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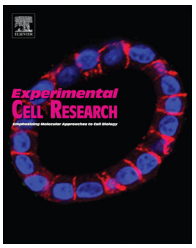

Research Article

# Contact guidance of smooth muscle cells is associated with tension-mediated adhesion maturation

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ABSTRACT

Contact guidance is a cellular phenomenon observed during wound healing and developmental patterning, in which adherent cells align in the same direction due to physical cues. Despite numerous studies, the molecular mechanism underlying the consistent cell orientation is poorly understood. Here we fabricated microgrooves with a pitch of submicrons to study contact guidance of smooth muscle cells. We show that both integrin-based cell–substrate adhesions and cellular tension are necessary to achieve contact guidance along microgrooves. We further show through analyses on paxillin that cell–substrate adhesions are more prone to become mature when they run along microgrooves than align at an angle to the direction of microgrooves. Because cellular tension promotes the maturation of cell–substrate adhesions, we propose that the adhesions aligning across microgrooves are not physically efficient for bearing cellular tension compared to those aligning along microgrooves. Thus, the proposed model describes a mechanism of contact guidance that cells would finally align preferentially along microgrooves because cellular tensions are more easily borne within the direction, and the direction of resulting mature adhesions determines the direction of the whole cells.





## Introduction

Contact guidance is a well-known phenomenon in which cells align consistently along the direction of microgrooves [1,2]. The ability of achieving contact guidance is important in many physiological processes including cell differentiation [4,5] and wound gape closure [6]. Morphological aspects of the phenomenon have been extensively described in numerous studies for decades, yet molecular mechanisms allowing for the consistent cell orientation remain unclear. Previous studies implicated the involvement of actin cytoskeleton [7,8], myosin II [9], integrin [5,10], focal adhesion kinase [5], and zyxin [10] in the cellular response to groove topography. Thus, synergetic effects of cellular tension and adhesions may be important in the process establishing contact guidance.

The first results presented here show that contact guidance is achieved by the environmental sensing through cell–substrate

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adhesions in a tension-dependent manner but not through cell–cell contacts. We then investigated the involvement of the adhesion-associated adapter protein paxillin. Paxillin functions as a scaffolding protein at the intersection of multiple adhesion-signaling pathways [11], and the approach with a focus on paxillin may lead to a better understanding of the mechanisms. We found from the paxillin analysis that the physical efficiency for bearing cellular tension at local cell–substrate interfaces influences the maturation of cell adhesions, which in turn determines the dominant direction of mature adhesions and finally that of cell orientation.

## Materials and methods

### Cell culture

Aortic smooth muscle cells derived from rat (A7r5, ATCC) and bovine (B354-05, Cell Applications) were cultured in low-glucose (1 g/L) DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences) and 1% penicillin–streptomycin (Invitrogen). Cells were maintained in a 5% $CO_2$ incubator at 37 °C.

### Fabrication of microgrooves

A prepolymer solution of polydimethylsiloxane (PDMS) was prepared at a 10:1 w/w ratio of base polymer to crosslinker (Sylgard 184, Dow Corning). The mixed solution was stirred, degassed, poured onto a custom-made acrylic mold to form a hollow chamber (hollow size: 20 mm in width, 20 mm in thickness, and 11 mm in height), and oven cured at 55 °C for 2 h. After removing the mold, the chamber was further oven cured at 125 °C for 15 min. A PDMS sheet, prepared to have an approximate thickness of 90 μm as measured by a micrometer caliper, was attached to the bottom of the chamber and oven cured at 125 °C for 15 min. The PDMS chamber was stretched uniaxially using a custom-made stretcher to produce a fixed amount of prestretch $\varepsilon$=0%, 10%, 20%, or 30% (Fig. 1A). The stretched sheet was treated with oxygen plasma (600 V in voltage, 4 mA in current, and 2.4 W in power) using a plasma generator (SEDE-GE, Meiwafosis) for a fixed period of time $\tau$=0, 50, 100, 200, 300, 400, or 500 s. Straight wrinkles aligning perpendicular to the prestretch direction were produced on the entire surface of the sheet after releasing the stretch.

### Observation of microgrooves

The surface of plasma-treated and untreated PDMS sheets was observed with scanning electron microscopy (SEM; VE-8800, Keyence; or JSM-6500F, JEOL) and atomic force microscopy (AFM; NanoWizard II, JPK Instruments). For SEM, plasma-treated PDMS sheets were cut from the stretch chambers and sputter-coated with ~20 nm layers of carbon using a carbon coater (CC-40F, Meiwafosis). The surface of the PDMS sheets was observed with SEM for each combination of plasma treatment time $\tau$ and prestretch $\varepsilon$. The pitch (the distance between two adjacent intensity peaks) of the grooves was obtained using a fast Fourier transform program of LabVIEW (National Instruments). For AFM, plasma-treated PDMS sheets ($\tau$=500 s, $\varepsilon$=20%) were coated with either 0.1% gelatin (Sigma) in PBS for 4 h at 37 °C, 0.1 mg/ml poly-D-lysine (PDL, Sigma) in distilled water for 3 h at 37 °C, 50 μg/ml fibronectin (Sigma) in PBS for 4 h at 37 °C, or 0.2 μg/ml vitronectin (Sigma) in PBS for 4 h at 37 °C, and then cut from the stretch chambers, placed on a glass plate, and mounted on the stage of AFM. Tapping-mode AFM was conducted using a silicon nitride cantilever (OMCL-TR800, Olympus) to observe the surface.

### Stiffness measurement

The stiffness of the PDMS sheets was measured with contact-mode AFM. Using a silicon nitride cantilever (OMCL-RC800PB-1, Olympus), force curves were acquired for a PDMS sheet treated with plasma for $\tau$=0, 50, 300, or 500 s with $\varepsilon$=0%. In a separate measurement, PDMS sheets were treated with plasma for $\tau$=500 s with $\varepsilon$=0% and coated with either 0.1% gelatin in PBS for 4 h at 37 °C, 0.1 mg/ml PDL in distilled water for 3 h at 37 °C, 50 μg/ml fibronectin in PBS for 4 h at 37 °C, or 0.2 μg/ml vitronectin in PBS for 4 h at 37 °C to perform contact-mode AFM. The elastic modulus (Young's modulus) of these PDMS sheets was determined using the Hertz model from the force curves, the opening angle of the pyramid on the cantilever that the manufacturer provided, and Poisson's ratio of the PDMS, 0.5.

### Cell imaging

The pretreated PDMS sheets or 12-mm glass-bottom dishes were coated with either 0.1% gelatin in PBS for 4 h at 37 °C, 0.1 mg/ml PDL in distilled water for 3 h at 37 °C, 50 μg/ml fibronectin in PBS for 4 h at 37 °C, or 0.2 μg/ml vitronectin in PBS for 4 h at 37 °C. The cells were cultured on them for 24 h, fixed with 1% paraformaldehyde for 30 min, washed with PBS, permeabilized with 0.1% Triton X-100 for 5 min, and incubated with Alexa-488 phalloidin (Molecular Probes) for F-actin staining and Hoechst-33342 (Molecular Probes) for DNA staining. Epifluorescence images or phase-contrast images of the cells present on the entire surface of the substrates were captured using a camera (ORCA-R2, Hamamatsu) under a microscope (IX-71, Olympus).

### Cell orientation angle

The angle of cell orientation was quantified for each $\tau$ and $\varepsilon$ using ImageJ (NIH). The outline of cells was extracted from the phase-contrast images. An equivalent ellipse, defined as one with the same second-order moments as the cell shape outlined, was determined for each cell. The angle of cell orientation was defined as the absolute value of the deviation of the major axis of the equivalent ellipse from the prestretch direction. High orientation angles denote an alignment perpendicular to the prestretch, with perfect cell alignment resulting in a 90° orientation angle. Randomly oriented cells have an average angle of 45°.

### Immunostaining

Cells were cultured on the gelatin-coated PDMS substrates with ($\tau$=500 s, $\varepsilon$=10%) or without ($\tau$=500 s, $\varepsilon$=0%) microgrooves for 2 days. Cells were fixed with 1% paraformaldehyde for 30 min, washed with PBS, permeabilized with 0.1% Triton X-100 for 5 min, treated with 10% normal goat serum in PBS for 1 h at room temperature, incubated with anti-paxillin (1:100, BD Biosciences)



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