Biochemical and Biophysical Research Communications 436 (2013) 246–251

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Influence of focal adhesion kinase on the mechanical behavior of cell populations

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ARTICLE INFO

Article history:
Received 17 May 2013
Available online 30 May 2013

Keywords:
Focal adhesion kinase
Cadherins
Cell–cell contacts
Mouse embryonic fibroblasts
Collective cell migration

ABSTRACT

Mechanical forces play an important role in the organization, growth, maturation, and function of living tissues. At the cellular level, the transmission of forces from outside the cell through cell-matrix and cell–cell contacts are believed to control spreading, motility, maturation as well as intracellular signaling cascades that may change many characteristics in cells. We looked at cell populations of mouse embryonic fibroblasts that are deficient of focal adhesion kinase (FAK) and examined their mechanical profile. We observed that the lack of FAK induces a mesenchymal-epithelial switch including the regulation of adherens junctions *via* E-cadherin, leading to increased cell–cell-cohesion. Our results show that the absence of FAK influences the macroscopic cell colony spreading in two (2D) and three (3D) dimensions as well as the velocity fields of the tissue, the single cell persistence and correlation length, changing from an independent to a collective mode of migration. Additionally, the single cell size in the sheet decreases significantly.

1. Introduction

After adhesion to the extracellular matrix, cells form a highly complex cytoskeleton whose major components are actin and myosin connecting to focal adhesions (FAs). The highly regulated interplay of actin stress fibers and FAs essentially defines the mechanics of cells and thus their motility, morphology, and function [1–3]. Focal adhesion kinase (FAK) is a central protein of FAs and is known to have a regulatory role on several cytoskeletal proteins. Among these are integrin activation, the linkage from FAs to actin, FA turnover, and actomyosin contractility [4–7]. The mechanical stability is dependent on the proper integration and regulation of all these cellular processes [8,9]. To date, it has not been unambiguous how FAK influences the overall cellular mechanics, which is defined by the interaction of all the different cytoskeletal components.

We have recently examined the mechanical properties of single wildtype (FAK+/+) and FAK-deficient (FAK−/−) mouse embryonic fibroblasts (MEFs) using a magnetic tweezer, 2D traction, -and atomic force microscopy [10]. We showed that MEF FAK−/− cells, when sparsely seeded on the extracellular matrix, were more easily deformed, cytoskeletal dynamics was dramatically increased, and cytoskeletal remodeling processes were less organized compared to MEF FAK+/+ cells. Western blot analysis revealed that FAK−/− cells had increased activity of myosin light chain kinase (MLCK), an activator of actomyosin contractility. These observations implied instability of the force-transmitting connection of the cytoskeletal network in MEF FAK−/− cells, which was reflected in increased cytoskeletal dynamics. Immunofluorescence analysis indicated that the actin cytoskeleton had an altered spatial distribution in MEF FAK−/− compared to MEF FAK+/+ cells, which was assumed to contribute to the mechanical changes [11–14]. We concluded that the mechanical changes of MEF FAK−/− cells were caused by an unstable and unorganized cytoskeleton.

Previously, it was reported that FAK affects the cytoskeleton not only *via* the focal adhesions (FAs) but also *via* the adherens junctions (AJs) in densely seeded cells, both of which exhibit many striking similarities [15,16]. For instance, they consist of dense clusters of transmembrane receptors (integrins/cadherins) and large numbers of similar signaling and structural molecules that provide a highly dynamic and responsive mechanical link to the actin cytoskeleton [17]. It was previously shown that loss of FAK in MEF cells led to an epithelial phenotype where cells expressed E-cadherin, Cytokeratin-18, and Desmoplakin [18]. Furthermore, FAK rescue of MEF FAK−/− cells restored the mesenchymal phenotype *via* protein kinase B (AKT) signal transduction and regulation of Snail1, a key regulator of the epithelial to mesenchymal transition (EMT) [18].

Abbreviations: EMT, epithelial to mesenchymal transition; AJs, adherens junctions; FAs, focal adhesions.

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http://dx.doi.org/10.1016/j.bbrc.2013.05.084

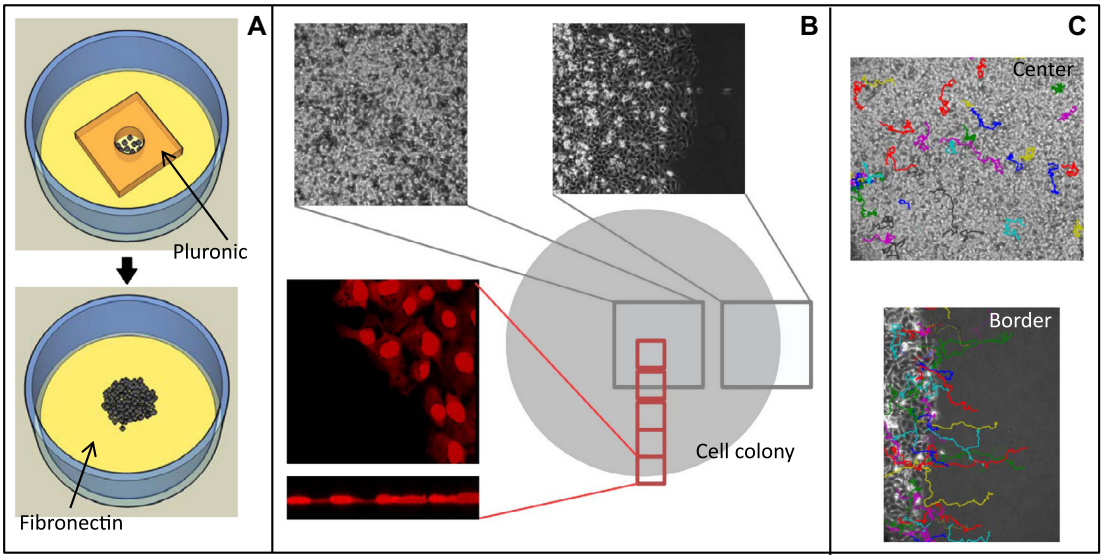
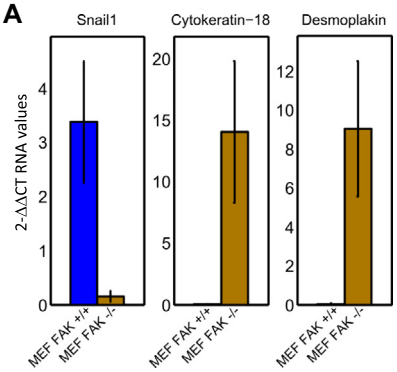


Fig. 1. Cells were seeded in a circular mask on a flat substrate coated with fibronectin and then allowed to form a monolayer. The mask was removed after 24 h (A). Single cells at the center and at the cellular front were tracked every 3 min for 24 h at 5× magnification. The 3D surface profile of the colony was reconstructed from confocal fluorescent image stacks (B). Examples of trajectories of individual cells at the center and cellular front are shown in color (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Based on these results, we elucidated the mechanical properties of MEF FAK−/− cells in a collective environment by using a combined macroscopic growth and microscopic migration assay in cell colonies. Cells were grown to confluency in a circular PDMS mask and continuously tracked by phase contrast -and fluorescence microscopy. The 3D shape of the cell colony was measured by confocal microscopy. Results indicated a different growth pattern in 2D and 3D for MEF FAK+/+ cells and MEF FAK−/− cells. MEF FAK+/+ cells showed a rapid increase in circular colony diameter of no more than 1–2 cell layers over 24 h; in contrast the colony diameter of FAK-deficient cells was smaller, of irregular shape, and dome-like structure of about eight layers. Furthermore, the migration pattern at the colony center and border of these cell lines was different. Due to the lack of cell–cell contacts, MEF FAK+/+ cells moved independently and at lower persistence compared to MEF FAK−/− cells, which migrated collectively at lower speed. In addition, the single cell size of MEF FAK−/− cells was decreased up to a factor of four when cells were in contact within cellular sheets.

Table 1
Semi-quantitative real time gene expression analyses showing a comparison of 2-ΔΔCT values between MEF FAK+/+ and MEF FAK−/− cells for the mouse genes Snail1, Cytokeratin-18 and Desmoplakin. (A) Graph shows the mean 2-ΔΔCT values for RNA as represented in (C) below of two independent cell cultures of MEF FAK+/+ and MEF FAK−/− cells. Note that MEF FAK−/− cells show low gene expression of Snail1 and high expression of Cytokeratin-18, thus confirming a more epithelial phenotype. In contrast, MEF FAK+/+ cells showed no or very low expression of both Cytokeratin-18 and Desmoplakin and high expression of the EMT regulator Snail1, supporting a mesenchymal phenotype. (B) Table shows mouse primer sequences designed from the NCBI mouse data base. Accession numbers are the following: Snai1 -31981483:64-858 Mus musculus (mSnai1) Snail homolog 1; Cytokeratin-18 – 254540067:64-1335 Mus musculus keratin 18 (mKrt18); Desmoplakin -190194417:296-8947 Mus musculus desmoplakin (mDsp). C) Table shows the mean 2-ΔΔCT values and s.e.m. for RNA (n=2) of Snail1, Cytokeratin-18, and Desmoplakin for MEF FAK+/+ and MEF FAK−/− cells.

A



B

Primer sequences

mSnai1-sybr-TF	5'TCAACTGCAAATATTGTAAGGA
mSnai1-sybr-BR	5' ACAGGTCGTGCAGACACAAG
mKRT18-sybr-TF	5' AACTGAGAACAGGAGACTGGAGAG
mKRT18-sybr-BR	5' TCTTCGATGATCTTGAAGTAGTGG
mDsp-sybr-TF	5' ATGATTGACATCGAGAAGATCAGA
mDsp-sybr-BR	5' TCTTGATGAAGTCTTGGTAATGGA

C

Cell type/ mean 2-ΔΔCT RNA values	Snail 1	Cytokeratin-18	Desmoplakin
MEF FAK +/+	3.38 ±1.13	0.02 ±0.01	0.02 ±0.01
MEF FAK -/-	0.15 ±0.10	14.06 ±5.76	9.04 ±3.49

2. Materials and methods

2.1. Cell lines and culture

Wildtype (FAK+/+) and FAK-deficient (FAK−/−) mouse embryonic fibroblasts (MEFs) from ATCC (CRL-2645 and CRL-2644) were cultured in DMEM 4.5 g/L D-glucose, 1% penicillin/streptomycin, 10% FCS (Biochrom) at 37 °C and 5% CO_2 on cell culture-treated plastic ware (Nunclon Surface, Nunc). Trypsin was used for routine passaging of cells. Prior to measurements, dishes of 35 mm diameter were coated with 5 μm/cm^2 fibronectin and cells were seeded in a circular PDMS mask of 4 mm diameter for 24 h to form a monolayer of about 15,000 cells and only then the mask was removed (Fig. 1A). Note that the mask had been coated with Pluronic-F127 (Invitrogen) to avoid interaction between the mask and the coating of the petri dish.

2.2. Growth assay

The 2D projection of the colony shape was reconstructed by segmentation and thresholding analysis of phase contrast images. The 3D surface of the colony was extracted from confocal fluorescent image stacks after staining the fixed colonies with Draq5 (Biostatus; Fig. 1B).

2.3. Migration assay

After 24 h, the cells were placed in a microscope incubation chamber (37 °C, 5% CO_2), and phase contrast images were recorded every 3 min for 24 h at 5× magnification (Fig. 1B and C). Fluorescence images were taken in parallel, imaging fibronectin-coated fluorescent beads of 1 μm diameter which were incorporated by the cells. Bead tracking allowed the reconstruction of individual cell tracks. The cells moved spontaneously by a mean squared displacement (MSD) that followed a power law with time, MSD (δt) = $\alpha * (\delta t/t_o)^{\beta}$, where t_o is the time interval of image recordings and α is called diffusivity. The power law exponent β is a measure of the persistence, with $\beta < 1$ for sub-diffusive and $\beta = 1$ for random migration, and $\beta > 1$ for directed and $\beta = 2$ for ballistic motion along a straight path [19].

2.4. RNA analyses

Using MEF FAK+/+ and MEF FAK−/− cells, RNA was fractionated, purified, DNAse I-treated and then cDNA was synthesized according to Strissel et al. [20]. Semi-quantitative real time PCR (Applied BioSystems 7300) using primers and a Syber green based master mix (Thermo Scientific) was performed with cDNA 40 ng/well, for three genes (Snail1, Cytokeratin-18 and Desmoplakin; Table 1). Mouse placenta RNA was also used as a positive control. Normalized 2-ΔΔCT values for RNA gene expression were calculated, where co-amplification of the housekeeping gene 18S ribosomal RNA was used as a standard curve in semi-quantitative analysis [20]. The results of Li et al. [18] could be reproduced, confirming the mechanism of a mesenchymal-epithelial switch in MEF FAK−/− cells.

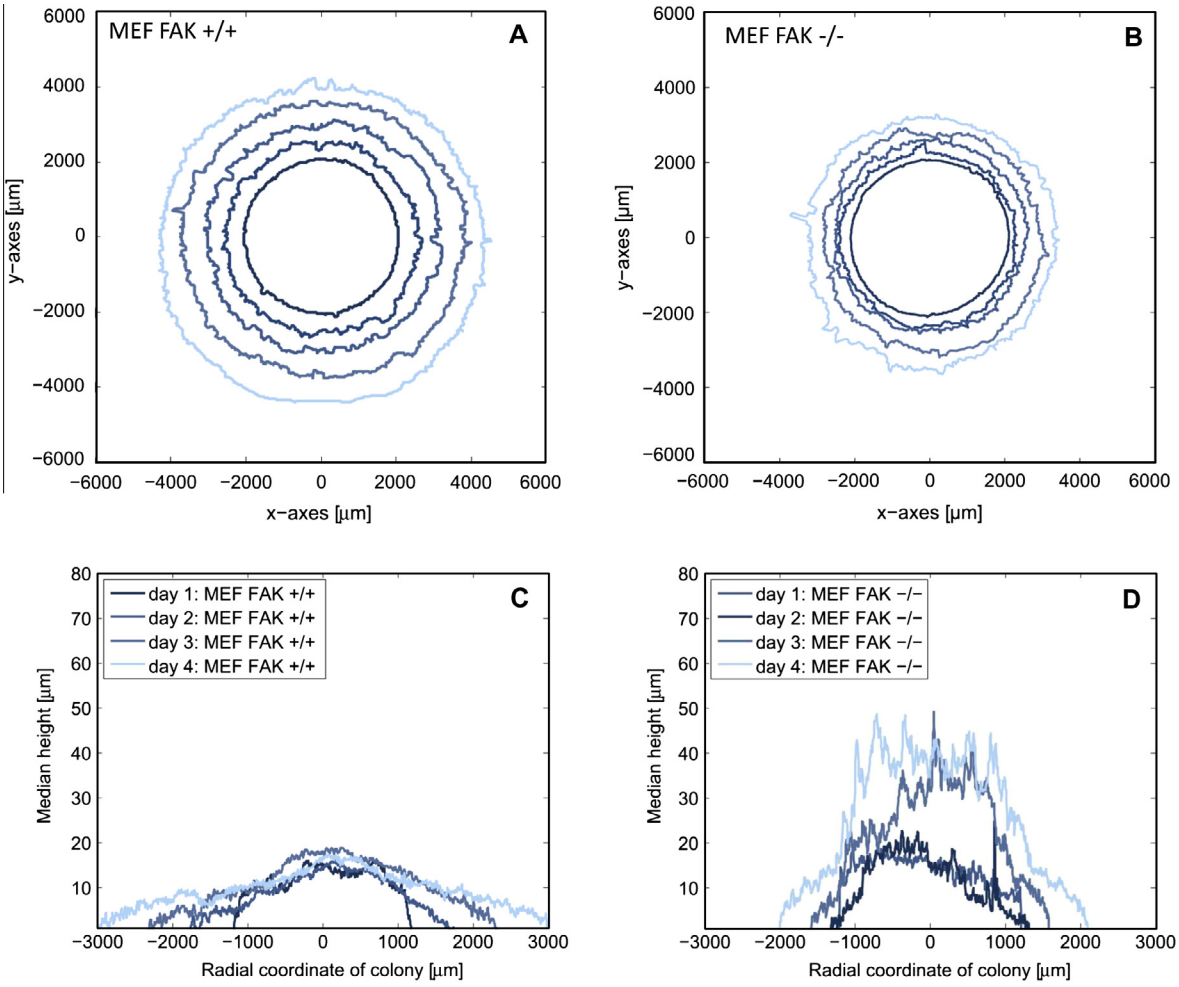


Fig. 2. 2D projection of the colony front of MEF FAK+/+ and MEF FAK−/− cells over 5 days. The colony shape remained circular. Cell colony radii increased at different speeds, for MEF FAK+/+ cells at 0.45 mm/day and MEF FAK−/− cells at 0.27 mm/day (A and B). Topology of MEF FAK colonies are presented by averaged height profiles: MEF FAK +/+ cells established up to 1–2 cell layers of growth (C), MEF FAK−/− cells increased their height to an irregular formed dome-shaped colony of up to 8 layers (D).

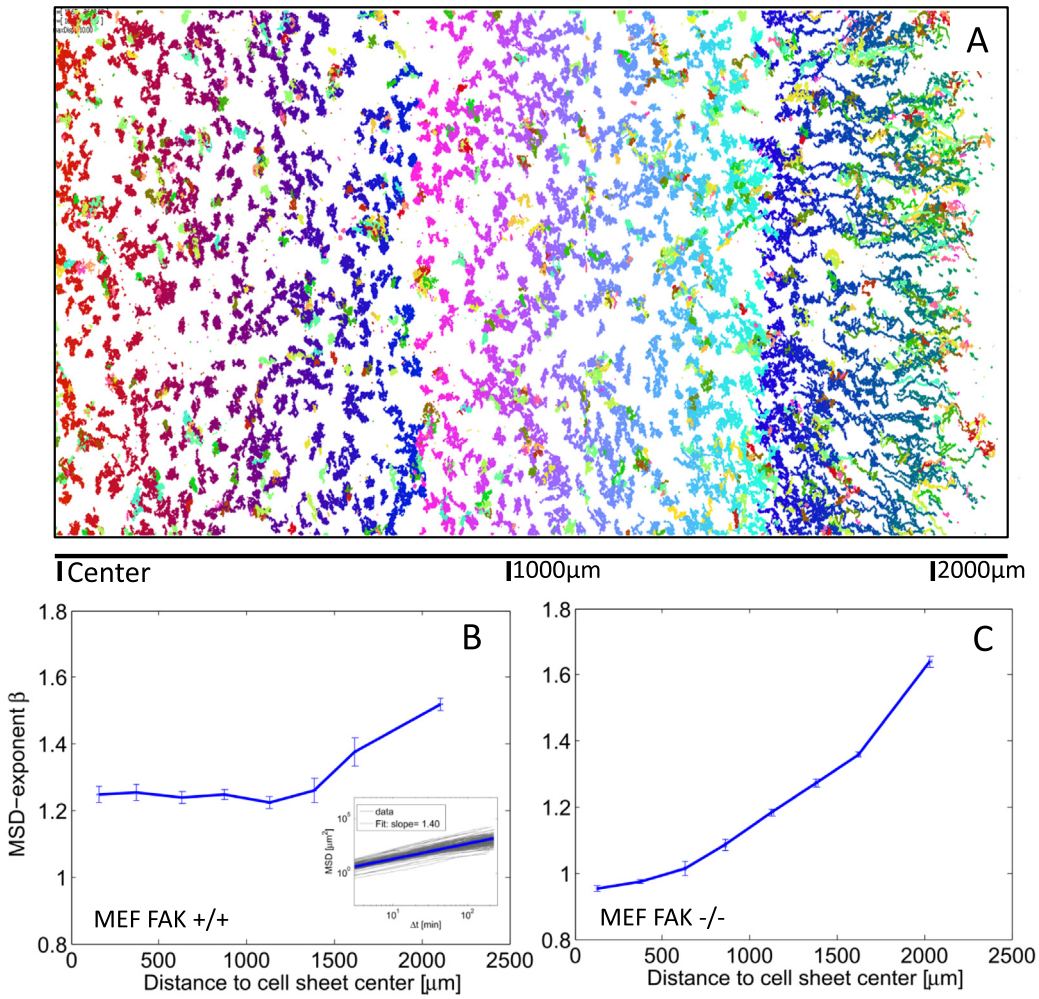


Fig. 3. Cell trajectories from fluorescent bead tracking of the top most cell layers in the colony of MEF FAK−/− cells. Cells at the border (>2 mm) migrated at higher persistence. Cells at the colony center (<1 mm) lost their contact to the substrate and were additionally confined by their neighbors. They migrated more randomly (A). The mean squared displacement is fitted to the power-law, MSD $(\delta t) = \alpha * (\delta t/t_0)^{\beta}$ (B, inset). The exponent β decreased from 1.4 at the cell front to around 1.2 at the center of the colony (B, FAK+/+ cells), and for FAK−/− cells (C), the exponent fell from 1.6 at the front to below 1, indicating sub-diffusive cell behavior.

3. Results

By comparing the cellular growth and sheet migration of MEF FAK+/+ mesenchymal and MEF FAK−/− epithelial cells [18], we tested the hypothesis whether contrasting differences would be found between these cell lines.

3.1. Macroscopic growth assay

On the macroscopic level, we found that over 5 days of cell colony growth, the 2D projection of the shape of the colony remained perfectly circular for both cell lines, however, they proliferated at different speeds (data not shown). MEF FAK+/+ cells increased their radius by 0.45 mm/day and MEF FAK−/− cells showed a colony radius increase of 0.27 mm/day (Fig. 2A and B). At the same time, a complex 3D topology emerged for both cell lines. MEF FAK+/+ cells formed a 'dome-like' colony by increasing the radius linearly, but only established 1–2 cellular growth layers. This supports the notion that MEF FAK+/+ cells may be unable to proliferate without ECM contact. In contrast, MEF FAK−/− cells formed up to eight layers of growth with irregular fluctuations in 3D topology (Fig. 2 C and D). We assume that the altered spreading behavior of these cell lines result from a different ratio between cohesion and cell adhesion to the substrate. It seems that MEF FAK−/− cells stick together due to their epithelial status and enhanced amount of adherens junctions [19], whereas the MEF FAK+/+ mesenchymal cells prefer spreading on substrates.

3.2. Single cell migration in cellular sheets

On the microscopic level, we analyzed the sheet velocity pattern and single cell movement by phase contrast and fluorescent bead imaging. We observed radial and tangential velocity fields for both cell lines and higher motility for MEF FAK+/+ compared to MEF FAK−/− cells. The persistence of cell movement was investigated by analyzing the time evolution of the mean squared displacement (MSD) of beads incorporated in cells (Fig. 3A), which increased with time according to a power law relationship [21] (Fig. 3B inset). The emergent single cell persistence was highest for FAK-deficient cells close to the colony front. These cells migrated tightly connected to their neighbors, whereas the wildtype FAK cells lost contact to the surrounding cells in the first rows (Fig. 3B and C).

For larger distances to the front of the colony, both MEF FAK+/+ and MEF FAK−/− cells migrated in a collective manner due to the high density in the sheet. Generally, we found that the persistence of cells decreased with increasing distance to the colony front due to over-crowding and collisions between neighboring cells. The

persistence of the MEF FAK−/− cells was additionally decreased as they lost contact to the substrate while piling up. Cells without contact to the substrate migrate sub-diffusively as reported by Chen et al. [22].

3.3. Velocity fields, correlation lengths, and step width distribution

The radial velocity fields of cell colonies also showed great differences. Not only was the maximum speed at the cell front higher for MEF FAK+/+ cells, but also the region of radially migrating cells was broader than in MEF FAK−/− cells, which was probably due to the epithelial nature of MEF FAK−/− cells (Fig. 4A). Moreover, in MEF FAK+/+ cells the averaged radial velocity fluctuated more compared to MEF FAK−/− as these cells migrated independently from each other.

The directional velocity correlation between the cells was also radially dependent (Fig. 4 B). We found that MEF FAK−/− cells correlated above three cells at the front of cellular sheets, rising with increasing cell density up to six cell diameters. MEF FAK+/+ cells, however, correlated only above two cells at the sheet border, which is probably due to less coordinated motion because of the absence of AJs.

3.4. Cell size

We also elucidated the projected two-dimensional single cell size in cell colonies. The cell shape of MEF FAK+/+ cells at the border was determined by the loss of contact to the cellular sheet. The cells produced pseudopods and showed an irregular cell form. With decreasing distance to the sheet center, the cell size became smaller, and the cells adopted a more ellipsoid cell shape. MEF FAK−/− cells showed a smaller cell area which remained constant over the entire sheet as the cells were adherent at all radial positions. The distribution of the cell sizes resembled a Rayleigh-distribution, the median values differed by a factor of four (Fig. 4 C and D).

4. Discussion

The mesenchymal or epithelial cellular phenotype of MEF cells was previously reported to be regulated by FAK [18]. Importantly, FAK was demonstrated as a key regulator of the epithelial to mesenchymal transition, involving Snail1, which led to controlling the expression of key proteins involved in adherence junctions (AJs). In our study, the differences observed between cell colony spreading, velocity, and single cell persistence or collective mode of migration were clearly linked to the epithelial (FAK−/−) or mesenchymal (FAK+/+) cellular states. Therefore, we support the idea that the presence or absence of cell–cell junctions in MEFs, affect many aspects of cellular behavior ranging from spreading, velocity, migration patterns and persistence to intracellular force distribution.

A link between focal adhesions (FAs) and adherens junctions (AJs) in controlling cell behavior has been proposed [23,24]. However, the extent to which interplays result from the redistribution of cytoskeletal tension to FAs and AJs or from the release or sequestration of soluble factors that interact with both types, is unknown. When localized to AJs, FAK is believed to play a structural role in

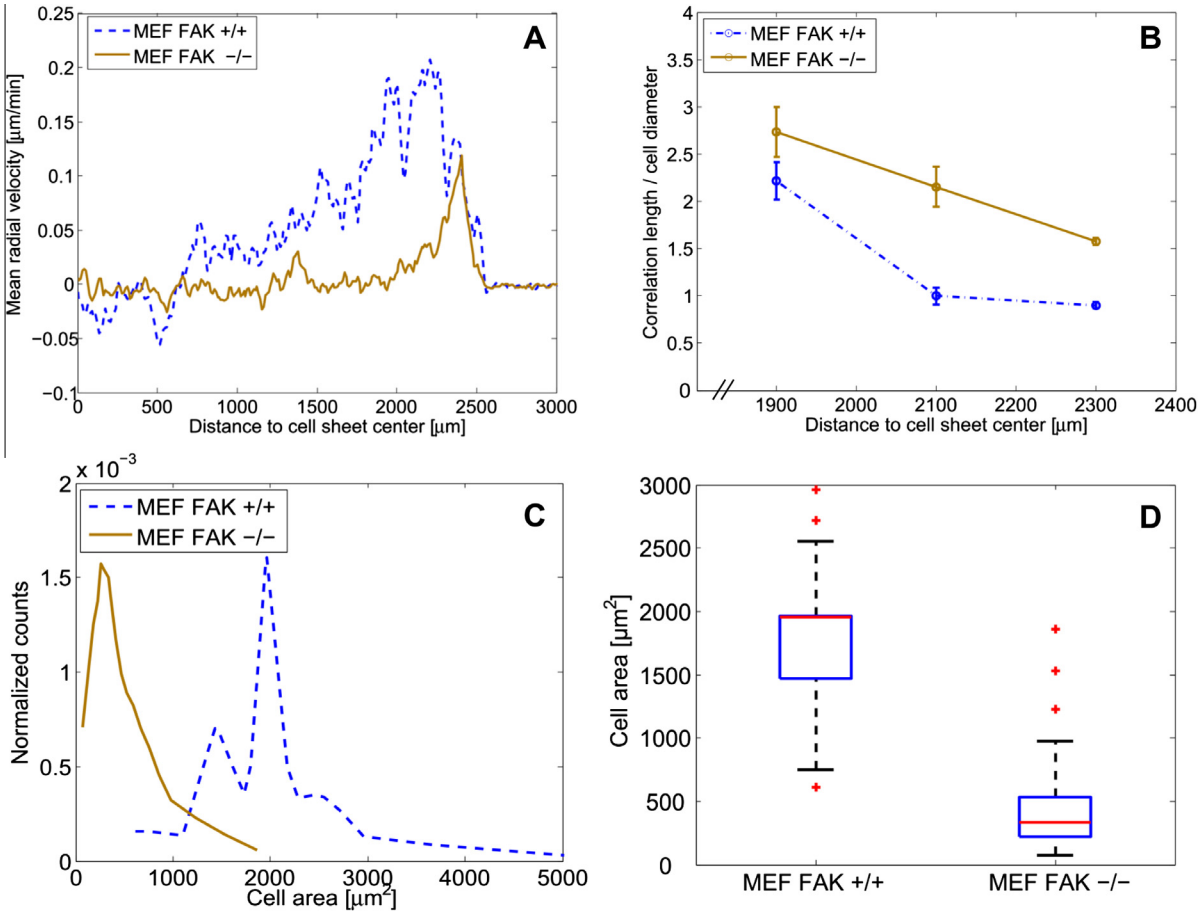


Fig. 4. Data computed from displacement fields of the radial section of cell colonies by MatLab particle image velocimetry. (A) Averaged radial cell speed for MEF FAK+/+ and MEF FAK−/− cells. (B) Directional correlation length as a function of distance (center → front). (C) Distribution of the different cell areas. (D) The average cell size of MEF FAK−/− cells was by a factor of four smaller compared to MEF FAK+/+ cells.

linking cadherin to the actin cytoskeleton [25]. Tension occurring at AJs may therefore also be important in stabilizing AJs and controlling downstream proliferative signaling pathways by regulating the fraction of FAK that is bound to AJs or freely diffusing in the cytoplasm. Although direct evidence for the effects of mechanical force at AJs remains elusive, evidence for the effects of cadherin engagement on cytoskeletal organization and cell function is accumulating. Chris Chen and others recently showed that cadherin-mediated cell–cell contacts decreased cell-ECM adhesions[26–28]. These investigators implemented lithographic and micro-patterning methods to demonstrate that, when preventing contact inhibition of cell spreading by holding cell-matrix contacts constant, cadherin mediated cell–cell contacts no longer inhibited proliferation but instead acted as stimulatory signals for cell proliferation in vascular cells. These results imply that AJs can generate opposing signals that affect cell behavior which needs to be further evaluated.

In conclusion, understanding how cells sense their environment by responding to external forces originating from cell-matrix and cell–cell contacts, which affect cell spreading, velocity, and the mode of migration, is not only important for embryonic development but also tumorigenesis.

Acknowledgment

We thank Drs. Ben Fabry and Claus Metzner for stimulating discussions. This work was supported by grants from Deutscher Akademischer Austauschdienst and Deutsche Forschungsgemeinschaft.

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