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Research Article

Quantitative evaluation of single cell spread on collagen matrices

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ABSTRACT

Cells change their morphology as a response to environmental cues. The quantitative evaluation of single cell spread on extracellular matrices, such as type I collagen, is a key tool in cancer research. Inherent to the manual scoring of cellular spread is inter-observer but also intra-observer variation. To overcome these problems, we have developed the Morphology Analysis Software (MAS). MAS scores phase-contrast images of cells on native type I collagen gels and identifies whether a cell has a spread or round morphology using a combination of four unique parameters: the presence of a cellular extension, the cell area, the cell eccentricity and cell circularity. The MAS software scores are equivalent to the average score of five independent observers but MAS is faster, more objective and standardized. A functional screening assay using six cytokines identified TGFα as a stimulator of HCT8/E11 and SK-BR-3 single cell spreading on top of type I collagen gels. This change in morphology correlates with increased migration potential as evidenced by xCELLigence migration assays and are counteracted by EGFR signaling pathway inhibitors. This underscores the use of morphology classification on a population of unlabeled cells as read-out of an important cancer cell property and the potential for the MAS software in drug screening strategies.

1. Introduction

The tumor environment plays a determining role in cancer progression and in the response to cancer management protocols. The environment includes multiple ostensibly normal cells such as endothelial cells, fibroblasts, immune cells and the extracellular matrix scaffold. In vitro models that mimic the matrix scaffold allow cell-matrix interactions that are largely lacking in 2D models [1]. Native type I collagen matrices have been widely used to study the morphology of single cancer cells in vitro for over 35 years [2–6] and several variations of this assay have been applied. Stiffness of the collagen gels is managed by using a range of different concentrations of type I collagen [7–9] and by addition of contractile fibroblasts [10]. The fibril diameter and pore size are controlled by the polymerization conditions (e.g. pH) [11]. Tracking morphological changes at a single cell resolution is of great importance to reveal spatially separated driver events as well as the temporal dynamics of mutational processes in tumor heterogeneity [12]. When single cell suspensions are seeded on top of the collagen matrix [7–9,13,14], the morphology of the single cells is usually scored 24 h later. In general, a round or an elongated/spread morphology is observed [6,8] (Fig. 1). Longer time-point experiments may allow the infiltration of cells into the matrix by measuring the depth of invasion into the gel by confocal microscopy or through H & E analysis [7–9]. Alternatively, multicellular spheroids can be incorporated into the gel [7,8,15,16]. Blacher et al. computerized the evaluation of the spheroid sprouting assay, where starburst migration of cells out of the spheroid is scored by phase-contrast images [16–18]. A type I collagen gel on top of a transwell filter allows to investigate invasion towards a gradient, and invasion can be monitored in real time through confocal microscopy [19], xCELLigence technology [20] or can be evaluated through end-point analysis of H & E stained sections [8].

In this paper we propose to score morphological changes in a population of unlabeled single cells seeded on top of native type I collagen gels and to use this score as a tool to evaluate treatment responses. We compare manual versus computerized scoring of the phase-contrast images. A low throughput screening identified TGFα as a major stimulator of morphological changes in HCT8/E11 colon

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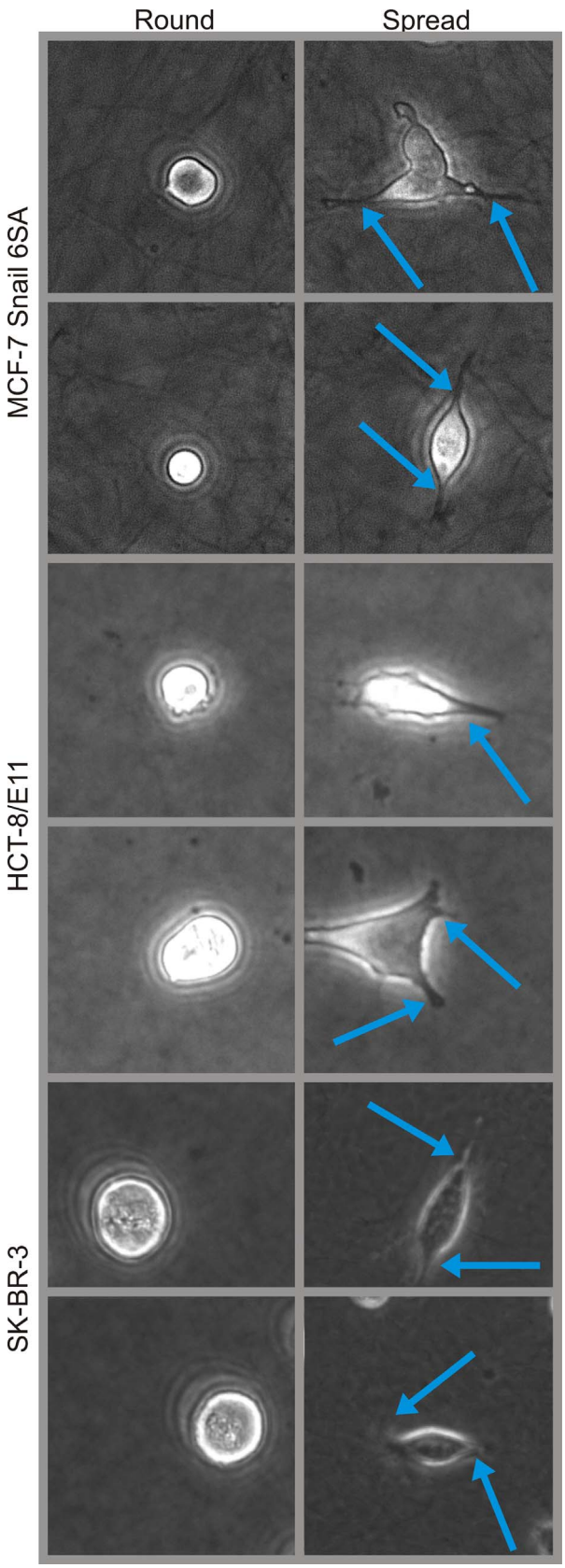

Fig. 1. Round versus spread cell morphology. Phase contrast images of MCF-7 Snail 6SA, HCT8/E11 and SK-BR3 single cells with a round or spread morphology and a type I collagen gel. Spread cells have a more elongate an irregular shape compared to round cells. Spread cells have protrusions/extensions (blue arrows) that, on phase-contrast they appear darker due to their slightly different focus field.

cancer and SK-BR-3 breast cancer cells. Morphological changes are linked with increased migration (as shown for HCT8/E11) and are counteracted by EGFR-signaling pathway inhibitors.

2. Materials and methods

2.1. Cell culture

SK-BR-3 (ATCC number: HTB-30), and MCF-7 Snail 6SA, are human breast cancer cell lines. The Snail 6SA is an epithelial-to-mesenchymal transition model and was established by constitutive activation of the Snail transcription factor, by replacement of six Serines to Alanines in the GSK3β-phosphorylation consensus motif. Non-treated MCF-7 Snail 6SA has a moderately spread morphology, when these cells are treated with Src-inhibitor (M475, 1 μM) or RhoA inhibitor (C_3T, 3 μM) they show a round and an elongated/spread morphology, respectively [21]. HCT-8/E11 (ATCC number: CCL-244) is a human colon cancer cell line. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 U/ml penicillin/0.1 mg/ml streptomycin) (Life Technologies, Ghent, Belgium), and incubated at 37 °C with 10% CO_2 in air (SKBR-3 in 5% CO_2). SK-BR-3 has a round morphology on type I collagen gel.

2.2. Morphology assay

The morphology assay is performed on a native type I collagen gel seeded with a single cell suspension according to De Wever et al. [8]. We performed the assay and reported accordingly to the 2014 cell migration workshop guidelines [22]. Briefly, a single cell suspension was seeded on top of a 0.1% type I collagen gel (Santa Cruz, Santa Cruz, California, USA) of 1.25 ml and 0.3 ml for a 6 well plate (1×10^4 MCF-7 Snail 6SA cells) and 24 well plate (5×10^3 HCT8/E11 or SK-BR-3 cells) respectively. Cytokines and inhibitors were added during cell seeding. Cytokines: transforming growth factor alfa (TGFα R & D systems, Oxon, United Kingdom), scatter factor (SF, Sigma-Aldrich, Diegem, Belgium), basic fibroblast growth factor (bFGF, R & D systems), interleukin 6 (IL6, R & D systems), transforming growth factor beta (TGFβ, R & D systems) and stromal-cell derived factor (SDF, R & D systems). Inhibitors: trametinib (GSK1220212, MEK1/2 inhibitor, GSK, Zeist, The Netherlands) and lapatinib (GSK572016, Her2 inhibitor, GSK).

2.3. Image acquisition

Following microscopes were used: phase-contrast microscope Leica DMI3000B with the LAS4.1 software packet for manual image acquisition (MCF-7 Snail 6SA and HCT8/E11), microscope Zeiss Axiovert 200M with the software package Axiovision 4.1 for automated image acquisition using autofocus before every phase-contrast image (SK-BR-3). Autofocus acquired images also allow Morphology Analysis Software (MAS) analysis. All images are exported as TIFF without scale bars, to prevent interference with MAS analysis.

2.4. Morphological analysis software (MAS)

The cell-segmentation is not straight-forward since the foreground-background contrast is low and cells contain both regions darker and brighter than the background intensity (Fig. 3 original image). Therefore a pre-processing step is first applied by MAS to generate a contrast image, making the cells brighter than the background intensity. This is achieved using a morphological gray-scale dilate operator followed by morphological image gray-scale fill operation and an erode operation [23] (Fig. 3 step 1). From this pre-processed image the cells are segmented. For this, a Laplace filter is applied and the local minima of the result image are used as the basins for a segmentation using the watershed algorithm [24]. Each watershed