Semaphorin3A Increases Focal Adhesion Formation to Shift the Relationship Between Cell Migration and Substratum Concentration Through a ROCK-dependent Mechanism

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Interactions between integrin-mediated adhesions and the extracellular matrix (ECM) are important regulators of cell migration and cell spreading. Studies have shown that cells exhibit a biphasic relationship between cell migration speed or cell area and substratum concentration, suggesting cells experience an optimal level of cell-substratum adhesive strength to facilitate maximal cell migration and spreading (DiMilla et, al. 1993; Gaudet et al., 2003). However, mechanisms by which extracellular ligands regulate cell migration and spreading in response to changes in ECM concentration are not clearly understood. Semaphorin3A (Sema3A) has been found to increase integrin receptor expression in breast cancer cells as well as inhibit breast cancer cell motility (Pan et, al. 2009). Therefore, we propose Sema3A alters cell adhesion dynamics to influence breast epithelial cell migration and spreading on different concentrations of various ECM. First, MDA-MB-231 breast epithelial cell migration and spreading were measured on various concentrations of collagen type I, fibronectin, and laminin I. Our results demonstrate that Sema3A inhibits cell migration and spreading at high concentrations of collagen, but enhances cell migration and spreading at lower collagen concentrations. In addition, analysis of cell morphology demonstrates that Sema3A-treated cells were more elongated on all concentrations of collagen. However, Sema3A has less robust effects on cell migration, spreading, and morphology when cultured on fibronectin and laminin. Second, inhibition of Rho-associated protein kinase (ROCK) blocks the Sema3A-mediated effects on cell migration and spreading when cultured on all concentrations of collagen. Interestingly, inhibition of ROCK alone results in more elongated cells. Third, Sema3A increases focal adhesion formation on all concentrations of collagen and fibronectin, but not laminin. However, inhibition of ROCK blocks Sema3A-enhanced focal adhesion formation on collagen. These results suggest Sema3A shifts the optimal level of cell-matrix adhesions to a non-optimal ECM concentration, in particular collagen, to yield maximal cell migration and spreading that is mediated through a ROCK-dependent mechanism.

Introduction

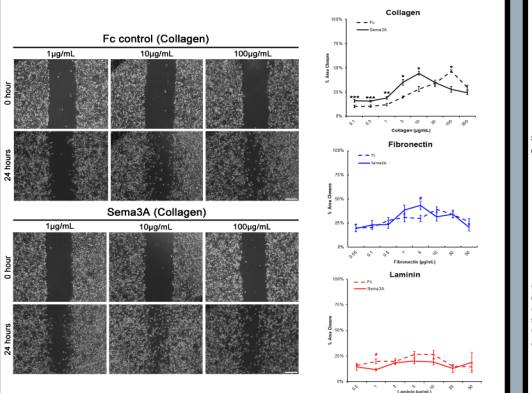
Cell migration is critical to normal and pathological processes, including embryogenesis, wound healing, angiogenesis, and tumor metastasis (Trinkaus, 1984). Cell migration requires integrins to form new attachments to the ECM in the direction of movement, while simultaneously breaking old adhesions made at the trailing edge of the cell (Hood and Cheresh, 2002). Various studies have demonstrated that surface concentrations of ECM alters cell spreading, adhesion, and motility (Gaudet et al., 2003; Mooney et al., 1995). Furthermore, studies have shown that cells exhibit a biphasic relationship between cell migration speed and substratum concentration, suggesting cells experience an optimal level of cell-substratum adhesiveness to facilitate maximal cell migration (DiMilla et, al. 1993; Gupton and Waterman-Storer, 2006; Palecek et al., 1997). When the ECM concentration is reduced or increased beyond the optimal concentration range, then cell motility is reduced. Although these observations are well documented, how changes in cell adhesion and ECM concentration alter cell motility in response to extracellular cues are not well understood.

Semaphorins are factors that were originally characterized as playing a role in axon pathfinding during neural development (Neufeld and Kessler, 2008). In addition, semaphorins demonstrate effects on non-neuronal cells as well. For instance, semaphorins have been shown to inhibit tumor progression and metastasis (Neufeld and Kessler, 2008). Specifically, semaphorin 3A (Sema3A) has inhibitory effects on prostate and breast carcinoma cell migration and metastasis (Herman and Meadows, 2007; Pan et al. 2009). Interestingly, Sema3A increases integrin expression and cell adhesion in breast epithelial cells (Pan et al, 2009). These findings suggest Sema3A may inhibit breast epithelial cell motility by altering the adhesion strength of cells, however, this remains to be determined.

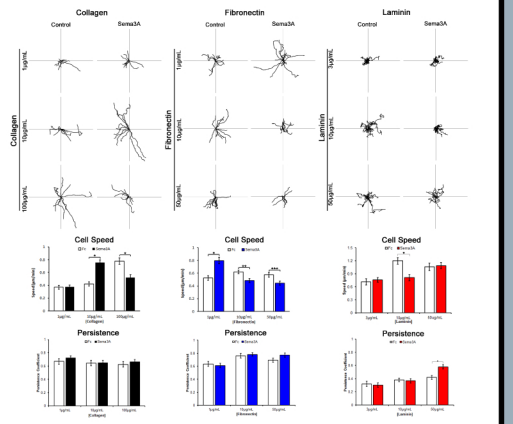
Because maximal cell motility is regulated by a balance between ECM concentration and cell-substratum adhesions, we assessed whether Sema3A alters cell motility on different concentrations of ECM through changes in cell adhesion dynamics. If Sema3A increases integrin expression in breast epithelial cells, then we predict that Sema3A treatment should enhance cell motility on suboptimal concentrations of ECM through increased cell-ECM attachments.

Rationale: Mechanisms by which extracellular ligands regulate the relationship between cell migration speed and substratum concentration are not clearly understood. Because Sema3A has been found to increase integrin receptor expression in breast cancer cells as well as inhibit breast cancer cell motility (Pan et, al. 2009), **we determined whether Sema3A alters the motility response of breast epithelial cells on increasing concentrations of collagen I, fibronectin, and laminin.**

Methods: Serum-starved MDA-MB-231 breast epithelial cells cultured on different concentrations of collagen I, fibronectin, or laminin were treated with 100ng/mL IgG1 Fc or Sema3A for 24 hours following scratch formation. Phase-contrast images were captured immediately following scratch formation (0 hour) and at 24 hours. Cell migration (% Area Closure) was quantified using ImageJ analysis software (imagej.nih.gov/ij) Scale bar = 100μm. For videomicroscopy, serum-starved MDA-MB-231 cells were treated with Fc or Sema3A for 30 minutes prior to image acquisition. Images were captured every 1 minute for a total of 60 minutes. MTrackJ analysis software (www.imagescience.org) was used to measure cell speed and directional persistence. Rose plots of cell migration trajectories of 10 representative cells grown on each ECM treated with Fc control or Sema3A.



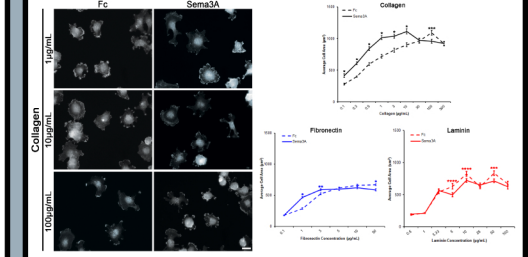
Time-lapse Videomicroscopy



Results: Sema3A shifts the motility response of MDA-MB-231 cells by enhancing motility on lower concentrations of collagen and fibronectin, but not laminin, while inhibiting motility at higher concentrations of ECM. Sema3A appears to influence cell speed but not directional persistence. *p>0.001, **p>0.005, ***p>0.01,

Rationale: Cell spreading is a process that requires integrin-based adhesion to the ECM. It has been shown that cells exhibit a biphasic spreading response to increasing concentrations of collagen I (Gaudet et, al. 2003). Because Sema3A has been shown to increase integrin expression in breast epithelial cells (Pan et al., 2009), **we sought to determine whether Sema3A could alter the spreading response to increasing concentrations of collagen I, fibronectin, and laminin.**

Methods: Serum-starved MDA-MB-231 cells were detached and pre-treated with 100ng/mL Fc or Sema3A for 30 minutes. Following Fc/Sema3A pre-treatment, cells were seeded onto different concentrations of collagen I, fibronectin, or laminin and allowed to attach and spread for 30 minutes at 37°C/5%CO2. Following incubation, cells were fixed with 4% paraformaldehyde. Cells were labelled with rhodamine-phalloidin and images were captured using an Zeiss Axiovert fluorescent microscope. Cell area was quantified using ImageJ analysis software. Scale bar = 20μm

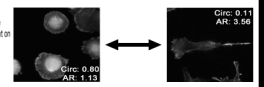


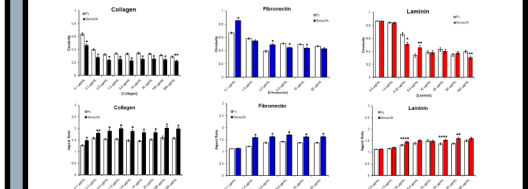
Cell Shape Analysis

Cell morphology is influenced by interactions between the extracellular environment and the cytoskeleton. We measured different aspects of cell shape in response to Sema3A treatment on different ECM concentrations. Both circularity and aspect ratio were used to measure cell shape.

Circularity describes the roundness of a cell [Circularity (Circ) = 4π x area/(perimeter)^2]

Aspect Ratio describes the elongation of a cell [Aspect Ratio (AR) = Major axis/minor axis]

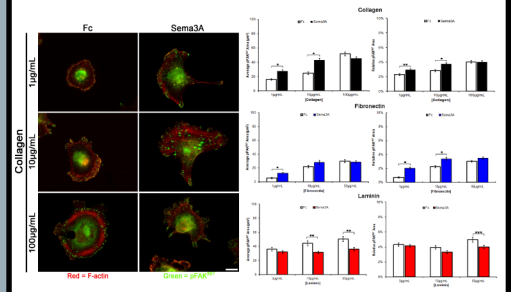




Results: Sema3A increased cell spreading at lower concentrations of both collagen I and fibronectin, but not laminin. In addition, cells grown on collagen and fibronectin were more elongated following Sema3A treatment. *p>0.001, **p>0.005, ***p>0.01, ****p>0.05

Rationale: In order to measure the effects of Sema3A on integrin signaling and cell-substratum attachments, **we assessed focal adhesion formation on increasing concentrations of collagen I, fibronectin, and laminin.**

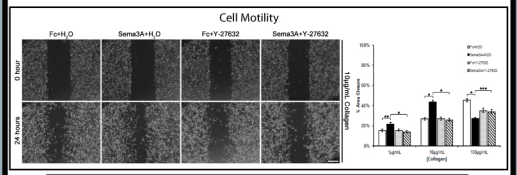
Methods: Serum-starved MDA-MB-231 cells were detached and pre-treated with 100ng/mL Fc or Sema3A for 30 minutes. Following Fc/Sema3A pre-treatment, cells were seeded onto different concentrations of collagen I, fibronectin, or laminin and allowed to attach and spread for 30 minutes at 37°C/5%CO2. Following incubation, cells were fixed with 4% paraformaldehyde and stained for phosphorylated FAK^{397} and F-actin (rhodamine phalloidin). Fluorescence images were captured using a Zeiss Axiovert microscope. Scale bar = 10m. Focal adhesions and cell area were quantified using ImageJ analysis software. pFAK^{397} area was measured for each cell (Average pFAK^{397} Area) and was normalized to total cell area (Relative pFAK^{397} Area).

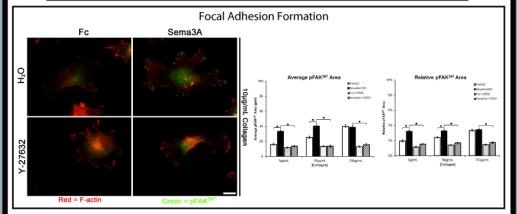


Results: Sema3A enhances focal adhesion formation in response to increasing concentrations of both collagen I and fibronectin, while Sema3A decreases focal adhesion formation on laminin. *p>0.001, **p>0.005, ***p>0.05

Rationale: The mechanism by which Sema3A alters integrin-based adhesions to regulate cell motility and spreading on different ECM concentrations is not well understood. Both integrin signaling and Sema3A can regulate Rho kinase (ROCK) to alter cell behavior. Therefore, **we determined whether Sema3A mediates its effects through a ROCK-dependent mechanism.**

Methods: Serum-starved MDA-MB-231 breast epithelial cells were treated with 100ng/mL IgG1 Fc or Sema3A ± 10μM Y-27632 on different concentrations of collagen I. Cell migration (scratch assay), cell area, cell shape, and focal adhesion formation were measured as previously described. Scale bar (scratch assay) = 100μm. Scale bar (FAK^{397} staining) = 10μm.





Results: Inhibition of ROCK blocked the Sema3A-mediated effects on cell migration, cell area, and focal adhesion formation on collagen. *p>0.001, **p>0.005, ***p>0.01

Conclusions:

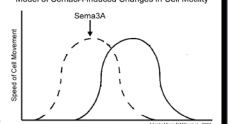
1.) Sema3A shifts the optimal level of cell-substratum adhesiveness to lower concentrations of collagen I and fibronectin, but not laminin, to yield maximal cell migration and spreading.

2.) Sema3A-treated cells were more elongated on all concentrations of collagen and fibronectin, but not laminin.

3.) Sema3A increased focal adhesion formation on all concentrations of collagen I and fibronectin, suggesting Sema3A regulation of integrin adhesions are responsible for the altered motility and spreading response to changes in ECM concentrations.

4.) Sema3A requires ROCK to alter cell migration, cell area, and focal adhesion formation on different concentrations of collagen.

Model of Sema3A-induced Changes in Cell Motility



Adapted from DiMilla et al., 2003

Cells exhibit a biphasic relationship between cell migration speed and substratum concentration, suggesting cells experience an optimal level of cell-substratum adhesiveness to facilitate maximal cell migration (DiMilla et, al. 1993; solid line). Our results suggest Sema3A shifts the motility response to the left (dashed line) such that cell motility is enhanced at lower concentrations of ECM, with more pronounced effects on collagen, as a result of increased cell-ECM adhesions.

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