Molecular Pathogenesis of Genetic and Inherited Diseases

Focal Adhesion Kinase Controls pH-Dependent Epidermal Barrier Homeostasis by Regulating Actin-Directed Na⁺/H⁺ Exchanger 1 Plasma Membrane Localization

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Ubiquitously expressed focal adhesion kinase (FAK), linked to multiple intracellular signaling pathways, has previously been shown to control cell motility, invasion, proliferation, and survival. Using mice with a keratinocyte-restricted deletion of *fak* (FAK^{K5 KO}), we report here a novel role for FAK: maintenance of adult epidermal permeability barrier homeostasis. Abundant lacunae of unprocessed lipids in stratum corneum (SC) of FAK^{K5 KO} mice and delayed barrier recovery pointed to malfunction of pH-dependent enzymes active in extracellular space of SC. Measuring the SC pH gradient showed significantly more neutral pH values in FAK^{K5 KO} mice, suggesting the importance of FAK for acidification. Moreover, normal functions were restored when FAK^{K5 KO} mice were exposed to a surface pH typical of mouse SC (pH = 5.5). Baseline levels and response to barrier disruption of secretory phospholipase A2 isoforms, enzymes that mediate generation of free fatty acids in epidermis, appeared similar in both FAK^{K5 KO} and control littermates. We found that the critical SC acidification regulator Na⁺/H⁺ exchanger 1 failed to localize to the plasma membrane in FAK-deficient keratinocytes both *in vivo* and *in vitro*. Thus, for plasma membrane localization in terminally differentiated keratinocytes, Na⁺/H⁺ exchanger 1 requires an intact

actin cytoskeleton, which is impaired in FAK-deficient cells. (*Am J Pathol* 2007, 170:2055–2067; DOI: 10.2353/ajpath.2007.061277)

Although ubiquitously expressed, focal adhesion kinase (FAK) has been most intensively studied as a major structural and enzymatic component of focal adhesion structures primarily in cells of mesenchymal origin.^{1,2} Deletion of FAK in mouse embryonic stem cells,³ endothelial cells,⁴ or neuronal tissue⁵ interferes with cell proliferation, migration, and survival, resulting in severe developmental abnormalities. Although FAK displays such protean effects on mesenchymal tissue constituents, whether it plays an important role in epithelial tissues is still unclear, because epithelial cells, particularly those in stratified epidermis, display very different types of adhesion structures, and their contact with the extracellular matrix is relatively restricted.

In recent studies, transgenic mice with deletions of FAK limited to the epidermis (FAK^{K5 KO}) have displayed epidermal thinning, an abnormal hair cycle, and sebaceous gland hypoplasia, pointing to possible roles for FAK in keratinocyte and appendage structure and function.⁶ Lack of FAK in epidermis was also associated with growth suppression of chemically induced skin tumors.⁷

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Finally, FAK was shown to be essential for keratinocyte survival and proliferation *in vitro*,^{6,7} but the responsible mechanisms are as yet not elucidated.

These studies suggest that FAK could regulate epithelial structure and function. Here, using FAK^{K5 KO} mice as a model, we sought evidence for more direct effects of FAK, solely on the epidermis, and investigated the responsible mechanism.

The final step of keratinocyte differentiation in multilayered epidermis is formation of an epidermal permeability barrier. The epidermal permeability barrier consists of terminally differentiated keratinocytes, corneocytes, embedded in a lipid matrix that is organized as stacked and patterned membrane sheets in the intercellular spaces (Figure 1A). This barrier, once built, is analogous to bricks and mortar, with the keratin macrofibrils and corneocytes forming the bricks, and the extruded lipids forming the mortar to seal together the cornified envelope that protects organism from the loss of body fluids and excludes foreign substances from the environment.^{8–14} As recently reported,¹⁵ cell-cell adhesions within the granular layer are also important for epidermal permeability barrier.

Our present study demonstrates that FAK exerts important effects on both the structure and function of epidermal permeability barrier based on a newly identified role in a posttranscriptional regulation of critical acidification mechanisms. Because acidification of the epidermal surface, in turn, regulates several important cutaneous functions including permeability barrier homeostasis, antimicrobial competence, and cytokine signaling, our studies provide a new mechanism that could impact the therapy of a wide variety of inflammatory dermatoses.

Materials and Methods

Mice

Mice were maintained and bred at the University of California-San Francisco Laboratory Animal Research Center and Veteran Affairs Medical Center facility in accordance with institutional guidance and National Institutes of Health standards. They were regularly monitored and had free access to standard mice chow and water. Mouse genotype was determined as previously described.⁶ Animals were individually caged for 3 weeks before experiments to avoid artifactual barrier perturbation due to trauma from scratching or biting.

Stratum Corneum Integrity and pH Measurements

Transepidermal water loss (TEWL) was measured with an electrolytic water analyzer (MEECO, Warrington, PA) either immediately after tape stripping (D-Squame, CuDerm Corporation, Dallas, TX) or at different time points. Surface pH measurements were performed using a flat glass-surface electrode (Mettler-Toledo, Giesen, Germany) with a skin pH meter PH900 (Courage&Khazaka, Cologne, Germany). In the rescue

experiment, tape-stripped mice were placed in bath filled with either vehicle or pH 5.5 buffer at 37°C. TEWL was determined at 0-, 3-, and 6-hour time points. At least five FAK^{loxP/+} and five FAK^{K5 KO} animals, 2 to 4 months old, were used in each experiment.

Electron Microscopy

For transmitted electron microscopy, skin biopsies were fixed in modified Karnovsky fixative overnight and post-fixed in either 0.2% RuO₄ or 1% OsO₄. Dehydrated samples were embedded in Epon-epoxy mixture, and ultrathin sections were examined in a Zeiss 10A electron microscope (Zeiss, Jena, Germany).

In Situ Zymography

β -Glucocerebrosidase (β -GlcCer'ase) and serine protease (SP) activity in the outer epidermis was determined as described previously.¹⁶ Serine protease inhibitor cocktail contained 1 μ g/ml aprotinin and 1 mmol/L phenylmethyl sulfonyl fluoride were purchased from Sigma (St. Louis, MO). Resorufin- β -D-glucopyranoside and BODIPY FL casein were purchased from Molecular Probes (Eugene, OR), whereas conduritol B epoxide was from Axxora (San Diego, CA). All images were taken at a fixed 500-millisecond exposure on a Axiophot epifluorescence microscope (Zeiss).

Nile Red Staining

Before each staining, the stock solution containing 0.05% (w/v) Nile red in acetone, stored at 4°C and protected from light, was diluted to 2.5 μ g/ml with 75:25 (v/v) glycerol/water, followed by brisk vortexing. A drop of the glycerol-dye solution was applied to each tissue section and immediately covered with a coverslip. Nile red fluoresces yellow-gold in the presence of neutral lipids and red in the presence of polar lipids.^{17–20} All images were taken with appropriate filters at fixed 240-millisecond (yellow-gold) and 75-millisecond (red) exposure on a Axiophot epifluorescence microscope (Zeiss).

Fluorescence Lifetime Imaging

Animals were anesthetized with chloral hydrate, and 20 to 30 μ l of 50 μ mol/L 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (Molecular Probes) was applied to a $\sim$ 0.25-cm² region on the flank skin of the animal four times at 10-minute intervals. The animal was then sacrificed, and the skin incubated with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein was harvested. Samples were viewed on a Zeiss LSM 510 (Zeiss) inverted microscope equipped with Ti:Sapphire (Spectra-Physics, Newport Corporation, Irvine, CA) laser as two-photon source. A single photon-counting module SPC830 (Becker Hickl GmbH, Berlin, Germany) was used for fluorescence lifetime measurement. A long-pass dichroic blocking infrared light and a BG-39 interference filter

(Chroma Technology, Rockingham, VT) were added in the epifluorescence light path. The laser was operated mode-locked at 800 nm for two-photon excitation. Images were collected at 512×512 pixels. To obtain sufficient photons at each pixel for the exponential decay curve, an average of 20 scans were accumulated for each image. Stratum corneum (SC) location was monitored both by measuring depth from the skin surface and by noting the characteristic morphology of nucleated epidermal cells. Calibration was performed with powdered SC, suspended in aqueous solution at a range of pH. SC was obtained by incubation of full-thickness mouse skin in 0.5% trypsin (in H_2O) at $4^\circ C$ overnight. Dermis and nucleated layers of epidermis came off sequentially; the remaining SC was then immersed in liquid nitrogen and powdered. A drop of suspension at each pH was mounted on microscope slides and analyzed. The resulting calibration curve of fluorescence lifetime versus pH showed a nonlinear sigmoidal form. Image J (FreeWare; Research Services Branch, National Institutes of Health, Bethesda, MD) was used for postexperimental data processing. Intra- and extracellular regions of interest were selected, and five regions of each were averaged to derive the mean lifetime.

Real-Time PCR

Total RNA isolation and real-time PCR were performed as described previously.⁶ In brief, epidermal samples were processed according to manufacturer's recommendations for isolation of RNA from fibrous tissue using the RNeasy columns (Qiagen). RNA quality and quantity were analyzed using the Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA). Only samples with the highest quality of RNA were used for real-time PCR analyses. Gene-specific primers for multiplex real-time RT-PCR for this study were designed for each gene of interest using "Primer Express" software (Perkin Elmer, Boston, MA) based on sequencing data from National Center for Biotechnology Information databases and purchased from Biosearch Technologies, Inc. (Novato, CA). Sequence data for all of the oligos (Supplemental Table 1) may be viewed at <http://ajp.amjpathol.org>. Reverse transcription was performed using PowerScript (Clontech, Mountain View, CA). This was followed by real-time PCR, which was performed in duplicate using the ABIPrizm 7900HT sequence detection system. Calibration curves were done for every gene targeted using at least five RNA serial dilutions covering a range of gene expression from 10 to several million copies. Reactions were incubated at $50^\circ C$ for 2 minutes and then $95^\circ C$ for 10 minutes. This was followed with 40 cycles of $95^\circ C$ for 15 seconds and $60^\circ C$ for 1 minute.

Western Blotting and Immunostaining

Keratinocytes or epidermis were lysed in modified radio-immunoprecipitation assay buffer.²¹ Equilibrated lysates were incubated at $37^\circ C$ for 5 minutes,²¹ separated by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose (Schleicher & Schuell, Keene, NH), and blotted with anti-FAK (BD Biosciences Transduction Laboratories, Lexington, KY), anti- Na^+/H^+ exchanger 1 (NHE1) 4E9 (Chemicon, Temecula, CA), anti-actin (Sigma), anti-loricrin, and anti-involucrin (Covance, Princeton, NJ) antibodies as described previously.²¹ All secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

For NHE1 and E-cadherin immunolocalization, cells or 5- μm -thick fresh frozen tissue sections were fixed in cold methanol for 10 minutes, rehydrated in phosphate-buffered saline (PBS), and incubated overnight at $4^\circ C$ with 10 $\mu g/ml$ anti-NHE1 monoclonal 4E9 antibody and rat anti-E-cadherin antibody (Zymed Laboratories, South San Francisco, CA) mixed with 10 $\mu g/ml$ ChromPure donkey whole IgG in PBS (Jackson ImmunoResearch). After three washes in PBS, samples were incubated with a mixture of fluorescein- and rhodamine-X-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch) for 40 minutes at room temperature, washed again, mounted in Vectashield (Vector, Burlingame, CA), and analyzed. For staining of actin stress fibers, cells were fixed in 3.8% paraformaldehyde/PBS for 20 minutes, permeabilized with 0.2% Triton-X/PBS for 2 minutes, and incubated with 10 nmol/L rhodamine-conjugated phalloidin (Molecular Probes) for 30 minutes at room temperature, washed, mounted in Vectashield (Vector), and analyzed.

Cell Culture and Viral Transduction

Primary mouse keratinocytes were isolated from 3-day-old FAK^{loxP/loxP}p53^{-/-} keratinocytes. Remaining fibroblasts were removed by brief trypsinization of 50% confluent monolayer culture. After three to four passages, only keratinocytes remained in the culture as confirmed with keratin-5 immunostaining (data not shown). To excise the floxed region of *fak*, the cells were transduced with 20 multiplicity of infection/cell adenovirus encoding green fluorescent protein (GFP) and Cre recombinase. Nearly all cells became green after 24 hours. GFP was not detectable after 7 to 10 days. To induce terminal differentiation, the cells were cultured for 1 to 5 days in the presence of 2 mmol/L $CaCl_2$. We initially performed Ca^{2+} -induced differentiation in the presence of lower $CaCl_2$ concentrations. However, genetically modified keratinocytes did not respond well. As suggested by other reports using models of primary mouse keratinocytes lacking molecules involved in cellular adhesion,²² we started to increase Ca^{2+} concentration. A more uniform NHE1 membrane pattern was obtained when Ca^{2+} concentration was increased to 2 mmol/L.

The sequence used to inhibit human FAK expression and scrambled control was cloned into pLentiLox3.7.²³ Sense oligo sequences were 5'-tGAACCTCGCAGTCATTATTtcaagagAATAAATGACTGCGAGGTTctttttc-3' (human FAK, 1057–1072 from start codon) and 5'-tGTCTCCGAACGTGTCTCACGTTtcaagagAACGTGACACGTTCCGGA-GACtttttc-3' (scrambled). The 5' T belongs to U6 promoter,

Figure 1. The epidermal permeability barrier is abnormal in FAK^{K5 KO} mice. **A:** Schematic drawing of mouse skin depicting epidermal permeability barrier within SC (inset). **B:** Barrier recovery, as measured by TEWL, in the first 6 hours after tape stripping is approximately threefold slower in FAK^{K5 KO} mice. **Asterisks** show significantly different values. $n = 5$ animals/each time point. Data are presented $\pm$ SD.

and lowercase letters represent components of short hairpin (sh) RNA. The underlined sequence forms the loop, and the italicized sequence is a terminator sequence. The 3' C was added to generate an XbaI site. Antisense oligo se-

Figure 2. Defective lipid processing into lamellar bilayers in FAK^{K5 KO} SC is pH-dependent. **A:** FAK^{K5 KO} mice demonstrate defective SC lipid processing. Electron micrographs of P7 FAK^{loxP/+} mouse skin compared with FAK^{K5 KO} littermates. RuO₄ postfixation. **Arrowheads**, normal structure of parallel lipid lamellae in normal littermates. **Arrows**, lacunae of unprocessed lipid in FAK^{K5 KO} mice. **B:** Decreased lipid processing results in an abnormal increase in the substrate polar lipids. Nile Red staining demonstrates more abundant polar lipids (**arrows**) in SC of FAK^{K5 KO} mice. **C:** Impaired lipid processing is due to decreased β -GlcCer'ase activity in FAK^{K5 KO} mice, compared with their FAK^{loxP/+} littermates. Resorufin- β -D-glucopyranoside was used as a fluorescent substrate. As a control, fluorescence was blocked in the presence of conduritol B epoxide, a high-potency β -GlcCer'ase inhibitor. Decreased β -GlcCer'ase activity in SC of FAK^{K5 KO} mice was reversed by exogenous acidification. **D:** Decreased β -GlcCer'ase activity is linked to impaired SC acidification. The pH gradient within SC of 4- to 6-month-old FAK^{K5 KO} and FAK^{loxP/+} mice was determined by measuring surface pH after each of six consecutive strippings with 22-mm D-Squame disks. Comparison of SC surface pH showed significantly (*) higher values in FAK^{K5 KO} mice after three to five strippings. **E:** Fluorescence lifetime imaging of epidermis in FAK^{loxP/+} and FAK^{K5 KO} littermates. The decrease in acidity throughout the SC of FAK^{K5 KO} mice confirms the importance of FAK for SC acidification. Fluorescence lifetime imaging measurements were obtained in paired FAK^{loxP/+} and FAK^{K5 KO} littermates, and measurements of at least five extracellular domains were taken at each depth. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, determined using a Student's *t*-test.

quences were 5'-tcgagaaaaaGAACCTCGCAGTCATTTA-TTcttgaAATAAATGACTGCGAGGTTCa-3' (human FAK) and 5'-tcgagaaaaaGTCTCCGAACGTGTCACGTTcttga-AACGTGACACGTTCCGAGACa-3' (scrambled). An additional four nucleotides at the 5' end were added to generate the *Xba*I overhang. The oligos were annealed and ligated into the *Hpa*I/*Xba*I site of pLentilox 3.7. This vector is bicistronic and also expresses GFP from a separate cytomegalovirus promoter.

For production of lentiviruses, 293T cells were transfected by the calcium-phosphate method using 20 μ g of plentilox3.7 or FAK short hairpin RNA construct, 10 μ g of cytomegalovirus-VSVg envelope vector pMD.G, 10 μ g of RSV-Rev, and 10 μ g of packaging vector pMDL g/p RRE.²⁴ Lentivirus-containing supernatants were harvested 48 hours after transfection and filtered through a 0.45- μ m filter (Millipore, Billerica, MA) to remove cells. Primary human keratinocytes were infected with the supernatants in the presence of polybrene for 2 days.

Results

Generalized FAK deletion leads to embryonic lethal mutants.^{3,25} To study a role of FAK in epidermis, we generated a mouse model with a keratinocyte-restricted deletion of *fak* (FAK^{K5 KO} mice) by crossing mice carrying a floxed *fak* gene⁵ with transgenic mice expressing Cre recombinase driven by the keratin-5 promoter.²⁶ DNA, RNA, and protein analyses confirmed disruption of full-length FAK in FAK^{K5 KO} mice.⁶ To determine whether barrier abnormalities are present, we assessed epidermal permeability barrier function in adult FAK^{K5 KO} mice. We assessed SC epidermal permeability barrier homeostasis as the kinetics of barrier recovery, measured as TEWL levels, after acute barrier disruption. Whereas basal barrier function was not significantly different between normal and mutant mice (data not shown), barrier recovery rates after acute barrier perturbation by tape stripping were significantly delayed in FAK^{K5 KO} mice at both 3 ($P < 0.05$) and 6 hours ($P < 0.01$, Figure 1B). These findings show that FAK is required for normal epidermal permeability barrier homeostasis.

We further defined what pathophysiological mechanisms might underlie these barrier defects. Because epidermal permeability barrier formation and repair involve interlocking of protein (cellular) and lipid (extracellular) compartments, we next examined whether abnormalities in one or both of these compartments account for the barrier anomaly.

The permeability barrier depends not only on the presence of multiple layers of fully differentiated corneocytes but also on the secretion and postsecreting extracellular reorganization of polar lipids into neutral hydrophobic, multilamellar lipid membrane arrays within the SC interstices.^{9,27} Electron microscopy comparison of FAK^{K5 KO} mice with their normal FAK^{loxP/+} littermates showed similar number of lamellar bodies fusing to plasma membrane in both animals, demonstrating that lipid secretion was normal in the FAK^{K5 KO} animals (data not shown). In contrast, lipid processing was delayed in FAK^{K5 KO} epi-

dermis; ie, much of the secreted lipids remained incompletely processed into lamellar membranes in the SC (Figure 2A), whereas, as in normal epidermis, such extracellular processing is completed in FAK^{loxP/+} mice by the second intercellular space above the outermost nucleated cell layer [the stratum granulosum (SG); Figure 2A]. Defective lipid processing from polar to neutral lipids also was reflected by increased polar lipids, as demonstrated by increased Nile red staining in FAK^{K5 KO} versus control FAK^{loxP/+} SC (Figure 2B).

The extracellular processing of secreted lipids is dependent on lipid hydrolases that convert sphingomyelin and glucosylceramides into ceramides.^{28,29} Therefore, we next compared the activity of the acidic pH-dependent, extracellular hydrolase β -GlcCer'ase in the epidermis of FAK^{loxP/+} and FAK^{K5 KO} littermates. β -GlcCer'ase activity was markedly reduced in both outer epidermis and SC in FAK^{K5 KO} versus FAK^{loxP/+} mice, as assessed by *in situ* zymography (Figure 2C). β -GlcCer'ase activation requires an acidic pH environment. To assess whether an acidification abnormality decreases β -GlcCer'ase activity, we first assessed whether β -GlcCer'ase is present but inactive in FAK^{K5 KO} epidermis. Because exogenous re-acidification of frozen sections of FAK^{K5 KO} epidermis normalized enzyme activity, the reduction in β -GlcCer'ase activity reflects pH-dependent alteration in enzyme activity, rather than decreased enzyme protein levels, in FAK^{K5 KO} animals.

To test further the role of pH in the barrier abnormality in FAK^{K5 KO} mice, we next compared pH at different levels of SC. We found that the pH gradient across the SC, assessed by a surface electrode after sequential stripping, displayed subtle but consistent abnormalities in FAK^{K5 KO} mice (Figure 2D). Because sequential tape stripping could disrupt tissue pH equilibria, we next used fluorescence lifetime imaging to visualize pH in intact, unperturbed SC as a function of depth. As with tape stripping, fluorescence lifetime imaging analysis revealed an elevated pH across the entire SC of FAK^{K5 KO} mice (Figure 2E), further confirming the importance of FAK for SC acidification. Together, these results provide a functional and structural basis for the permeability barrier abnormality in FAK^{K5 KO} mice.

Whereas an elevated surface pH down-regulates lipid-processing enzymes that generate ceramides for the permeability barrier, a pH increase would conversely increase the total activity of SPs in SC.³⁰⁻³³ Accordingly, *in situ* zymography revealed higher total SP activity in both the outer epidermis and SC of FAK^{K5 KO} than in FAK^{loxP/+} mice (Figure 3A). Both the increased SP activity in FAK^{K5 KO} mice and the reduced activity in FAK^{loxP/+} mice could be reversed by *in situ* buffering to pH 7.4 and 5.5, respectively, showing that increased SP activity in FAK^{K5 KO} mice again likely reflects pH-sensitive alterations in enzyme activity, rather than altered protein expression. In the presence of SP inhibitor cocktail, protease-catalyzed hydrolytic release of fluorescent dye was abolished in all conditions examined.

As previously reported for mice with a normal, acidic SC pH,³⁰⁻³³ FAK^{loxP/+} mice displayed both a normal density and a normal length of corneodesmosomes be-

A in situ zymography: serine proteases

tween adjacent corneocytes within the lowermost two to three layers of SC. In contrast, corneodesmosomes in FAK^{K5 KO} mice were 1) smaller, 2) present at lower densities, and 3) prematurely degraded (ie, by the first and second layer of SC) (Figure 3, B and C). Together, these results demonstrate that the surface pH abnormality in FAK^{K5 KO} mice correlates with SP-mediated degradation of corneodesmosomes within the SC. This accelerated loss of SC could also contribute to the permeability barrier abnormality.

Finally, we assessed directly whether the acidification defect is important by testing whether acute exogenous acidification, which restores a normal physiological pH without affecting the underlying nucleated layer,^{30,31} normalizes permeability barrier homeostasis. As seen in Figure 4, exposure of 4- to 6-month-old FAK^{K5 KO} mice to unbuffered solution after barrier perturbation reproduced the barrier recovery delay seen in air-exposed mice (compare with Figure 1B). In contrast, exposure to solutions buffered to the ambient acidic pH (ie, pH 5.5) of normal SC completely normalized barrier recovery kinetics (Figure 4A). Although exposure to unbuffered solution also reproduced the abnormal lipid processing seen in FAK^{K5 KO} air-exposed mice, exposure of FAK^{K5 KO} mice to an acidic pH buffer resulted in the appearance of mature lamellar bilayers in the lower SC of FAK^{K5 KO} mice similar to those seen in normal littermates (Figure 4B; data for FAK^{loxP/+} not shown). These results provide direct evidence that the permeability barrier abnormality in FAK^{K5 KO} mice can be attributed to an abnormality in SC acidification.

A number of exogenous and endogenous mechanisms contribute to SC acidic mantle. Because lack of FAK is an endogenous defect, we focused on endogenous mechanisms. Several endogenous processes have been implicated in acidification of adult SC: 1) generation of *cis*-urocanic acid from histidine,³⁴ 2) free fatty acids generation from phospholipid hydrolysis mediated by secretory phospholipases A2 (sPLA2) isoforms,³⁵ and 3) NHE1.³⁶ The histidase pathway is not essential for bulk SC acidification because adult *his/his* mice still display normal acidic pH of SC even though they have lost >90% of histidase function.³³

sPLA2 represent a large family of low-molecular weight, Ca²⁺-requiring secretory enzymes that catalyze the hydrolysis of phospholipids at the sn-2 position, leading to the generation of free fatty acids and lysophospholipids. We compared levels of epidermal sPLA2 isoforms in FAK^{loxP/+} and FAK^{K5 KO} mice using real-time PCR (Figure 5). Both baseline levels and the response to barrier disruption of sPLA2 isoforms were similar in both backgrounds, except

Figure 3. Decreased numbers of SC desmosomes are linked with increased SP activity in FAK^{K5 KO} mice. **A:** Serine protease activity was higher in FAK^{K5 KO} versus FAK^{loxP/+} SC. BODIPY FL was used as a fluorescent substrate. Activity was blocked by acidification of tissue sections or serine protease inhibitors. **B:** Reduced number of corneodesmosomes (gray circles) led to corneocyte detachment and cleft formation (*) in SC of FAK^{K5 KO} mice. Black circles, normal-appearing corneodesmosomes in SC of FAK^{loxP/+} littermates. **C:** SC of FAK^{K5 KO} mice has significantly (*) fewer corneocyte layers and fewer corneodesmosomes in comparison with FAK^{loxP/+} littermates. Data are presented as the mean $\pm$ SD. **P* < 0.05, determined using a Student's *t*-test.

Figure 4. Exogenous acidification rescues defective barrier recovery in FAK^{K5 KO} mice. Eight- to 12-week-old FAK^{loxP/+} or FAK^{K5 KO} mice were subjected to barrier perturbation using tape stripping (see Materials and Methods) and then exposed to either unbuffered solution, as a control, or solution buffered to pH 5.5, to exogenously acidify the SC. TEWL was measured at 3 and 6 hours after tape stripping, and biopsies were obtained after the 6-hour measurement. Biopsies also were obtained at baseline (time 0) as a control. Time 0 animals were not used in subsequent experiments. **A:** TEWL as a measure of SC integrity showed comparable barrier recovery in FAK^{K5 KO} and FAK^{loxP/+} mice 3 and 6 hours after disruption when the stripped area was acidified. *n* = 5 animals/each time point. Data are presented $\pm$ SD. Asterisks show significantly different values. **B:** Processing of lipid lamellae (arrows) was likewise normalized when SC of FAK^{K5 KO} was exposed to HEPES buffer pH 5.5 after barrier break.

sPLA2-IIF and -V baselines, which were somewhat higher in FAK^{K5 KO} mice, suggesting that sPLA2 were not involved in SC acidification defect of FAK^{K5 KO} mice.

Figure 5. Real-time PCR analyses of sPLA2 isoforms expression. **A:** Baseline level of sPLA2 expressed in epidermis. Data are shown as fold increase/decrease of mRNA levels in skin samples of FAK^{K5 KO} mice (*n* = 6) versus average of sPLA2 levels in FAK^{loxP/+} animals (*n* = 5) using log₁₀ scale. Baseline levels of sPLA2 isoforms were similar in both backgrounds, except sPLA2-IIF and -V, which were increased in FAK^{K5 KO} mice. **B:** Schematic drawing describing the experiment. **C:** Induction of sPLA2 isoforms and inflammatory response 9 hours after tape stripping is similar in epidermis of 4- to 6-month-old FAK^{loxP/+} and FAK^{K5 KO} mice. Data are shown as fold increase/decrease of mRNA levels in samples from tape-stripped skin versus control sample from the same animal using log₁₀ scale. *n* of FAK^{loxP/+} = 5; *n* of FAK^{K5 KO} = 6. **D:** Real-time PCR analyses of IL-1β and IL-8 expression shown as in **C**. Induction of these cytokines is a sign of typical inflammatory response that follows the epidermal barrier disruption by tape stripping.

NHE1 acidifies the SC by extruding H⁺ ions into extra-cellular space primarily at the SG-SC interface.^{30,31,36,37} Therefore, we next assessed whether the acidification defect could be linked to decreased NHE1 expression. We first examined whether NHE1 protein synthesis was abnormal in FAK^{-/-} epidermis. Paradoxically, we found that NHE1 protein expression in 2-month-old FAK^{K5 KO}

Figure 6. NHE1 plasma membrane localization, not protein levels, differs in epidermis of FAK^{K5 KO} mice. **A:** NHE1 levels are either slightly higher (2-month-old) or similar (6-month-old) in epidermis of FAK^{K5 KO} mice. Diffuse appearance of NHE1 bands interferes with accurate quantification by densitometry. **B:** Normal NHE1 plasma membrane localization in upper SG (arrows) of FAK^{loxP/+} mice is diminished in FAK^{K5 KO} animals (arrowheads).

animals was higher than in their normal littermates, suggesting a possible compensatory up-regulation. This difference diminished by the age of 6 months (Figure 6A).

Because FAK links both signaling and structural molecules, we next examined whether FAK deletion impaired NHE1 transport to the plasma membrane. NHE1 must be localized at the plasma membrane of terminally differentiated keratinocytes to regulate extracellular pH.^{33,36} Immunolocalization studies confirmed that very little of NHE1 translocated from the cytosol to the plasma membrane in the upper SG of FAK^{K5 KO} mice (Figure 6B).

We next used two *in vitro* systems to confirm that FAK expression controls recruitment of NHE1 to the plasma membrane. Whereas primary keratinocytes isolated from FAK^{K5 KO} mice did not proliferate in culture and died, under the same conditions, isolated FAK^{loxP/loxP} or FAK^{loxP/+} keratinocytes survived and proliferated (see also Ref. 6). From our previous work, we knew that FAK-null cells could survive *in vitro* on p53-null background.³ Therefore, to allow us to culture mouse FAK^{K5 KO} keratinocytes, we introduced p53-null mutation in FAK^{loxP/loxP} mice. Primary keratinocytes were isolated from FAK^{loxP/loxP}p53^{-/-} mice, and the floxed region of *fak* was knocked out using an adenovirus carrying the gene

Figure 7. Deleting FAK in mouse keratinocytes *in vitro* mimics NHE1 mislocalization phenotype *in vivo*. **A:** Cre recombinase encoded by adenovirus excised floxed region of *fak* and depleted FAK expression 72 hours on transduction. Actin served as loading control. **B:** Primary p53^{-/-} mouse keratinocytes with (FAK^{loxP/loxP}p53^{-/-}) or without (FAK^{-/-}p53^{-/-}) FAK cultured in 2 mmol/L Ca²⁺ for 5 days to induce differentiation synthesize similar levels of both an early (involucrin) and late (loricrin) differentiation markers. **C:** Deletion of FAK *in vitro* does not change overall NHE1 protein levels in differentiated keratinocytes. Results presented are from three independent experiments. Actin served as loading control. **D:** In terminally differentiated FAK^{loxP/loxP}p53^{-/-} keratinocytes, NHE1 is primarily localized on plasma membrane (arrows). Cre-mediated deletion of FAK resulted in diminished plasma membrane-localized NHE1 in FAK^{-/-}p53^{-/-} keratinocytes (arrowheads).

for Cre recombinase (Figure 7A). Five-day culture of FAK^{loxP/loxP}p53^{-/-} and FAK^{-/-}p53^{-/-} keratinocytes in the presence of 2 mmol/L Ca²⁺ resulted in comparable

Figure 8. NHE1 mislocalization phenotype of FAK^{K5 KO} mouse is reproduced in FAK-depleted primary human keratinocytes. Primary human keratinocytes were left untreated or transduced with a lentivirus encoding GFP (green) and either scrambled sequence or short hairpin RNA FAK (shFAK). Three days later, cells were exposed to 2 mmol/L Ca²⁺ for 5 days. NHE1 was localized mostly at plasma membrane in untreated (arrowheads) and scrambled sequence/GFP-transduced keratinocytes (short arrows), whereas shFAK/GFP-transduced keratinocytes had very little NHE1 left at the plasma membrane (long arrows).

levels of terminal differentiation, assessed as expression levels of early (involucrin) and late (loricrin) differentiation markers (Figure 7B). As in our *in vivo* studies presented above, NHE1 translocation was defective in differentiated keratinocytes lacking functional FAK, whereas NHE1 production was not significantly affected. NHE1 levels were similar in differentiated FAK^{loxP/loxP}p53^{-/-} and FAK^{-/-}p53^{-/-} keratinocytes (Figure 7C), whereas NHE1 immunolocalization showed very little of NHE1 translocated from the cytosol to the plasma membrane in differentiated FAK^{-/-}p53^{-/-} keratinocytes (Figure 7D).

Because of the potential importance of this finding, we used an alternate, unrelated approach to assess how FAK controls NHE1 localization. FAK expression can be down-regulated in primary human keratinocytes with normal p53, using lentivirus carrying both GFP and FAK short hairpin RNA. Using short hairpin RNA to down-regulate FAK produced the same result: less NHE1 was detected on the plasma membrane of differentiated

Figure 9. Intact actin cytoskeleton is required for localization of NHE1 at the plasma membrane. **A:** Prominent actin stress fibers (arrows) present in primary p53^{-/-} mouse keratinocytes with FAK (FAK^{loxP/loxP}p53^{-/-}) were not detected in keratinocytes without FAK (FAK^{-/-}p53^{-/-}) cultured under the same conditions, in 2 mmol/L Ca²⁺ for 2 days. **B:** Disruption of actin stress fibers with 2 μmol/L cytochalasin D interfered with plasma membrane localization of NHE1, but not E-cadherin, in primary p53^{-/-} mouse keratinocytes even when FAK is present (FAK^{loxP/loxP}p53^{-/-}). Colocalization of NHE1 and E-cadherin in FAK^{loxP/loxP}p53^{-/-} keratinocytes cultured for 2 days in medium supplemented with 2 mmol/L Ca²⁺ and 0.2% dimethylsulfoxide (arrows) or 2 μmol/L cytochalasin D (arrowheads).

GFP-positive cells by immunolocalization. Control, scrambled sequences did not affect NHE1 localization in differentiated GFP-positive keratinocytes (Figure 8). Taken together, our results strongly suggest that the pH abnormality in the SC of FAK^{K5 KO} mice can be attributed to a failure of FAK-dependent localization of NHE1 to the plasma membrane in terminally differentiated keratinocytes.

We next examined the organization of actin cytoskeleton in FAK-deficient keratinocytes. Rhodamine-phalloidin staining showed that FAK^{-/-} keratinocytes were unable to organize actin stress fibers in a parallel pattern. The only stress fibers present were at the cell periphery, organized in a circular fashion resembling the actin cytoskeleton defect in FAK^{-/-} fibroblasts (Figure 9A).^{3,38} An intact actin network is required not only for cellular contractility and motility but also for the correct intracellular localization of glucose transporters, as well as for their incorporation into the plasma membrane in re-

sponse to insulin.³⁹ To determine whether actin stress fibers are required for NHE1 distribution to the plasma membrane, we disrupted the cytoskeleton with fungal toxin cytochalasin D, a potent inhibitor of actin polymerization. NHE1 immunolocalization showed that very little NHE1 translocated from the cytosol to the plasma membrane in differentiated FAK^{loxP/loxP}p53^{-/-} keratinocytes when the actin cytoskeleton is disassembled. This, however, did not affect localization of E-cadherin at the plasma membrane, suggesting that actin-dependent transport to plasma membrane is specific for a subset of proteins, including NHE1.

Discussion

The early embryonic lethality of FAK^{-/-} mice indicated an indispensable role for this nonreceptor protein tyrosine kinase in development.^{3,25} The defects affected mostly mesoderm-derived structures. Subsequent work with cells of mesodermal origin linked FAK tightly with extracellular matrix signaling. In this study, using FAK^{K5 KO} mice, we investigated a role of FAK in epidermis, particularly in upper layers that do not have direct contact with extracellular matrix in basement membrane. We found that epidermal permeability barrier function is abnormal in these mice. Epidermal barrier function depends on both lipid secretion, which was normal in FAK^{K5 KO} mice, and lipid processing, which was abnormal in FAK^{K5 KO} mice. Lipid processing, in turn, is regulated by the pH of SC, which at a normal, acidic pH activates pH-dependent lipid-processing enzymes. SC acidification also down-regulates pH-dependent SC proteases, which degrade corneodesmosomes, thereby reducing SC integrity and leading to desquamation.

Because the epidermal permeability barrier abnormality is detectable as early as 1 week after birth (data not shown), it is likely that this barrier dysfunction is an intrinsic abnormality, rather than secondary to other alterations produced in FAK^{K5 KO} epidermis. In addition to its control of NHE1 localization and barrier homeostasis, FAK also may affect other aspects of epidermal function.

Injuries localized to the SC alone initiate inflammatory responses (eg, irritant contact dermatitis). In addition, the severity of disease phenotype correlates with the extent of barrier abnormality (eg, psoriasis, atopic dermatitis, and various ichthyoses), and improvement in SC function alone improves inflammatory dermatoses (eg, psoriasis and atopic dermatitis).⁴⁰⁻⁴³ Therefore, innate or acquired abnormalities of epidermal permeability barrier function are not just a secondary phenomenon to inflammatory dermatoses; in many cases, these abnormalities are a primary initiator of the skin pathogenesis.⁴⁴ Effective barrier repair strategies are arising as a necessity for successful treatment of inflammatory dermatoses.¹⁰ Formation and maintenance of the epidermal permeability barrier requires hydrolytic processing of relatively polar, secreted lipid mixture of lamellar bodies into their less polar lipid products. Two enzymes essential for this processing, β -GlcCer'ase and acidic sphingomyelinase, require acidic milieu for optimal activity,^{29,45} suggesting

that epidermal permeability barrier homeostasis is dependent on SC pH.^{46,47} When exposed to alkaline or neutral solutions, skin exhibits greater susceptibility to develop irritant/allergic contact dermatitis.⁴⁶⁻⁴⁸

A number of exogenous and intrinsic mechanisms have been proposed to contribute to SC acidity. Behne et al^{36,37} have shown that H⁺ generated by NHE1 in SG are important source of acidity in extracellular microdomains at the SG/SC interface where lipid processing occurs. Lack of NHE1 or its pharmacological inhibition impairs barrier development or recovery. NHE1 expression levels are regulated by changes in extracellular pH. Whereas in myocytes,⁴⁹ esophageal mucosa,⁵¹ and renal epithelia,⁵¹⁻⁵³ extracellular acidity leads to suppression of NHE1 activity and mRNA levels, in keratinocytes the situation is completely opposite: NHE1 expression is decreased in response to extracellular acidity and increased under alkalinizing conditions. In response to acute alkalinization as in barrier disruption, NHE1 up-regulation allows a quick response to restore epidermal permeability barrier homeostasis.^{32,33} Therefore, alkalinization of extracellular space might explain the compensatory up-regulation of NHE1 protein levels that we observed in SC of FAK^{K5 KO} mice. Yet, even higher total NHE1 protein levels did not suffice to bring pH down to normal acidic values, because NHE1 failed to translocate to the plasma membrane in terminally differentiated FAK^{-/-} keratinocytes. Hence, NHE1, although present, is not functional. There are several possible explanations for the failure of NHE1 to translocate normally. Coupled to Cl⁻/HCO₃⁻ exchange, increased NHE1 activity results in cellular uptake of NaCl and cell swelling consequent to the influx of osmotically obliged water.⁵⁴⁻⁵⁶ Because both mouse and human keratinocytes with lowered FAK expression are bigger (data not shown), this increase in their size might block NHE1 activity by keeping it away from plasma membrane and therefore preventing further cell enlargement. Using NHE-deficient Chinese hamster ovary activator protein-1 cells, Bullis et al⁵⁷ found that exogenously expressed hemagglutinin-tagged NHE1 is mostly localized in lipid rafts and that interruption of actin cytoskeleton for 2 hours with cytochalasin D does not affect its localization in lipid rafts. Because keratinocytes in our system were p53-deficient and therefore insensitive to cytochalasin D-dependent apoptosis, we could expose them to cytochalasin D for 2 to 5 days, allowing a complete degradation of NHE1 already present at the plasma membrane and preventing transport of newly synthesized NHE1 protein. Therefore, we could demonstrate that the translocation of endogenous NHE1 to plasma membrane of terminally differentiated keratinocytes requires an intact and fully functional cytoskeleton, which is impaired in FAK^{-/-} cells.³

NHE1 is also directly involved in alterations of the metabolic microenvironment and cell invasiveness that contribute to tumor formation and progress. In tumors, the extracellular space is more acidic (pH 6.2 to 6.9) than the cytosol (pH 7.12 to 7.65), whereas in normal cells, this pH gradient is reverse (extracellular, pH 7.3 to 7.4; cytosolic, pH 6.99 to 7.20). Further studies have shown that the development and maintenance of this gradient pH

change is driven primarily by the stimulation of NHE1 activity (reviewed in Ref. 58). Cellular alkalization induces a switch to aerobic glycolysis followed by serum- and anchorage-independent cell proliferation, leading to the growth of disorganized poorly vascularized masses.

There is likely a cross talk between NHE1 and FAK other than presented here.⁵⁹ NHE1 is predominantly localized in lamellipodia, where it anchors actin cytoskeleton to the plasma membrane by its direct binding of ezrin/radixin/moesin actin-binding proteins.⁶⁰ Both NHE1 and FAK reportedly have direct interaction with ezrin.^{60,61} However, we examined and found no difference in epidermal barrier between ezrin-deficient and normal littermates, indicating that NHE1 connection to actin cytoskeleton might be different in keratinocytes than in fibroblasts. The most plausible explanation is that fibroblasts have an extensive contact with extracellular matrix through focal adhesions, whereas in natural surroundings, only a subset of keratinocytes sees extracellular matrix at the same extent. Therefore, adhesion structures in fibroblasts are very different from adhesion structures in keratinocytes.

Furthermore, an NHE1 mutant with impaired cytoskeletal anchoring and binding to ezrin/radixin/moesin actin-binding proteins was uniformly distributed along the plasma membrane in fibroblasts.⁶⁰ For example, a 45-residue-long region of the NHE2 cytosolic domain was found to be critical for targeting NHE2 to the apical membrane. Deletion of these residues, which bound specifically *in vitro* to the SH3 domain of the cytoskeletal protein α -spectrin, resulted in NHE2 mislocalization to basolateral membrane.⁶² Spectrin is also implicated in cytoskeleton-dependent delivery of glucose transporters to the cell surface in response to insulin.³⁹ Ion transport by NHE1 is stimulated by various membrane receptors that respond to migratory cues (integrins, receptor tyrosine kinases, and G-protein-coupled receptors). Disrupting either ion transport or cytoskeleton anchoring function of NHE1 impairs cell polarity, and cells lose directionality during migration.⁶⁰ Whether NHE1 activity is indispensable for tumor formation and progression is not yet clear.

Studies by McLean et al.^{63,64} demonstrated that loss of FAK inhibits malignant tumor progression and reduces keratinocyte migration. However, the mechanism is still unknown. Our finding that FAK is required for localization of NHE1 to the plasma membrane raises an interesting possibility. Mislocalized NHE1 in FAK-deficient keratinocytes might remain inactive, and therefore transformed cells might not be able to either undergo directional migration or achieve the reverse pH gradient required for tumor growth. FAK-dependent NHE1 localization to plasma membrane, therefore, may impact not only in epidermal barrier homeostasis but also skin malignancies.

There are a number of inherited disorders of the cornification, including ichthyosis vulgaris; lamellar, X-linked, and harlequin ichthyosis; Sjögren-Larsson's syndrome; and Gaucher, Refsum, and Maple syrup urine diseases; etc. For some of these diseases, the underlying genetic defects have been identified as mutations in well-known components of the process.

For most, however, the defects are either pleiotropic or map to regions that do not contain obvious candidate genes. The past few years have provided many breakthroughs by studying epidermal barrier formation in the mouse as an animal model. Mouse mutants with genetically altered levels of catalyzing enzymes, lipid biosynthesis, protein precursors, intercellular junctions and transcriptional regulators have mimicked some of the phenotypic presentations, but they have also revealed subtle defects in upstream parts of the pathways that might help to unravel the underlying genetic program and mechanisms of epidermal barrier formation. Deciphering mechanisms of the epidermal barrier defect in FAK^{K5 KO} mice will shed a light to yet unknown regulatory pathways and bring a new level of understanding how the barrier is formed.

Conclusions

We found that 1) epidermal permeability barrier recovery, as measured by TEWL, is about threefold slower in FAK^{K5 KO} mice in the first 6 hours after tape stripping; 2) lacunae of unprocessed lipids are abundant in SC of FAK^{K5 KO} mice; 3) as determined with *in vivo* zymography, β -GlcCer'ase and SP, pH-regulated enzymes active in extracellular space of SC, have altered activity in epidermis of FAK^{K5 KO} mice; 4) the SC pH gradient, measured by two different methods, showed significantly higher values in FAK^{K5 KO} mice, suggesting the importance of FAK for acidification; 5) exogenous acidification rescues defective barrier recovery in FAK^{K5 KO} mice; 6) sPLA2 isoforms expressed in epidermis respond in a similar way to barrier disruption in both FAK^{K5 KO} and control littermates; 7) NHE1, an important component in SC acidification, is not localized at the plasma membrane of terminally differentiated FAK^{-/-} keratinocytes; 8) FAK^{-/-} keratinocytes have an abnormal actin cytoskeleton; and 9) intact actin cytoskeleton is required for NHE1, but not E-cadherin, plasma membrane localization. Taken together, these findings demonstrate that FAK is required for optimum epidermal permeability function at least in part because of its control of SC acidification by regulating NHE1 localization.

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