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The influence of Pyk2 on the mechanical properties in fibroblasts

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ABSTRACT

The cell surface receptor integrin is involved in signaling mechanical stresses *via* the focal adhesion complex (FAC) into the cell. Within FAC, the focal adhesion kinase (FAK) and Pyk2 are believed to act as important scaffolding proteins. Based on the knowledge that many signal transducing molecules are transiently immobilized within FAC connecting the cytoskeleton with integrins, we applied magnetic tweezer and atomic force microscopic measurements to determine the influence of FAK and Pyk2 in cells mechanically. Using mouse embryonic fibroblasts (MEF; $FAK^{+/+}$, $FAK^{-/-}$, and siRNA-Pyk2 treated $FAK^{-/-}$ cells) provided a unique opportunity to describe the function of FAK and Pyk2 in more detail and to define their influence on FAC and actin distribution.

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Introduction

Cell adhesion and cell–cell contacts critically influence cell metabolism, protein synthesis, cell survival, cytoskeletal architecture, and consequently cell mechanical properties like migration, spreading, and contraction [1,2]. An important group of adhesive transmembrane receptors that mechanically link the extracellular matrix (ECM) with the internal cytoskeleton are the integrins [3]. Integrins are intimately connected with the focal adhesion complex (FAC) which consists of proteins, including talin, vinculin, α-actinin, paxillin, and kinases such as focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2). The signal transduction pathways that are activated in response to mechanical forces include many unique components as well as elements shared by other signaling pathways [4–6].

The formation of the focal adhesion complex (FAC) is greatly augmented either through externally applied tension to the cell or through intracellularly driven myosin-II contractility. FAC functions *via* other mechanically linked structure(s) that sense the mechanical stress, regardless of an intracellular *versus* extracellular origin [7–9]. Exactly which structure can act as mechanosensor/regulator/coupler, however, is currently debated. Focal adhesion kinase (FAK), which is located within the FAC, has been proposed to be one of the principal mechanosensors of adherent cells.

In addition to FAK, many cells express a structurally related kinase known as Pyk2 [also known as *c*ell *a*dhesion *k*inase-(CAK-), *r*elated *a*dhesion *f*ocal *t*yrosine *k*inase (RAFTK) or *c*ell *a*dhesion *t*yrosine *k*inase (CADTK) [10]. Pyk2 is a Ca^{2+}-dependent non-receptor protein kinase that undergoes bimolecular transphosphorylation [11] in response to integrin engagement, increased intracellular Ca^{2+}, and/or activation of PKCs. Although Pyk2 is predominantly localized in the cytoplasm [12], a minor component of the enzyme co-localizes with paxillin in focal adhesions of cultured cells. Like FAK, Pyk2 acts as an important scaffolding protein and transduces signals from G-protein-coupled receptors to downstream MAPK signaling pathways depending on which signaling kinase and/or adaptor protein binds to the phosphorylated enzyme. Pyk2 has also been shown to link a variety of stressful stimuli, including Ca^{2+} overload, UV irradiation, and tumor necrosis factor (TNF-α) treatment to MAPK activation in several cell types [13]. Recently, Hirotani et al. demonstrated that Pyk2 is an essential signaling component in cardiomyocyte hypertrophy [14].

It has also been reported that the absence of FAK in mouse embryonic fibroblasts (MEFs) leads to overexpression of Pyk2 [15,16]. As a highly homologous protein to FAK, Pyk2 is believed to be expressed in $FAK^{-/-}$ cells to compensate the loss of FAK [17]. Moreover, it is known that overexpression of Pyk2 can influence the distribution of actin under the cellular membrane cortex [18,19]. Therefore, it is possible that the overexpression of Pyk2 in the FAK deficient cells induces the reorganization of the cytoskel-

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eton to (over)compensate the mechanical defect in FAK$^{-/-}$ cells. Elucidating the detailed mechanism by which forces are generated and transmitted in relation to Pyk2 expression and cytoskeletal organization is the basis of this investigation.

Materials and methods

Cell lines and culture. Mouse embryonic fibroblasts (MEFs) were purchased from ATCC (CRL-2644, FAK$^{-/-}$ (=FAK deficient cell) and CRL-2645, FAK$^{+/+}$ (=FAK wildtype cell)). Both cell lines were maintained in low-glucose (1 g/L) Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin (DMEM complete medium). Downregulation of Pyk2 expression was achieved by using siRNA against Pyk2 in FAK$^{-/-}$ cells. Specifically, hundred thousand MEF FAK$^{-/-}$ cells were transfected with siRNA against Pyk2 "*siPyk2*" (Qiagen) or a non-silencing control siRNA "*siControl*" (Allstar siRNA, Qiagen) in 35 mm wells by using 6 μL HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol and siRNA in a final concentration of 20 nM. Cells were treated with siRNA 72 h before the measurements. Sequence of siPyk2: *sense*: R[GGG ACA UUG CUG CUC GGA A]dTdT; *antisense*: R[UUC CGA GCA GCA AUG UCC C]dTdG.

Gel-electrophoresis and Western blotting. Adherent FAK$^{+/+}$ and FAK$^{-/-}$ cells were washed with PBS, lysed with ice-cold RIPA buffer containing protease inhibitors (complete, EDTA-free, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma) and then boiled with Laemmli-buffer directly after protein extraction. An aliquot of pure protein lysate was used for protein quantification in a Bradford assay (Roti-Nanoquant). Equal amounts of protein extracts (12–50 μg) were loaded on 12% Tris–Glycine SDS–Polyacrylamide gels and separated at 130 V. PVDF-membranes were equilibrated for 20 min in transfer buffer (25 mM Tris-base, 192 mM glycine, 10% methanol) before the transfer which was accomplished at 15 V constant for 25 min. The membrane was blocked with dry milk buffer (5% dry milk powder, 0.1% Tween, TBS). Antibodies were diluted in blocking milk at given ratios. The membrane was incubated for at least 1 h with the antibody dilution. ECL plus (Amersham) was used for chemoluminescence reaction and detection was carried out in a dark room with chemiluminescence hypersensitive films (Amersham).

Magnetic tweezer. The principle of the magnetic tweezer device used has been described by Kollmannsberger and Fabry [20]. In brief, superparamagnetic 4.5 μm epoxylated beads (Invitrogen) were coated with fibronectin (100 μg/ml, Roche) in PBS at 4 °C for 24 h. Prior to measurements, fibronectin-coated beads were sonicated, added to cells (100,000 beads/dish) and incubated for 30 min at 5% CO_2 and 37 °C. A magnetic field with a high field gradient was generated by a needle-shaped tip. Bright-field images of the cell, bead and needle tip were taken by a CCD camera at a rate of 40 frames/s. The bead position was tracked on-line, using an intensity-weighted center-of mass algorithm. When a force step with an amplitude, ΔF was applied to a bead, it moved with a displacement, $d(t)$ towards the needle tip. The ratio, $d(t)/\Delta F$ defines a creep response, $J(t)$ that is for all force amplitudes well described by a power-law, $J(t) = a(t/t_0)^b$, where, t_0 is the reference time. The parameter, a (units of μm/nN) characterizes the elastic cell properties and corresponds to the compliance (i.e. inverse of stiffness), and the power-law exponent, b describes the dissipative (i.e. frictional) cell properties.

Immunofluorescence. Ten to fifty thousand cells were seeded on 5 μg/ml fibronectin-coated glass slides and incubated overnight at 37 °C and 5% CO_2. Adherent cells were fixed for 20 min with 3% paraformaldehyde and lysed for 5 min with 0.2% Triton-X-100. Cells were then blocked for 1 h in 0.5% BSA in PBS at room temperature. The first and second antibodies were both diluted in 0.5% BSA in PBS at given ratios and incubated for 1 h at RT each. AlexaFlour546-Phalloidin (Molecular Probes) was added simultaneously with the second antibody for actin staining. Samples were mounted with Mowiol (Sigma) solution. Microscopy was carried out on a LEICA microscope DMI6000 equipped with phase contrast and fluorescence. Objectives used were 63×/1.3 NA. Data were acquired by a CCD camera, ORCA ER (Hamamatsu).

Atomic force microscopy (AFM). Measurements were performed on a MFP-3D Stand Alone AFM (Asylum Research). All cell lines were seeded on fibronectin-coated (50 μg/ml) cell culture dishes (Nunc) in CO_2-independent medium at 37 °C for 24 h before the measurements to obtain low cell densities. The force mapping mode was used to measure the cell stiffness. With this method, the deflection of the cantilever is recorded while pushing the tip into the cell. The spring constant of the cantilever (Bio-Lever, OLYMPUS) was determined as $k = 6.7 \pm 1.3$ pN/nm by the thermal noise method [21] before force mapping. During force mapping mode imaging, 20×20 force curves were acquired in an area of 80×80 μm where the cell was located. The force of maximum indentation was 165 pN. The elastic modulus was calculated according to the Hertz model [22]. To reduce the influence of the underlying substrate, only regions of the cell that were 60% of the maximal cell height and higher were analyzed. A typical indentation depth was on the order of 150 nm.

Results and discussion

It was previously shown by Lim et al. [19] that Pyk2 levels increase in siRNA-FAK silenced cells, an observation we cannot confirm. Our siRNA-FAK treated MEF wildtype cells from Dr. E.D. Adamson expressed a reduced level of FAK, but did not overexpress the protein Pyk2 (data not shown). If our understanding is correct that the loss of FAK and not Pyk2 has a mechanical effect on FAK$^{-/-}$ cells then silencing of Pyk2 in FAK$^{-/-}$ cells should prove this. On the other hand, if the overexpression of Pyk2 in FAK$^{-/-}$ cells has a mechanical influence on FAK$^{-/-}$ cells then siPyk2 treated FAK$^{-/-}$ cells should differ mechanically from siControl treated FAK$^{-/-}$ cells. Note, that mouse embryonic fibroblasts from ATCC carry an additional p53 knock-out mutation [23] which may also influence the mechanical properties [24].

As shown in Fig. 1A, FAK$^{-/-}$ cells overexpress Pyk2, a protein highly homologous to FAK compared to FAK$^{+/+}$ cells. To analyze these differences in mechanical terms, we subjected the cells to magnetic tweezer measurements. The cell stiffness value $1/a$ of FAK$^{-/-}$ cells was about 2-fold lower than in FAK$^{+/+}$ cells at nearly all force steps (0.5–10 nN) (Fig. 1B), while the b value of the relation, $J(t) = a(t/t_0)^b$ points to higher internal cytoskeletal actin dynamics in FAK$^{-/-}$ cells (Fig. 1C).

Based on these observations, we focused first on the cell size, the structure of the actin cytoskeleton, and FAC distribution. FAK$^{+/+}$, FAK$^{-/-}$, siPyk2 and siControl treated FAK$^{-/-}$ cells were fixed and antibody-stained against vinculin (green) to visualize the focal adhesions and stained with phalloidin-AlexaFlour546-Phalloidin (red) to detect the actin cytoskeleton (Fig. 2). Compared to FAK$^{+/+}$ cells, the actin cytoskeleton was more cortically distributed and focal adhesions showed a higher intracellular density in FAK$^{-/-}$, siPyk2 treated FAK$^{-/-}$ and siControl treated FAK$^{-/-}$ cells, and their overall cell size was reduced.

To analyze these findings in mechanical terms, we used atomic force microscopy and determined the elastic modulus of these cells. In force mapping mode, we measured the overall cell stiffness of these cell lines and found that FAK$^{+/+}$ cells were about 6-fold stiffer compared to FAK$^{-/-}$, siRNA treated FAK$^{-/-}$ and siControl treated FAK$^{-/-}$ cells (Fig. 3). These results support cell stiffness

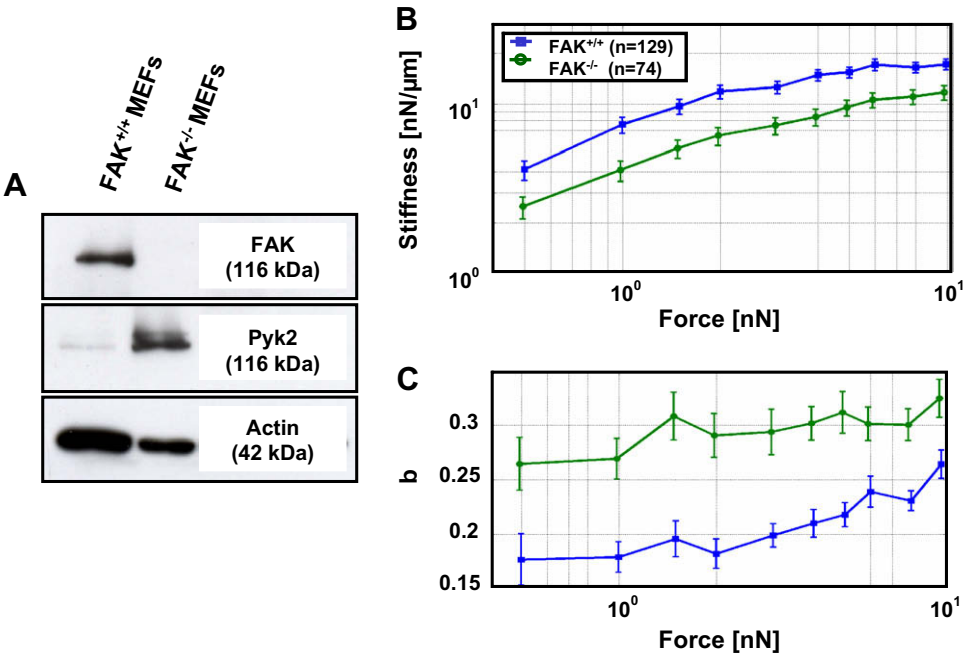

Fig. 1. (A) Gel-electrophorese and Western blotting. $FAK^{+/+}$ and $FAK^{-/-}$ cells were lysed and blotted against FAK, Pyk2 and actin. Pyk2 protein levels increased in $FAK^{-/-}$ cells. Actin was used as loading control. (B) Magnetic tweezer measurements of $FAK^{+/+}$ and $FAK^{-/-}$ cells. The cell lines were incubated with fibronectin-coated paramagnetic beads of 4.5 µm diameter for 30 min and then displaced from their original position by forces from the magnetic tweezer. The prefactor *a* (units of µm/nN) characterizes the elastic cell properties and corresponds to a compliance, i.e. inverse of stiffness. *b* reflects the dynamics of the force-bearing structures of the cell that are connected to the bead. A power-law exponent of $b = 0$ is indicative of a purely elastic solid, and $b = 1$ is indicative of a purely viscous fluid. $FAK^{-/-}$ cells (green) were less stiff (B) and more viscous (C) than $FAK^{+/+}$ cells (blue) at all force steps. The standard error of the *mean* (SEM) is indicated by the bar; *n* = number of cells. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

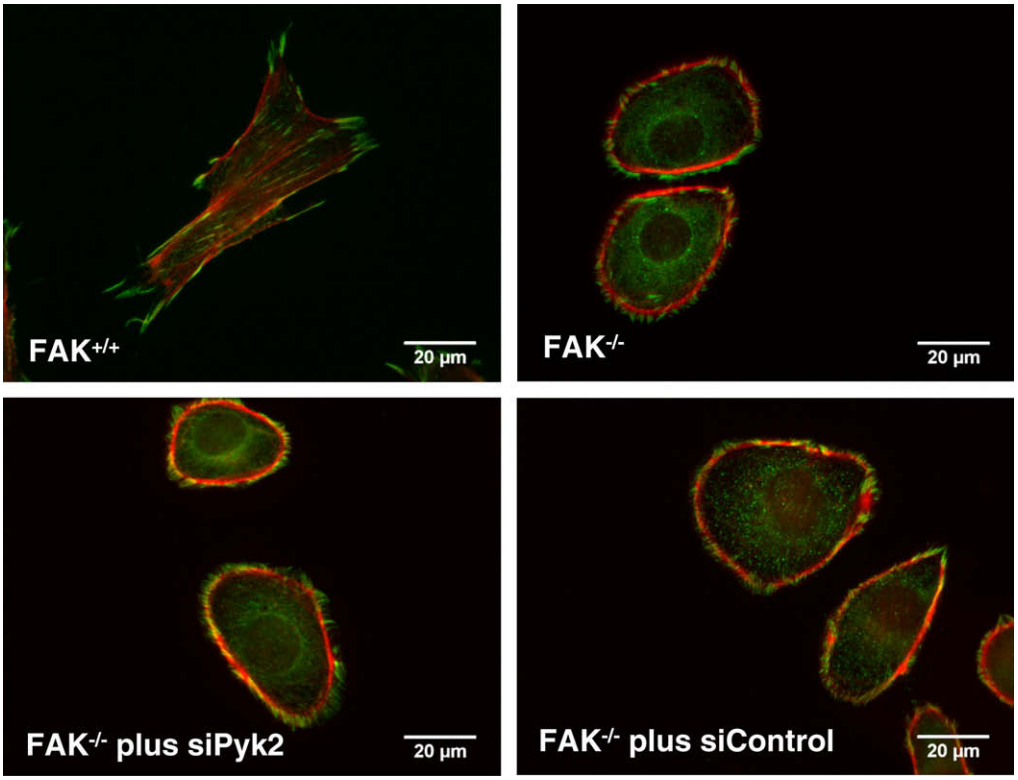

Fig. 2. Immunofluorescence images of MEF cells: $FAK^{+/+}$, $FAK^{-/-}$, $FAK^{-/-}$ with siRNA against Pyk2 and with siControl. Actin was fluorescently labeled with phalloidin (red) and vinculin was fluorescently marked (green) by antibody-staining. $FAK^{+/+}$ cells are larger in area, distribute a wider intracellular actin network and show fewer focal adhesions compared to $FAK^{-/-}$ cell lines. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

measurements carried out by the magnetic tweezer device for $FAK^{+/+}$ and $FAK^{-/-}$ cells.

The protein Pyk2 has been associated with distributional changes of the cytoskeleton [16] and could therefore be responsi-

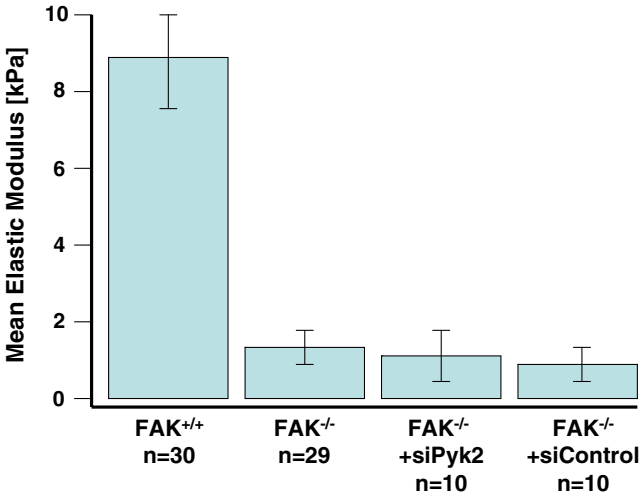

Fig. 3. Atomic force microscope measurements of $FAK^{+/+}$, $FAK^{-/-}$, $FAK^{-/-}$ with siPyk2 and siControl treated cells. The mean elastic modulus determined by force mapping mode is given in [kPa], and the standard error *of* the mean (SEM) is indicated by the bar; *n* = number of cells. $FAK^{+/+}$ cells show about a 6-fold higher elastic modulus than the $FAK^{-/-}$ cell lines.

ble for the phenomelogical and mechanical changes observed in $FAK^{-/-}$ compared to $FAK^{+/+}$ cells. According to Du et al. [18] microinjection of Pyk2 leads to reorganization of the cytoskeleton in cells. These researchers reported cortical actin distribution with less stress fibers inside the cell body after microinjecting Pyk2 into Swiss 3T3 fibroblasts. Lim et al. [19] also described an effect of Pyk2 on the organization of the cytoskeleton and additionally on Rho-kinase. The silencing of Pyk2 in (Pyk2 overexpressing) $FAK^{-/-}$ cells resulted in normal levels of Rho-kinase and thereby to a more structured organization of the cytoskeleton. It was therefore necessary to verify whether the mechanical defects in $FAK^{-/-}$ cells were either due to the deletion of FAK or the overexpression of Pyk2.

Analysis of $FAK^{-/-}$, siPyk2 -and siControl treated $FAK^{-/-}$ cells by atomic force microscopy revealed that these cell lines differed in an almost similar way to $FAK^{+/+}$ cells, i.e. their ability to resist against external forces. $FAK^{+/+}$ cells were consistently stiffer and showed a lower intracellular actin dynamic (*b* value in magnetic tweezer measurements). Therefore, FAK but not Pyk2 must be responsible for the changes in cell stiffness and actin dynamics in $FAK^{-/-}$ cells. Immunofluorescence images support this view that the absence of FAK induces the reorganization of the actin cytoskeleton and that the downregulation of Pyk2 is correlated with additional cytoskeletal changes, i.e. from cortical to a slightly more intracellularly distributed actin. Data from Schober et al. [25] strengthen this notion partially that the reorganization of the cytoskeleton can be triggered by the deletion of FAK, but must not necessarily be caused by an overexpression of Pyk2. It was hypothesized that the deletion of FAK and not of Pyk2 overactivates the Rho-pathway in some ways. Therefore, future experiments should clarify whether Rho-kinase is upregulated in the siFAK treated cells (that do not overexpress Pyk2) and whether Rho-kinase could be the inducer of mechanical defects also in siFAK treated cells.

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