM L-glutamine, 1% penicillin/streptomycin and 10% foetal bovine serum (FBS) (Gibco, Invitrogen Ltd, VWR)
International, Ireland) at 37 °C and 5% CO₂. The A549 cell line was authenticated using Short Tandem Repeat (STR) profiling (LGC Standards) showing that our A549 batch is an exact match for the ATCC human cell line CCL-185 (A549) (with 100% match between the submitted sample and the database profile). Mycoplasma and phenotypic responses were regularly checked for contamination as part of the laboratory GLP. The passage number was restricted between five and twelve: cell morphology and mechanical properties were measured and compared among A549 cells from the same passage. At 80% confluence, A549 cells were detached from T75 flask substrate with TrypLE™ (Gibco, Invitrogen, Oregon, USA), centrifuged and diluted in supplemented F12K medium at concentrations appropriate for each experimental procedure.

2.3. 2D cell models

A549 cells were counted using a Countess™ Automated Cell Counter (Invitrogen, Oregon, USA) and plated in 4-well Millicell® EZ slide (Millipore®, MA, USA) at a concentration of 10⁶ cells/ml (500 μl/well). Cells were incubated for 4 d at 37 °C (5% CO₂) to allow cell attachment onto the glass substrate and cell growth. Cell medium was regularly changed every 3 d.

2.4. 3D cell models

A549 cells were seeded onto layers (thickness around 1 mm) of Matrigel™ Basement Membrane Matrix or PuraMatrix™ Peptide Hydrogel (both from BD Biosciences, UK). Matrigel™ is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins; PuraMatrix™ is a synthetic matrix that is used to create defined 3D microenvironments for a variety of cell culture experiments. Briefly, Matrigel™ Basement Membrane Matrix was thawed by submerging the vial in ice in a 4 °C refrigerator overnight. The vial was then swirled to ensure that the matrix was evenly dispersed and, using cooled pipette tips and keeping the glass slides on ice, the Matrigel™ Basement Membrane Matrix was added to the surface of a 4-well Millicell® EZ slide following the Thick Gel Method reported by the supplier. This method is recommended by BD Biosciences to grow cells within a 3D matrix. Slides were incubated at 37 °C for 30 min prior cell seeding. For PuraMatrix™, the stock solution (1% w/v) was diluted to the appropriate working concentrations (0.15%, 0.25% and 0.30%) with sterile DI water. PuraMatrix™ can be used in the undiluted form at a concentration of 1% (w/v) or diluted. In our study, selection of PuraMatrix™ concentrations was based on the manufacturer’s guidelines for use. According to the latter, concentrations below 0.5% are suitable for most applications. Thus, a concentration of 0.30% was selected to form the 3D cell models. In addition, concentrations equal to 0.25% are recommended for surface plating of adherent cells (such as the A549 cell line), and a final concentration of 0.15% for cell encapsulation. Hence, we decided to include also these concentrations in our experimental design. 250 μl of PuraMatrix™ dilutions were then added to the surface of a 4-well Millicell® EZ slide and gelation was promoted by carefully and slowly adding supplemented F12K medium to each well (500 μl/well). The glass slide was incubated at 37 °C for 1 h to complete the gelation of the hydrogel. After the hydrogel has assembled, the medium was changed twice over a period of 1 h to equilibrate the environment to reach physiological pH. A549 cells were carefully seeded at the top of the Matrigel™ or PuraMatrix™ layers at a concentration of 10⁶ cells/ml (500 μl/well). Cell cultures were grown for 4 d and cell medium was changed every 3 d.

2.5. Laser scanning confocal microscopy (LSCM)

Immunostaining and LSCM imaging were performed on the 2D/3D models without the need of cell isolation. After fixation with 4% paraformaldehyde (PFA) for 10 min at room temperature, cell cultures were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 min and stained with Hoechst 33342 for nuclei and Oregon Green-phalloidin, Alexa Fluor 488®-phalloidin or rhodamine phalloidin (1:50) (all supplied by Invitrogen, Fisher Scientific, Ireland) for F-actin. The slides were incubated at room temperature for 1 h in the dark, rinsed with PBS and mounted in transparent mounting medium (VECTASHIELD, Vector Laboratories Inc., CA, USA) prior to LSCM imaging by a ZEISS 510 Meta confocal microscope equipped with a Zen imaging software (Carl Zeiss, Aixiowitz, Germany). An overnight blocking step (1% bovine serum albumin (BSA) in PBS) was carried out at 4 °C prior to staining when probing for: cortactin (Cortactin (H222 Mouse mAb; 1:100), E-cadherin (E-Cadherin (4A2) Mouse mAb; 1:200), vimentin (Vimentin (D21H3) Rabbit mAb; 1:100) (all purchased from Cell Signaling Technology Inc., Brennan & Company, Ireland), and fibronectin (Fibronectin Rabbit mAb; 1:200) (Abcam, WVR International, Ireland). These primary antibodies were diluted in 0.1% Triton X-100 in PBS, added to the cultures overnight at 4 °C, followed by a washing step and incubation with the appropriate secondary antibody (Goat anti-Rabbit IgG (H + L) Secondary Antibody Alexa Fluor® 488, or Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody Alexa Fluor® 488/568) (supplied by Invitrogen, Fisher Scientific, Ireland). Qualitative LSCM imaging was possible by acquiring a series of z-stack images. Surface rendering of z-stack images was carried out by the open-source software ImageJ (Kankaanpaa et al., 2012) and by the Volocity 3D Image Analysis Software (PerkinElmer Inc., Massachusetts, USA) in order to further elucidate the F-actin organization. ImageJ software was used for quantifying the fluorescence intensity of the F-actin stain in a single z section.

2.6. Atomic force microscopy (AFM) – imaging and measurements of fixed specimens

Cell cultures were fixed with 2.5% glutaraldehyde (GA) for 30 min at 37 °C. The samples were washed with PBS three times and then stored in PBS at 4 °C. AFM measurements were performed on the in vitro models without the need of cell isolation, by means of a NTEGRA Spectra instrument (NT-MDT, Russia) using liquid cell configurations. Soft cantilevers (MLCT probe, Bruker, USA) with nominal values of f₀ = 15 kHz, k = 0.02 N/m and tip radius = 20 nm were used to probe the cells. Cantilevers were calibrated using the Sader method (Sader et al., 1999).

Topography images were recorded in tapping mode with a resolution of 512 × 512 pixels and a scan rate of 0.4 Hz. For the 3D cell cultures, topography images were acquired only for the cells located on top of the hydrogels substrates. Data analysis for AFM imaging and surface roughness was carried out by NOVA Image Analysis Software (NT-MDT, Russia). Cell roughness data was averaged (n_cells = 20) for each sample.

Force mapping was performed in contact mode at 1 Hz. Deflection-height curves represents the movement of piezo (x-axis) during force curves against the deflection of the cantilever (y-axis) (Supplementary Fig. S1). Young’s modulus (stiffness) was determined by fitting with Hertz model using Protein unfolding and nano-indentation software “PUNIAS” [http://site.voila.fr/punias]. When analysing the 3D cell cultures, force mapping was carried out on cells located on top of hydrogels substrates, as well as on cells that were partially embedded into the matrix. Force spectroscopy data are reported as average (n_curves = 500/sample) ± standard deviation.

2.7. Atomic force microscopy (AFM) – force measurements of live specimens

An AFM (MFP-3D, Asylum Research, USA) was used to quantify the mechanical properties of A549 cells forming 2D/3D cultures. Measurements were carried out at room temperature in pre-warmed
supplemented Hams F12 K culture media. Soft cantilevers (MLCT, Bruker, USA) with nominal values of $f_0 = 15$ kHz, $k = 0.02$ N/m and tip radius $r = 20$ nm were used to probe the cells. The exact spring constant was determined before each experiment for each lever using the Sader method (Sader et al., 1999) in air. Inverse Optical Lever Sensitivity (InvOLS) was subsequently measured in pre-warmed cell medium using a glass substrate as a reference. For 3D cell cultures, force mapping was carried out on cells located on top of hydrogels, as well as on cells that partially penetrated into the matrix. Force maps of arrays of $6 \times 6$ points were recorded for each cell analysed in a $20 \times 20 \mu m$ area above the cell cytoplasm (rate = 1 Hz; displacement $= 5 \mu m$; max force $= 2$ nN) (Supplementary Fig. S2). For A549 cells grown on glass, arrays of $16 \times 16$ points were acquired in a $30 \times 30 \mu m$ area due to the more spread out morphology of the cells. The elastic Young’s modulus $E$, (reported as mean $\pm$ relative error and extrapolated from fitting Log(E) distributions) was determined by fitting the force vs indentation curve to the Sneddon Model (Sneddon, 1965) using Igor Pro (WaveMetrics, USA) and assumed a conical shaped indenter with $\alpha = 21.46$, Poisson’s ratio $\nu = 0.5$ and a maximum indentation depth $\delta = 200$–300 nm to ensure that data was free from any substrate influence. Data that did not conform to the Sneddon Model were manually excluded from analysis along with any statistical outliers.

2.8. He-ion microscopy (HIM)

2D and 3D cell cultures were fixed at room temperature in 2.5% GA in 0.1 M Sørensen’s phosphate buffer (pH 7.3) and rinsed with Sørensen’s phosphate buffer. After dehydration in increasing concentrations of EtOH (from 70% up to 100%), the samples were immersed in hexamethyldisilazane (HDMS) 30% in EtOH for 5 min before being transferred to HDMS 60% in EtOH for 5 min. The final wash was carried out in pure HDMS for 10 min. The samples were air dried and imaged by a Zeiss Orion Plus He-ion microscope (Carl Zeiss, Oberkochen, Germany) using an accelerating voltage of 30 kV. Samples were transferred into the chamber, which had undergone plasma clean overnight prior to loading samples, using a load lock. The working distance was 8 mm and a 10 $\mu m$ beam limiting aperture was used. The probe current was between 0.5 and 1.5 pA. Images were acquired by collecting the secondary electrons emitted by the interaction between the He-ion beam and the specimen with an Everhart-Thornley detector (part of the He-ion microscope system). The image signal was acquired in a 32- or 64-line integration to each contributing line of the image.

He-ion microscopy (HIM) is a relativistic new imaging technique in which a focused beam of He $^+$ ions is directed onto the sample surface, which liberates secondary electrons that are collected forming detailed images of the sample surface topography (Bell, 2009). In addressing topics of the biological sciences, HIM offers various advantages over conventional SEM approaches, such as a high spatial resolution (with better material contrast and improved depth of focus) and the ability to image uncoated, non-conductive samples without the deposition of a metal (or other conductive) overcoat (Rice et al., 2013), which has been shown to reduce and/or completely mask cell surface details (Bazou et al., 2011). This opens up a whole new range of surface details in biological specimens that can be examined rapidly and with less risk of artefacts. Here, we exploited the high-resolution nature of HIM to image the shape, membrane texture and membranous projections of A549 cells cultured in 2D or 3D with unsurpassed image resolution and detail. The authors recognize that the preparation methods of biological specimens for HIM imaging can also introduce artefacts. To avoid this and to preserve the cells architecture, a drying method using HMDS was used. HMDS represents a cost- and time-efficient alternative to critical point drying (CPD) in the preparation of cells for SEM imaging (Braet et al., 1997), and recent studies suggest that HMDS-based methods are optimal to dehydrate cells on 3D scaffolds for SEM examination (Lee and Chow, 2012). Care was taken during sample preparation to ensure that artefacts were negligible.

2.9. Cell lysis, SDS-PAGE and western immuno-blotting

Cell cultures grown for 4 d were washed with ice-cold PBS. RIPA buffer (Santa Cruz Biotechnology Inc., Fannin Limited, Dublin, Ireland) supplemented with sodium orthovanadate, protease inhibitor, and PMSF (Santa Cruz Biotechnology Inc., Fannin Limited, Dublin, Ireland) was used as lysis buffer. For cell cultures grown on glass substrates, 500 $\mu l$/well of supplemented RIPA buffer was added. 1 ml/well of Corning™ Cell Recovery Solution (Becton Dickinson, Ireland) was added to allow recovery of cells cultured into Matrigel™. Cell cultures were then scraped, transferred into a plastic tube, and placed on ice for 1 h. The solution thus obtained was then centrifuged twice at 5000 rpm for 5 min, and supernatant decanted after each centrifugation step. The precipitate (containing the recovered cells) was re-suspended in 1 ml of supplemented RIPA lysis buffer. Cell cultures grown into PuraMatrix™ were recovered with a cell scraper, and the suspensions thus obtained transferred to a clean Eppendorf tube. After centrifugation at 5000 rpm for 5 min, the supernatant was decanted and 300 $\mu l$ of supplemented RIPA lysis buffer added. Following sonication for 15 min in a sonic bath to favour cell lysis, all lysates were centrifuged for 15 min at 15,000 rpm at 4 °C. The protein content of each lysate thus obtained was quantified using the Pierce BCA Protein Assay Kit (Product no 23225; Thermo Scientific, Fisher Scientific, Ireland), per manufacturer’s protocol.

For SDS-PAGE, the appropriate lysates volume was diluted in supplemented RIPA lysis buffer and NuPAGE® LDS Sample Buffer supplemented with NuPAGE® Sample Reducing Agent (both supplied by Thermo Scientific, Fisher Scientific, Ireland). Samples were heated for 10 min at 70 °C, and resolved on pre-cast NuPAGE® 4–12% Bis–Tris Gels (Novex, Life Technologies, Fisher Scientific, Dublin, Ireland) at 200 V. A biotinylated protein ladder (Cell Signaling Technology Inc, Brennan & Company, Ireland) was also resolved within the same gel as control. Resolved proteins and molecular weight marker were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon P transfer membrane, Merck Millipore, Ireland) by wet transfer for 2 h at 30 V. Polyvinylidene difluoride membranes were then blocked in either 5% non-fat dry milk in TBS-T 1 × (0.1% Tween20 in TBS) or 5% BSA TBS-T 1 × for 1 h at RT. TBS 10 × was purchased from Santa Cruz Biotechnology (Ireland), diluted in DI water or 0.1% Tween20-DI water to obtain TBS 1 × and TBS-T 1 ×, respectively. Four wash steps (2 × TBS-T 1 ×, 2 × TBS 1 ×, 5 min each in gentle agitation at RT) were carried out before staining with the primary antibody (overnight; 4 °C). A list of the primary antibodies used within this study (all purchased from Cell Signaling Technology Inc, Brennan & Company, Ireland) is reported in Table 1. Staining of GADPH or $\alpha$-tubulin proteins was used as loading control.

The membranes were then washed and incubated with their respective HRP-linked secondary antibodies (anti-rabbit IgG HRP-linked antibody or anti-mouse IgG HRP-linked antibody, both from Cell Signalling Technology Inc, Brennan & Company) (1 h, RT, with gentle agitation). Anti-biotin, HRP-linked Antibody (Cell Signalling Technology Inc, Brennan & Company) (1 h, RT, with gentle agitation). Anti-biotin, HRP-linked Antibody (Cell Signalling Technology Inc, Brennan & Company, Ireland) was used to stain the protein ladder. Probed membranes were then washed, incubated with HRP substrate (Luminata™ Forte Western HRP Substrate, Merck Millipore, Ireland) and protein bands visualised by chemiluminescent detection on CL-XPosure Film (Thermo Scientific, Fisher Scientific, Ireland). Relative proteins’ expression levels were quantified by ImageJ software.

2.10. Quantification of secreted cytokine/chemokines, EGF and TGF-β1/TGF-β1

Supernatants were harvested from cell cultures at t = 3 d and t = 4 d, combined and tested by Enzyme ImmunoSorbent Assays (ELISAs).
Supernatants were harvested from two independent experiments and tested in duplicate. The amount of human interleukin-1 beta (IL-1β), chemokine (C-X-C Motif) ligand 1 (CXCL-1), human epidermal growth factor (EGF), latent transforming growth factor beta (TGF-β), total TGF-β1, and free-active TGF-β1 were measured by the following kits: Human IL-1β ELISA MAX™ Deluxe (BioLegend, Medical Supply Co. Ltd, Ireland), Human CXCL-1/GRO-α DuoSet ELISA (R&D Systems Eire, UK), Human EGF ELISA kit (Sigma Aldrich, Ireland), LEGEND MAX™ Human Latent TGF-β ELISA, LEGEND MAX™ Total TGF-β1 ELISA, and LEGEND MAX™ Free Active TGF-β1 ELISA (purchased from BioLegend, Medical Supply Co. Ltd, Ireland). Assays were carried out as for manufacturers’ protocols. Optical density of each well at 450 nm was determined using an Epoch microplate reader (Biotek, Mason Technologies, Ireland), and was corrected by subtracting the optical absorption of the 96-well plastic plate at 570 nm.

The cell culture supernatants were also tested by means of PathScan Antibody Array Kit Th1/Th2/Th17 (Cell Signalling Technology Inc, Brennan & Company, Ireland), to simultaneously detect the secretion of multiple extracellular signalling molecules. These were: IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-17A, TNF-α, GM-CSF and IFN-γ. The assay was carried out as for supplier’s protocol: the array was then incubated with HRP substrate (Luminata™ Forte Western HRP Substrate, Merck Millipore, Ireland) and the chemiluminescence signal was detected by FUSION-FX7, equipped with Capt Advance FX7 software (Vilber Lourmat, France).

### 2.11. Biological response to docetaxel

After 4 d, 2D and 3D cell cultures were exposed to 10 increasing concentrations of docetaxel (0.001, 0.05, 0.1, 0.5, 5, 50, 100, 500 and 1000 nM) prepared in fresh media. Untreated cultures were included in the experimental design and used as negative controls (NT). As positive control (PT), cell cultures were exposed to LDH lysis buffer (Vilber Lourmat, France).

Substrate, Merck Millipore, Ireland) and the chemiluminescence signal was detected by FUSION-FX7, equipped with Capt Advance FX7 software (Vilber Lourmat, France).

### 2.12. Statistical analysis

Unless differently stated in the text, Graph-Pad Prism (Graph-Pad Software Inc., La Jolla, CA, USA) was used to carry out the statistical analysis. A p value < 0.05 was considered statistically significant. The statistical tests used are specified in the corresponding figure caption.

### 3. Results

In this study, we characterized various in vitro models of lung adenocarcinoma grown in 3D within matrices (Matrigel™ and PuraMatrix™). Once seeded on Matrigel™ or PuraMatrix™, cells of the A549 lung adenocarcinoma line invaded the hydrogels (as schematically shown in Fig. 1a) to form 3D cell cultures. For comparison, A549 cells were also cultured on glass substrates, obtaining 2D cell cultures.

#### 3.1. Morphology of 3D in vitro models as compared to 2D cell cultures

##### 3.1.1. Cell shape

It is well documented in the literature that cell-cell and cell-extra- cellular microenvironment interactions mediate, among others, shape-induced effects (Buxboim et al., 2010; Pickup et al., 2014; Rehfeldt et al., 2007). Accordingly, our results showed that A549 cells remodelled their shape when cultured in 3D (Fig. 1b). In detail, LSCM analysis demonstrated that the cellular morphology shifted towards spheroid geometry in 3D cell models.

##### 3.1.2. Cytoskeleton organization and formation of invadopodia

A549 cells re-organized their cytoskeleton as a consequence of the 3D microenvironment (Fig. 1b). Cytoskeletal F-actin was organized into stress-fibres when A549 cells were cultured in 2D, as shown in the fluorescence intensity profiles reported in Fig. 1b, whereas it was cortical when cells were cultured in 3D. Interestingly, cytoskeletal F-actin projections were visible on the cells’ edges only in 3D cell cultures.

Cancer cell invasion is a dynamic process that involves both alterations in cell shape and formation of membrane protrusions (invadopodia), to facilitate invasive growth and migration (Jaafar et al., 2012; Yilmaz and Christofori, 2009). Our morphological data could therefore suggest that A549 cells turned to a phenotype with a higher invasive potential when shifting from 2D to 3D cell organization. Indeed, the changes evidenced herein in the cytoskeletal organization of A549 cells could also be solely or partially due to the cell adaptation to the 3D microenvironment itself, and not correlated to a phenotypic transformation. To elucidate this, we evaluated the expression of cortactin (Fig. 2), which is a prominent component and a specific marker of invadopodia (Clark et al., 2007), in 2D and 3D cultures. Invadopodia are specialized extracellular matrix (ECM)-degrading, actin-rich membrane protrusions formed by aggressive (mesenchymal) cancer cells, and are the main responsible for tumour cell invasion. Both 2D and 3D cultures expressed cortactin (Fig. 2a): the highest expression levels of this protein were detected in 3D cell cultures grown in Matrigel™ and PuraMatrix™ 0.15% (Fig. 2b). However, careful analysis of LSCM images showed that, cortactin expression was mainly cortical in 3D cell cultures, while the staining resulted punctuated and intracellular in 2D cell cultures (Fig. 2a). Notably, in 3D cultures, cortactin strongly co-localized with the F-actin enriched protrusions present on the cell membrane (as highlighted by arrows in Fig. 2a). This proved that such protrusions could indeed be identified as invadopodia.

Cortactin assembly seemed to be regulated through RhoA-GTPase in all cell cultures, while activation of the Rac-GTPase could not be detected (Fig. 2b). Finally, expression of MT1-MMP, which is known to correlate closely with cortactin expression levels and invadopodia formation (Clark et al., 2007), was found to be expressed in A549 cells grown in 3D in Matrigel™ and PuraMatrix™ 0.15% (Fig. 4c), thus supporting the conclusion that A549 cells formed invadopodia when cultured in 3D.

It should be highlighted that, in some instances, our experimental...
results displayed differences in the protein expression levels of pairs of replicates (Fig. 4b and c). When working with 3D cell cultures, it is common to experience variability. This is considered one of the main issues preventing the successful integration of these models in the routine preclinical screening of anti-cancer drugs. Though it is difficult to pinpoint the exact causes, a factor that could have contributed to differences in protein expression in our study was the batch-to-batch variability in hydrogel composition (Matrigel™ or PuraMatrix™). A deeper level of detail could be obtained by studying the effect of these parameters on the 3D cultures. A better understanding on the influences that these parameters could have on the 3D cultures’ characteristics would be the matter of a further in depth study.

3.1.3. Expression of EMT biomarkers

To further elucidate the changes in the cell invasive potential of A549 cells as a consequence of the microenvironment, we evaluated the expression of Epithelial-to-Mesenchymal (EMT) protein markers in 2D or 3D cultures (Fig. 3).

Loss of E-cadherin is one of the best indicators of EMT transition in
epithelial cells (Thiery, 2002). Our results showed that E-cadherin was expressed only in 3D cultures grown in Matrigel™ and PuraMatrix™ (at three different concentrations: 0.15%, 0.25% and 0.30%). Projections of series of z-stack images are reported. Cells were stained with Cortactin (H222) Mouse mAb followed by Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody Alexa Fluor® 488 (cortactin, in green), Hoechst 33342 (nuclei, in blue), and rhodamine-phalloidin (F-actin, in red). Arrows highlight the co-localization of cortactin in F-actin enriched protrusions of the cell membrane. Scale bars: 20 μm (63 × objective lens). (b,c) Western blot showing the expression of: (b) cortactin, RhoA-GTPase, Rac1/2/3-GTPase and (c) MT1-MMP in A549 cells cultured in (from left to right): 2D on glass substrates (G), or 3D in Matrigel™ (M) or PuraMatrix™ (PM) at three different concentrations (0.15%, 0.25% and 0.30%). Two individual experiments (n1 and n2) are presented for each culture. The relative expression levels of cortactin, RhoA/GTPase and MT1-MMP in each sample was calculated as the ratio between the protein band intensity and that of the loading control (GADPH). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Detailed topographical images recorded by AFM (Fig. 4a), showed that fixed A549 cells cultured on glass (2D) had flattened morphology and presented actin-enriched regions at cells’ edges with a height of about 0.5–0.8 μm. This correlated with a rather smooth cell surface, as determined from surface roughness measurements. The round cell aggregates formed by A549 cells cultured in Matrigel™ were characterized by a smooth cell surface, comparable to that of 2D cell cultures. This result agreed with previous scientific literature (Cichon et al., 2012). On the other hand, AFM topographic images recorded an increase in surface roughness for A549 cells cultured in PuraMatrix™, with insignificant differences among the three PuraMatrix™ concentrations tested.

The AFM topographical results were confirmed by HIM images (Fig. 4b), which evidenced the presence of unique membrane ruffles and a multitude of filopodia/invadopodia (highlighted by arrows) on the surface of A549 cells cultured within PuraMatrix™. In this instance, HIM allowed avoiding conventional Scanning Emission Microscopy.

### 3.2. Topographical characterization of 3D cell cultures

#### 3.2.1. Cell surface roughness

Atomic Force Microscopy (AFM) and He-Ion Microscopy (HIM) were used to characterize the cell topography and describe in detail the subtle cell membrane structural changes evidenced by the morphological analysis (Section 3.1).
(SEM) sample preparation artefacts associated with metal coating procedures. The low sputter yield rate of the helium ions beam for organic materials (Joens et al., 2013), in addition to the increased HIM resolution versus SEM, the advanced charge reduction and the high contrast secondary electron emission (Bell, 2009), enabled the repeated imaging of small and delicate surface features and structure of completely unmodified 3D cell cultures at the nanometre scale with no discernible damage by the beam. In our study, HIM represented a time-efficient methodological approach that could perfectly match and/or complement time-consuming AFM topographical measurements, for identifying the subtle cell morphological changes induced by the 3D microenvironment. Nevertheless, we recognize that the methodology described here has some limitations, since the AFM/HIM approach taken allowed recording the cellular morphological properties only of

Fig. 3. Expression of EMT markers in 2D and 3D cell cultures. Representative LSCM images of A549 cells cultured on (from left to right) glass, in Matrigel™ and in PuraMatrix™ (at three different concentrations: 0.15%, 0.25% and 0.30%). Projections of series of z-stack images are reported. Cells were stained with: (a) E-Cadherin (4A2) Mouse mAb followed by Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody Alexa Fluor® 568 (E-cadherin, in red), Hoechst 33342 (nuclei, in blue), and Alexa Fluor 488®-phalloidin (F-actin, in green). (b) Vimentin (D21H3) Rabbit mAb coupled to Goat anti-Rabbit IgG (H + L) Secondary Antibody Alexa Fluor® 488 (vimentin, in green), Hoechst 33342 (nuclei, in blue), and rhodamine-phalloidin (F-actin, in red). (c) Fibronectin Rabbit mAb followed by Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody Alexa Fluor® 568 (fibronectin, in red), Hoechst 33342 (nuclei, in blue), and Alexa Fluor 488®-phalloidin (F-actin, in green). Scale bars: 20 μm (63× objective lens). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
those cells located on top of hydrogels substrates, and not of the entire 3D cell population.

### 3.3. Mechanical properties of 2D and 3D cell cultures

#### 3.3.1. Cell sti
dness

Cellular stiffness was investigated in both fixed and live specimens by AFM nanoindentation (Radmacher, 1997; Touhami et al., 2003), to further elucidate the effect of the 3D microenvironment on the cellular phenotype and invasive potential of A549 cells. The micro-fabricated probe used in AFM offers force sensitivity on the order of 10 pN, to probe the elasticity of very soft and inhomogeneous specimens (such as 3D cell cultures) with a spatial resolution at molecular or atomic scales (Dufrene et al., 2013). To date, AFM nanoindentation studies (Lekka and Laidler, 2009; Morton and Baker, 2014; Sokolov, 2007) have shown that cancer cells of various origins are generally characterized by decreased stiffness (due to changes in the cytoskeletal organization) as compared to healthy samples (Cross et al., 2007; Cross et al., 2008; Dokukin et al., 2011; Iyer et al., 2009), with sensitivity and specificity up to 99% (Guz et al., 2015). Additionally, AFM data has demonstrated that changes in cancer cell stiffness could distinguish cancer cells with high metastatic potential from less invasive types (Liu et al., 2014; Watanabe et al., 2012; Xu et al., 2012; Zhou et al., 2013). In this study, analysis of force-distance curves allowed us to determine the stiffness (here reported as Young’s modulus) of A549 cells in the different 2D/3D microenvironments. Our results on fixed specimens showed that, cells were stiffer when cultured on 2D glass substrates compared to 3D models (Fig. 5).
Since the sample fixation procedures could indeed alter the mechanical properties of the cells and of the surrounding hydrogel-based matrix (Matrigel™ and PuraMatrix™), AFM force-distance curve analysis was used to also investigate the characteristics of live specimens. The resulting elastic Young’s modulus (E) of live A549 cells is shown in Fig. 6 as histograms of the logarithmic function of E (Log10(E)). For cells grown in 2D on a glass surfaces, the histogram of Log10(E) could be fitted with a single Gaussian distribution centred at 3.05 ± 0.49 kPa (Fig. 6a). It can be hypothesized that the broad distribution is associated with the variance in stiffness of the different components of the cells (such as nucleus and a highly-organized cytoskeleton). Log10(E) histograms were narrowly distributed for live A549 cells grown within Matrigel™, with a maximum peak at around 2.8 kPa (Fig. 6b). In contrast, cells cultured within PuraMatrix™ 0.15% exhibited a bimodal distribution for the Log10(E) histogram, with a dominant peak centred at 1.25 ± 0.09 kPa, implying a soft cell response, and a second peak exhibiting a stiffer cell response at 20.80 ± 1.56 kPa (Fig. 6c). Such bimodal Log10(E) distribution suggested the coexistence of cells with different cytoskeletal organizations (and therefore mechanical properties) within the same specimen. We suggest that the cells’ spatial localization within the 3D cultures played a major role in defining the Log10(E) distribution. We hypothesise that cells partially embedded into the hydrogel-based matrix were more rigid as compared to the cells located on the surface of the hydrogel substrate, in response to their closer proximity to the glass substrate. Due to the semi-liquid nature of PuraMatrix™ at very low concentrations (e.g. 0.15%), such cells might “feel” the glass substrate and respond by adapting their mechanical properties. A transition from the bimodal distribution to a normal distribution was in fact evident when shifting to a more viscous (and therefore stiffer) substrate, such as PuraMatrix™ at higher percentages (0.25% and 0.30%). For A549 cells grown within PuraMatrix™ 0.25% (Fig. 6d), a dominant response occurred at 0.50 ± 0.05 kPa with a small tail at 3.34 ± 0.12 kPa, whereas cell cultures formed within PuraMatrix™ 0.30% showed a single peak at 2.44 ± 0.16 kPa (Fig. 6e). On average, live A549 cells grown in 3D were found to be less stiff than those cells cultured in 2D on glass (Fig. 6f). This confirmed the AFM nanoindentation results obtained on fixed specimens.

3.4. Biochemical signatures of 3D cell cultures of A549 cells

3.4.1. Cell viability

We evaluated the ATP levels in untreated 2D and 3D cell cultures as an indicator of viability. No significant differences could be detected, with exception of A549 cells cultured on PuraMatrix™ 0.30% that showed an increased cell viability (Fig. 7).

3.4.2. Secretion of cytokines/chemokines, EGF and TGFβ-1 and subsequent activation of specific cellular pathways

Cancer cells are known to increase the secretion of specific factors, including cytokines/chemokines and growth factors (e.g. EGF and TGFβ-1), that play an important role in tumour progression and chemoresistance. In our study, no significant secretion of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-13, IL-17A, TNF-α, GM-CSF, IFN-γ (Fig. 8a) and IL-1β (Fig. 8b) could be detected in supernatants harvested from 2D or 3D cell cultures.

On the other hand, IL-8 (also known as CXCL-8) and CXCL-1 (or Fig. 6. Stiffness of live cells. (a-e) Distribution of the logarithmic function of the elastic modulus (Log10(E)) measured by AFM of live A549 cells grown on (a) glass substrates in 2D (nreplicates = 2135), or in 3D within (b) Matrigel™ (nreplicates = 133) and (c-e) PuraMatrix™ (nreplicates = 429, 164 and 126 for 0.15%, 0.25% and 0.30%, respectively). The Kruskal-Wallis (KW) test for nonparametric samples (which does not assume any specific distribution) showed that all the distributions observed were statistically independent (P = 0.0). (f) Box-and-whisker plot of Log10(E) of live A549 cells grown on (from left to right) glass (G), Matrigel™ (M) and PuraMatrix™ (PM) at three concentrations (0.15%, 0.25% and 0.30%). The median E value (indicated by a blue line) for A549 cells grown in 2D on glass is superimposed with a grey dashed horizontal line, and highlights that cells cultured in 3D within Matrigel™ or PuraMatrix™ had a lower stiffness. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
growth-related oncogene-α, GRO-α), were found in the supernatants of both 2D and 3D cell cultures (Fig. 8a and c, respectively). Notably, the levels of IL-8/CXCL-8 detected were higher when A549 cells were grown in 3D culturing conditions, with the highest amounts found in supernatants harvested from 3D in vitro models grown in Matrigel™ (M) and PuraMatrix™ (PM). Data are normalized to the ATP levels in 2D cultures. Data are reported as average ± standard error of the mean (n=5). A p value > 0.05 was considered not statistically significant (ns) when compared to the ATP levels in A549 cells grown on glass (two-way ANOVA with Dunnet post-test).

3.5. Chemoresistance

Data on the ATP levels in untreated (NT) and docetaxel-treated 2D/3D in vitro models showed that a decrease in the cell viability close to the half-maximal lethal concentration (LC50) could be detected only in 3D cell cultures grown in PuraMatrix™ 0.30% (p < 0.05 as compared to 2D cell cultures). Both 2D and 3D cell cultures showed expression of phospho-Akt and phospho-mTOR (Fig. 8d): phosphorylation of such proteins is known to be triggered by, among other factors, IL-8 through phosphatidylinositol-3 kinase (PI3K) activation (Liu et al., 2016; Waugh and Wilson, 2008). PI3K activation induces phosphorylation of its substrate, Akt, and subsequently of mTOR: phospho-mTOR is involved in cancer cells survival through autophagy inhibition. While no significant differences were found in the expression levels of phospho-Akt among cultures, the highest amounts of phospho-mTOR were detected in 3D cell cultures grown in Matrigel™, CXCL-1 release to the extracellular space, which can ultimately trigger cancer cells’ tumorigenicity (Zhong et al., 2008), is also thought to be regulated through PI3K/Akt pathway activation and subsequent phosphorylation of mTOR (Lo et al., 2014; Shieh et al., 2014). Accordingly, Matrigel™-based 3D cultures showed an increase (although not significant) in CXCL-1 secretion levels (Fig. 8c). IL-8 signalling also activates mitogen-activated protein kinase (MAPK) cascade, with downstream phosphorylation of Erk1/2 (Liu et al., 2016; Waugh and Wilson, 2008). In our study, the protein bands corresponding to phospho-Erk1/2 were clearly detectable in all cultures (Fig. 8f). Interestingly, no EGF could be detected in the supernatants of 2D/3D models (Fig. 8e). Activation of Erk1/2 independently from EGF ultimately describes a pathway linking IL-8 signalling to the phosphorylation of such proteins. 2D cell cultures and 3D models grown in Matrigel™ shared similar expression levels of phospho-Erk1/2; whereas, an increased phosphorylation of these proteins was clearly detected in 3D cultures grown in PuraMatrix™ (Fig. 8f). Significant differences could be also found when varying the matrix concentration, from 0.15% to 0.30%, with the highest expression levels found in 3D cultures grown in PuraMatrix™ 0.30%. IL-8/CXCL-8 can also trigger activation of Rho-GTPase, promoting invasion and motility through the polymerization of cortactin (Liu et al., 2016; Waugh and Wilson, 2008). In section 3.1.2 we have shown that RhoA-GTPase, as well as cortactin, expression could be detected in all cell cultures (Fig. 2).

Finally, a decrease in free-active TGFβ-1 levels were evidenced in supernatants harvested from 3D cell cultures (Fig. 8g), with the most significant decrease associated to cells cultured in Matrigel™. The secretion levels of latent TGFβ and total TGFβ-1 were also quantified as a control (Supplementary Fig. S4). Latent TGFβ secretion levels followed a trend similar to that described for free-active TGFβ-1; whereas, the detectable amounts of total TGFβ-1 were similar in all cell cultures. This suggested that the enzymatic activity necessary to activate TGFβ-1 varied among 2D and 3D culturing conditions. A549 cells cultured in 2D responded by phosphorylating SMAD2 when stimulated with recombinant human (rh)-TGFβ-1 (5 ng/ml) for 24 h (Fig. 8h) or as response to the autogenous levels of TGF-β1 detected in culture after 4 d (Fig. 8i). No activation of the SMAD2 signalling cascade was evidenced in 3D cultures (Fig. 8i).

4. Discussion

It is well recognized that cancer cells respond to 3D architecture and stiffness of culturing substrates. In agreement with the scientific literature (Barthes et al., 2015; Cichon et al., 2012; Shen et al., 2014; Strehmel et al., 2013; Sunami et al., 2014), our results on the morphological properties of A549 cells in 2D/3D cultures indeed confirmed the influence of three-dimensional spatial organization in defining the cell shape. In detail, shifting from 2D to 3D, cancer cells switched into a spheroidal shape (Fig. 1b). Not surprisingly, A549 cells responded also to differences in substrate stiffness by re-organizing their cytoskeleton. Consistent with previous studies (Engler et al., 2006; Shukla et al., 2016), A549 cells cultured on a stiff substrate (glass) showed actin stress fibres formation; whereas, cells grown of softer substrates (Matrigel™ and PuraMatrix™) exhibited cortical F-actin. Such cytoskeleton re-organization translated into changes in the mechanical properties of A549 cells, which showed a smaller Young’s modulus (i.e., stiffness) when cultured in soft matrices (Matrigel™ and PuraMatrix™) (Figs. 5 and 6). This result is consistent with previous studies on A549 cells (Shukla et al., 2016) and fibroblasts (Solon et al., 2007). As previously elucidated by other researchers (Tee et al., 2011), we suggest that the reduced stiffness detected in cells cultured in 3D soft matrices in our study, was due to the decreased cortical tension associated with the F-actin re-distribution.

Our results on the mechanical properties of A549 cells in 2D/3D microenvironment seemed to highlight that A549 cells cultured in 3D possessed higher mesenchymal characteristics. In fact, cell stiffness/Young’s modulus is known to decrease with the gain of metastatic potential (Xu et al., 2012) through a power-law relation (Swaminathan et al., 2011). Since decreased substrate stiffness and 3D architecture resulted in a reduction of A549 cells’ Young’s modulus (both in fixed and live specimens) (Figs. 5 and 6), we originally hypothesised that...
these parameters would also alter the expression of EMT markers, such as E-cadherin, vimentin and fibronectin, towards a mesenchymal phenotype. However, as shown in Fig. 3, decreasing cell substrate stiffness or shifting from 2D to 3D cultures did not trigger spontaneous EMT. E-cadherin expression (epithelial marker) in 3D cultures grown in Matrigel™ or PuraMatrix™ at three different concentrations (0.15%, 0.25% and 0.30%), as detected by PathScan Antibody Array Kit Th1/Th2/Th17. Positive (PT) and negative (NT) controls are included in the array kit. The spot intensity is directly proportional to the amount of target molecule detected. Spot intensity quantification was carried out for evaluating differences in IL-8 secretion levels among the different cell cultures. For each culture, IL-8 levels are reported as average ± standard deviation (nspots quantified = 2). (b,c,e,g) Amount of (b) IL-1β, (c) CXCL-1, (e) EGF and (g) TGF-β-1 detected by ELISAs in supernatants harvested from A549 cell cultures grown (from left to right) on glass (G), in Matrigel™ (M) or in PuraMatrix™ (PM) at three different concentrations (0.15%, 0.25% and 0.30%). Data are reported as average ± standard error of the mean (ntests = 2; nreplicates = 2). (d,f,h) Western blot showing the expression of: (d) phospho-Akt (p-Akt), Akt, phosphor-mTOR (p-mTOR), (f) phospho-Erk1/2 (p-Erk1/2), Erk1/2, and (h) phospho-SMAD2 (p-SMAD2) in A549 cells cultured in (from left to right): 2D on glass substrates (G), or 3D in Matrigel™ (M) or PuraMatrix™ (PM) at three different concentrations (0.15%, 0.25% and 0.30%). Two individual experiments (n1 and n2) are presented for each culture. The relative expression levels of p-Akt, p-mTOR and p-Erk1/2 in each sample was calculated as the ratio between the protein band intensity and that of the loading control (GADPH). (i) Western blot showing the expression of p-SMAD2 in A549 cells grown on glass in the absence (−) and in the presence (+) of recombinant human TGF-β1 (rh TGF-(1) (5 ng/ml). (a–c, e, g) p values > 0.05 were considered not statistical significant (ns) when compared to values found for A549 cells grown on glass (2D) (one-way ANOVA followed by Turkey’s multiple comparison test).
expression of fibronectin in 3D cultures grown in Matrigel™ and PuraMatrix™ 0.15%, which are indeed softer than glass substrates, seemed in conflict with previous studies reporting that soft matrices do not induce expression of mesenchymal markers (Brown et al., 2013; Markowski et al., 2012; Mason et al., 2012; Tilghman et al., 2010). According to recent studies (Brown et al., 2013; Markowski et al., 2012; Wei and Yang, 2016), EMT is triggered by high substrate stiffness through TGFβ signalling. In our study, the total levels of latent TGFβ (Supplementary Fig. S4) and free-active TGFβ-1 (which actually binds to the TGFβ receptor and exerts biological functions) (Fig. 8g) were found to be lower in 3D cell cultures than in 2D. Accordingly, phosphorylation of SMAD2, which is triggered by TGFβ signalling and induces EMT through SNAIL activation, was detected only in 2D cultures (Fig. 8d). Other physical properties of the hydrogels, such as crosslinking and pore size (which can be altered by modifying factors as polymerization conditions), may therefore have affected the expression of fibronectin in 3D cell cultures grown in Matrigel™ and PuraMatrix™ 0.15%. In addition, physical cues are not the only parameters influencing the cell phenotype in 3D cultures. According to the manufacturer (Corning), for example, trace amounts of fibronectin are present in the Matrigel™ matrix; thus, the high levels of fibronectin detected by LSCM in 3D cultures grown in such hydrogel could be also partly or completely due to the composition of the culturing microenvironment. Thus, it must be acknowledged that the stiffness of the bulk substrate may not be the only cause of the observed change in EMT biomarkers expression.

Although no EMT was detected, A549 cells’ membrane was characterized by F-actin projections when cultured in 3D (Fig. 1b). Such cytoskeletal protrusions were more evident in those cultures grown in PuraMatrix™ (Fig. 4b). Topographical data showed that this translated into a higher cell surface roughness (as measured by AFM) of A549 cells grown in 3D in PuraMatrix™ (Fig. 4a). Since PuraMatrix™ is significantly less stiff than the other substrates tested of at least 75 Pa (Allen et al., 2011), we could conclude that an inverse correlation between the stiffness of the substrate and the surface membrane structures/roughness of the cancer cells grown in 3D was observed. Hence, we hypothesized that, the surface membrane structures found in 3D cultures grown in the soft matrices (Matrigel™ and PuraMatrix™) were associated with the process of migration and invasion. Shulka et al. have in fact shown that matrix stiffness can influence A549 cells migration even when it does not alter the expression of EMT biochemical markers (Shulka et al., 2016). Our results showed that, although expressed in both 2D and 3D cell cultures (Fig. 2b), cortactin was highly co-localized in correspondence of the F-actin-enriched protrusions only in Matrigel™/PuraMatrix™-based cultures (Fig. 2a). The ECM-degrading activity of invadopodia is due to the presence of matrix metalloproteinases (MMPs), and in particular MT1-MMP (Chen and Wang, 1999; Poincloux et al., 2009). We found that MT1-MMP was indeed expressed in 3D cultures grown in Matrigel™ and PuraMatrix™ 0.15% (Fig. 2c). These results, together with those on cells’ stiffness/Young’s modulus, seem indeed to indicate that 3D cultures were characterized by the formation of invadopodia and therefore by a higher invasion potential, although this was not linked to EMT phenotypic changes.

Since induction of EMT could not explain the formation of invadopodia in 3D cultures, we investigated the biochemical signature of A549 cells in 2D/3D culturing microenvironments. Our ELISAs results (Fig. 8) were consistent with scientific literature showing that, A549 cells are not capable to produce GM-CSF, a mediator that can stimulate the invasive capacity of human lung cancer cells (Uemura et al., 2006). IL-6 secretion by A549 cells is known to be induced by IL-1β and TNF-α (Crestani et al., 1994), which could not be detected in the cell culture media of any of our in vitro model (Fig. 8a). Accordingly, no IL-6
secretion was found in 2D or 3D cultures (Fig. 8a), supporting our data showing that spontaneous EMT did not occur in response to changes in substrate/architecture. IL-6 does in fact trigger EMT in A549 cell line (Shintani et al., 2016). Similarly, we could not detect secretion of IL-17 p70 (Fig. 8a), which once again induces EMT in NSCLC cell lines (Gu et al., 2015; Huang et al., 2016). In addition, in A549 cell line, IFN-γ is reported to efficiently reduce IL-8 secretion under the influence of IL-1β (Boost et al., 2008). In our study, we could not detect any secretion of IFN-γ (Fig. 8a) or IL-1β (Fig. 8b); accordingly, IL-8 secretion levels were remarkable, with a spot intensity comparable to that of the assay positive control (PT) (Fig. 8a). Autocrine IL8/CXCL-8, which is known to be especially elevated in NSCLC cell lines (Liu et al., 2016), facilitates invasion, EMT and survival in A549 cells (Desai et al., 2013) and other cancer cells (David et al., 2016; Waugh and Wilson, 2008). The key pathways that are activated in cancer cells in response to IL-8 are three: PI3K, MAPK and Rho-GTPase signalling cascades (Liu et al., 2016; Waugh and Wilson, 2008). The presence of IL-8 supported our conclusion that contractin synthesis (and the subsequent formation of invadopodia) was activated through Rho-GTPase signalling cascade (Fig. 2b). Notably, IL-8 levels were higher in 3D cell cultures than in 2D models, which made us conclude that IL-8 was responsible for promoting the cancer cell invasive potential in 3D cultures independently from EMT. We proved also that such high IL-8 amounts correlated to a preferential activation of the PI3K/Akt signalling cascade in in vitro models grown within Matrigel™ and of the MAPK/Erk1/2 pathway in PuraMatrix™-based 3D cultures (Fig. 8d and f, respectively). Both cultures promote cancer cells proliferation and survival. This might explain the increased cell viability of untreated 3D cultures grown on PuraMatrix™ 0.30% as compared to 2D models (Fig. 7). Notably, A549 cells cultured in 3D in PuraMatrix™ 0.25% and 0.30% showed a lower chemoresistance to docetaxel, a chemotherapeutic drug used in clinical setting for NSCLC treatment, than those cells cultured on glass (2D) (Fig. 9). This is in contradiction with the concept that, on average, the setting for NSCLC treatment, than those cells cultured on glass (2D)

5. Conclusions

In conclusion, in this study we evaluated a range of cellular properties within 3D models (morphology, topography, mechanical and biochemical signatures) that are key determinants in tumour cells invasion and cancer progression and survival in human patients. The differences among cell models described herein, highlight the importance of characterizing the properties of 3D cell models used in preclinical testing, as alternatives to animal studies. As also suggested by other experts in the field (Berg et al., 2014; Edmondson et al., 2014), we strongly believe that dissecting and quantifying the effect of 3D cell culture substrates on cancer cells phenotype transformations is indeed critical for drug efficacy assays. 3D cell models that show a satisfactory correlation to clinical scenarios could in fact be used for an effective preclinical efficacy validation of novel anticancer drugs, ultimately providing a perspective platform for personalised medicine approaches. Our study can therefore find direct application in any attempt to relate the pathological condition/process of interest with the appropriate 3D system.

Conflict of interest statement

The authors disclose no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tice.2017.11.003.

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