Culture phases, cytotoxicity and protein expressions of agarose hydrogel induced Sp2/0, A549, MCF-7 cell line 3D cultures

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Abstract Advancements in cell cultures are occurring at a rapid pace, an important direction is culturing cells in 3D conditions. We demonstrate the usefulness of agarose hydrogels in obtaining 3 dimensional aggregates of three cell lines, A549, MCF-7 and Sp2/0. The differences in culture phases, susceptibility to cisplatin-induced cytotoxicity are studied. Also, the 3D aggregates of the three cell lines were reverted into 2D cultures and the protein profile differences among the 2D, 3D and revert cultures were studied. The analysis of protein profile differences using UniProt data base further augment the usefulness of agarose hydrogels for obtaining 3D cell cultures.

Keywords Agarose hydrogels · 3D aggregates · Cell culture phases · Cytotoxicity · Protein expressions

Introduction

Cells in culture are important material for many applications. There is a constant change in the way cells are cultured, as augmented by advancements in cell culture material, media, instrumentation and

imaging technology. Cell cultures have a wide range of applications from simple applications such as testing the cytotoxic effects of candidate compounds to complex tissue engineering applications. Cells in culture are increasingly being used for studies that reflect a realistic in vivo condition rather than just cells of one type grown as monolayers in isolation. The trend in terms of increasing cell culture complexity is towards 3 dimensional (3D) cultures that make it possible to create ex vivo conditions in the lab. 3D cell cultures have proven to be very useful for several studies including cell physiology, cell behaviour, cellular metabolism, cytotoxicity, genotoxicity, bio-marker discovery, cell development and differentiation, protein and gene expression and tissue engineering applications (Pampaloni et al. 2007; Longati et al. 2013; Vidi et al. 2013).

The culture phases include the lag, log, plateau and the decline phases. Similar to the unique doubling time and the seeding densities as required for a particular cell type, there is a marked difference in the duration of each of the culture phases for the same cell type as 2D and 3D cultures. The number of cells and the time period of the healthy culture phases that the 3D system can sustain is much more than the 2D culture system for almost all cell lines (Cukierman et al. 2001; Li et al. 2002; Xu et al. 2009). This feature can be useful for understanding the tumor establishment and growth in vivo. Cells grown as 3D aggregates are known to be more resistant to drug-induced genotoxicity and cytotoxicity (Meli et al. 2012). This attribute is

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important to obtain more realistic data that can be translational for drug discovery and therapeutic applications. Many studies have highlighted the importance of the 3D cell culture systems in inducing a differential gene and protein expression for several cell lines (Zschenker et al. 2012). This is of significance in utilizing this differential expression for cancer research and biomarker discovery (Bazou 2010; Lai et al. 2011).

In our own earlier studies, we observed that SiHa (human cancer of the cervix cell line) and BMG-1 (human brain glioblastoma cell line) and cells grown as 3D aggregates showed marked differences in the cell culture phases, their susceptibility to genotoxic drug and protein expressions when compared to their 2D counterparts grown as monolayers (Ravi et al. 2014).

Several matrices and scaffolds of many types are available for culturing cells in 3D, as required by the study direction (Baker et al. 2011). These matrices and scaffolds range from simple hydrogels to complex natural and synthetic composites. In this study we highlight the usefulness of simple agarose hydrogels in obtaining 3D aggregates of three cell lines and the advantages that such aggregates offer for a variety of applications. We present our findings obtained from studies on the culture phases, cytotoxicity, protein and gene expression comparisons of agarose hydrogel induced 3D aggregates of Sp2/0, A549, MCF-7 cell lines with their 2D counterparts. Also, the induction of 3D spheroids and the formation of morphologically well defined extracellular matrix in the MCF-7 cell line using agarose hydrogels are highlighted.

As each cell line has unique optimal agarose hydrogel conditions for obtaining 3D aggregates, we have standardized the conditions to obtain floating 3D aggregates for the Sp2/0, A549, MCF-7 cell lines. The effect of such 3D cultures of human peripheral blood lymphocytes (HPBL) was also studied, with mitotic index (MI) as the end-point. The influence of the agarose hydrogel properties on the type of aggregates formed for a same cell line was also studied. We describe the 3D aggregate morphology and characteristics for the three cell lines studied and also their culture phases were compared with the 2D monolayer counterparts. The effect of a cytotoxic agent cisplatin on the 3D and 2D cultured cells, with cell viability as the end-point was studied along with the possible influence of agarose hydrogels in interfering with the activity of cisplatin on the cultured cells. The 3D

aggregates obtained from the three cell lines were reverted back into 2D culture system and their morphology and behavior were studied. This is to understand the changes in the cell physiology when they are reverted back as 2D cultures (3DRs) after converting them into 3D aggregates. The protein expression differences for the three cell lines as 2D, 3D and 3DRs were compared by SDS PAGE analysis. The proteins that showed expression differences by the 2D, 3D and 3DRs were traced to their genes and the possible utility of such genes and proteins for cancer biomarker discovery and intervention are explored.

Materials and methods

Establishment of 3D cultures

The cell lines Sp2/0, MCF-7 and A549 were maintained by standard methods in DMEM supplemented with 10 % FBS. For the 3D cultures, the optimal agarose concentrations for each cell line were standardized by culturing on 0.5, 0.75 and 1 % agarose. Required amount of agarose was melted in plain DMEM with care taken not to boil it. 1, 0.5 ml of molten agarose was added to 12 well culture plate and 24 well culture plate respectively. The seeding densities used for all experiments were 0.05×10^6 cells/ml, 0.15×10^6 cells/ml and 0.169×10^6 cells/ml for Sp2/0, MCF-7 and A549 respectively. The three continuous cell lines used in the study were obtained from the National Centre for Cell Sciences, India. Revert cultures of the three cell lines were setup by transferring healthy 3D aggregates into fresh wells with complete DMEM. The effect of 3D cultures on HPBL cultures was studied with the as the end point. Human Peripheral Blood Lymphocyte cultures were performed as per routine cytogenetic protocols 5 ml of peripheral blood was collected from a healthy human volunteer by venipuncture in heparinised container. 1 % of agarose was prepared in plain RPMI and care was taken to melt it without boiling. The dissolved agarose was poured onto six well plates and allowed to solidify under UV for 20 mins. After the gel was set, 5 ml of RPMI with 20 % FBS, 400 µl of PHA and 300 µl of whole blood was added to all the wells. The culture was incubated at 37°C at 5% CO₂ for 24 hours, after which 200 µl of colchicine was added to each well. After 24 hours incubation, the culture suspension was harvested after thorough disengaging

of the clot from the 3D and 2D wells into separate centrifuge tubes. After centrifugation at 1000 RPM for 10 mins, 8 ml of prewarmed hypotonic solution (0.75M Potassium Chloride solution) was added to each tube under constant vortex. The tubes were incubated at 37°C for 20mins after which they were spun at 1000 RPM for 10 mins. Under constant vortex, the cells were fixed by the dropwise addition of 8 ml Carnoy's fixative (Methanol: Acetic acid in the ratio 3:1). After incubation for 20mins at 37°C, the tubes were spun at 1000 RPM for 10mins after which a fixative change was done. The fixed cells were overnight incubated at 4°C and casted onto clean glass slides the following day. After overnight aging, slides were stained with 5 % Giemsa and viewed under the microscope. Under 10X magnification of the bright field microscope, in each field of a slide, the number of interphase cells and metaphase spreads were scored. The Mitotic Index was calculated according to the formula: $\text{Mitotic Index} = \frac{\text{No. of Metaphase spreads}}{\text{Total no. of cells}} \times 100$. The media and supplements used for cell cultures were obtained from GIBCO^R (Grand Island, NY, USA).

Culture phase analysis

2D and 3D cultures were initiated with the standardized 3D conditions as seven sets for the three cell lines. The cells were harvested at 24 h intervals till the 144th hour to ascertain the cell counts and viability for comparing the culture phases of cells in 2D and 3D conditions.

Cisplatin induced cytotoxicity

Cultures of Sp2/0, A549 and MCF-7 were setup in 2D and 3D conditions with the optimized parameters in 24 well plates. After 48 h, cells were subjected to cisplatin (Pharmaceuticals Worldwide, United Bio-tech, New Delhi, India) exposure for 3 h at the following concentrations: 5, 10, 15, 20 and 25 µg/ml. The cells were harvested and the cell pellet was suspended in fresh medium. The percentage viability was estimated for all the three cell lines in 2D and 3D cultures using trypan blue assay. The possible influence of the agarose hydrogel on interfering with the activity of cisplatin was ascertained by incubating the drug along with the agarose hydrogel for 3 h and then exposing 2D cultures of Sp2/0 and A549 cells to the medium along with the drug thus incubated.

Cytotoxicity levels of the cells exposed to the drug previously incubated with the hydrogels was estimated to study the possible influence of the hydrogels in interfering with the drug activity on the cells.

Protein profiling for cancer biomarker expression

Cells were harvested from confluent 2D, 3D and 3D revert cultures of Sp2/0, A549 and MCF-7 and proteins were extracted. Radio Immuno Precipitation Assay buffer containing Tris, SDS and EDTA was used for obtaining protein extract. The cells were harvested from the flask and the cell suspension was centrifuged at 1000 RPM for 10mins. The supernatant was discarded and the pellet was resuspended in 5 ml of PBS. After centrifuging the suspension at 1000 RPM for 10mins, the PBS was completely blotted and 1 ml of RIPA buffer was added. The tube was centrifuged at 5000 RPM for 10mins at 4°C and the clear suspension was stored at - 4°C in a Cryovial. Protein extracts were analyzed by SDS PAGE at 15 and 30 µg/ml concentrations and the gel was stained with Coomassie Brilliant Blue. The gel pictures were documented using a standard gel doc system. Based on the molecular weights, the protein bands that showed differences in the 3D and 3DR cultures when compared with the 2D counterpart protein extracts were analyzed for the protein and gene identifications by gene ontology and UniProt analysis.

Results

Establishment of 3D aggregates and 3D reverts

The 3D aggregates and the aggregate reverts of A549, MCF-7 and Sp2/0 cell lines were obtained. The 3D cultures of all three cell lines had two distinct components, the extracellular matrix and the cells as grouped aggregates. All the three cell lines in 3D had the cell aggregates being covered with the extracellular matrix. The 2D, 3D and the 3DRs of the three cell lines are presented in Fig. 1. The Sp2/0 3D aggregates were more diffused with loosely aggregated cells within a jelly-like extracellular matrix. The A549 3D aggregates were more compact with lesser amount of the extracellular matrix keeping the cells within a single large aggregate. The 3D aggregates of MCF-7 cells showed distinct, well formed spheroids as the cultures

Fig. 1 The morphological features of the three cell lines as 2D, 3D and 3DRs showed unique characteristics for each of the cell line. While A549 exhibited a compact 3D structure with medium amount of extracellular matrix holding the single large aggregate together, the MCF-7 showed distinct spheroid structures. The 3D cultures of the suspension cell line Sp2/0

showed loosely formed aggregate with large amount of jelly-like extra cellular matrix. While the 3DRs of A-549 and Sp2/0 showed faster movement of cells from the aggregates, the MCF-7 took a longer time for the cells to migrate out of the spheroids to form a monolayer

progressed, till the 4th day of culture. The individual spheroids appeared to fuse towards the 7th day, forming larger aggregates. A feature of the MCF-7 3D cultures that was markedly unique was the presence of well formed spherical acellular structures during the 2nd day of culture into which the individual cells appear to accumulate, leading to the formation of the distinct, well formed spheroids as the cultures progressed to the 4th day. The formation of 3D aggregates, the acellular spherical structures and the formation of the cellular spheroids are presented in Fig. 2.

The HPBL 2D and 3D cultures were harvested after 48 h and the mitotic indices were calculated. The average MI for 2D culture was 44.9 and 3D culture

was 46.98 %. There is no significant difference observed between 2D and 3D cultures for the MI.

Culture phase analysis

Healthy 3D aggregates were seen at 24 h after culture initiation and started to decline after 96, 120 and 72 h for A549, MCF-7 and Sp2/0 cell lines, respectively. In both of the cultures, A549 showed 98 % viability till 48 h. The percentage viability dropped to 96, 91 and 33 % in 2D culture while 3D cultures showed a viability of 97.6, 97 and 94 % at 24, 72 and 120 h. While the seeding densities for 2D and 3D conditions were 0.169×10^6 cells/ml, at the end of 192 h, the 2D

Fig. 2 The human breast cancer cell line MCF-7 formed distinct spheroids, whose formation progress could be well observed and documented. The progress of the cultures towards the formation of the spheroids is presented as A1–A4 in the panel A. The B1 to B3 panels show the presence and the role of globular extracellular matrix entities towards the formation of spheroids. Arrows in B1 show the initial formation of globular structure, where the cells are surrounded with a spherical shaped extracellular matrix. Arrows in B2 show the cells dividing inside the globular structure leading to the formation of spheroids called as acinar structures. B3 shows the formation of multiacinar structures which is formed by attachment of several

acinar structures. The Panel B shows the progressive formation of the acinar structures from the initial well with formed extracellular matrix. The individual cells appear to be trapped into the extracellular matrix with a definite spherical structure, divide and form an aggregate of cells that is spherical. Many such individual aggregates form the multiacinar structures. In the panel B, the acellular spherical extracellular matrix is presented at the extreme left, the progressive development of the acinar structures are present through the panel, ending with the multiacinar structures presented at the extreme right of the panel B

cultures had less than 20 % viability whereas 3D cultures had 63 % viability.

MCF-7 showed 95 % viability till 96 h for both culture conditions. The viability dropped to 93.6, 95.6 and 85 % for the 2D cultures and the 3D cultures showed viability of 93.6, 96 and 95 % at 24, 72 and 120 h. Though the seeding densities for the 2D and the 3D conditions were 0.15×10^6 cells/ml, at the end of 168 h the 2D cultures had less than 82 % viability whereas 3D cultures had 90.42 % viability.

In the 2D cultures of SP2/0, the viability was observed as 81, 87 and 80.8 % and in the corresponding 3D cultures, the viability was 81, 87 and 85 % at 24, 72 and 120 h respectively. The seeding densities for 2D and 3D conditions were 0.05×10^6 cells/ml and at the end of 192 h, the 2D cultures had less than 82 % viability whereas 3D cultures had 90.42 % viability. The growth curve comparison between 2D and 3D cultures of A549, MCF-7 and Sp2/0 cells is given in panel A of Fig. 3.

Fig. 3 The 3D cultures of the three cell lines showed a marked difference in the cell viability over time. The 3D cultures showed better viability and tended to last longer when compared to their 2D counterparts. Also, the panels 1, 2 and 3 show that the 3D cultures of the three cell lines were less susceptible to the cisplatin induced cytotoxicity when compared to their 2D

counterparts. Also, the differences were much pronounced for the suspension cell line Sp2/0 (3) than the monolayers. Among the monolayers, the A549 cell line showed more differences in higher concentration of the drug when compared to the MCF-7 cell line

Cisplatin induced cytotoxicity

The cisplatin dose standardization was done by initially exposing cells to 1, 5, 10 and 12 μg/ml. As there was a significant reduction in the viability only above 5 μg/ml, the standardized doses used in the experiment were 5, 10, 15, 20 and 25 μg/ml. The cisplatin induced cytotoxicity revealed higher drug susceptibility by cells in 2D cultures compared to those in 3D cultures.

A dose dependent decrease in the cell viability for both 2D and 3D cultures was seen. For A549, the untreated cells showed a viability of 92 %. For the exposed cells, the viability dropped to 91, 90, 84, 76 and 69 % in 2D culture whereas the 3D culture viability dropped to 92, 90, 88, 87 and 86 %. While the

seeding densities for 2D and 3D conditions were 0.169×10^6 cells/ml, at 25 μg/ml, the 2D cultures had viability of 69 % whereas 3D condition had viability of 87 % (panel 1 of Fig. 3).

For MCF-7, the untreated cells showed a viability of 83.33 %. In 2D cultures, the viability of exposed cells dropped to 83.33, 82.90, 81.43 and 80 % and in 3D cultures, the viability dropped to 83.33, 83, 82.76 and 82 %. The seeding densities for 2D and 3D cultures were 0.15×10^6 cells/ml, at 25 μg/ml the 2D condition had viability of 80 % whereas 3D condition had viability of 82 % (2 of Fig. 3).

The untreated Sp2/0 cells showed a viability of 93 %. In 2D condition, viability of the exposed cells dropped to 91, 88, 82, 75 and 64 % and 3D condition the viability

dropped to 98.80, 98.18, 94.73, 94 and 89 %, respectively. The seeding densities for 2D and 3D conditions were 0.05×10^6 cells/ml and at 25 $\mu\text{g/ml}$ dose, the 2D cultures had viability of 64 % whereas 3D cultures had viability of 89 %. The dose dependent cisplatin induced cytotoxicity for the 3 cell lines is presented in the panel B of (3 Fig. 3).

Protein profiling

Protein harvest was done from 2D, 3D cultures and 3D reverts at the exponential culture phase, with >96 % viability. The total protein extracts were analyzed by SDS-PAGE for the profile comparisons, with a sample concentration of 15 and 30 $\mu\text{g/ml}$ uniformly for all extracts (Fig. 4).

SDS PAGE and UniProt analysis

Sp2/0

On comparing the protein expression profile of 2D with that of 3D and 3D reverts condition, cells cultured in 3D showed prominent expression of proteins in the 49–20.6 kDa range. In the same range, protein expression is more enhanced in 3D revert cultures than their 2D and 3D counterparts (right panel, Fig. 4). This range includes proteins such as RAN Binding Protein 1, SRC Like Adapter Protein1, Eukaryotic Translation Initiation Factor EIF-2B Subunit 1, DNA Fragmentation Factor Alpha, Eukaryotic Translation Initiation Factor EIF-2S subunit 1, Proto-oncogene MAS etc. Another observation to be noted is that the expression pattern is similar in 2D cultures and 3D reverts but more enhanced in the later, whereas 3D cultures show a different pattern altogether. Above 49 kDa, protein expression is comparatively higher in 2D and 3D revert cultures than in 3D. Some of the proteins expressed in myeloma cells that come under this range include DNA Primase small subunit PRIM, DNA polymerase Delta subunit 2 POLD2, Cell Division Control Protein 6 CDC6, X-ray Repair Cross complementing Protein G22P1, T-complex Protein 1 subunit delta CCT4, Mitochondrial Translation Initiation Factor MTIF2 etc.

Mcf-7

In the range of 50–7.1 kDa, cells from 2D showed higher expression of proteins than cells cultured in

3D or the 3D reverts condition. 3D reverts showed a very prominent band around 34.8–43 kDa and reduced expression of proteins in any other range (centre panel, Fig. 4). Proteins such as EPCAM, M4S1 (Epithelial cell adhesion molecule), Milk fat globule (Lactadherin), Maspin (Serpin) show enhanced expression whereas other proteins like VDUP1 Thioredoxin, TPD52L1 Tumor Protein D52, CLDN4 Claudin 4, GCL Grancalcin, Trans-acting T cell-specific transcription factor GATA3 etc. fall in the range of proteins that show reduced expression in reverts. On comparing the protein expression pattern of the cells from three different culture conditions, 2D was found to exhibit a pattern more close to 3D, in contrast to the results observed for suspension cell line, Sp2/0.

A549

Protein expression in A549 shows a similar pattern to that of MCF-7, with a prominent band observed around 38–45 kDa and a slightly enhanced expression around 80–100 kDa in 3D reverts. An overall reduced intensity of bands in 3D was seen when compared with that of the proteins from 2D conditions (left panel, Fig. 4). Proteins that have a molecular weight in the 38–45 kDa range include Cellular Tumor antigen p53, Repulsive Guidance Molecule C, L-selectin etc. and proteins like Cadherin-1 are in the range of 80–100 kDa. Macrophage Inflammatory Protein 1 alpha, Tumor necrosis factor receptor 1, Protein Kinase C 1, CD30 ligand, Carcinoembryonic antigen, Tyrosine kinase-type cell surface receptor Her2 etc. are some of the proteins that have a reduced expression in both 3D and 3D reverts in comparison with 2D.

Literature referred for tissue/cancer specific biomarkers (Ravi et al. 2014) and UniProt database were used for obtaining information about the proteins that were differently expressed by the 3D and the 3DRs. The information about the proteins included their molecular weights and their functions. This analysis was used to interpret the SDS-PAGE results, as specific for the three cell types used for the study, as presented in Table 1 Essentially the size and staining intensity of the bands were used to draw a conclusion on their expression levels.

Discussion

It is known that the matrix used for the 3D cultures have an important bearing on the way cells behave morphologically and physiologically. This reflects as a change in the gene/protein expressions when cells are subjected to growth on a 3D matrix. The profound influence of the 3D matrix physical properties on the cultured cells is comprehensively reviewed recently (Owen and Shoichet 2010).

Of the three cell lines used, the Sp2/0 is a suspension cell line and the other two are attached cell lines. Agarose hydrogels supported their growth as 3D aggregates, although each cell line had its own specific % agarose and seeding density requirements to achieve optimal growth, as can be used for further studies. HPBL cultures did not show any marked differences in the MI % as 2D and 3D cultures. This is due to their being short-time cultures and also that the cells are non-cancerous. In contrast, the Sp2/0 cells although being suspension cultures, showed marked differences among the 2D and 3D cultures. This, along with the results obtained for the two monolayers confirm the utility of agarose hydrogels in inducing a different environment for the growth of continuous cell lines as 3D aggregates.

The three cell lines exhibited unique features as 3D aggregates, with each having a distinct morphology. The differences are largely in the amount of the extracellular matrix formed and the extent of aggregation of cells within the aggregates, the size of the aggregates and the formation of compact spheroids by the MCF-7 cells. These differences might be due to the origin of the cell lines and the type of cancers that they represent. This feature can give insights into the *in vivo* tumor progression and behavior. The MCF-7 cell lines clearly showed their progressing into spheroids that were morphologically similar to the acinar and multiacinar structures (Debnath and Brugge 2005).

As with the differences with the 3D aggregate characteristics, the 3DRs of each of the cell lines too had distinct features. The differences were both morphological and physiological. These include time taken for the individual cells to migrate out of the aggregates as well as their protein profiles. The comparison of culture phases for the three cell lines studied reiterated the earlier observations that the 3D culture systems can prolong the culture phases. This is

because of the cells survive for much longer as 3D aggregates than as 2D monolayers or suspensions. Due to this, the number of cells that the 3D system can support is much higher than that of the 2D systems, for a given culture phase (Li et al. 2002). Similarly, the cisplatin induced cytotoxicity results indicate the higher tolerance to the drug by the 3D aggregates, in tune with earlier reports (Pampaloni and Stelzer 2009; Chitcholtan et al. 2012).

Protein biomarker discovery has gained substantial interest from researchers as they can reduce the mortality and incidence rates by aiding in the early detection of some of the aggressive cancers. With the advent of state-of-the-art technologies such as Next Generation Sequencing and protein SELDI arrays, screening for potential genetic and proteomic biomarkers has become less tedious and more promising. In this study, we have done a preliminary analysis for observing the differences in protein biomarker expressions between 2D and 3D cultures of three cell lines: Sp2/0, MCF-7 and A549. Apart from the biomarker expression studies, we also made a comparison of protein expression patterns in the cell lines as 2D, 3D and 3D revert cultures.

For the Sp2/0 cell line, a panel of proteins expressed in multiple myeloma were chosen and studied for differences between the three culture conditions. It was observed that protein expression was more enhanced in 2D and revert cultures than in 3D for all proteins. Also protein expression pattern in 2D was more similar to 3D reverts while 3D cultures showed a different pattern. This may be attributed to two reasons: HPBL cultures are short-term and are non-cancerous. Hence the effect of the matrix on these cultures are either negligible or entirely absent when quantified in terms of the Mitotic Index. In contrast, the Sp2/0 cells although being suspension cultures, showed marked differences when cultured in 2D and 3D conditions. Previous studies have reported that the gene and protein signature of immortalized suspension cell lines are a lot closer to *in vivo* cancerous conditions than observed for solid tumor types. Thus, even though 3D cultures of suspension cell lines may not contribute much to biomarker discovery, it will indeed be interesting to study how presence of a matrix can alter the inherent expression of proteins in them.

When standard protein biomarker expressions in the adherent cell types, MCF-7 and A549 were compared for the three culture conditions, the

Table 1 The whole protein extracts were obtained from the 2D, 3D and 3DR cultures of three cell lines A-549, MCF-7 and Sp2/0

S. no.	Protein name	Molecular weight (kDa)	Expression levels when compared with 2D cultures	
			In 3D cultures	In 3D reverts
A549				
1.	Cellular tumor antigen p53	43.653	↓	↑
2.	Macrophage inflammatory protein 1 alpha	49.842	↓	↓
3.	Repulsive guidance molecule C	45.08	↓	↑
4.	L- selectin	42.187	↓	↑
5.	Tumor necrosis factor receptor 1	50.945	↓	↓
6.	Protein kinase C 1PRKCI	68.262	↓	↓
7.	CD30 ligand	63.747	↓	↓
8.	Carcinoembryonic antigen	76.795	↓	↓
9.	Cadherin 1	97.456	↓	↑
10.	Tyrosine kinase-type cell surface receptor Her2	137.910	↓	↓
MCF-7				
1.	VDUP1 thioredoxin	43.661	↓	↓
2.	EPCAM, M4S1 (epithelial cell adhesion molecule)	34.932	↓	↑
3.	TPD52L1 tumor protein D52	22.49	↓	↓
4.	CLDN4 claudin 4	22.07	↓	↓
5.	GCL Grancalcin	24.010	↓	↓
6.	Milk fat globule (lactadherin)	43.128	↓	↑
7.	Maspin (serpin)	42.10	↓	↑
8.	Trans-acting T-cell-specific transcription factor GATA3	47.916	↓	↓
Sp2/0				
1.	RAN binding protein 1	23.596	↑	↑
2.	Caesin kinase 2 beta	24.942	↑	↑
3.	SRC like adapter protein1	31.158	↑	↓
4.	Eukaryotic translation initiation factor EIF-2B subunit 1	33.816	↑	↓
5.	Thymidylate synthase TYMS	34.958	↑	↓
6.	DNA fragmentation factor alpha	36.572	↑	↑
7.	Eukaryotic translation initiation factor EIF-2S subunit 1	36.108	↑	↑
8.	Proto-oncogene MAS	37.055		↑
9.	Protein farnesyltransferase FNTA	44.013	↑	↑
10.	DNA primase small subunit PRIM1	49.295	↓	↑
11.	DNA polymerase delta subunit 2 POLD2	51.355	↓	↑
12.	Cell division control protein 6 CDC6	62.614	↓	↑
13.	X-ray repair cross complementing protein G22P1	69.484	↓	↑
14.	T-complex protein 1 subunit delta CCT4	58.066	↓	↑
15.	RNA binding protein EWSR1	68.496	↓	↑
16.	Histone lysine N methyl transferase EZH2	85.292	↓	↑
17.	Mitochondrial translation initiation factor MTIF2	81.289	↓	↑
18.	1-aminocyclopropane-1-carboxylate oxidase 2 ACO2	85.464	↓	↑

The protein profile differences as obtained by SDS-page analysis were first identified based on the prominence of the bands of specific molecular weights. The proteins that showed differences in the 3D and 3DRs on comparison with their 2D counter parts were analyzed by gene ontology and UniProt tools. The data thus obtained was used to relate the differently expressed protein bands to the proteins for the three cell lines; as relevant to the cancer types the cell lines represent

Fig. 4 SDS-PAGE protein analysis of the whole protein extracts from the 2D, 3D and the 3DRs showed differences in the protein profiles. The bands that appear in higher intensity are marked with arrows pointing upwards and those bands that are comparatively of less intensity are marked with arrows pointing downwards. Specific molecular weight regions that show

marked differences are presented below each of the three gel images. These differences can be useful for several applications such as for cancer biomarker discovery. Also the induced differences highlight the usefulness of simple agarose hydrogels as useful matrices for 3D cell cultures owing to their ease of use

expression pattern showed similarity between both cell lines. The protein profile of cells from 2D and 3D cultures showed a similar expression pattern while that of the reverts was different from both. The reduced intensity of protein expression in 3D cultures may be closer to *in vivo* conditions; it is a known fact that 2D conditions promote abnormal proliferation, metabolism and reduced cell–cell interactions not seen in tumor tissues. We were able to observe that revert cultures had increased expression of L-selectin, Cadherin 1 and osteopontin for A549 cell line and Epithelial Cell Adhesion Molecule (EPCAM) Milk fat globule MFGE8 for MCF-7 cell line. These proteins are involved in cell–matrix adhesions and are more enhanced when 3D aggregates were made to adapt to 2D conditions and sustain as monolayers.

Response of cells to environmental cues from the culture conditions can be examined in various ways. In this study, we considered the protein expressions of cells from three different lineages cultured in the conventional 2 D way and in the presence of a matrix. When cells are grown on a flat culture dishes without any matrix, they develop cell anchorage receptors only on the ventral surface. When cells are grown in a 3 D environment, cell-cell and cell-matrix interaction is more profound as witnessed by the presence of cell adhesion receptors all over the cell surface. This results from the formation of aggregates or cell masses that are induced by the matrix. Thus, in 3D cultures, the adhesion proteins are seen all over the surface implying that the cells interact with the matrix analogous to their behaviour *in vivo* (Berardi et al. 2013).

An important protein in whose expression we could see a matrix-induced difference is Cadherin 1 in the A549 cell line. Cadherin 1 belongs to the family of transmembrane glycoproteins which mediate cell adhesion through calcium dependant signalling pathways. This protein is also a tumor suppressor protein which controls cell proliferation. These receptors are also implicated in various other processes through their association with the secondary messengers, Catenins. Catenins are central players of the Wnt signalling pathway which play a major role in early embryonic development and tissue homeostasis (Stockinger et al. 2001). Cadherin 1 was found to be less expressed in 3D A549 cultures when compared to their 2D counterparts. Similar results have been reported in 3D cultures of PrCa, a prostate cancer cell line (Harma et al. 2010). From these data and our results, we infer that down-regulation of Cadherin 1 is likely to be a characteristic feature of certain types of tumor cells in 3D cultures. This, while being different from the highly upregulated levels in 2D cultures, has been found analogous to *in vivo* conditions for this tumor type.

Previous reports show that the expression level of Cadherin 1 is variable, depending on the stage of cancer progression. Analysis of squamous cell carcinomas, breast and colon cancer reports that loss of cadherin 1 expression occurs when cells are starting to invade; these expression levels get restored on reaching different sites of metastasis (DesRochers et al. 2012). Hence, it is possible that in the 3D culture, expression levels of this protein might vary at different culture phases; reduced expression seen during the proliferative stage and restored levels likely observed during the stationary phase. This inference is augmented by the increased expression of the Cadherin 1 when the 3D aggregates are reverted back into a 2D condition (Table 1, point # 9 for the A549 cell line).

Maspin is a serine protease inhibitor well established for its inhibitory role in cancer progression. Maspin's biological function is fundamental in regulation of cell migration, invasion and angiogenesis (Cella et al. 2006). In the cultures of the breast cancer cell line MCF-7, we observed a reduced expression of Maspin in 3D conditions when compared to their 2D counterparts. The cells in 3D cultures exhibit slower progression when compared to their 2D counterparts (Figure 3, panel B). Maspin has been found to be down-regulated in certain cancer types (breast, gastric

and prostate) while being up-regulated in others (pancreatic, thyroid and colorectal). This suggests that the role of maspin differs from one tissue type to another one (Berardi et al. 2013). 3D cultures prove advantageous to study such proteins because cells in 2D cultures do not expand, differentiate and function according to their cell types. Irrespective of their different tissue types, fibroblast and epithelial cells were found to merely proliferate and migrate in culture dishes. But in 3D cultures, mammary epithelial cells were found to differentiate and secrete milk proteins unlike their 2D counterparts (Wozniak et al. 2004). We could see the matrix induced differences in maspin expression by the breast cancer cell line MCF-7 in the 3D cultures and not in the 2D and the 3D reverts (Table 1, point # 7 for the MCF-7 cell line). These observations on the maspin expression differences reiterate that 3D cultures are more realistic *in vitro* models to study breast tumor.

Cells growing in an artificial environment such as conventional culture flasks or plates spread out in an extreme manner due to lack of dorsal anchorage receptors. These unnatural geometric constraints make the cells behave in a different way from cells seen in their natural environment (Berardi et al. 2013). With the introduction of a matrix into the culture conditions, cells are observed to adopt a phenotype comparable to their *in vivo* conditions. Focal adhesions are sites where integrins and other proteins mediate adhesion to actin cytoskeleton. The protein constitution of these sites is modulated by the cells in response to the molecular composition and nature (2D or 3D) of their extra cellular environment. Focal adhesions have been preliminarily observed attached to the periphery or central region of stress fibres in cells cultured on two dimensional surfaces. We found that the level of grancalcin, a protein implicated in focal adhesion sites, to be down-regulated in 3D cultures of MCF-7 than in their 2D counterparts. This implies that the varied composition of the cell's cytoskeleton and its interaction with the matrix plays a key role in altering the signalling pathways which regulate cell behaviour. These interactions are in turn controlled by the mechanical/tensile properties of the matrix. For example, in our own studies we observed that either partially embedded aggregates or floating aggregates can be obtained from a same cell line by varying the type of agarose, the composition, the extent of melting and the gel volume.

Conclusion

Agarose hydrogels are probably the simplest materials available to obtain 3D aggregates and are easy to use, economical and time saving. They provide us with a matrix substance whose characteristics can be changed, by dissolving them in a variety of liquids, their composition and the extent of melting. These features have an effect on the types of 3D aggregates formed; even for the same cell line. Characteristic features of the 3D aggregates obtained, their culture phase features, the cytotoxic susceptibility and protein profiles are useful end points that can be applied for various studies including cell physiology, protein and gene expression, drug discovery and cancer research. Also, this cell culture model induced the formation of extracellular globules and acinar and multiacinar structures of the MCF-7 cell line proving its advantage in providing a more realistic picture when compared to the 2D cultures. We could demonstrate the effects of cell compaction as induced by the matrix on the morphological and protein expression changes in the 3 cell lines studied. Also, we demonstrate that agarose hydrogels do not interfere with the activity of the drugs such as cisplatin and bleomycin. This augments the advantages of the study approach, especially when the gels can be suitable modified for their physical properties to obtain a different type of cell aggregates in 3D cultures. The matrix induced morphological and physiological changes as can be analyzed in this paper through the altered culture phases study, cytotoxic susceptibility and protein expressions can be useful study approaches for a variety of applications.

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