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Fibronectin in Layer-by-Layer Assembled Films Switches Tumor Cells between 2D and 3D Morphology

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Abstract

Tumor cells showing a 3D morphology and in coculture with endothelial cells are a valuable in vitro model for studying cell–cell interactions and for the development of pharmaceuticals. Here, we found that HepG2 cells, unlike endothelial cells, show differences in adhesion to fibronectin alone, or in combination with poly(allylamine hydrochloride). This response allowed us to engineer micropatterned heterotypic cultures of the two cell types using microfluidics to pattern cell adhesion. The resulting cocultures exhibit spatially encoded and physiologically relevant cell function. Further, we found that the protrusive, migratory and 3D morphological responses of HepG2 are synergistically modulated by the constituents of the hybrid extracellular matrix. Treating the hybrid material with the cross-linking enzyme transglutaminase inhibited 3D morphogenesis of tumor cells. Our results extend previous work on the role of fibronectin in layer-by-layer assembled films, and demonstrate that cell-specific differences in adhesion to fibronectin can be used to engineer tumor cell cocultures.

Graphical Abstract

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Author Contributions

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Notes

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Supporting Information

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Figures S1–S3 and Supporting Methods(PDF)

Movie S1 ([AVI](#))

Movie S2 ([AVI](#))

Movie S3 ([AVI](#))

Movie S4 ([AVI](#))

Keywords

layer-by-layer; hepatocyte; microfluidics; fibronectin; transglutaminase; organoid

1. INTRODUCTION

Tumor cell cultures are commonly used as in vitro models for testing anticancer drugs.¹ Cell morphology appears to be a critical component for modeling solid-tissue tumors, as cultures that exhibit a 3D architecture, rather than grow as 2D monolayers, are physiologically more relevant to testing tumor drug responses.^{2,3} Therefore, developing functional cell adhesive materials which elicit tumor cell phenotypes that cannot be captured by conventional cell cultures is of great biomedical interest.^{4,5} Reflecting the complex microenvironmental cues that drive tumor cell function in vivo,² developing cell cultures that incorporate heterotypic interactions of tumor cells with other cell types, such as endothelial and stromal cells, can further increase the predictive power of in vitro anticancer drug screening assays.³

Additionally, when compared to random mixtures of cell types, the use of micropatterning approaches offers precise spatial control over cell location and phenotype.⁶ However, micropatterning two different cell types in the same culture dish is challenging, because many anchorage-dependent cells share the same adhesive ligands. Researchers have developed several methods that overcome these challenges and permit micro-patterning of cocultures.^{6–8} Yet, such methods have several practical limitations such as the need for serum-free media, the use of harsh solvent with biological proteins, the need for specialized equipment, substrates and/or chemistries for barrier removal, or barrier fragility when elastomeric membranes are used. While the use of discrete microfluidic channels for heterotypic tissue culture alleviates some of these limitations,⁹ the need for fluidic connections limits throughput. Hence, there is also a pressing need for developing robust and scalable approaches that can simplify heterotypic cell patterning involving tumor cells.

We demonstrate here that it is possible to robustly pattern cocultures of a liver tumor cell line with endothelial cells by sequential plating on adhesive surfaces prepared by modifying layer-by-layer assembled films of fibronectin and a synthetic polyelectrolyte, poly(allylamine hydrochloride), which we previously presented.¹⁰ In the resulting cocultures, both tumor and endothelial cells exhibit physiologically relevant differences in phenotype, depending on their spatial location and microenvironmental context. Additionally, we could switch tumor cells between a 2D and 3D phenotype by treating the layer-by-layer fabricated adhesive substrate with the ECM cross-linking enzyme,

Transglutaminase 2. Because of its simplicity, this approach for coculture is not only useful for modeling tumor cell interactions with other cell types, but may also allow the scale-up of these engineered microtissues for high-throughput assays.

2. MATERIALS AND METHODS

Note: Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

2.1. Materials.

Paraformaldehyde 16% (Electron Microscopy Sciences, Hatfield PA), fibronectin, poly(allylamine hydrochloride), bovine serum albumin (BSA), Triton X-100, PKH 26 red dye, guinea pig tissue transglutaminase 2 (TG2), PKH 67 green dye, Texas-red maleimide (all from Sigma, St. Louis MO), Dulbecco's phosphate buffered saline (DPBS; Mediatech, Manassas VA), Trypsin-EDTA 0.25%, Dulbecco's modified Eagle medium (DMEM) media with 10% FBS supplement, HepG2 cells (all from ATCC, Manassas VA), human umbilical vein endothelial cells (HUVEC; Lonza, Walkersville, MD), mouse antifibronectin antibody (clone FN12-8, Takara Bio Inc., Shiga, Japan), AlexaFluor 488 goat antimouse IgG (H+L), H33342 (all from Invitrogen, Carlsbad CA), Poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning, Midland, MI), SU-8 photoresist and developer (MicroChem Corp, Newton, MA), and glass-bottomed dishes (MatTek, Ashland, MA).

2.2. ECM Preparation.

The various cell adhesive substrates used in this study were prepared as follows: The "FN" substrate was prepared by incubating a 50 $\mu\text{g/mL}$ solution of fibronectin in DPBS on tissue culture polystyrene (TCPS) surfaces for 90 min. The solution was then aspirated and the surface rinsed twice with DPBS. The "PAH" substrate was prepared by incubating a 1 mg/mL poly(allylamine hydrochloride) solution (pH 5.0) in DI- H_2O on TCPS surfaces for 40 min, followed by aspiration and rinsing twice with DI- H_2O . The "hCAM" surface was prepared by incubating the "FN" substrate for 40 min with a 1 mg/mL poly(allylamine hydrochloride) solution as above at room temperature. The solution was then aspirated and the wells rinsed twice with DI- H_2O . Where transglutaminase type 2 (TG2) was used, hCAM substrates were first prepared as above, and incubated overnight with TG2 solution (0.1 U/mL TG2, 5 μM CaCl_2 , 2 mM DTT, and 137 mM NaCl, in 25 mM Tris buffer solution at pH 7.4). Controls were treated similarly without including TG2 in the buffer. The adhesive substrates (also interchangeably referred to as ECMs) were stored under DPBS at 4 °C up to 2 weeks until used.

2.3. HepG2 Attachment.

Cells were seeded at a density of 15 800 cells/ cm^2 in each well of 12-well plates modified by the various ECMs and images were acquired at the center position of each well. After 1 h, unattached cells were removed by aspirating media and rinsing twice with PBS, followed

by the addition of fresh media to the wells. Cells were then reimaged at the same positions to discern changes.

2.4. HepG2 Spreading.

Cells were plated on TCP, FN, PAH, and hCAM for 24 h, fixed, and labeled with DAPI for counting nuclei. Individual cell objects from 10× phase contrast images were outlined in ImageJ, and the measured areas were normalized to the corresponding number of nuclei to get single cell areas. Each measurement is an average of six replicate wells from a total number of cells of 301, 332, 172, and 269 for TCPS, FN, PAH, and FN/PAH, respectively.

2.5. Remodeling of Fibronectin.

To visualize fibronectin remodeling, we cultured cells on hCAM or FN for 3 days, fixed and labeled with an antifibronectin antibody. Antibody labeling was done as described previously.¹¹ Briefly, plates were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Wells were blocked with 10% goat serum/1% BSA blocking solution for 30 min at room temperature to block nonspecific antibody adsorption. They were then incubated with mouse monoclonal antifibronectin antibody solution prepared in blocking solution for 45 min at room temperature and washed three times to remove unbound primary antibody. The wells were reblocked with the blocking solution and finally incubated with goat antimouse secondary antibody labeled with Alexa 488, as well as H33342 to visualize nuclei. The grayscale fibronectin and nuclear images collected using a 10X 0.4 NA objective at 1392 × 1040-pixel full frame size, were overlaid in color using the “RGB Gray Merge” plug-in in ImageJ. Images were cropped to show detail of ECM reorganization.

2.6. TIRF Microscopy.

Total internal reflection microscopy (TIRF) was employed to visualize cell interactions with the different cell adhesion materials. Cell membranes were first stained with Cellmask Orange stain prior to trypsinization, following the manufacturer’s instructions. PAH, FN and hCAM surfaces were prepared on 35 mm glass bottom culture dishes with No. 1.5 cover glass using the same procedure as described above. HepG2 cells were seeded at a concentration of 20 000 cells/cm², and images were collected immediately after cell seeding on the culture dishes. Images were acquired at 37 °C using a Total Internal Reflection Zeiss Axiovert 200 Microscope (Zeiss, Thornwood, NY) equipped with a Lasos77 Argon ion laser light source (Lambda Photometrics Ltd., Hertford-shire, UK) with a 100×/1.45 NA Oil Plan-Fluar objective. Light was passed through a filter set with the following characteristics: excitation 514/10 nm, dichroic beamsplitter 540 nm, and emission 575/50 nm.

2.7. Microfluidic Patterning of HepG2-HUVEC Cocultures.

The PDMS microfluidic manifold used for microfluidic patterning of the cell adhesion materials in cells was prepared as previously described.¹⁰ The procedure for preparing the micropatterned surfaces was as follows: 100 mm TCPS dishes were coated for 90 min with 1 mg/mL solution of fibronectin in PBS. The fibronectin coated patch was then washed 2× with DI-H₂O and briefly dried and sealed against the PDMS microfluidic manifold. The

microfluidic channels were then rinsed by flowing in sterile filtered DI H₂O for 20 min using gravity flow, followed by a freshly prepared solution of poly(allylamine hydrochloride) for 40 min. The channels were rinsed again by flowing in DI H₂O for 20 min, the PDMS manifold removed, and the micropatterned dishes were finally rinsed twice with PBS and stored under PBS at 4 °C until further use. For patterning tumor-endothelial cocultures, we trypsinized and plated HepG2 on the dish for 30–60 min. Unattached cells were removed by washing 2× with PBS, fresh media added to the dish, and placed in the incubator. After 2 days, HUVEC were trypsinized and plated in the same dish and allowed to adhere for 30–60 min in 10 mL of media, composed of 5 mL each of HepG2 and HUVEC media. For visualizing individual cell types in mixed cultures, cells were first labeled with PKH 26 or PKH 67 live cell dyes, following the manufacturer's instructions. Samples were illuminated with a mercury arc lamp and the following filter sets were used for imaging the respective dyes: dye PKH 26 - excitation 546/12, dichroic beamsplitter 580, emission long pass 590, and dye PKH 67 - excitation 540/40, dichroic beamsplitter 510, emission 515/50.

2.8. Confocal Microscopy.

Cells were fixed and double-labeled with Texas-red maleimide to label cell bodies and antifibronectin antibody to label fibronectin as described above. For some samples, cells were colabeled with Texas-red maleimide, N-hydroxysuccinimidyl ester of fluorescein, and H33342. Some samples included the ECMs without any cells, labeled for fibronectin. Samples were imaged on a Zeiss LSM 800 laser scanning confocal microscope equipped with diode lasers at 405, 488, 561, and 640 nm, two-variable dichroic beamsplitters, and a 2-channel multialkali detector. Images were collected over 1023×1023 pixels with a slice thickness of 4.91 μm , using an Zeiss EC-Plan Neofluor 10× 0.3 NA objective (Zeiss). The NHS-fluorescein colabeled samples and the bare FN and hCAM samples were confocally imaged using a Zeiss 20X Plan-Apochromat 0.8 NA objective over 1024 × 1024 pixels with a slice thickness of 0.3 and 0.9 μm , respectively. Image stacks were opened in ImageJ and assembled in Inkscape except for Figure S3, which was assembled using Zeiss Zen software.

2.9. Quantification of HepG2 3D Morphogenesis.

Differences in brightness due to 3D cell assemblies in phase contrast images were used to threshold and segment areas with 3D structure. Phase contrast images obtained with a 10× 0.3NA objective were first smoothened by applying a Gaussian filter with a 2-pixel kernel iteratively 10 times. An intensity threshold for segmenting the objects of interest was then manually selected using the images on hCAM. The same threshold was then applied to cells on both surfaces compared, and areas quantified. Three frames were measured per sample and averaged over 3 samples.

2.10. Quantification of HepG2 Migration.

Cell migration was quantified as follows: Time-lapse phase contrast images of HepG2 cells were collected at 5 min intervals over a period of 200 min using a Zeiss 20X 0.4NA LD Plan Neofluar/Ph2 objective. Cells were segmented using the Empirical Gradient Thresholding plugin for ImageJ.¹² The segmentation masks were overlaid over the phase contrast images to select single cell objects in ImageJ. Cell migration rate and directional persistence were subsequently measured using the ADAPT plugin for imageJ.¹³

2.11. Statistical Analysis.

Student's t test was used to assess statistical significance at the $p = 0.05$ level. The HepG2 migration data was analyzed as follows: speed and directionality data from the cell migration experiments were analyzed respectively using a generalized mixed effects model, including a fixed effect for the mean of each ECM and random effects for experimental replicates within each ECM. The variance among individual observations was allowed to differ for each experimental replicate of each ECM to account for apparent heterogeneity. This model was evaluated in a Bayesian framework in R¹⁴ using the package *rjags*,¹⁵ resulting in posterior distributions from which uncertainties of interest were characterized. A detailed description of this model is provided in the Supporting Information.

To assess the robustness of results, we also conducted alternative analyses. For each experimental replicate in each ECM, the sample mean, sample median, and number of observed cells were determined. The sample means were used in an ANOVA model, in which each mean was weighted by the corresponding number of observed cells. The results of the ANOVA model were used to construct 90% confidence intervals for the mean of the distribution. Differences among the ECMs were assessed using Tukey's Honestly Significant Differences,¹⁶ which produces 90% confidence intervals that have been extended to account for the fact that six comparisons are conducted. The ANOVA assessment was also repeated using the sample median of each experimental replicate for each ECM in place of the sample means.

3. RESULTS

3.1. Micropatterning HepG2 and HUVEC Cocultures.

Oncogenically transformed cells have long been observed to exhibit decreased adhesion to fibronectin.^{17,18} On the other hand, many normal cells, including endothelial cells,¹⁹ show rapid adhesion to fibronectin. This difference in adhesion suggested to us a path to spatially engineer patterned cocultures of tumor and endothelial cells. We employed layer-by-layer assembly—an approach to generate adhesive nanofilms using charge interactions between polyvalent anions and cations, and can be used with naturally occurring ECM proteins.^{10,20} To test the feasibility of doing this, we first qualitatively examined how the human hepatoma cell line, HepG2, adhered to fibronectin and to the polycation, poly(allylamine hydrochloride). Four distinct adhesion substrates were prepared in 12-well tissue culture dishes, as described in Materials and Methods: bare tissue culture polystyrene (TCPS), TCPS coated with fibronectin (FN), TCPS coated with poly(allylamine hydrochloride) (PAH), and TCPS coated with both fibronectin and poly(allylamine hydrochloride) (hCAM), using layer-by-layer sequential adsorption in that order. One hour after plating, images of the same fields of view on the four surfaces were collected before (Figure S1A–D), and after (Figure S1E–H), washing with PBS to remove unattached cells.

We observed low cell attachment to TCPS, and little to no cell attachment onto FN (comparing Figure S1A, B to each other and to E, F) during the first hour after seeding. In contrast, both PAH and hCAM surfaces allowed almost complete cell attachment (comparing Figure S1C, D to G, H) in the same period. On the basis of these observations,

we conclude that at 60 min, fibronectin alone does not allow the attachment of this tumor cell line, while poly(allylamine hydrochloride) alone or in combination with fibronectin promotes rapid cell attachment. On the same time scale, we observed rapid endothelial cell attachment to FN (data not presented). Based on these results, micropatterning of tumor-endothelial cocultures was then attempted. Microfluidically patterned lines of hCAM over a background of FN were first created on tissue culture plastic plates, as described in Materials and Methods (Figure 1A). HepG2 were then plated on the surface in HepG2 media, and unattached cells were rinsed away after 40 min. HepG2 adhered and patterned on the hCAM lines but not on the surrounding regions of FN (Figure 1B). Forty-eight hours after plating, the HepG2 cells maintained the fidelity of the patterning, as observed by fluorescent imaging of Calcein AM label (Figure 1C). When HUVEC were then seeded onto the patterned HepG2 culture, they firmly attached and started spreading on FN areas within 30 min (Figure 1D). We expect that cells would have landed on hCAM areas as well, although we could not distinguish them in phase contrast.

3.2. Patterned Cocultures Show a Spatially Dependent Phenotype.

By 4 days in culture, both cell types exhibited distinct morphologies on hCAM lines compared to that on PAH. Corresponding to the boxed region on the micropattern outlined in Figure 2A, HepG2 on hCAM reorganized into 3D aggregates visible under phase contrast (Figure 2B). Cells on PAH regions did not show this morphological change and remained spread (Figure 2B). To verify the 3D nature of cell morphology on hCAM, we imaged cells by confocal microscopy after staining with Texas-red maleimide (Materials and Methods). HepG2 cells showing a 3D appearance in phase-bright regions on hCAM (Figure 2C), also had a raised, 3D morphology in confocal cross-section (Figure 2E), whereas cells on PAH with a monolayer appearance and a lower phase contrast signal (Figure 2D), also had a flat morphology in confocal cross-section (Figure 2F). The bare FN and hCAM ECMs in the absence of cells were planar and showed a uniform distribution of fibronectin (Figure S2). To gain further insight into cell morphology on both surfaces, cells on hCAM or PAH were labeled with fluorescein-NHS ester to label amines, and H33342 to label nuclei, in addition to the Texas-red maleimide stain, and imaged by confocal microscopy with a 20× 0.8 NA objective. Cells on hCAM showed an outer shell of Texas-red maleimide label with a 2–3 cell thick solid interior labeled with fluorescein and H33342 (Figure S3). Cells on PAH showed an outer shell of Texas-red maleimide label with a cell body monolayer labeled with fluorescein and H33342 (Figure S3).

We then examined if the HUVEC in coculture also showed any location-dependent differences in morphology. To visualize this, HUVEC were first labeled with the red fluorescent live cell dye PKH 26 and plated on the patterned HepG2 as before. Whereas the endothelial cells on FN regions formed a flat monolayer, those on hCAM organized within a day into microvessel-like cords (Figure 3A–D). The cords were not stable, and disassembled over a period of a further 2 days (data not shown). Although we focus the rest of the study on understanding tumor cell interactions and morphogenesis on hCAM, we note that it is significant that endothelial cells exhibit a cordlike morphology on hCAM, in the presence of 3D HepG2 cells because endothelial cells are critical for tumor growth and function in vivo in promoting tumor oxygenation by forming capillary blood vessels.^{21,22}

3.3. HepG2 Spreading.

HepG2 3D morphogenesis on hCAM (Figure 2) suggested a role for the fibronectin present within hCAM, in regulating this response. To gain further insight into this, we examined other HepG2 cell responses to these surfaces. When HepG2 cells were plated and left undisturbed for 24 h, all 4 substrates allowed cell attachment (Figure 4A–D). However, there were noticeable differences in cell morphology. By 24 h, cells on TCPS adopted a compact colony morphology with few extensions at the periphery (Figure 4A), while cells on FN displayed a more spread morphology and comparatively less colony compaction (Figure 4B). On the other hand, cells on PAH and hCAM both showed multiple filopodia at cell edges, and a well-spread morphology (Figure 4C and 4D). The average cell spread areas on TCPS, FN, PAH, and hCAM were respectively, $336 \pm 54 \mu\text{m}^2$, $638 \pm 211 \mu\text{m}^2$, $1555 \pm 203 \mu\text{m}^2$, and $1439 \pm 204 \mu\text{m}^2$ (Figure 4E). Cell spreading was significantly different between all pairs of the four surfaces, except for PAH and hCAM which had statistically similar spreading. Together, these results show that polyallylamine has a greater effect on promoting cell spreading than does fibronectin.

3.4. HepG2 Migration.

Because morphogenesis is often associated with cell migration,¹⁷ we then used time-lapse phase contrast microscopy to compare cell migration on hCAM to that on the other three surfaces. Cells on TCPS showed little migration (Movie S1). Surprisingly, there was also little observable migration on either FN (Movie S2) or PAH (Movie S3) surfaces, even though cells showed some protrusions on these substrates. There was evident cell migration only on hCAM (Movie S4). Image analysis was then employed as described in Materials and Methods, to quantify possible differences in cell migration speed and direction between cells on the four substrates. The original data and modeling results are presented in Figure 5, which was constructed using the R package *ggplot2*.²³ Results pertaining to cell speed, which was analyzed on the log-scale to reduce the influence of outliers, are presented in terms of the median of the distribution to facilitate translation to the original scale (i.e., the median of log-transformed observations is equal to log-transformation of the median observation, but this relationship is not true for averages).

The results in Figure 5E suggest that the median cell speeds on PAH or hCAM are between one to four times greater than the median speeds of cells on TCP or FN. The results do not suggest which of TCP or FN has greater median cell speed, with the statistical uncertainty allowing for the median speed for TCP to be roughly anywhere from half to twice the median speed for FN. Similarly, the results do not suggest which of PAH or hCAM has greater median cell speed, with the statistical uncertainty allowing for the median speed for PAH to be roughly anywhere from half to three times the median speed of hCAM.

Figure 5F suggests that the mean directionality on hCAM was greater than the average directionality of cells on TCP or FN. The results do not suggest which of TCP or FN has greater average directionality, since 0 falls within the uncertainty interval comparing average directionality for TCP and FN under all six of the implemented analyses. Observationally, the average directionality for PAH appears to fall between that of TCP or FN and that of

hCAM; however, these results do provide sufficient evidence to statistically confirm this ordering.

Although consideration of alternative modeling strategies may yield different results, the results presented here were in general agreement across the six implemented analyses, which indicates they are not specific to any one of the many ways this data may be analyzed. Together, these data suggest that an increase in cell protrusions is not sufficient to promote migration of these tumor cells under our experimental conditions. They also show a surprising synergy between PAH and FN in promoting cell migration, each of which independently did not have the effect.

3.5. Fibronectin in hCAM Alters Tumor Cell–Surface Interactions.

Because cell migration is mechanistically linked to adhesion,²⁴ we employed total internal reflection fluorescence (TIRF) microscopy to examine differences in cell-ECM interactions on hCAM, comparing it with cells on uncoated glass, FN, or PAH. To do so, cells were plated on glass-bottom dishes prepared as described in Materials and Methods, labeled with Cellmask Orange stain, and examined with a 100×/1.45 NA oil-immersion objective under TIRF conditions as described in Materials and Methods. During the first 5–6 min after plating, tumor cells on hCAM showed multiple thin, long filamentous cell membrane processes close to the substrate (Figure 6). This contrasted with that on FN and PAH, where they showed smaller, punctate interaction areas between the cells and the surfaces. No discernible membrane processes were observed on uncoated glass surface (Figure 6).

The above differences in cell morphogenesis, migration and protrusive activity on hCAM compared to PAH demonstrate that the presence of fibronectin in hCAM contributed to these differences. We then examined if HepG2 cells could directly interact with the fibronectin on hCAM. To determine this, cells were plated on either hCAM or FN, fixed after 72 h, and labeled for fibronectin with an antifibronectin antibody, as described in Materials and Methods. We had previously observed that fibronectin is homogeneously adsorbed in polyelectrolyte multilayers.¹⁰ Cells on hCAM (Figure 7B) locally degraded the fibronectin and remodeled it into bright focal densities of the protein, as those on FN (Figure 7A). Qualitatively, there appeared to be difference in the morphology of these fibronectin densities, although we have not quantified them. We conclude that these tumor cells physically interact with and remodel the fibronectin present within hCAM. We also noticed a decrease in overall fibronectin label intensity on hCAM compared to that on FN (Figure 7).

3.6. Transglutaminase Treatment Inhibits Tumor Cell 3D Morphogenesis on hCAM.

We then asked how the presence of fibronectin might drive the spontaneous 3D morphogenesis of tumor cells that we observe on hCAM. We hypothesized that the ability of tumor cells to remodel the fibronectin may be responsible for the 3D morphogenesis of tumor cells on hCAM. ECM cross-linking enzymes such as transglutaminase alter tumor cell responses by altering ECM stiffness and susceptibility to remodeling by extracellular proteases.²⁵ We had previously shown that transglutaminase type 2 (TG2) treatment leads to ECM stiffening and alterations in vascular smooth muscle cell morphology, and contractile

signaling on collagen type 2 ECM.²⁶ We reasoned that treating hCAM substrates with TG2 might inhibit HepG2 ability to remodel fibronectin by cross-linking it, and thereby block 3D morphogenesis.

Accordingly, hCAM surfaces were prepared and treated with TG2 as described in Materials and Methods. Control surfaces were similarly prepared without adding TG2 enzyme. HepG2 cells were plated on both substrates and cells were fixed 96 h after plating using paraformaldehyde and labeled by indirect immunofluorescence for fibronectin, and for cellular protein using Texas red maleimide. Confocal microscopy of the labeled fibronectin showed a dramatic change in the organization of fibronectin upon TG2 treatment. Whereas the fibronectin on hCAM was organized into bright, fibrillar focal densities (Figure 8A), the same on TG2-treated hCAM was diffusely organized (Figure 8B). Corresponding to the differences in fibronectin organization, confocal slicing of cellular protein labeled with Texas red maleimide showed cells on hCAM assembled into 3D structures (Figure 8C), while TG2 treatment suppressed this 3D morphogenesis (Figure 8D). To quantify the extent of this change in 3D morphogenesis, we took advantage of the fact that cells in 3D structures had a brighter phase contrast signal than those in monolayers (compare Figure 8E, F, or Figure 2C, D). Images were processed as described in Materials and Methods using ImageJ, to threshold and quantify the large brighter regions, which correspond to 3D cell areas. Results showed that there was a statistically significant suppression of 3D morphogenesis upon TG2 treatment of hCAM. Taken together, our results show that the fibronectin in hCAM is accessible to alteration both by cells and by tissue transglutaminase 2.

4. DISCUSSION

Here, we used differences in adhesive properties of tumor and endothelial cells to engineer a micropatterned coculture of tumor and endothelial on layer-by-layer assembled hybrid extracellular matrix (ECM) of fibronectin and poly(allylamine hydrochloride), and we examined several aspects of tumor cell-surface interactions with our hybrid ECM. Our coculture system evokes context-dependent, physiologically relevant, cell function in both tumor and endothelial cells, as well as suggests hypotheses for the regulation of tumor cell responses by the hybrid material.

Although endothelial cells formed cords on hCAM in the presence of HepG2 (Figure 3), they did not undergo such a morphological change on hCAM in the absence of HepG2, or on PAH even in the presence of HepG2 monolayers (data not shown). This suggests a specific microenvironmental requirement—either the interaction with HepG2 or the hCAM substrate, or a combination of the two—for the observed endothelial cell response. Further experiments are needed to understand the mechanistic basis for the response.

Of the four surface preparations tested, tumor cells show enhanced membrane protrusions close to the surface (Figure 6), show enhanced migration (Figure 5, Movies S1–S4), and form 3D morphologies (Figures 2 and 8 and Figure S3), only on hCAM, which is a combination of fibronectin and poly(allylamine hydrochloride). We hypothesize that this is related to the ability of tumor cells to sense and remodel fibronectin present in hCAM (Figures 7 and 8). We had previously exploited the fact that polycations promote rapid cell

adhesion²⁷ to capture cells on surfaces patterned by dielectrophoresis (DEP).¹⁰ The interaction of HepG2 cells with both components of the hCAM supports our previous perception that cells in contact with hCAM interact with both polyallylamine and fibronectin.¹⁰ Our working model is that cells interact with hCAM using a two-stage binding process. First, nonspecific electrostatic interactions occur immediately when cells first make contact with positively charged molecules of polyallylamine, perhaps through interactions with the negatively charged cell-surface carbohydrate moieties.²⁸ Second, specific interactions occur between integrins and fibronectin, which also trigger intracellular signaling pathways important for physiological cell functions.^{10,28,29}

Engineered ECMs offer an opportunity to experimentally control the geometry and composition of adhesive ligands, and when appropriately designed, allow one to model disease states and to distinguish cell responses to different extracellular perturbations such as therapeutic drug candidates.^{4,30} Specifically, in the context of cancer, tumor cell engineering to form 3D spheroids is of great interest for in vitro drug testing.³ We found here that the presence of fibronectin in combination with polyallylamine allows the HepG2 hepatoma cell line to spontaneously assemble into 3D structures unlike the planar monolayers on PAH (Figure 2). The experiments presented here suggest the hypothesis that fibronectin remodeling may be involved in the 3D morphogenesis since in the absence of fibronectin, i.e., plating cells on PAH (Figure 2), or by blocking FN remodeling by transglutaminase treatment (Figure 8), hepatoma cell 3D morphogenesis is blocked.

Because fibronectin is a highly extensible adhesive protein,³¹ it is likely that its presence affords mechanical flexibility to polyelectrolyte films, which can further be stiffened by cross-linking the fibronectin. Indeed, previous work using chemical cross-linkers on layer-by-layer films incorporating polypeptides suggests that such treatment alters the mechanical stiffness of the nanofilms.²⁰ It is reasonable to hypothesize that transglutaminase 2 increases in the mechanical stiffness of our hybrid ECM and thereby suppresses 3D morphogenesis, although further studies are required to distinguish this from other known functions of transglutaminase 2.³²

5. CONCLUSIONS

Our results demonstrate that combining synthetic and naturally derived cell adhesive materials provides synergistic advantages to engineer patterned microtissues of hepatoma with endothelial cells. The fibronectin-poly(allylamine hydrochloride) composite cell adhesion material (hCAM) allows rapid hepatoma cell attachment in the presence of polyallylamine. When fibronectin was present as a component of the adhesive substrate, hCAM also promoted tumor cell migration and a 3D morphology. PAH was observed to promote a spread, mesenchymal phenotype. These observations, combined with the fact that endothelial cells formed microvessel-like cords in coculture, suggest that this is a useful coculture system to study tumor-endothelial cell interactions in a physiologically relevant context, and may be useful for building model tissues in vitro. This approach could be useful for cancer drug screening applications because of its simplicity, robustness, and scalability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

TCPS	tissue culture polystyrene substrate
FN	fibronectin-coated TCPS
PAH	poly(allylamine hydrochloride)-coated TCPS
hCAM	hybrid cell adhesive material prepared by layer-by-layer assembly of FN followed by poly(allylamine hydro-chloride) on TCPS

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Figure 1.

A) Schematic of the microfluidic patterning procedure: (a) A patch of fibronectin is adsorbed on TCPS. (b) PDMS microfluidic manifold is overlaid on the adsorbed fibronectin patch and polyallylamine solution is flowed through. (c) PDMS manifold is removed to reveal the final heterogeneous pattern with the substrates indicated in the legend. (B) Phase contrast image, 40 min after plating, shows HepG2 cells adherent to a line on the hCAM pattern (yellow arrows) avoiding the neighboring FN regions (green regions in A). (C) Epifluorescence and (D) phase contrast images, of the same field of view of HepG2 labeled with calcein AM, 48 h after HepG2 plating and 40 min after plating HUVEC. Red arrows in D point to newly adherent HUVEC. Scale bar = 100 μm .

Figure 2. hCAM promotes 3D morphogenesis in HepG2 cells. (A) Schematic of the microfluidic ECM pattern used for coculture. (B) Bright-field image of a patterned coculture of tumor and endothelial cells in a region corresponding to the dotted box in A, 4 days after plating the HepG2. The dark line in the middle of the image in B was marked by hand on the bottom of the dish at the time of preparing the substrate, to distinguish hCAM and PAH regions. (C, D) Phase contrast images of hCAM and PAH regions, respectively, showing a monoculture of HepG2 cells alone 4 days after plating; (E, F) corresponding laser scanning confocal microscopy images of Texas-Red maleimide labeled HepG2 cells on hCAM and PAH, respectively. The confocal cross-sections in E and F are each 191.2 μm thick. Scale bar = 100 μm .

Figure 3.

(A) Phase contrast and (B) fluorescence images using a 10× objective of HepG2 and HUVEC cocultures, imaged 6 days after plating the HepG2. The HUVEC were labeled with PKH 26 dye prior to plating. The large arrowheads in (B) point to spread endothelial cells on FN. The small arrowheads in (B) point to endothelial cords on hCAM. (C, D) Digitally enlarged details from B showing HUVEC on FN and hCAM regions, respectively. Scale bars = 100 μm .

Figure 4.

(A–D) HepG2 cell morphology on the four cell adhesion materials. Cells were plated on the indicated surfaces for 24 h and imaged under phase contrast optics using a 10× objective. (E) Comparison of HepG2 cells spread area/cell on the four cell adhesion materials. All indicated pairwise comparisons were significantly different from each other at $p = 0.05$ level. Areas of cells on PAH and hCAM, were statistically similar. Scale bar = 100 μm , $n = 6$. Error bars indicate standard deviation.

Figure 5.

Analysis of cell speed and directionality. (A) Raw data and box plots summarizing observed cell speeds. Four observations lying above $0.3 \mu\text{m}/\text{min}$ are given as text. Note that y-axis is shown on a log-scale. (B) Raw data and box plots summarizing observed cell directionality. For A and B, no single cells were observed during the third experimental replicate for either PAH or hCAM. (C) Uncertainty intervals for the median speed under each ECM. (D) Uncertainty intervals for the median directionality under each ECM. In C and D, vertical line segments corresponding to ANOVA Mean and ANOVA Median depict 90% confidence intervals; for vertical line segments corresponding to MCMC depict 90% posterior credible intervals for the median. (E) Uncertainty intervals for the ratio of median speeds for each pairwise comparison among ECMs. (F) Uncertainty intervals for the difference in median directionality for each pairwise comparison among ECMs. In E and F, vertical line segments corresponding to ANOVA Mean and ANOVA Median depict intervals from Tukey's Honestly Significant Difference at a 90% confidence level; vertical line segments corresponding to MCMC depict 90% posterior credible intervals.

Figure 6.

Membrane protrusions close to the substrate in the first few minutes after seeding HepG2 cells on the indicated substrates, imaged by total internal reflection fluorescence (TIRF) and bright field microscopy. The images were taken using a 100 $\times$ TIRF oil-immersion between 5 to 6 min after cell seeding, whereas cells were maintained in the microscope incubation chamber at 37 $^{\circ}$ C, as described in Materials and Methods. Scale bar = 10 μ m.

Figure 7. Remodeling of fibronectin on hCAM by HepG2 cells. Cells on (A) FN and (B) hCAM were fixed and processed to label fibronectin (green), and H32342 labeling for the nuclei (red) 72 h after plating. Small arrows point to bright focal densities of fibronectin labeling and large arrows point to regions with loss of fibronectin labeling. Scale bar = 100 μm

Figure 8.

Effect of TG2 treatment on hepatoma 3D morphology. Confocal microscopy images of fibronectin and Texas red maleimide respectively of HepG2 cells 96 h after plating on: (A, C) hCAM and (B, D) TG2-treated hCAM. The boxed areas in C and D correspond to the region shown in A and B, respectively. Phase contrast microscopy images of cells on (E) hCAM and (F) TG2-treated hCAM. Boxplot and overlaid scatterplot of 3D cell area in

pixels on hCAM and Tg2-treated hCAM. (G) The confocal cross-sections are 108 μm thick. Scale bar = 100 μm ; ‘*’ indicates $p < 0.05$; $n = 3$.