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ScienceDirect
Biomaterials 29 (2008) 282–289
Biomaterials
www.elsevier.com/locate/biomaterials

# The effect of the RACK1 signalling protein on the regulation of cell adhesion and cell contact guidance on nanometric grooves

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Received 3 July 2007; accepted 21 September 2007
Available online 22 October 2007

**Abstract**

A wide variety of different cell types have been shown to respond to nanofabricated growth surfaces via the process of contact guidance, however little is known about the intracellular mechanisms that control these events. In the present study we have identified the multi-functional signalling adaptor protein, RACK1, as a novel negative regulator of contact guidance on custom-engineered nanometric grooves. We found that over-expression of RACK1 in human breast cancer cells leads to a pro-adherent morphology characterised by the formation of stress fibres and focal adhesions. Enforced expression of RACK1 also limits the response of cells to contact guidance on nanometric grooves. In contrast, ablation of RACK1 protein with specific anti-sense oligonucleotides led to a dramatic enhancement of bi-directional extension of cells on nanometrically deep grooved surfaces, with a corresponding loss of focal adhesions and stress fibres. RACK1 therefore exerts a tonic inhibitory effect on cell contact guidance, while positively promoting an adhesive phenotype. This is the first example of an intracellular signalling molecule involved in the regulation of cell contact guidance on nanometric growth surfaces.


*Keywords:* Nanotopography; Contact guidance; Cell spreading; Cell morphology; Signal transducing mediator; Antisense

## 1. Introduction

The process of cell contact guidance is essential for every physiological process involving cell migration. For example, contact guidance has been implied in various morphogenetic movements including neural crest cell migration, primordial germ cell migration and guidance of axonal growth cone and is also implicated as an important component of several homoeostatic processes, such as wound healing and tissue repair [1]. Moreover, every cell type tested to date has displayed contact guidance when cultured on groove and ridge patterns with lateral dimensions in the micrometre range [2,3]. The movement of cells exhibiting contact guidance is characterised as 'bi-directional' with a cell extending in opposite directions simultaneously. For example, contact guidance of fibroblasts along an axis of oriented extracellular matrix fibres leads to bi-directional migration of cells and wound closure [4]. This directionality is associated with chemical, structural and/or mechanical anisotropies of the substratum. The nanostructured nature of the basement membrane led to the hypothesis that nanoscale substrate topography is a relevant stimulus for cells, independent from chemistry [5]. Indeed, various cell types display contact guidance when grown on nanometre high grooved and ridge patterns [6]. In the present study we investigate the role of the multi-functional signalling adaptor protein, RACK1 in regulating the response of human breast cancer cells to contact guidance on custom-engineered nanometrically deep grooves.

By virtue of its ability to coordinate the interaction of key signalling molecules, RACK1 is becoming widely perceived as playing a central role in critical biological responses, such as cell growth and migration [7]. For example, RACK1 has been shown to be important for linking IGF-1 receptor activation to the integrin-mediated promotion of cell migration [8,9]. RACK1 is a ubiquitously

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doi:10.1016/j.biomaterials.2007.09.030

expressed 36-kDa protein containing seven internal Trp-Asp 40 (WD40) repeats, with a consensus $X^{6-94}$-[GH-$X^{23-41}$-WD]$^{N4-8}$ (where $N$ = number of WD repeats) that are predicted to form a seven-bladed propeller structure [10]. RACK1 was recently identified in a yeast two-hybrid screen to identify proteins that interact with the cytoplasmic domain of $\beta$-integrins, which appears to be mediated by WD repeats 5–7 of RACK1 [11]. In this context the role of RACK1 seems to be as a protein adaptor, linking protein kinase Cε (PKC)ε to integrin $\beta$-chains [12]. Disruption of the PKCε targeting to integrin receptors, by antisense depletion of RACK1 or over-expression of a truncated form of RACK1 that lacks WD repeats 5–7, leads to impaired adhesion and migration of cells [12]. One of the functions of RACK1 therefore may be to control the interactions of signalling pathways involved in the co-ordination of cell adhesion, movement, and division. Consequently, RACK1 may play an important role in tissue remodelling processes such as wound healing. Certainly, RACK1 mRNA and protein is up-regulated in damaged and repairing segments of proximal kidney tubules within 12 h after acute ischaemic renal injury in rats [13].

In this current study, the effects of RACK 1 on cell contact guidance has been investigated using photolithography to produce regular grooves with micrometre scale pitch and nanometre scale depth. Fluorescent microscopy has been used to investigate the effects of over- and under-expression of RACK1 in transfected human breast cancer epithelial cells (MCF-7). In this study contact guidance of MCF-7 cells is promoted by growth on nanometric grooved surfaces of depth ~350 nm. This groove depth, and shallower depths, have previously been shown to efficiently promote contact guidance in epithelial cells [14].

## 2. Experimental procedures

### 2.1. Materials

MCF-7 cells stably over-expressing pcDNA3 vector (NEO cells) and over-expressing HA-tagged RACK1 (RACK1 cells) were generously provided by Professor Rosemary O'Connor, University of Cork, Ireland.

### 2.2. Fabrication of cell growth surfaces

Quartz slides were cleaned in 7 parts sulphuric acid and 1 part hydrogen peroxide for 5 min. The slides were then spin coated with AZ primer at 4000 rpm for 30 s. Shipley S1818 photoresist was next added, and the slides spun for a further 30 s. After spinning, the slides were soft baked at 90 °C for 30 min. The samples were then exposed to UV light through a chrome mask (Hoya), patterned with 12.5 μm wide line pattern. The exposed resist was developed using 1:1 (v/v) Shipley AZ developer:-water. The slides were next etched to produce 361 nm deep grooves in a plasma technology RIE80 unit (tri-chloromethane environment, pressure of 15 mTorr (1 Torr = 133.322 Pa), RF power of 100 W, giving an etch rate of 25 nm/min). The mastering resist was then removed and the whole slide was etched for a further minute to produce a uniform chemistry. These slides were used as a master against which polycaprolactone was embossed in order to provide the required numbers of replicates. Surface profilometry measurements of the replicates showed that the 12.5 μm line pattern was preserved and that the embossed grooves were 353 nm deep. Control materials were embossed against planar quartz slides.

### 2.3. Cell culture and antisense treatment

Clones of MCF-7 cells stably over-expressing pcDNA3 (NEO cells) or pcDNA3 containing HA-tagged RACK1 (RACK1 cells) were maintained in Dulbecco's modified Eagle's medium supplemented with 1 mg/ml G418. Morpholino oligonucleotides of the murine RACK1 sequence were generated by Gene Tools. The sequence of the antisense oligonucleotide is 5′-CACGAAGGGTCATTTGCTCGGTCAT-3′ and that of the standard control oligonucleotide is 5′-CCTCTTACCTCAGTTACAATTTA-TA-3′. The manufacturer's protocol was used to deliver the morpholino oligonucleotides. Antisense or control morpholino oligonucleotides were added to a final concentration of 10 μM and swirled for 10 s. Cells were then gently scraped off the growth surface and transferred to a new culture plate. This process was then repeated 4 h later. Cells were then maintained in culture for subsequent analysis.

### 2.4. Western blotting

Samples of cell lysate were run on 10% (w/v) polyacrylamide gels, transferred to nitrocellulose membranes and then immunoblotted with anti-RACK1 (Santa Cruz) and anti-GAPDH antibodies (Abcam). Immunoblots were developed with ECL reagent (Amersham) according to manufacturer's instructions.

### 2.5. Immunofluoresence

After 48 h of culture, the cells on the test materials were fixed in 4% formaldehyde/PBS, with 1% (w/v) sucrose at 37 °C for 15 min. Once fixed, the cells were stained for immunofluorescence. Briefly, the cells were permeabilised and stained using phalloidin-rhodamine (Invitrogen) and a primary monoclonal antibody for vinculin (hvin1, Sigma). A biotinylated anti-mouse secondary antibody and a FITC-streptavidin tertiary layer were then used (Vector Laboratories, UK). Nuclei were counterstained with DAPI.

### 2.6. Scanning electron microscopy

Cells were fixed with 1% glutaraldehyde (Sigma, UK) buffered in 0.1 M sodium cacodylate (Agar, UK) (4 °C, 1 h) after a 48 h incubation period to allow the viewing of individual cells. The cells were then post-fixed in 1% osmium tetroxide (Agar, UK) and 1% tannic acid (Agar, UK) was used as a mordant, then dehydrated through a series of alcohol concentrations (20%, 30%, 40%, 50%, 60%, and 70%), stained in 0.5% uranyl acetate, followed by further dehydration (90%, 96%, and 100% alcohol). The final dehydration was in hexamethyl-disilazane (Sigma, UK), followed by air drying. Once dry, the samples were sputter coated with gold before examination with either a Hitachi S800 or S4700 field emission SEM (both used at an accelerating voltage of 10 keV).

### 2.7. Quantification and statistics

Images of phalloidin stained actin cytoskeleton were used inside ImageJ (free download from NIH USA) to automatically calculate the length of the longest cell axis. From here, mean cell lengths could be calculated. All results were $\log_2$ transformed before use of one-way ANOVA (Tukey) using SigmaStat® software. Between 30 and 50 cells for each treatment from three material replicates were considered.