Published in final edited form as:

Lab Chip. 2012 November 7; 12(21): 4244–4248. doi:10.1039/c2lc40819h.

Patterning microscale extracellular matrices to study endothelial and cancer cell interactions *in vitro*

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Abstract

The extracellular matrix (ECM) of the tumor niche provides support to residing and migrating cells and presents instructive cues that influence cellular behaviours. The ECM protein fibronectin (Fn) enables vascular network formation, while hyaluronic acid (HA) is known to facilitate breast tumor development. To recapitulate aspects of the tumor microenvironment, we developed systems of spatially defined Fn and HA for the co-culture of endothelial colony forming cells (ECFCs) and breast cancer cells (BCCs). A micropatterned system was developed using sequential microcontact printing of HA and Fn. This approach supported the preferential adhesion of ECFCs to Fn, but did not support the preferential adhesion of BCCs to HA. Thus, we developed a microstructured analog to spatially organize BCC-laden HA micromolded hydrogels adjacent to ECFCs in fibrin hydrogels. These novel, miniaturized systems allow the analysis of the spatial and temporal mechanisms regulating tumor angiogenesis, and can be applied to mimic other microenvironments of healthy and diseased tissues.

Introduction

The development of highly organized and functional vascular networks is fundamental for healthy tissue growth and homeostasis. When unregulated, the formation of vascular networks becomes involved in the pathogenesis of cancer by supplying nutrients and oxygen to enhance tumor growth.¹ In fact, the ability of a tumor to achieve vascularization is an essential hallmark of cancer biology.² For primary tumors to grow beyond 1- 2 mm,³ they must invade the surrounding extracellular matrix (ECM) and recruit endothelial cells (ECs) to initiate the formation of new blood vessels (i.e. vascularization).⁴ Thus, cancer growth and vascularization is a tightly regulated and complex process modulated by cytokines and a myriad of cell phenotypes and ECM components within the tumor microenvironment.⁵ Hyaluronic acid (HA), has been specifically associated with facilitating tumor cell growth by enhancing breast cancer cell (BCC) motility, invasion, and angiogenesis.⁶⁻⁷ The deposition of ECM components from BCCs, such as fibronectin (Fn), have even been shown to facilitate capillary-like structure formation of ECs.⁸

Methods to investigate cancer-EC interactions include adherent 2-D co-cultures,⁹⁻¹³ or culture within collagen-coated microfluidic channels¹⁴ to observe the effects of direct and/or indirect cell contact. Three-dimensional methods include co-culturing cells within ECM-based scaffolds.¹⁵⁻¹⁹ Verbridge *et al.* and Cross *et al.* used a collagen-based gel to encapsulate cancer cells and then top-seeded ECs to analyze invasion behaviour,¹⁷⁻¹⁸ whereas Stahl *et al.*, used a collagen-based spheroidal co-culture system to study cell contact mediated interactions.¹⁹ However, these approaches present difficulties for studying the distinct effect of cell-ECM and cell-cell interactions. Thus, the development of *in vitro* model systems that recapitulate aspects of the unique tumor microenvironment has become an emergent field in cancer research.^{9, 17}

Microfabrication technology allows precise control over the spatial orientation of cells, and the presentation of biochemical and biophysical cues. Micropatterning is a versatile

technique and has been harnessed to pattern various ECM components to study cell behavior.²⁰⁻²⁵ Specifically, Fn patterned substrates have been developed to study cell behavior such as tumor cell secretion,²⁰ vascular structure formation,^{22, 24} or EC migration.²⁵ Hyaluronic acid patterns have also been created to study EC alignment²⁶ or cancer cell adhesion.²¹ As patterning of single cell types has been extensively studied, current research aims to pattern multiple cell types and is reviewed in.²⁷ However, there have been limited studies on developing patterned co-cultures of endothelial and cancer cells. A relevant study developed a substrate of alternating photoimmobilized micropatterns of HA and glass. Endothelial cell and tumoral fibroblast adhesion was inhibited on HA patterned regions, with both cell types adhering to glass patterned regions, allowing heterotypic cell interactions.²⁸ Additional studies used microfluidics to pattern ECs and cancer cells on planar surfaces,²⁹ or pattern ECM hydrogel constructs to co-culture macrophages and cancer cells.³⁰ We believe developing novel microscale co-culture systems may provide effective and tunable biological tools to investigate the cell-cell and cell-ECM interactions that regulate tumor angiogenesis.

Here, we aimed to control the spatial adhesion of ECFCs and BCCs on a 2-D surface using patterned HA and Fn. We developed dual-patterned surfaces and exploited the preferential adhesion of ECFCs to Fn. We then developed a patterned microstructured system to control the growth of metastatic BCCs in micromolded arrays of HA hydrogels adjacent to unidirectional stripes of ECFCs in fibrin hydrogels. To our knowledge, this is the first culture system that incorporates these two critical tumor ECM components to study the spatial and temporal interactions between endothelial progenitors and cancer cells with their surroundings. Such co-cultures are also envisioned to fundamentally advance the development of culture systems that better recapitulate the cellular interactions in a relevant ECM surrounding in health and disease.

Experimental

Micropatterned HA-Fn surfaces

A sequential patterning approach using microcontact printing (μ CP) and silane chemistry was developed to present Fn and HA adjacent to cell resistant polyethylene glycol (PEG)-ylated regions²¹⁻²² for the co-culture of human ECFCs and BCCs (Fig. 1A). Detailed methods are provided in the ESI.

Patterned microstructured HA-fibrin co-culture systems

Acrylated HA (AHA) polymer (~30% modified, corresponding to 30% of HA macromer repeating groups contain acrylates) was dissolved in sodium phosphate buffer (0.2M Na_3PO_4 , pH 8.0) overnight with agitation, and modified with 4.7mM of adhesive (RGD) peptides (GenScript). To encapsulate BCCs, MDA-MB-231s at a density of 6×10^6 cells/mL were resuspended in HA polymer solution. Next, HA polymer solution was crosslinked with 5.83mM of dithiol MMP-cleavable peptide crosslinkers (MMP; GenScript) susceptible to matrix metalloproteinase (MMP)-1 and MMP-2 degradation.³³ The HA polymer solution was immediately added to sterile PDMS micromolds, inverted on acrylated glass substrates, and reacted for 10-20 minutes before removing PDMS molds. Meanwhile, ECFCs at a density of 12,500cells/cm² were cultured on 2-D 50mm wide Fn patterned glass coverslips as previously described.²² At 12 hours, a fibrinogen:thrombin solution (3:2, Millipore) was added directly atop the MDA-MB-231 BCCs encapsulated in the micromolded HA hydrogels to form a fibrin gel. After curing fibrin gel for 20 minutes at 37°C, the ECFCs on the 2-D 50mm Fn patterned coverslips were carefully inverted on top of the fibrin gel and cultured for an additional 24 hours²² (Fig. 1C). Further details are included in the ESI.

Results and Discussion

HA and Fn co-patterned substrates

First, we developed a 2-D micropatterned co-culture surface based on our established techniques.²¹⁻²² Using a sequential μ CP approach, we were able to present discrete patterns of HA and Fn adjacent to cell resistant PEG-ylated regions (Fig. 1A). Silane chemistry is widely used to functionalize inorganic materials with biological molecule,³⁴ and previously we used an amine-terminated silane (APTMS) to covalently attach HA to glass.^{21, 35} However, Fn adsorption is strongly dependent on surface functional group, with greater Fn adsorption occurring through hydrophobic interactions with a methyl-terminated silane, than an amine terminated silane; $\text{CH}_3 > \text{NH}_2$.³⁶ Due to the diversity of silane molecules, we were able to select appropriate silanes to pattern the biomolecule of interest (OTS for Fn; APTMS for HA). However, since both silane molecules (APTMS and OTS) supported the adsorption of HA and Fn, molecules were patterned sequentially (ESI, Fig. S2A-B).

Immunofluorescent (IF) imaging confirms the presentation of HA and Fn with various dimensions and spacing (Fig. 1B). Separate channel images verify that HA is restricted to square features and Fn to striped features (ESI, Fig. S2C).

Co-cultures on HA-Fn patterned substrates

We cultured ECFCs on HA-Fn patterned substrates and hypothesized that ECFCs would preferentially attach to Fn patterned regions, as Fn enhances EC adhesion³⁷ and stimulates tubulogenesis.³⁸ Indeed, ECFCs preferentially attached to the Fn presenting stripes within 12 hours, and remain restricted over 24 hours (Fig. 2A). When cultured on surfaces presenting Fn and collagen 1, ECFCs showed no preferential adhesion (Fig. 2B) indicating the selection of HA and Fn was critical to enable selective ECFC adhesion on the micropatterned surfaces.

We previously demonstrated that HA patterned surfaces support the cancer cell adhesion²¹ and hypothesized that BCCs would also preferentially attach to HA regions of our micropatterned HA-Fn surfaces. We investigated the adhesion of three BCC lines representing distinct levels of tumorigenicity (non-tumorigenic MCF10A; tumorigenic, non-metastatic MCF7; and metastatic MDA-MB-231) on HA-Fn surfaces, but observed no preferential adhesion of any BCC line to the HA patterned regions after 24 hours. All BCC lines attached to either Fn or Fn and HA patterned regions (Fig. 2C). This is not unexpected as we have shown that cell adhesion to HA is dependent on cell type and adjacent molecules. Also, integrin-Fn interactions are stronger³⁹ than cell receptor-HA interactions.⁴⁰

Next, we examined whether seeding BCCs after ECFC culture would direct BCC attachment to HA. In this manner, ECFCs were cultured on HA-Fn substrates for 12 hours, followed by seeding of a BCC line. None of the BCC lines attached to HA patterns. Interestingly, after 24 hours, BCCs aligned in striped patterns with MDA-MB-231 cells exhibiting the greatest preference to Fn or pre-seeded ECFCs. After 24 hours, the ECFCs were no longer aligned on Fn stripes, but displaced to surrounding areas (Fig. 2D). It seems that co-culturing affects the behavior of aligned ECFCs, resulting in cell growth outside the Fn regions. It has been reported that BCCs exhibit enhanced ECM deposition when co-cultured with another cell type.⁴¹ We speculate that BCCs attached to the Fn-ECFC striped patterns and deposited ECM that enabled their growth, and the growth of ECFCs, outside Fn regions. Future studies using the 2-D micropatterned surfaces would facilitate the study of the dynamic interactions at the tumor-vasculature interface.

Patterned microstructured arrays of cancer cell-laden HA hydrogels

Recently, we determined the optimal parameters of a HA matrix to support vascular network formation.⁴² To encapsulate BCCs in micromolded HA hydrogels, AHA with 30% modification enabled optimal hydrogel micromolding (ESI, Table S1). Cell viability for all three BCC lines was affected following encapsulation in HA hydrogels (Fig. 3A). We observed that the survival rate depended strongly on the BCC line. Both the non-tumorigenic MCF10As and the tumorigenic, non-metastatic MCF7s demonstrated a low percentage of viable cells after just 1 day in culture, ~65% viable cells, which decreased to ~45% viable cells after 2 additional days in culture. The highly metastatic MDA-MB-231s maintained ~75% cell viability after 3 days in culture. We speculate that the disparity is due to differential MMP expression.⁴⁴ MMPs degrade the ECM to promote cancer cell migration and metastasis.⁴⁵ Metastatic BCCs (i.e. MDA-MB-231s) have enhanced synthesis and secretion of MMPs as compared to less malignant BCCs (i.e. MCF7).⁴⁹ Thus, MMP expression is required to degrade the MMP-cleavable crosslinks in the HA hydrogels and facilitate migration and survival. Further studies that quantify MMP expression of BCCs cultured in our HA hydrogels are required to confirm this hypothesis. Additionally, we observed spreading of the MDA-MB-231 cells in the HA hydrogels after 3 days of culture (ESI, Fig. S3). All further studies use MDA-MB-231 BCCs.

We used micromolding⁴³ (Fig. 3B) to form arrays of HA hydrogels of various dimensions and heights ranging from 50-100 μ m (Fig. 3C). We encapsulated MDA-MB-231 cells within micromolded HA hydrogels (Fig 3D). Initially, MDA-MB-231 cells displayed a circular morphology, but after 48 hours spread and elongate (Fig. 3D). Further analysis indicates that ~76% of encapsulated MDA-MB-231 cells expressed proliferation marker Ki67 (Fig. 3Ei) indicating that micromolding did not compromise proliferative capacity (~80.5% in culture; data not shown). Moreover, ~74% of the MDA-MB-231 cells remained viable (Fig. 3Eii).

Patterned microstructured HA-fibrin co-culture systems

We previously controlled the unidirectional assembly of ECFCs via patterned Fn and a 3-D fibrin milieu.²² For a microstructured co-culture system, we organized MDA-MB-231 BCCs in micromolded HA hydrogels, and separately, ECFCs on 2-D Fn patterns (Fig. 4A). After 12 hours fibrin components were added to micromolded HA hydrogels and polymerized within the void space. The 2-D ECFC patterned substrates were then inverted atop the fibrin gel (Fig. 1D). Images confirm that: (1) MDA-MB-231s remain confined to HA micromolded hydrogels of 300 μ m $\times$ 300 μ m for 48 hours, and (2) ECFCs elongate and remain patterned and restricted within fibrin gel for 24 hours during the co-culture period (Fig. 4B). After 24 hours of co-culture, we begin to observe the degradation of both fibrin and micromolded HA hydrogels. Future studies utilizing our microstructured co-culture system will allow us to visualize all the dynamic interactions occurring between ECFCs and cancer cells.

Conclusions

The ability to confine multiple cell types using lithographic methods provides a unique platform to investigate various cell interactions on one substrate while maintaining spatial control over cell location. To investigate the interactions of the tumor-vascular niche *in vitro*, we designed spatially defined ECM-based microenvironments. We successfully developed a multifaceted microstructured co-culture system of patterned BCC-laden HA microgels next to ECFC chains within fibrin gels. Because we incorporated well-established microfabrication technologies in an innovative way, our approach is extremely versatile and can easily be applied to design co-culture systems using a wide range of polymeric hydrogels to investigate the heterotypic interactions between many different cell types. This

approach permits the precise control over the presentation of ECM molecules enabling investigators to address the complex interactions taking place between various cell types in their particular environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sudhir Khetan and Jason Burdick of the University of Pennsylvania for kindly providing AHA polymer, and Donny Hanjaya-Putra for expertise on polymerization. L.E.D. is an IGERT trainee and NSF Graduate Fellow. This research was supported by: NSF Grant 1054415 & NIH Grant U54CA143868.

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Fig. 1. (A) Development of 2-D micropatterned HA-Fn surfaces (B) IF images of HA (green)/Fn (red) surfaces (C) Development of microstructured co-culture systems. Scale bars=100 μ m.

Fig. 2.

Cell culture on 2-D micropatterned HA-Fn surfaces (A) ECFCs on HA/Fn surfaces; light (i) and IF (ii); phalloidin/HA(green), Fn (red), nuclei (blue). (B) ECFCs cultured on collagen 1 and Fn-coated surfaces; light microscope (i) and IF (ii); phalloidin(green), Fn(red), nuclei(blue). (C) BCCs on HA/Fn surfaces. Arrows indicate adhesion: MCF10A to Fn and HA, MCF7 to Fn, and MDA-MB-231 outside HA; HA(green), Fn/CD44(red), nuclei(blue). (D) ECFCs with BCCs after 24 hrs; phalloidin/HA(green), CD31(red), nuclei(blue). Scale bars=100 μm.

Fig. 3.

Micromolding HA hydrogels for BCC encapsulation.

(A) Quantification of viable BCCs after 1 and 3 days culture in bulk HA hydrogels. (B) Development of HA hydrogel arrays. (C) Micromolded HA hydrogels (top: 200 μ m x 200 μ m with 200 μ m spacing; lower: 100 μ m x 100 μ m with 200 μ m spacing) at low (*left*) and high (*right*) magnification (i). Cross-section (ii). MDA-MB-231s encapsulated in HA hydrogels (D) at low (*left*), and high (*right*) magnification. Arrow indicates spreading. (E) MDA-MB-231 cell proliferation (i); Ki67(green), nuclei(blue) and viability (in green; nonviable in red) (ii). Scale bars = 100 μ m.

Fig. 4. Microstructured co-culture systems. (A) Illustration of intended system. (B) Images at different z-positions after 24 hours of co-culture; MDA-MB-231s encapsulated in micromolded HA hydrogels and ECFCs in fibrin. Scale bars = 100 μ m.