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Short communication

Cellular distribution of invadopodia is regulated by nanometer scale surface protein patterns

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A B S T R A C T

Invadopodia are proteolytic structures formed by cancer cells. It is not known whether their cellular distribution can be regulated by the organization of the extracellular matrix or the organization of the golgi complex or whether they have an adhesion requirement. Here, we used electron beam lithography to fabricate fibronectin (FN) nanodots with isotropic and gradient micrometer scale spacings on K-casein and laminin backgrounds. Investigating cancer cells cultured on protein nanopatterns, we showed that (i) presence of FN nanodots on a K-casein background decreased percent of cells with neutral invadopodia polarization compared to FN control surfaces; (ii) presence of a gradient of FN nanodots on a K-casein background increased percent of cells with negative invadopodia polarization compared to FN control surfaces; (iii) polarization of the golgi complex was similar to that of invadopodia in agreement with a spatial link; (iv) local adhesion did not necessarily appear to be a prerequisite for invadopodia formation.

1. Introduction

The leading cause of death for cancer patients is metastasis. One of the early steps in metastasis is that tumor cells invade the surrounding matrix using their proteolytic feet, namely invadopodia. Invadopodia are actin rich cellular structures formed by invasive cancer cells (Bati and Okvur, 2014; Bowden et al., 2006; Buccione et al., 2009; Linder, 2009; Linder and Aepfelbacher, 2003). Growth factor receptor tyrosine kinase and integrin initiated signaling pathways are known to activate cortactin and induce invadopodia (Beaty et al., 2013; Destaing et al., 2011; MacGrath and Koleske, 2012; Mader et al., 2011; Oser et al., 2010; Stylli et al., 2008; Yamaguchi et al., 2005). Invadopodia can appear as structures of a few hundred nanometers and extend up to eight micrometers if the underlying matrix is thick enough (Baldassarre et al., 2003). Invadopodia also form on stiff substrates such as glass where they can be identified by molecular markers such as actin, cortactin and/or MT1-MMP (Artym et al., 2006; DesMarais et al., 2009; Oser et al., 2009; Stylli et al., 2008). Invadopodia appear to be in close proximity to the golgi complex suggesting a link between proteolytic activity and membrane transport (Baldassarre et al., 2003; Buccione et al., 2009; Caldieri and Buccione, 2010). In addition, position and orientation of golgi is modulated by micrometer scale surface patterns (Thery et al., 2006). However, it is not known whether the organization of the extracellular matrix can regulate cellular distribution of invadopodia or whether polarization of the golgi complex and invadopodia correlate. Furthermore, it is still unclear if invadopodia have an adhesive function as they lack vinculin (Gimona et al., 2008; Linder, 2009; Linder et al., 2011); it is not known whether invadopodia require local adhesion at the sites of formation.

Surface patterning techniques are diverse and their applications in cell biology have mainly focused on cell adhesion and motility (Agheli et al., 2006; Bat et al., 2015; Cavalcanti-Adam et al., 2007; Horzum et al., 2014; Pesen and Haviland, 2009). Micropatterned surfaces have been used to investigate dynamics of mechanical properties of invadopodia/podosomes (Anderegg et al., 2011; Labernadie et al., 2010; van den Dries et al., 2012). However, nano- and micro-patterned substrates are barely exploited to reveal important insights into cell biology and in particular cancer cell biology.

Here, we used electron beam lithography to fabricate surface immobilized protein nanopatterns that can mimic the *in vivo* organization of the extracellular matrix. We fabricated nanometer scale protein patterns of homogenous and gradient micrometer scale spacings. We used the patterned surfaces to determine whether the cellular distribution of invadopodia can be regulated by the organization of the

Abbreviations: FN, fibronectin; Lam, laminin; KcasFN, FN nanopatterns on a K casein background; LamFN, FN nanopatterns on a laminin background; KcasFNg, gradient FN nanopatterns on a K-casein background; LamFNg, gradient FN nanopatterns on a laminin background

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extracellular matrix or the organization of the golgi complex or whether they have an adhesion requirement. Our results showed that both composition and organization of surface protein nanopatterns regulated invadopodia formation and distribution; polarization of invadopodia was similar to that of the golgi complex, and local adhesion did not necessarily appear to be a prerequisite for invadopodia formation.

2. Materials and methods

Unless otherwise noted, materials were obtained from Sigma, Germany.

2.1. Fabrication of protein nanopatterns

K-casein coating of silicon wafers (University Wafer, MA, USA) was performed as previously described (Pesen et al., 2007; Pesen and Haviland, 2009) with the following modifications: Silicon chips were coated with APTES (3-aminopropylethoxysilane) followed by glutaraldehyde coating and incubated with 2 mg/ml K-casein for 24 h or 0.025 mg/ml laminin for 1 h. Both unlabelled and DyLight350 or 650 (Thermofisher Scientific, Waltham, MA, USA) conjugated K-casein were used. Surfaces were patterned by electron beam lithography (eLine, Raith GmbH, Dortmund, Germany) at an accelerating voltage of 5 kV and an aperture of 30 µm. The line exposure mode was used with step size of 5 micrometer instead of the dot exposure mode to reduce exposure times. Uniform (5 micrometer spaced dots) and gradient (1–10 micrometers spaced dots) patterns were designed using the Raith software in GDSII format. After exposure, surfaces were backfilled with 0.05 mg/ ml unlabeled or DyLight350 or 650 (Thermofisher Scientific, Waltham, MA, USA) conjugated fibronectin for 1 h. Control samples contained only one type of protein: K-casein, fibronectin or laminin and were not exposed to an electron beam

2.2. Cell culture

Unless otherwise noted, cell culture materials were obtained from Biological Industries, Israel. MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were passaged every 2-3 days. For culture on nanopatterns, cells were first starved in Leibovitz's medium supplemented with 0.35% bovine serum albumin (GIBCO/Invitrogen, Karlsruhe, Germany) for 2 h. Then, cells were lifted with cell dissociation buffer and cultured on nanopatterns in Leibovitz's medium supplemented with 5 nM EGF for 24 h at 37 °C and 5% CO2. Cells were starved and stimulated with EGF to induce invadopodia formation. The experimental culture medium did not contain serum which contains fibronectin and other extracellular matrix proteins, and therefore can overwrite the surface protein nanopatterns.

2.3. Immunofluorescence

Cells were first fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and then permeabilized with 0.1% Triton X-100 in PBS. After blocking with 1% bovine serum albumin in PBS, cells were stained with cortactin (4F11, Millipore or H222 Cell Signaling), fibronectin (F3648 and F0791, Sigma, Germany), laminin (L9393, Sigma, Germany), golgi (A21270, Invitrogen), vinculin (V9131, Sigma, Germany) specific primary antibodies. Then cells were stained with the Alexa350, Alexa488, Alexa555 or Alexa647 fluorophore conjugated secondary antibodies and phalloidin (Molecular Probes, Eugene, OR, USA). The cells were imaged by an Olympus epifluorescence microscope with a 100X oil immersion objective or a Zeiss epifluorescence microscope with a 63X oil immersion objective.

2.4. Image analysis

Image analysis was performed by ImageJ program. Invadopodia analysis was performed using a previously described approach 39. Detection of invadopodia from immunofluorescence images was performed in a semi-automatic way. An ImageJ macro was used to threshold and detect cortactin rich spots in the immunofluorescence images for cortactin. The ROIs (region of interests) for these spots were overlaid onto the immunofluorescence images of actin and double positive spots were counted as invadopodia. In addition, all invadopodia were detected by eye in the immunofluorescence images for cortactin. The manual and macro counts of invadopodia were compared. The macro originally developed for the detection of focal adhesions identified almost all of the invadopodia per cell (R^2 = 0.9445, Fig. S1), there was no false positive invadopodium out of 955 cortactin spots detected by the macro in 217 cells analyzed. Yet manual inspection was used to include false negatives. The total number of invadopodia detected manually was 1065 for 217 cells. Only single cells were analyzed to exclude input from cell–cell interactions.

For polarization analysis, the partitioning of cells into two parts was performed using an ImageJ macro to draw a separation line in combination with the functions of the ROI manager tool of ImageJ (Fig. S2, Appendix). The cell was divided into two parts through a line passing through its geometric center. For gradient surfaces, the separation line aligned perpendicular to the gradient pattern. For isotropic patterned surfaces, it was parallel to any line of the nanodots. For control surfaces, there was no specific alignment of the separation line. The two parts of the cell were randomly marked as "up" and "down" except that for gradient surfaces, where "up" was for the part on the higher density of fibronectin nanodots and "down" was for the part on the lower density of fibronectin nanodots. Polarization was calculated as

$$P = 5 * [(Value_up - Value_down)/(Value_up + Value_down)].$$

The "value" was either cell area, sum of the distances of invadopodia to the cell center or the mean fluorescence intensity of the golgi complex.

Cells with polarization values between (−5 and −3.3), (−3.3 and 3.3) and (3.3 and 5) were also classified into negative, neutral and positive polarization groups, respectively.

We used sum of the distances to cell center instead of mean intensity for invadopodia because cortactin staining shows not only invadopodia but also vesicles containing cortactin in the cytosol and the cortactin at the cell membrane.

2.5. Statistical analysis

Data were processed by Excel. The number of cells per condition

Table 1
Cell area, invadopodia number per cell and invadopodia density.

	FN	Lam	KcasFN	LamFN	KcasFNg	LamFNg
Cell area (µm^2)	1234 ± 63	851 ± 56	1051 ± 74	649 ± 113	896 ± 42	561 ± 43
Inv. number per cell	4.8 ± 0.45	6.97 ± 1.16	3.62 ± 0.43	7.23 ± 1.39	4.54 ± 0.71	4.5 ± 0.68
Inv. density (1/µm^2)	0.0042 ± 0.0004	0.0076 ± 0.0012	0.004 ± 0.0005	0.0123 ± 0.0019	0.0047 ± 0.0006	0.008 ± 0.0009
n	56	31	47	13	54	16

Mean ± s.e.m values are reported. Please refer to Fig. S3 for the graphical presentation and statistically significant differences.

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