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Myosin phosphorylation on stress fibers predicts contact guidance behavior across diverse breast cancer cells

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ABSTRACT

During cancer progression the extracellular matrix is remodeled, forming aligned collagen fibers that proceed radially from the tumor, resulting in invasion. We have recently shown that different invasive breast cancer cells respond to epitaxially grown, aligned collagen fibrils differently. This article develops insight into why these cells differ in their contact guidance fidelity. Small changes in contractility or adhesion dramatically alter directional persistence on aligned collagen fibrils, while migration speed remains constant. The directionality of highly contractile and adhesive MDA-MB-231 cells can be diminished by inhibiting Rho kinase or β1 integrin binding. Inversely, the directionality of less contractile and adhesive MTLn3 cells can be enhanced by activating contractility or integrins. Subtle, but quantifiable alterations in myosin II regulatory light chain phosphorylation on stress fibers explain the tuning of contact guidance fidelity, separate from migration per se indicating that the contractile and adhesive state of the cell in combination with collagen organization in the tumor microenvironment determine the efficiency of migration. Understanding how distinct cells respond to contact guidance cues will not only illuminate mechanisms for cancer invasion, but will also allow for the design of environments to separate specific subpopulations of cells from patient-derived tissues by leveraging differences in responses to directional migration cues.

1. Introduction

Migration is an important cell behavior that occurs during many pathological and physiological processes. For instance, during cancer invasion and metastasis, faster migration occurs through altered signaling pathways, cytoskeletal dynamics and adhesive structures. In addition to faster migration, it is well known that the tumor microenvironment (TME) presents directional cues for cancer cells, allowing for more efficient cell migration towards blood vessels, lymph vessels and along nerve fibers. Directed migration comes in various flavors, one of which is contact guidance, or directed cell migration along aligned fibers or fiber-like structures. Contact guidance has been shown to be a powerful modulator of breast cancer metastasis, due to the robustly aligned collagen emanating radially from the TME [1], but other cancers likely share similar signatures [2]. Furthermore, this aligned fiber structure likely enhances stromal and immune cell migration towards the tumor [3,4]. Understanding how contact guidance operates across different cells is a critical aspect of understanding the biology of tumor invasion and metastasis. In addition, fabricating tumor mimicking environments that allow for the separation and expansion of patient-derived cells for drug screening applications will require both the ability to make complex structures as well as the understanding of how cells respond to those structures.

There have been numerous approaches for fabricating contact guidance cues in 2D and 3D environments [5–8]. Controlling 3D contact guidance cues is more difficult and imaging cells embedded within these environments can pose challenges, so most of the work has been conducted in 2D environments. Most contact guidance work has been carried out on gratings [9–12] that present either micro- or nano-sized grooves and ridges from 50 nm to 50 μm in width with depths ranging from 30 nm to 3 μm. In addition to gratings, lines of extracellular matrix (ECM) have been printed such that cells occupy one line and move randomly in 1D [13,14] or span several lines [15–18]. Finally, aligned collagen fibrils

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have been epitaxially grown on mica [19]. Epitaxial growth of aligned collagen has several advantages. This contact guidance cue is formed using a native ECM protein and certain *in vivo* structural characteristics of collagen fibrils like D-banding are retained [20]. These substrates have been used to assess contact guidance in fibroblasts [21]. In addition, we recently used these substrates to show that cancer cells that migrate with similar speed, but different migration mode, sense contact guidance cues with vastly different directional fidelity [22].

While all cells share basic migration steps including adhesion and contractility that results in traction generation or tail retraction, these steps are regulated differently among cells. Migration phenotypes have begun to be more rigorously defined and are commonly referred to as migration modes. Different signaling pathways are required for each migrational mode and blocking or enhancing certain pathways can allow a cell to switch between modes. Adhesion is regulated by integrin binding to ECM proteins like collagen. Integrins are activated either by intracellular focal adhesion (FA) molecules or manganese ions (Mn^{2+}), resulting in higher affinity interactions with the ECM [23]. In particular, β1 integrin activation seems to increase traction force [24]. Attenuating integrin interactions with the ECM switches cells from a mesenchymal to amoeboid mode of migration [25]. Integrins initiate the assembly of FAs and recruitment of FA proteins such as paxillin. Paxillin is phosphorylated on several sites including pY118 that leads to FA turnover and maturation [26,27]. Paxillin phosphorylation and the accompanying FA turnover and maturation are brought on by myosin-mediated contractility that is controlled by the phosphorylation of myosin II regulatory light chain (MRLC) by myosin light chain kinase (MLCK) and Rho kinase (ROCK) [26–28]. In the context of migration modes, the amoeboid mode depends on Rho/ROCK signaling to generate cortical contractility and blocking ROCK activity can switch cells from amoeboid to mesenchymal migration [29,30]. However, much of the work outlining the differences in migration modes has been carried out in randomly migrating cells with no external directional cue.

Both adhesion and contractility are important during contact guidance. Work on gratings has shown that directional fidelity is dependent on FA maturation [31,32], however the role of paxillin phosphorylation in contact guidance is not clear. Cell spreading on gratings requires myosin contractility through the Rho/ROCK pathway for directional alignment [33,34], but other Rho GTPases like Cdc42 and Rac [35,36] and MLCK [37] are dispensable. In addition, contact guidance in 3D systems depends on ROCK, but not MLCK phosphorylation of MRLC [6]. However, no study has examined the role of MRLC phosphorylation on stress fibers or paxillin phosphorylation in FAs during contact guidance. Furthermore, no study has examined contact guidance fidelity in response to adhesion and contractility perturbations across cell lines that migrate with different migration modes.

In this paper, we benchmark contact guidance on epitaxially grown collagen fibrils with two other common contact guidance cues (μCP lines and gratings) in MDA-MB-231 (mesenchymal) and MTLn3 (amoeboid) cells. We show that both cell lines migrate similarly on μCP collagen and gratings. However, epitaxial grown, aligned collagen fibrils generated distinct migration behavior as we have demonstrated before [22]. MDA-MB-231 cells sense contact guidance with high fidelity, but MTLn3 cells, sense contact guidance cues with low fidelity. We then perturb ROCK-mediated contractility and β1 integrin-mediated adhesion and show that directionality can be tuned up or down in cells roughly independent of cell speed. MDA-MB-231 cells can be made to be less directional after contractility or adhesion inhibition, whereas MTLn3 cells can be made to be more directional after contractility or adhesion enhancement. The perturbations that generate these dramatic changes in directionality alter MRLC and paxillin phosphorylation when localized to stress fibers and in FAs. Additionally, while dual perturbations of contraction and adhesion yield synergistic migration speed responses, additive or saturated directionality responses are observed, suggesting that contraction and adhesion have overlapping roles in modulating cell directionality. Finally, while MRLC phosphorylation on stress fibers is poorly predictive of cell migration speed on contact guidance cues, directionality is directly proportional to MRLC phosphorylation on stress fibers across numerous conditions and cell lines.

2. Materials and methods

2.1. Cell culture and reagents

A human mammary basal/claudin low carcinoma cell line (MDA-MB-231, ATCC, Manassas, VA, USA) was cultured in Dulbecco's Modified Eagles Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York, USA) and 1% penicillin-streptomycin (pen-strep) (Gibco) at 37 °C in 5% CO_2. A rat mammary basal adenocarcinoma cell line (MTLn3, Jeffrey E. Segall, Albert Einstein College of Medicine) was authenticated using IDEXX BioResearch (Westbrook, Maine, USA) and cultured in MEMα (Gibco) supplemented with 5% FBS (Gibco) and 1% pen-strep (Gibco) at 37 °C in 5% CO_2. Imaging media for MDA-MB-231 and MTLn3 cells was the same as the subculturing media, with the exception that no phenol red was included and that 12 mM HEPES (Sigma Aldrich) was included.

2.2. Collagen substrate treatment

High concentration non pepsin treated rat tail collagen type I (Corning, Corning, NY, USA) was used for the contact guidance cues. Four different types of contact guidance cues were made. The first two substrates involved depositing heterotrimeric collagen onto functionalized glass. Collagen was adsorbed (3 μg ml^{-1} in solution for 1 h) or microcontact printed onto no. 1 1/2–22 mm square coverslips (Corning). Cover slips were cleaned [38] and functionalized with 1% aminopropyltriethylsilane (Fisher Scientific, Hampton, New Hampshire, USA) in 10 mM acetic acid (Alfa Aesar, Ward Hill, MA, USA) and 6% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate buffered saline (PBS) without calcium and magnesium (Gibco). Polydimethylsiloxane (PDMS) stamps were made by mixing 184 Silicone Elastomer Base (Dow Corning, Midland, MI, USA) with its curing agent in a 10:1 wt ratio and then allowing it to spread on top of a silicon master fabricated at the Minnesota Nanocenter (University of Minnesota, Minneapolis, MN, USA). The master coated with PDMS was exposed to a vacuum to remove any air bubbles and then cured for 1 h at 60 °C. PDMS stamps were sonicated in double distilled water and in 100% ethanol. A 200 μl collagen solution of 60 μg ml^{-1} collagen I in 0.5 M acetic acid was applied to each stamp. After 40 min incubation, the collagen solution was removed and then the stamp was placed on the functionalized coverslip and allowed to incubate for 15 min. Later, the stamp was removed generating lines 3 μm in width spaced 3 μm apart (Fig. 1C) [15].

The third substrate involved generating a grating. Shallow and deep gratings containing ridges and grooves (shallow: 0.1 μm deep with 1.5 μm pitch and deep: 3 μm deep with 6 μm pitch, Fig. 1D–H) were prepared from CD-Rs and the masters used for μCP mentioned above. Commercial CD-Rs consist of layers including polycarbonate, dye, gold, lacquer and polymer coatings. A thin layer (50–100 nm) of gold is coated on the dye and polycarbonate layers which have a spiraling pregroove. To expose the pregroove, the lacquer and polymer coatings on the CD-Rs were removed [39]. The CD-Rs were