Wounding activates p38 map kinase and activation transcription factor 3 in leading keratinocytes

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Summary

Quiescent epidermis anchors to laminin 5 in the basement membrane via integrin α6β4. Wounding elevates expression of laminin 5, generating leading keratinocytes (LKs) that migrate via β1 integrins. Laminin 5 was evaluated as a regulator of cell signaling, and mRNA and protein expression in LKs. An in vitro wound model was developed based on suspension and re-adhesion of quiescent human keratinocytes (HKs). DNA microarrays identified multiple mRNAs elevated 1.5 hours after suspension and re-adhesion including activation transcription factor 3 (ATF3). In vitro and in vivo, levels of ATF3 protein elevate in nuclei of LKs, but not in nuclei of the following cells, 2 hours after suspension or wounding but decline by 12-18 hours post injury. Significantly, null defects in laminin 5 or integrin β4 that inhibit anchorage chronically elevate ATF3 in vivo. This suggests that adhesion to laminin 5, but not other ligands, suppresses activation. On suspension, ATF3 and other transcripts in the microarrays are elevated by phosphorylated p38 mitogen-activated protein kinase (P-p38), a stress kinase that regulates mRNA and cell motility. Inhibition of P-p38 with SB203580 prevents phosphorylation of ATF2, a transcription factor for ATF3 in LKs. Re-adhesion to laminin 5 via α6β4 dephosphorylates P-p38 and suppresses ATF3 protein relative to cells in suspension. Thus, wounding of quiescent HKs disrupts laminin 5 adhesion to activate p38, generating mRNA transcripts that define LKs. Adhesion to deposits of laminin 5 via α6β4 suppresses P-p38 and activation mRNAs including ATF3. Defects in laminin 5 and α6β4 sustain P-p38 with probable pathological effects on transcription and migration.

Key words: Laminin 5, p38 MAPK, ATF3, Epidermal Wounds

Introduction

Wounding of quiescent epidermis disrupts the basement membrane, changes cell-cell and cell-substrate adhesion and cell signaling (Borradori and Sonnenberg, 1999; Fuchs et al., 1997; Goldfinger et al., 1999; Martin, 1997; Nguyen et al., 2000a; Woodley et al., 1999). Initial changes in adhesion and signaling are necessary for subsequent changes in gene transcription and protein translation required for repair of the basement membrane and migration (Frank, 2004). These changes generate a subpopulation of activated leading keratinocytes (LKs) at the wound edge that are distinct from either quiescent keratinocytes or following keratinocytes in the outgrowth (Lampe et al., 1998; Li et al., 2003; Nguyen et al., 2000a; Wood et al., 2002). Here, we investigated the role of laminin 5, a basement membrane adhesive ligand (Nguyen et al., 2000a; Ryan et al., 1999), in regulating cell signaling and protein expression in LKs generated by wounding or adhesion defects in laminin 5.

Changes in outside-in signals through integrin receptors in epidermal wounds contribute to changes in cell motility and protein expression that define LKs. For example, quiescent epidermal keratinocytes adhere to laminin 5 via integrin α6β4 in hemidesmosomes (Carter et al., 1991; Gipson et al., 1993; Goldfinger et al., 1999; Ryan et al., 1999). Adhesion via α6β4 does not require actin-dependent interactions that mediate cell motility (Frank, 2004; Xia et al., 1996). In contrast, wounding exposes the dermis and activates adhesion to dermal collagen via integrin α2β1 or to fibronectin via integrin α5β1, which require actin rearrangements to mediate cell motility. Targeted disruption of laminin 5 in mice generates epithelial blisters caused by failure of laminin 5 to bind integrin α6β4 in hemidesmosomes and/or integrin α3β1 (Nguyen et al., 2000a; Ryan et al., 1999). In the absence of laminin 5, integrin α3β1 interacts with an alternative basement membrane ligand, probably laminin 10. The switch from β4 to β1 integrins correlates with the discontinuous 'beads on a string' organization of α6β4 in the basement membrane zone and may generate changes in integrin-ligand interactions, cell signals and/or protein expression similar to wounds and tumors (Nguyen et al., 2000a; Ryan et al., 1999). For example, suspension and re-adhesion of keratinocytes via α6β4 and α3β1 to laminin 5 in vitro activates phosphoinositide 3-OH kinase (PI3K), which regulates epithelial motility and mRNA transcription (Li et al., 2003; Xia and Karin, 2004). The PI3K-Rac-JNK/p38 pathway participates in initial adhesion of quiescent human keratinocytes (HKs) to laminin 5 via α6β4 and α3β1 (Nguyen et al., 2000b; Xia and Karin, 2004). Initial adhesion on laminin 5 elevates GTP-bound Rho allowing for subsequent Rho-dependent adhesion on collagen via α2β1 (Nguyen et al., 2000b). Integrin α3β1 directs the stabilization

of polarized lamellipodium in epithelial cells through activation of Rac1 (Choma et al., 2004). This sequence of adhesion and signaling changes allows HKs to make the transition from quiescence to activation in wounds. Similarly, ligation of α3β1 in A549 adenocarcinoma cells by laminin 10/11 or laminin 5 preferentially activates the Rac-MKK-JNK/p38 stress pathway that mediates epithelial migration (Gu et al., 2001; Ono and Han, 2000; Xia and Karin, 2004). In addition to adhesion, interaction of cells with integrin ligands also regulates mRNA transcription and protein translation through activation of p38 MAPK (Balda and Matter, 2003). Ligation of α6β4 activates the Rac-PAK-MKK-p38 pathway to promote expression of IL-6 in thymic epithelial cells (Mainiero et al., 2003). Thus, cell suspension and re-adhesion onto dermal collagen or laminin 5 via integrins activates JNK and/or p38 by phosphorylation to alter protein expression and/or cell motility (Clark et al., 2003; Ono and Han, 2000).

Here, we sought to: (1) identify changes in mRNA transcription and protein translation in quiescent keratinocytes as they transition into wound LKs; (2) identify cell signals necessary for the transcriptional changes in LKs; (3) understand the role of laminin 5 adhesion in regulating the signaling and transcriptional changes. We found that suspension or wounding of HKs activates p38 in LKs to promote motility and transcription of activation transcription factor 3 (ATF3), a stress-response transcription factor. Significantly, in vivo defects in laminin 5 or α6β4 inhibit quiescent anchorage and chronically activate expression of ATF3. These results suggest that wounding activates p38 as a significant regulator of adhesion and transcription in LKs. Defects in laminin 5, like wounding, activate p38 leading to pathological expression of wound transcripts and migration.

Materials and Methods

Cells and cell culture

Primary HKs were prepared as described (Boyce and Ham, 1985) and were grown in keratinocyte growth media (Clonetics). All experiments were performed using HKs between passages one and three. Keratinocytes from individual JF/VS9-3-96 with junctional epidermolysis bullosa-pyloric atresia (JEB-PA) have premature termination mutations in both alleles of the ITGB4 gene encoding the β4 integrin subunit and were derived from tissue from family two described earlier (Pulkkinen et al., 1998). Keratinocytes from an individual with gravis JEB (JEBG) have null defects in the laminin β3 chain and do not secrete laminin 5 (Lim et al., 1996) and were from M. Pittelkow (Mayo Clinic College of Medicine, Rochester, MN). Keratinocytes from mice (MKs) with homozygous null mutations in the LAMA3 gene ($\alpha3^{-/-}$ MKs) encoding the α3 chain of laminin 5 or wild-type mice ($\alpha3^{+/+}$ MKs) were immortalized with E6 and E7 oncogenes from human papilloma virus (Ryan et al., 1999). $\alpha3^{-/-}$ MKs require an exogenous adhesive ligand because they do not deposit laminin 5 and were maintained on collagen-coated culture dishes (rat tail collagen I, Becton-Dickinson) (Sigle et al., 2004). MKs were maintained in KGM containing 60 μM calcium.

Reagents and antibodies

The mouse monoclonal antibody (mAb) P1B5 against integrin α3, P1H5 against integrin α2, P4C10 against integrin β1, P4C11 against a 47 kDa non-integrin membrane glycoprotein and P1C12 against the CD44 antigen were previously described (Wayner and Carter, 1987). Rat mAb P4G11 against integrin β4 was prepared by Tai Mei Yang in the Carter lab. Rat mAb GoH3 against integrin α6 was from BD Biosciences Pharmingen, San Diego, CA. Purified rabbit polyclonal antibody (pAb) against ATF3 (sc-188) was from Santa Cruz Biotechnology, Santa Cruz, CA. Purified rabbit pAbs against p38 map kinase (cat. no. 9212), phosphorylated p38 map kinase (Thr180, Tyr182; cat. no. 9211), activating transcription factor 2 (ATF2; cat. no. 9222) and phosphorylated ATF2 (Thr71; cat. no. 9221) were from Cell Signaling Technology, Beverly, MA. Purified rabbit pAb against focal adhesion kinase phosphorylated at Tyr397 and FITC or rhodamine-conjugated goat anti-mouse IgG were from Biosource International, Camarillo, CA. Peroxidase-conjugated goat affinity-purified antibody to rabbit IgG was from Cappel Pharmaceuticals, Aurora, OH. The membrane permeable jun N-terminal kinase peptide inhibitor 1, L stereoisomer (L-JNKI1) (Bonny et al., 2001) was obtained from Alexis Biochemicals (San Diego, CA) or Calbiochem (La Jolla, CA). The p38 inhibitors SB203580 and SB202190 were from Calbiochem.

Wound activation of keratinocytes in human skin in vivo

Simplate II bleeding-time devices (Oranon Teknika) were used to create uniform incisional wounds (5 mm long and 1 mm deep) on the forearm of a normal volunteer as previously described (Olerud et al., 1995). Wounds were harvested as 4 mm punch biopsies at the indicated times after wounding, using local 1% lidocaine for anesthesia; immediately frozen in OCT (optimal cutting temperature compound) and stored at –70°C until use. The recruitment of volunteers and the method used for collection of biopsies were approved by the University of Washington Institutional Review Board. Cryostat sections (6 μm) were cut, mounted on glass slides, fixed (2% v/v formaldehyde for 10 minutes), permeabilized (0.5% v/v Triton X-100 in PBS for 10 minutes) and reacted with the indicated primary antibodies.

Wound activation of keratinocytes in mouse skin in vivo

Procedures for skin wounds in mice were approved by the Fred Hutchinson Cancer Research Center Animal Care Committee. Mice (C57BL/Ks) were anesthetized with isofluorane and shaved. Full thickness punch biopsies were created on the dorsal surface of the mice. Mice were euthanized with intraperitoneal injections of sodium pentobarbital after wounding. Punch biopsies were taken which contained both wounded epidermis and surrounding unwounded epidermis at 0, 4, 8 and 18 hours post wounding. Wounds were harvested, embedded in OCT and sectioned at 6 μm on a cryostat.

Human keratinocyte scrape wounds in vitro

HKs were grown to confluence and the monolayer was mechanically wounded using a pipette tip. Single wounds were generated in cells plated onto coverslips for immunofluorescence analysis. Eighteen wounds of equal area were generated on 10 cm plates using a comb-like device, for examination of protein expression through immunoblotting.

Suspension and re-adhesion on immobilized laminin 5 or collagen

Confluent cultures of HKs were suspended by digestion with trypsin-EDTA, washed with PBS containing soybean trypsin inhibitor and re-adhered to surfaces coated with laminin 5 or collagen (see preparation of laminin 5 below). This approach generated populations of activated LKs sufficiently large for mRNA and protein expression studies through DNA microarrays and immunoblotting.

Laminin 5-coated surfaces were prepared as previously described (Gil et al., 2002; Xia et al., 1996). Collagen surfaces were prepared by adsorbing human placental type I collagen (Wayner and Carter, 1987) at 10 μg/ml for 2 hours at 24°C.

BSA adhesion assay

The BSA adhesion assay couples deposition of laminin 5 to subsequent adhesion to the deposits and was performed as previously described (Frank, 2004; Gil et al., 2002). MKs from laminin 5 null mice ($\alpha3^{-/-}$ MKs) and from a wild-type mouse ($\alpha3^{+/+}$ MKs) were grown to confluence, suspended and re-plated onto petri dishes coated with HD-BSA, a non-adhesive surface, or onto laminin 5 surfaces. Triton and SDS protein extracts were collected at 2 hour intervals from 0 to 10 hours after re-plating. The quiescent cell populations established baseline levels of protein expression and p38 phosphorylation. Extracts were examined by immunoblotting with anti-p38 map kinase and anti-phosphorylated and p38 map kinase antibodies.

Immunofluorescence microscopy

Scrape wounds in vitro were fixed with 2% formaldehyde, 0.1 M cacodylate and 0.1 M sucrose and tissue sections were fixed with 2% formaldehyde in PBS for 15 minutes. Coverslips and tissue sections were permeabilized with 0.5% Triton X-100 detergent in PBS and blocked with 0.5% HD-BSA for 30 minutes. Wounds were stained with the indicated antibodies. Samples were washed with PBS and incubated with affinity-purified FITC or Rhodamine-conjugated species-specific secondary antibodies. Coverslips were mounted with Prolong (Molecular Probes, Eugene, OR) and analyzed with a Zeiss fluorescent microscope.

Immunoblotting

Keratinocyte populations were sequentially extracted with 1% Triton X-100 in PBS for 10 minutes, followed by extraction with 1% SDS in PBS. Both lysis buffers contained 1 mM PMSF and 2 mM *N*-ethyl maleimide, 1 mM sodium fluoride and 1 mM sodium orthovanadate in PBS. Triton-soluble and Triton-insoluble protein fractions were separated using 12% SDS-PAGE gels (Laemmli, 1970), transferred to nitrocellulose membranes and immunoblotted with the indicated antibodies. Blots were developed using the Enhanced Chemiluminescence kit (Amersham).

Assay of p38 kinase activity

P38 MAP kinase was assayed as recommended (Cell Signaling Technology) as follows: immobilized mAb against P-p38 (Thr180/Tyr182) was used to immunoprecipitate P-p38 from cell extracts as indicated in Fig. 7B. An in vitro kinase assay was performed using ATF-2 as substrate. P-ATF-2 product was detected by western blotting using P-ATF-2 (Thr71) antibody.

DNA microarray analysis of mRNA from wounded and quiescent keratinocytes

Total RNA was isolated from the quiescent and activated cell populations using a Totally RNA kit (Ambion). Generation of Cy fluor-labeled cDNA was according to a published method (Fazzio et al., 2001) and was co-hybridized to spotted human cDNA microarray chips. cDNA microarray chips were produced by the FHCRC microarray facility (Seattle, WA) under direction of Jeff Delrow. cDNA chips were produced by PCR amplification of each cDNA clone in a human cDNA library (18,000 genes) representing various tissues and cell types (Research Genetics). A fluorescent image was generated using a GenePix 4000 fluorescent scanner (Axon Instruments). The image was analyzed using GenePix Pro microarray acquisition and analysis software. Five separate analyses were performed and data was statistically analyzed using the Student's paired *t*-test.

Semi-quantitative reverse-transcription PCR analyses

Adherent quiescent confluent HKs were suspended with trypsin-EDTA and either re-adhered to laminin 5 or kept in suspension over agarose to prevent adhesion for 2, 9, 24 and 48 hours. RNA was extracted from the cells with the RNeasy Midi Kit (Qiagen). 2 μg of each RNA sample was then reverse-transcribed using the SuperScript™ First-Strand Synthesis System (Invitrogen). To amplify ATF3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in semi-quantitative PCR, the following primers were used (Syed et al., 2005): ATF3, 5′-CTCCTGGGTCACTGGTGTTT-3′ (forward) and 5′-GTCGCCTCTTTTTCCTTTCA-3′ (reverse); GAPDH, 5′-CAT-CACCATCTTCCAGGAGC-3′ (forward) and 5′-GGATGATGTT-CTGGAGAGCC-3′ (reverse). 2 μl aliquots of cDNA were amplified by PCR as described (Syed et al., 2005) except that annealing temperature was 55°C for both ATF3 and GAPDH primers and the number of amplification cycles was 23 for both ATF3 and GAPDH. The PCR products were fractionated on a 2% agarose gel and visualized after ethidium bromide staining. The results were obtained using two independent cDNA syntheses from each RNA sample.

Results

Comparison of quiescent keratinocytes to wound-activated LKs by DNA microarray

Microarrays of cDNA were used to compare mRNA transcript levels of quiescent adherent HKs to wound-activated LKs. A diagram of the manipulations used to activate the quiescent keratinocytes is presented in Fig. 1A. Quiescent basal keratinocytes in epidermis were modeled with confluent cultures of primary HKs. Early transcriptional changes in wound-activated LKs were modeled by suspending the quiescent HKs and then re-adhering cells onto laminin 5 surfaces. HKs were re-adhered for 1.5 hours prior to purifying total RNA. Using cDNA microarrays, mRNA transcripts in the quiescent HKs were compared to transcripts in HKs re-adherent and spread on laminin 5 (Fig. 1B). Prior studies have shown that re-adhesion to laminin 5 is mediated by integrin $\alpha6\beta4$ and $\alpha3\beta1$ whereas spreading and motility are mediated by $\alpha3\beta1$ (Frank, 2004; Xia et al., 1996). The predictive capability of the wound model and DNA microarray were validated by examining transcripts known to be elevated in wounds in vivo (Fig. 1B). The array correctly reported mRNA upregulation of urokinase plasminogen activator (Morioka et al., 1987), α3 chain of laminin 5 (Lampe et al., 1998; Ryan et al., 1994), CD9 (Penas et al., 2000) and ezrin (Crepaldi et al., 1997), each involved in epithelial cell motility. The arrays correctly predicted a downregulation of mRNAs for desmoglien and plakoglobin (Okada et al., 2001), corneodesmosin (Haftek et al., 1997) and serine protease inhibitor (Scott et al., 1998), characteristic of quiescent or differentiated keratinocytes. We concluded that the in vitro wound model coupled with cDNA microarray comparisons were capable of accurately reporting physiologically significant changes in equilibrium levels of mRNA transcripts occurring in epidermal wounds.

In five separate experiments, the arrays consistently reported fivefold increases ($P<0.05$) or higher in levels of four mRNA transcripts in re-adherent HKs compared to levels in quiescent HKs. These transcripts included activating transcription factor 3 (ATF3), tumor necrosis factor α-induced protein 3 (TNFαIP3), growth-related oncogene-1 (Gro-1), and urokinase plasminogen activator. Reproducible increases in mRNA expression were also detected for syndecan 4, a transmembrane glycoprotein reported to interact with the G-4 domain of

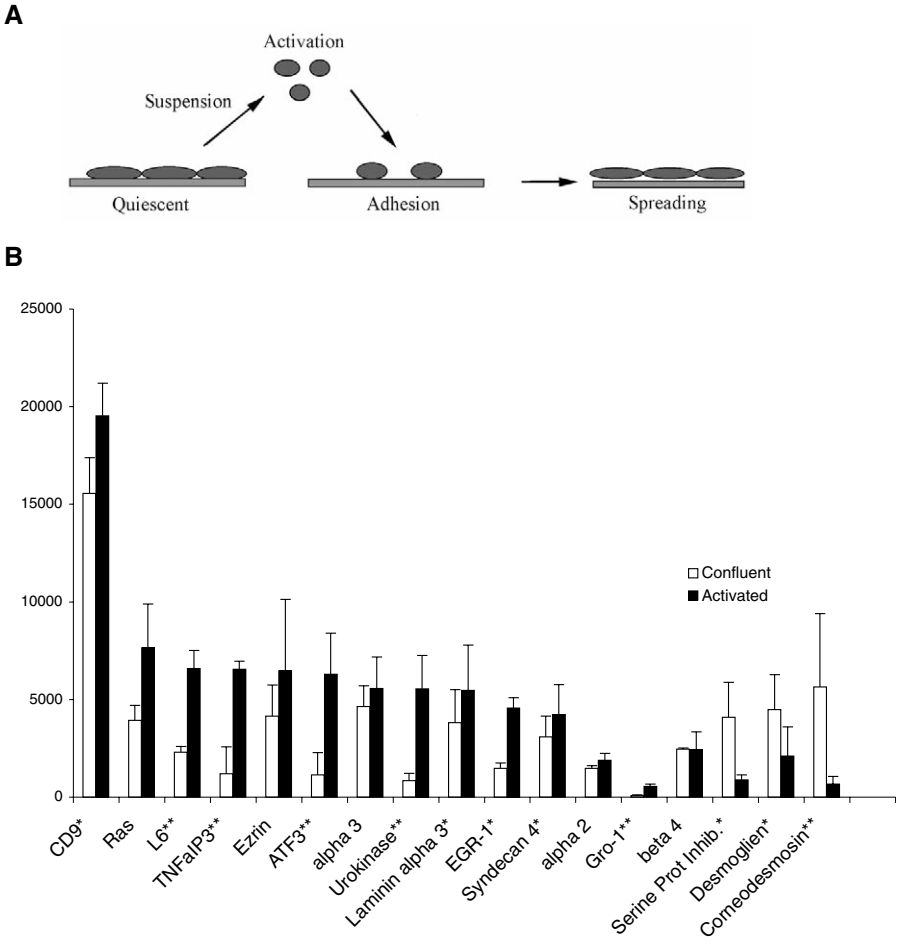

Fig. 1. (A) Suspension and re-adhesion of quiescent HKs activates leading keratinocytes for a wound model. HKs were grown at confluence to generate quiescence. Suspension with trypsin/EDTA activated the keratinocytes. Re-adhesion onto surfaces coated with laminin 5 was mediated by integrins α6β4 and α3β1. Re-adhesion was followed by cell spreading via integrin α3β1. Spreading, but not adhesion, was blocked with Cytochalasin D. (B) cDNA microarray analysis of quiescent and suspended/re-adherent HKs. Levels of mRNA transcripts in quiescent and suspended/re-adherent HKs were compared by cDNA microarray analysis 1.5 hours post suspension/re-adhesion with spreading. Solid bars represent transcript levels of activated suspended/re-adherent HKs, whereas open bars represent transcript levels in quiescent cells. Transcript levels are reported as mean (±s.e.m.) fluorescent units (*y*-axis). Significant differences $^{**}P<0.05$ and $^{*}P<0.10$ in fluorescence levels were found between the groups indicated and the control. Microarray analysis was performed in five separate suspension/re-adhesion experiments.

laminin 5 (Utani et al., 2003; Utani et al., 2001); EGR-1, a transcription factor encoded by an immediate early response gene (Sukhatme et al., 1988); Ras, a GTP binding protein and oncogene (Charvat et al., 1999); L6, a member of the transmembrane 4 super family and a tumor antigen (Marken et al., 1992; our unpublished work); SOX9, a developmental transcription factor (Smith and Koopman, 2004) and PMAIP1, a PMA-induced protein 1. Reproducible but small increases in transcripts for integrins α3, α2 but not β4, were observed within 1.5 hours of wounding. In controls (results not shown), suspension and re-adhesion for 3 days further elevated transcripts for laminin α3 chain, and β1 integrins. Here, we evaluate cell signals and adhesion events that regulate early expression of ATF3 in wounds.

ATF3 protein is transiently expressed in LKs of wounds in vivo and in vitro

ATF3 is a member of the ATF/Creb family of transcription factors that is upregulated in response to injury or cell stress (Hai and Hartman, 2001; Hai et al., 1999). Expression of ATF3 protein was examined in LKs by immunofluorescence microscopy in timed scrape wounds in confluent quiescent HKs (Fig. 2A). Cells were fixed and permeabilized 0, 3, 6 and 12 hours post wounding. LKs at the wound margins, but not following cells, upregulated and translocated ATF3 protein to the nucleus within 3 hours of wounding (Fig. 2Ab), followed by a decrease within 6 hours (Fig. 2Ac) and a return to baseline levels within 12 hours of wounding (Fig. 2Ad). Treatment of scrape wounds with actinomycin D, an inhibitor of mRNA transcription, or cycloheximide, an inhibitor of protein translation, prevented ATF3 protein expression by LKs, confirming regulation at the transcriptional level. LKs expressing ATF3, but not following cells, spread over the exposed wound edge and localized paxillin in prominent focal adhesions (Fig. 2B). Thus changes in cell adhesion and spreading are concurrent with changes in transcription and translation of ATF3 in LKs. In principle, signals that regulate ATF3 may also regulate adhesion. Results in Figs 1 and 2 establish that wound stress induced by scraping or by detachment with trypsin elevates levels of ATF3 mRNA and protein in LKs.

Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was used to confirm the elevation of ATF3 mRNA during suspension (Fig. 3A). Quiescent adherent HKs expressed low levels of ATF3 mRNA that increased by 2 hours after suspension confirming the results in Fig. 1B. Levels of ATF3 mRNA returned to baseline within 8 hours after suspension with or without re-adhesion.

ATF3 protein in quiescent confluent HKs was also compared to levels in suspended HKs and suspended/re-adherent HKs (Fig. 3B). ATF3 protein was not detected in quiescent adherent HKs. In suspended HKs, ATF3 was maximal by 2 hours after suspension and remained elevated for 6 hours (Fig. 3A) and

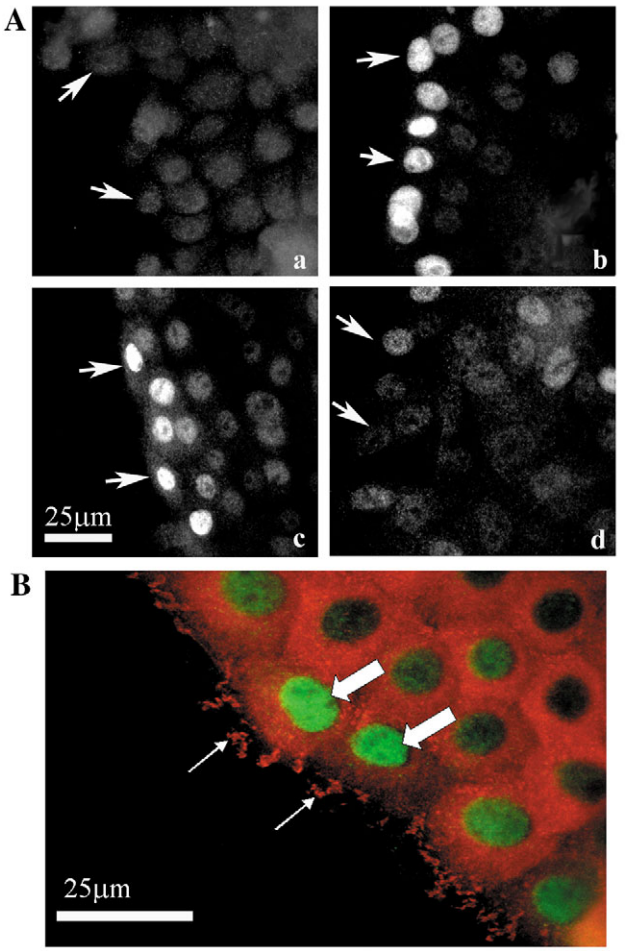

Fig. 2. (A) ATF3 is upregulated in LKs of in vitro scrape wounds. Monolayer cultures of quiescent HKs were fixed at (a) 0, (b) 3, (c) 6 and (d) 12 hours after scrape wounding. ATF3 protein was undetectable in quiescent unwounded cells at 0 hour. ATF3 protein expression levels are elevated and the protein is localized to the nucleus of LKs within 3 to 6 hours and returned to baseline levels within 12 hours. Following keratinocytes distant from the wound margin did not upregulate ATF3 protein expression. White arrows identify LKs. (B) ATF3 is upregulated in LKs that assemble focal adhesions at the wound edge. After wounding (2 hours), ATF3 was selectively expressed in nuclei (large white arrows) of LKs at the wound margin but not following cells. The LKs expressing ATF3 also assembled focal adhesions detected with anti-paxillin antibody (small white arrows). Bar, 25 μm.

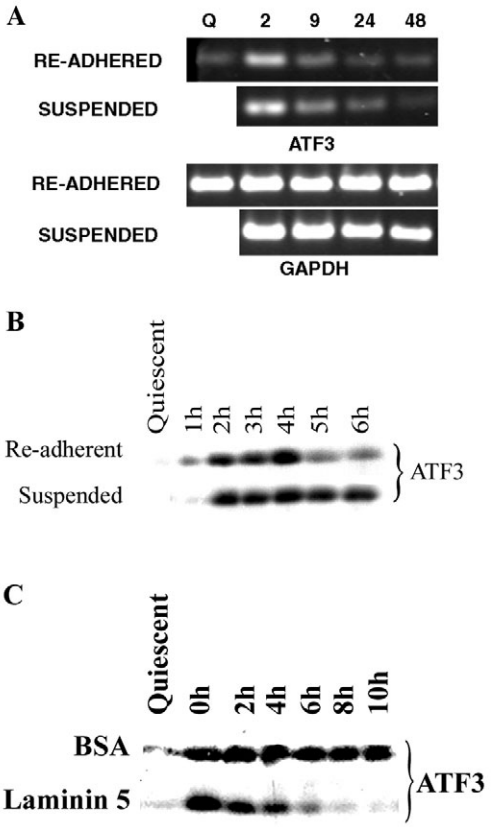

Fig. 3. (A) Levels of ATF3 mRNA increase on suspension but decline with or without re-adhesion. Semi-quantitative reverse-transcription PCR was used to evaluate levels of ATF3 mRNA in adherent quiescent HKs (Q), Quiescent HKs were suspended with trypsin EDTA or re-adhered to laminin 5 for the indicated times (2, 9, 24 and 48 hours). After 2 hours of suspension or re-adhesion, levels of ATF3 mRNA increased but subsequently declined over time. In controls, levels of GAPDH remained constant. (B) ATF3 protein is upregulated in suspended HKs but declines upon re-adhesion. Confluent quiescent HKs were suspended with trypsin-EDTA and re-adhered onto laminin 5 or held in suspension. Extracts were collected from the quiescent parent population (labeled Quiescent) and from suspended and re-adherent HKs 1-6 hours post suspension. Extracts were immunoblotted with an anti-ATF3 antibody. ATF3 protein was not detectable in quiescent cells, but was elevated in suspended cells throughout the assay (hours 1-6). Levels of ATF3 protein in adherent cells were maximal at 4 hours post activation, after which they declined. (C) Re-adhesion of MKs on laminin 5 suppresses ATF3 protein expression. Laminin 5 null MKs were grown to confluence, suspended and re-plated onto a non-adhesive BSA coated surface or onto a laminin 5-coated surface. Extracts were collected from the quiescent cell population, and from cells plated onto BSA or Laminin 5 at 2 hour intervals from 0-10 hours. Levels of ATF3 in the extracts were examined by immunoblotting. MKs plated onto BSA failed to adhere, upregulated ATF3 protein expression and maintained elevated ATF3 expression for the duration of the assay. MKs plated onto laminin 5 adhered, upregulated ATF3 initially but then suppressed ATF3 expression to baseline by 6 hours. The time course for the downregulation of ATF3 protein was confirmed in at least seven different blot experiments.

beyond 12 hours (results not shown). In re-adherent cells, ATF3 protein was maximal by 4 hours after re-adhesion but declined significantly after 4 hours. The failure of ATF3 protein to decline in suspended HKs, raised the possibility that suspension elevates and re-adhesion suppresses ATF3 protein expression.

The role of adhesion in downregulating levels of ATF3 protein was confirmed (Fig. 3C). To evaluate the role of adhesion in regulating ATF3 protein, we employed keratinocytes from mice with null defects in the α3 chain of laminin 5 (Sigle et al., 2004) (Fig. 3C). It was necessary to use laminin 5 null keratinocytes because laminin 5 deposited onto surfaces of exogenous ligands contributes to adhesion and signaling (Frank, 2004; Nguyen et al., 2000b). ATF3 protein expression was examined in MKs plated onto either a non-adhesive BSA-coated surface or onto exogenous laminin 5.

Suspended $\alpha3^{-/-}$ MK upregulated ATF3 protein, but failed to deposit laminin 5 and therefore did not adhere to BSA-coated surfaces during the assay (0-10 hour) (Sigle et al., 2004). In contrast, $\alpha3^{-/-}$ MK plated onto an exogenous laminin 5 surface adhered and downregulated ATF3 (Fig. 3C). Similar results were also obtained using HKs with null defects in laminin 5 (Results not shown). This confirmed that adhesion to laminin 5, and probably other adhesive surfaces, suppressed ATF3 protein expression elevated by suspension.

To ensure that the elevation in ATF3 was physiologically relevant, incision wounds in mouse skin were examined for ATF3 expression at 0, 4 and 18 hours post injury. ATF3 protein was undetectable in LKs at the wound edge in the zero time wound (Fig. 4a). Within 4 hours of the wounding, ATF3 was elevated and translocated to the nucleus in LKs (Fig. 4b) whereas expression in adjacent quiescent following cells remained undetectable. ATF3 returned to the baseline level of quiescent cells by 18 hours (Fig. 4c). A composite image (Fig. 4d) shows expression of ATF3 in LKs at the wound margin 4 hours after injury, but absent from the quiescent cells distant to the injury.

In summary, wounding in vivo or in vitro transiently upregulates ATF3 protein and mRNA in LKs, but ATF3 remained at baseline levels in adjacent following or quiescent keratinocytes. Significantly, suspension activates ATF3 expression at both the mRNA and protein levels whereas re-adhesion suppresses the duration of ATF3 expression particularly at the protein level. This suggests that adhesion in general, or adhesion to laminin 5, may limit ATF3 expression to LKs. The role of adhesion in downregulating expression of ATF3 protein will be evaluated elsewhere. Here, we determine what upstream cell signals activate expression of ATF3 mRNA and protein.

Wound activation of p38 MAPK phosphorylates downstream ATF2 to elevate ATF3

We sought to identify cell signals activated by scrape wounding or suspension of HKs that elevate ATF3 in LKs and that may be suppressed by re-adhesion to laminin 5. Interestingly, p38 mitogen-activated kinase (MAPK) has previously been reported to transcriptionally and/or post-transcriptionally regulate at least seven out of the 50 mRNA transcripts elevated at least 1.5 times or higher in LKs in our wound screens (Fig. 2, ATF3, TNFαIP3, uPA, GRO1, IL1β, PMAIP1 and SOX9) (Frevel et al., 2003). Thus, p38 is a major contributor to increases in mRNA levels that define LKs and is also a possible upstream regulator of ATF3. Phosphorylation of activation transcription factor 2 (ATF2) by JNK (Cai et al., 2000; Yin et al., 1997; Zhang et al., 2001) and/or by p38 MAPK (Fan et al., 2002) is reported to upregulate ATF3 expression in cell lines. We determined if activation of JNK or p38 MAPK was necessary for phosphorylation of ATF2 and elevation of ATF3 in LKs of scrape wounds. Confluent cultures of HKs were pretreated with or without specific inhibitors of p38 kinase activity, SB203580 or SB202190 (50 μm each, 30 minutes prior to wounding) or membrane-permeable forms of the JNK peptide inhibitor 1, L stereoisomer (50 μm each, 30 minutes prior to wounding) (Bonny et al., 2001). Following scrape wounding, HKs were incubated in the presence or absence of inhibitors for 2.5 hours to allow for upregulation of phosphorylated ATF2 (P-ATF2) and ATF3 by LKs. LKs in scrape wounds pretreated with either SB203580 (Fig. 5Ab) or SB202190 (not shown) failed to upregulated P-ATF2 or ATF3 (Fig. 5Ad). In contrast, inhibitors of JNK failed to suppress expression of ATF3 (Fig. 5Bd) under conditions where the inhibitors did prevent JNK-mediated phosphorylation of Jun (Fig. 5Bb).

Levels of P-p38 were transiently increased in LKs at the edge of in vivo wounds 1 hour after injury but declined within 4 hours of injury (Fig. 6B). Activation of p38 requires phosphorylation of Thr180 and Tyr182 by dual specificity mitogen-activated kinase kinase 3/6 or 4 (Ono and Han, 2000). The anti-P-p38 antibody was specific for p38 phosphorylated on both Thr180 and Tyr182. Both P-p38 and P-ATF2 were selectively increased in LKs of in vitro wounds (Fig. 6A). The role of p38 was confirmed by immunoblotting of scrape wounds (Fig. 7A). Confluent plates of HKs were wounded with

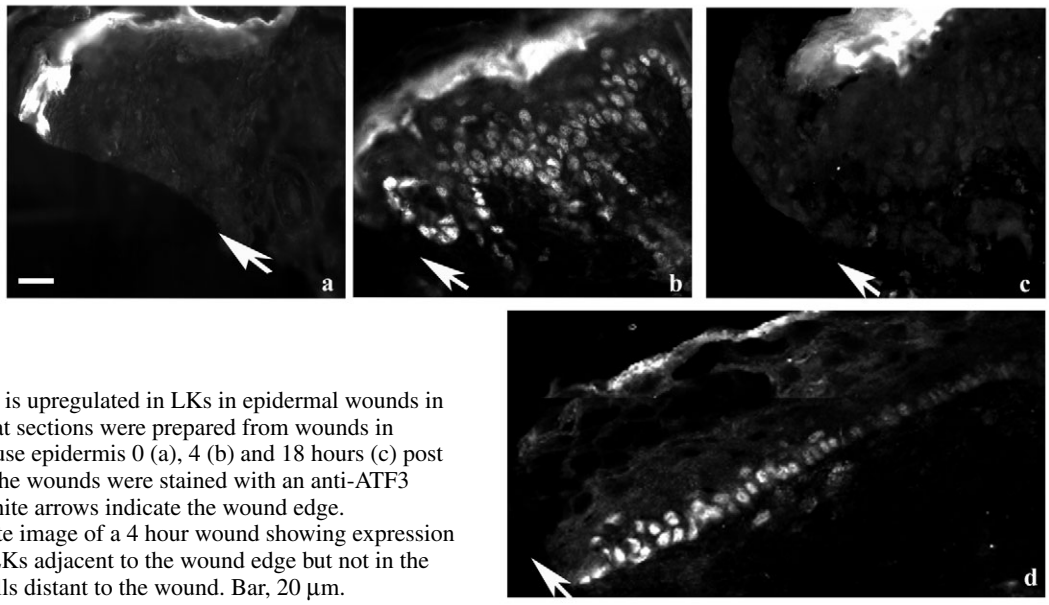

Fig. 4. ATF3 is upregulated in LKs in epidermal wounds in vivo. Cryostat sections were prepared from wounds in neonatal mouse epidermis 0 (a), 4 (b) and 18 hours (c) post wounding. The wounds were stained with an anti-ATF3 antibody. White arrows indicate the wound edge. (d) Composite image of a 4 hour wound showing expression of ATF3 in LKs adjacent to the wound edge but not in the following cells distant to the wound. Bar, 20 μm.

a comb-like device, removing equal numbers of adherent cells (50%), to generate adherent activated LKs at the wound edge. Triton-soluble extracts were prepared from the quiescent HKs prior to wounding and at 0, 0.5, 1, 2 and 3 hours post wounding. The extracts were immunoblotted with anti-phospho-p38 (P-p38), and anti-p38 antibodies (Fig. 7A). P-p38 was elevated above levels in the quiescent cells 0.5 hours post injury and was still elevated after 3 hours. Levels of total p38 protein remained constant, indicating that neither protein synthesis nor catabolism accounted for the changes in P-p38. Phosphorylated ATF2 (P-ATF2) was not detected in adherent quiescent HKs (Fig. 7A). However, levels of P-ATF2 rapidly elevated in suspended cells suggesting an increase in p38 kinase activity. Consistently, assay of p38 kinase activity utilizing ATF2 as a substrate failed to detect p38 kinase activity in quiescent adherent HKs (Fig. 7B). Suspension of the HKs elevated p38 kinase activity (Fig. 7B). Scrape wounding of the adherent HKs also increased p38 kinase activity (Fig. 7B). In summary, p38 is phosphorylated and p38 kinase activity is activated by scrape wounding or suspension. Inhibition of P-p38 is sufficient to block phosphorylation of ATF2 and elevation of ATF3.

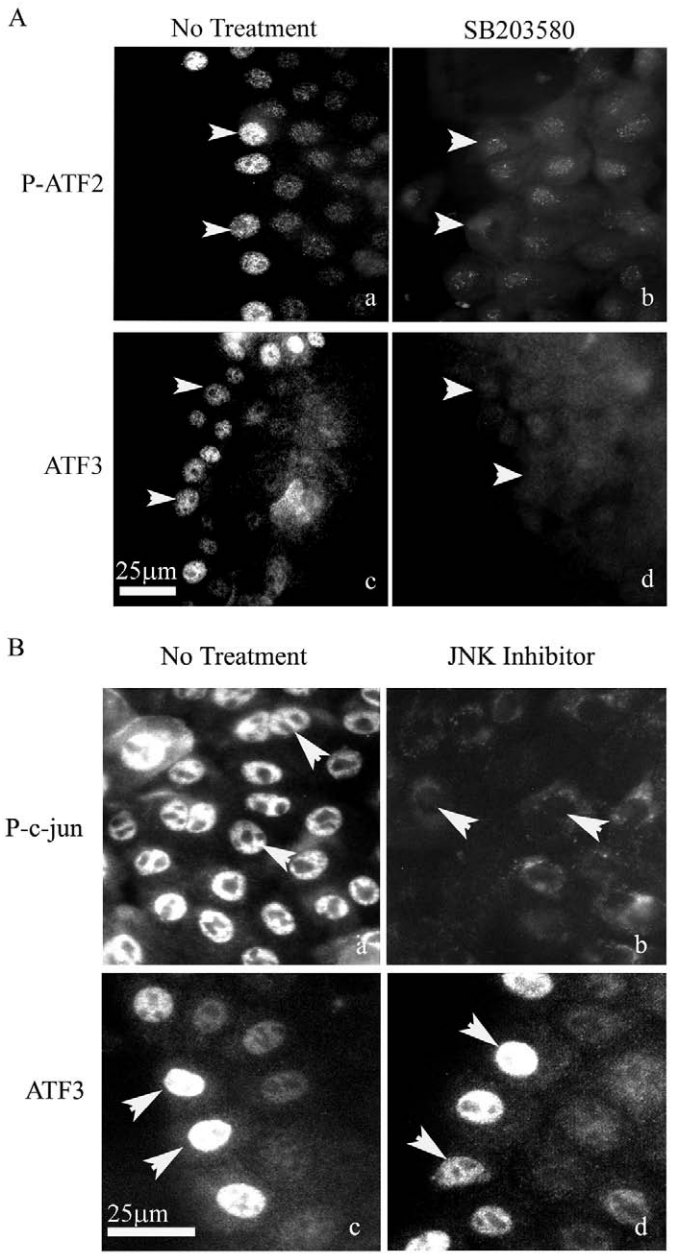

Fig. 5. (A) Inhibition of P-p38 with SB203580 prevents phosphorylation of ATF2 and up-regulation of ATF3. Confluent HK monolayers were either untreated to serve as a control, or pretreated with SB203580 for 1 hour. Scrape wounds were generated and allowed to incubate for 2.5 hours in the presence or absence of inhibitor. ATF2 phosphorylation (P-ATF-2; a,b) and ATF3 (c,d) protein expression levels were examined by immunofluorescent staining. SB203580 blocked expression of P-ATF-2 and ATF-3. (B) Pretreatment of scrape wounds with the membrane-permeable form of the JNK peptide inhibitor (JNK Peptide Inhibitor 1, L stereoisomer; 50 μM, 30 minutes prior to wounding) (Bonny et al., 2001) prevents phosphorylation of jun but not expression of ATF3. Bar, 25 μm.

Re-adhesion to laminin 5 suppresses P-p38 whereas re-adhesion to collagen maintains P-p38

Here, we used three approaches to evaluate the adhesion requirements for dephosphorylation of P-p38. In the first approach, HKs were suspended and then incubated with immobilized antibodies against integrin subunits α3, α2, α6, β1 or against non-integrin receptors (CD44, P4C11 antigen, a 45 kDa cell surface protein recognized by mAb P4C11) (Fig. 8A). We used laminin 5 null HKs for these studies to avoid deposits of laminin 5 that would complicate the adhesion signals. The adherent cells were spread prior to suspension and phosphorylated focal adhesion kinase on Tyr397 (P-FAK; Fig. 8A, Adherent). Suspension of the adherent HKs (Suspended) phosphorylated p38 and dephosphorylated P-FAK. Incubation of the suspended HKs on non-adhesive control antibody SP2 did not induce adhesion or spreading and did not dephosphorylate P-p38. However, re-adhesion of suspended HKs on adhesive laminin 5 or collagen or immobilized adhesive mAbs dephosphorylated P-p38. Therefore, adhesion-dependent dephosphorylation of P-p38 did not require integrins, cell spreading or phosphorylation of FAK. In further controls (Results not shown) addition of the soluble monoclonal antibodies to suspended HKs failed to dephosphorylate P-p38. We conclude that in vitro cell adhesion, not cell spreading, is required for dephosphorylation of P-p38. This suggests that the primary mediator of cell adhesion in vivo, laminin 5 and α6β4, are sufficient to dephosphorylate P-p38.

In the second approach, we tried to distinguish between laminin 5 and collagen in their roles in regulating dephosphorylation of P-p38. Cytochalasin D, an inhibitor of the actin cytoskeleton, blocks cell spreading on laminin 5 but not adhesion: both integrins α6β4 (Xia et al., 1996) and α3β1 (Frank, 2004) adhere HKs on laminin 5 in the presence of cytochalasin D and dephosphorylate P-p38 (Fig. 8B). In contrast, cytochalasin D blocks adhesion, spreading and dephosphorylation of P-p38 on collagen and fibronectin (Fig. 8B) (Xia et al., 1996). Thus, dephosphorylation of P-p38 required re-adhesion but re-adhesion on laminin 5 is independent of the actin cytoskeleton whereas re-adhesion on collagen requires the actin cytoskeleton. This suggests that

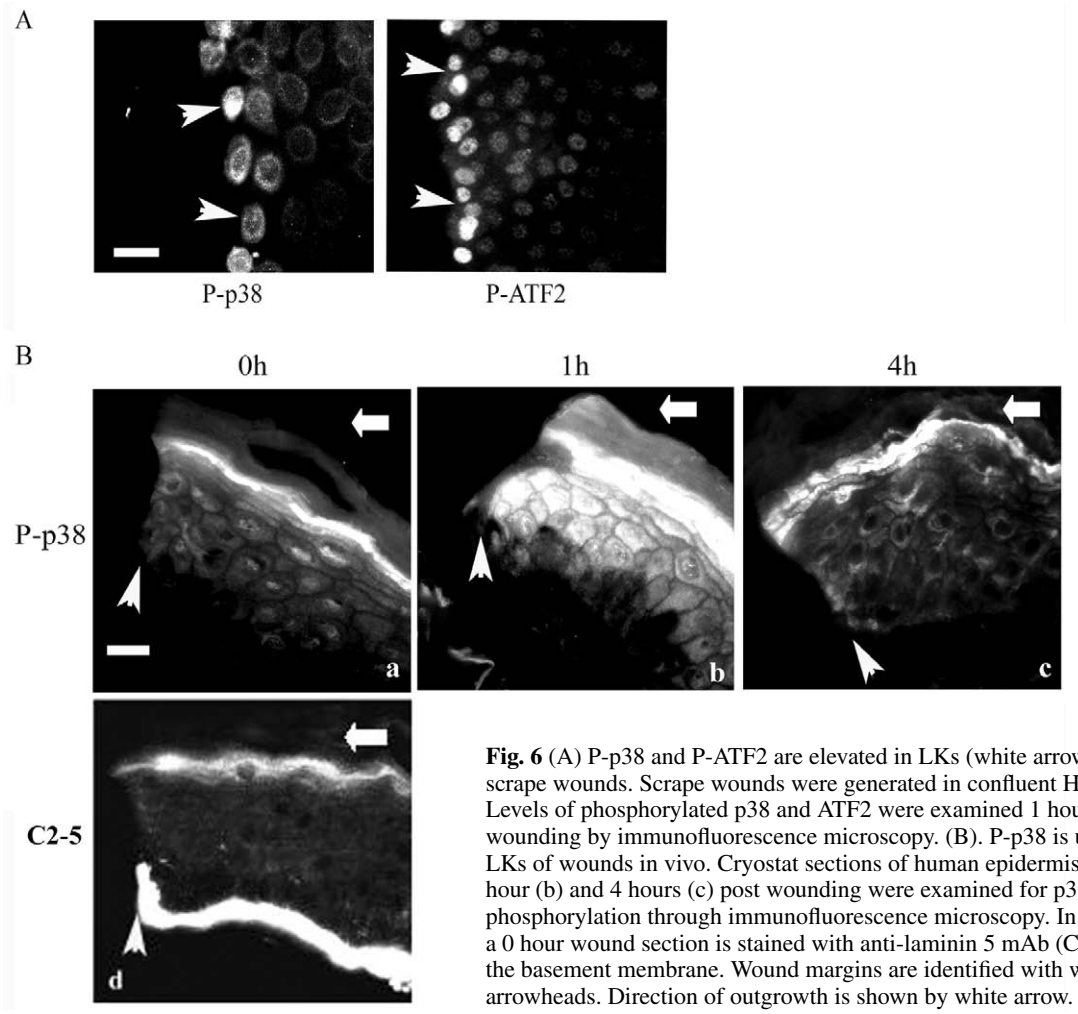

Fig. 6 (A) P-p38 and P-ATF2 are elevated in LKs (white arrowheads) of scrape wounds. Scrape wounds were generated in confluent HK monolayers. Levels of phosphorylated p38 and ATF2 were examined 1 hour post wounding by immunofluorescence microscopy. (B). P-p38 is upregulated in LKs of wounds in vivo. Cryostat sections of human epidermis 0 hours (a), 1 hour (b) and 4 hours (c) post wounding were examined for p38 phosphorylation through immunofluorescence microscopy. In the control (d), a 0 hour wound section is stained with anti-laminin 5 mAb (C2-5) to identify the basement membrane. Wound margins are identified with white arrowheads. Direction of outgrowth is shown by white arrow. Bar, 20 μm.

adhesion to laminin 5 could dephosphorylate P-p38 under conditions when activation integrins like α2β1 and α5β1 do not contribute to adhesion or spreading, as in quiescence.

In the third approach, we compared re-adhesion to laminin 5 with re-adhesion to collagen in dephosphorylation of P-p38. Published reports have established that suspension and re-adhesion of cells on collagen activates phosphorylation of p38 that regulates cell motility and transcription (Clark et al., 2003; Ono and Han, 2000). We emphasize that these published studies required suspension of the cells prior to re-adhesion. However, studies here, and elsewhere (Frisch et al., 1996; Khwaja and Downward, 1997), indicate that suspension of HKs or scrape wounding is sufficient to phosphorylate p38 whereas re-adhesion dephosphorylates P-p38 relative to cells in suspension. As a possible explanation for the difference in results, we determined if re-adhesion to laminin 5 and collagen differ in dephosphorylating P-p38.

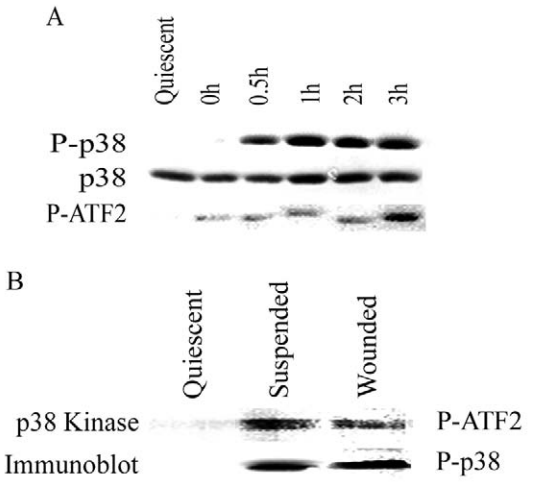

Fig. 7. (A) Scrape wounding increases phosphorylation of p38 and ATF2. HKs were grown to confluence and multiple scrape wounds were generated in the monolayer. Triton-soluble extracts were collected in non-wound HKs (Quiescent), or in wounded HKs at the time of wounding, (0 hour), and 30 minutes, 1 hour, 2 hours and 3 hours post wounding. Levels of p38 remained constant but P-p38 increased by 0.5 hours after wounding and P-ATF2 increased immediately after wounding. (B) p38 kinase activity is elevated in suspended or wounded HKs. Confluent quiescent HKs (Quiescent) were assayed for p38 kinase after suspension for 3 hours (Suspended) or scrape wounding for 6 hours (Wounded). In vitro assay of p38 kinase activity was performed as described (see Materials and Methods) using ATF2 as substrate and immunoblotting for P-ATF2. Aliquots of the cell extracts were also immunoblotted (Immunoblot) for P-p38 to confirm that levels of P-p38 correlated with p38 kinase activity.

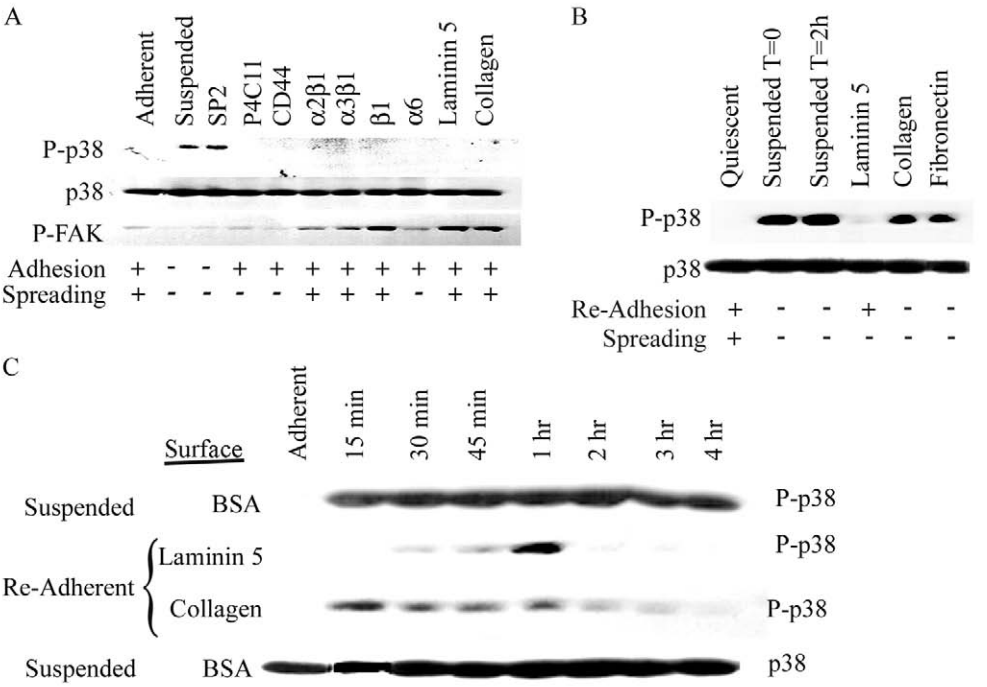

Fig. 8. (A) Adhesion, not cell spreading, dephosphorylates P-p38. Human keratinocytes null for laminin 5 were suspended and re-adhered to the indicated ligands (laminin 5, collagen) or immobilized mAbs: SP2, an irrelevant non-adhesive mAb; P4C11, a mAb against a 47 kDa non-integrin surface glycoprotein; CD44, a non-integrin cell surface antigen; mAbs against the indicated integrin subunits α2, α3,α6, β1. Cell adhesion and cell spreading induced by each ligand is indicated. Triton-soluble extracts were prepared from the adherent cells after washing then fractionated by SDS-PAGE and immunoblotted as indicated with antibodies against P-p38, p38 and focal adhesion kinase phosphorylated on Tyr397 (P-FAK). (B) Cytochalasin D inhibits adhesion and dephosphorylation of P-p38 on collagen and fibronectin but not laminin 5. Quiescent laminin 5 null HKs were suspended, treated with cytochalasin D (10 μM), then either left in suspension for 2 hours or incubated on laminin 5, collagen or fibronectin surfaces for 2 hours. Adhesion and spreading of the cells is indicated. Extracts of the indicated cells were blotted with anti-P-p38 and anti-p38 Abs. (C) Re-adhesion of suspended HKs on laminin 5 dephosphorylates P-p38 followed by transient re-phosphorylation. Confluent HKs (with null defects in laminin 5) were suspended using trypsin and either re-adhered to laminin 5 or collagen or suspended over BSA. Triton-soluble extracts were collected from the quiescent adherent HKs, suspended HKs and suspended/re-adherent cells at the indicated intervals (15 minutes, 30 minutes, etc). Expression of P-p38 was examined by immunoblotting. Blots for cells re-adhered on laminin 5 were re-probed with anti-p38 to control for levels of total p38 and are representative of all the blots.

The adherent quiescent HKs did not express detectable P-p38 by immunoblotting (Fig. 8C, Adherent). Upon suspension, P-p38 increased dramatically and stayed phosphorylated in suspension when incubated on non-adhesive surfaces coated with BSA (Fig. 8C, BSA, 15 minutes to 4 hours). However, re-adhesion of the suspended HKs on laminin 5 or collagen surfaces decreased phosphorylation within 15 minutes (Fig. 8C, laminin 5 or collagen). Surprisingly, the initial dephosphorylation of P-p38 was followed by a transient increase in phosphorylation that was maximal by 1 hour on laminin 5 but decreased over the next 4 hours. In contrast, initial adhesion (15 minutes) on collagen, decreased P-p38 but not to the basal level observed on laminin 5. Furthermore, the levels of P-p38 on collagen remained above baseline throughout the assay (4 hours). This suggests that adhesion to laminin 5 can be distinguished from adhesion to collagen based on dephosphorylation of P-p38.

ATF3 is chronically elevated in laminin 5 and β4 null epidermis

We determined if adhesion defects in laminin 5 or α6β4 increase P-p38 and ATF3 expression. Basal keratinocytes in mice with null defects in laminin 5 assemble immature hemidesmosomes with lethal blistering (Ryan et al., 1999). These abnormalities are detectable even in non-blistered regions in the basement membrane zone as discontinuities in hemidesmosome components including integrin α6β4 (Ryan et al., 1999). Similar discontinuities are also apparent in the basement membrane zone of LKs in epidermal wounds (Nguyen et al., 2000a) and prostate cancer (Klezovitch et al., 2004; Yu et al., 2004). This suggests that LKs in wounds that display elevated ATF3 (Fig. 4d) may display similarities to basal cells of individuals with defects in laminin 5. Skin from wild-type and laminin 5 null mice were examined by immunofluorescence microscopy for expression of ATF3 (Fig. 9A). ATF3 was undetectable in cryostat sections of wild-type mouse epidermis (Fig. 9Aa). In the same field, staining of integrin β4 was polarized to the basement membrane zone of basal keratinocytes and appeared as a continuous ribbon (Fig. 9Ad). ATF3 was chronically expressed in nuclei of basal and suprabasal epithelial cells of the laminin 5 null mouse (Fig. 9Ab and Ac) where the integrin β4 appeared as discontinuous ‘beads on a string’ in the basement membrane zone of the same basal keratinocytes (Fig. 9Ae and 9Af). Similar elevations in ATF3 were also detected in the epidermis from individuals

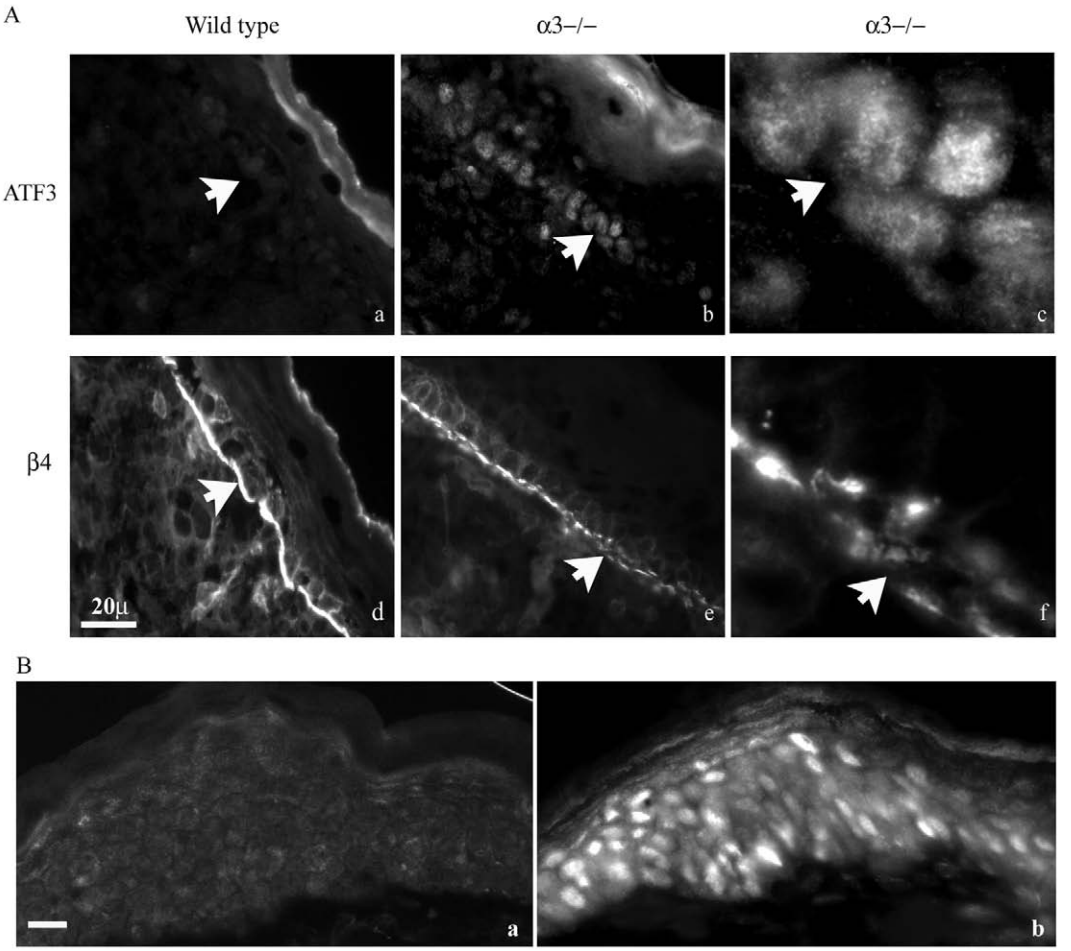

Fig. 9. Adhesion defects in laminin 5 or integrin β4 chronically elevate ATF3. (A) Cryostat sections of skin from wild type neonatal mouse (a,d) and laminin 5 null neonatal mouse (b,c,e,f,) were stained with an anti-ATF3 (a,b,c) or anti-integrin β4 antibody (d,e,f). ATF3 protein is not detectable in wild type mouse epidermis (arrows identify epidermal basal cells). β4 was continuous and polarized to the basement membrane (d, arrow identifies β4 staining in the basement membrane zone). In contrast, laminin 5 null mouse skin ($\alpha3^{-/-}$) exhibited increased levels of nuclear ATF3 protein expression (b, c, arrow identifies ATF3 staining). Integrin β4 was polarized to the basement membrane zone in laminin 5 null keratinocytes but was discontinuous (e,f) (arrows identify discontinuities in β4 integrin). (B) Cryostat sections of skin from a normal control individual (a) and from an individual with null defects in the INTB4 gene encoding the integrin β4 subunit (b) were stained with anti-ATF3 antibodies. ATF3 was elevated in most cell layers in the β4 null epidermis. Bar, 20 μm (A); 20 μm (B).

with null defects in the ITGB4 gene encoding the β4 subunit of integrin α6β4 (9Bb). Based on elevated ATF3 expression, inherited adhesion defects in laminin 5 or integrin β4 result in the activation of ATF3 similar to LKs in wounds.

MKs that are null for laminin 5 ($\alpha3^{-/-}$) fail to adhere to BSA because they cannot deposit laminin 5 and as a result, they express ATF3 protein in suspension (Fig. 3C). Here, we determined if deposition of laminin 5 is sufficient to promote adhesion and adhesion-dependent dephosphorylation of P-p38 that regulates ATF3 (Fig. 10). Many studies have reported that P-p38 is required for cell migration (see Discussion). Furthermore, we have previously shown that deposition of laminin 5 at the rear of migrating LKs is necessary for polarized migration of LKs (Frank, 2004). First, we determined if null defects in laminin 5 would elevate levels of P-p38 in vitro (Fig. 10). MKs with the wild-type α3 chain of laminin 5 ($\alpha3^{+/+}$ MKs) and mutants null for the α3 chain of laminin 5 ($\alpha3^{-/-}$ MKs) were compared for deposition of laminin 5 and adhesion to the deposits in the BSA adhesion assay (Gil et al., 2002; Sigle et al., 2004). The $\alpha3^{+/+}$ and $\alpha3^{-/-}$ MKs were suspended and re-plated onto either a non-adhesive surface coated with BSA or an adhesive laminin 5 surface (Fig. 10). Both the $\alpha3^{+/+}$ and $\alpha3^{-/-}$ MKs adhered on the laminin 5 surfaces and dephosphorylated P-p38 with similar kinetics. However, on the non-adhesive BSA surface, the $\alpha3^{+/+}$ MKs deposited laminin 5, adhered and dephosphorylated P-p38 within 4 hours of contact with the substratum. In contrast, $\alpha3^{-/-}$ keratinocytes failed to adhere on the BSA surface because they did not deposit laminin 5 and P-p38 remained phosphorylated throughout the assay. We conclude that deposition of endogenous laminin 5 followed by adhesion to the deposits is sufficient to dephosphorylate P-p38. This suggests that deposition of laminin 5 by LKs at the rear of the migrating cell may dephosphorylate p38 at the rear of the migrating LK and in following cells that migrate over the path of deposited laminin 5. Thus deposition of endogenous laminin 5 is

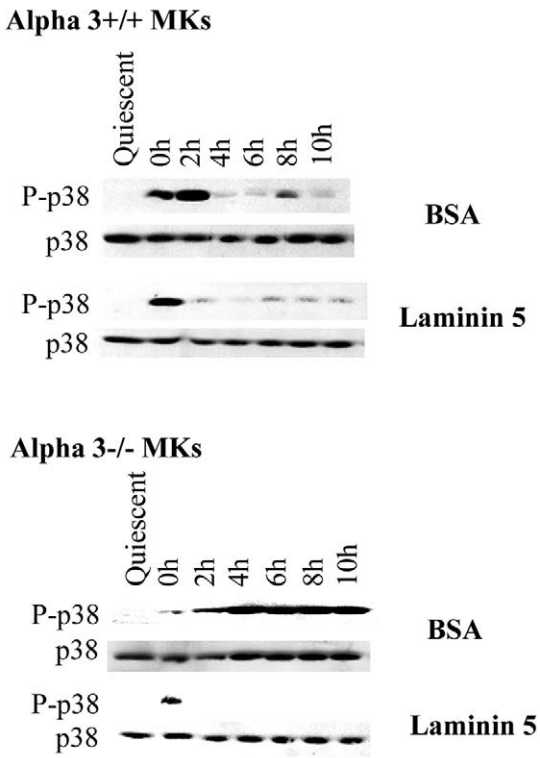

Fig. 10. Defects in laminin 5 deposition, prevent adhesion and dephosphorylation of P-p38. MKs from laminin 5 null mice ($\alpha3^{-/-}$) and wild-type mice ($\alpha3^{+/+}$) were suspended and replated onto either a BSA- or Laminin 5-coated surface. Triton-soluble extracts were collected at 2 hour intervals from 0 to 10 hours and immunoblotted with anti-P-p38 and anti-p38 antibodies. Quiescent $\alpha3^{+/+}$ and $\alpha3^{-/-}$ MKs did not express phosphorylated p38. When plated onto a BSA-coated surface, $\alpha3^{+/+}$ MKs deposited laminin 5, adhered to the deposits on the BSA surface and dephosphorylated p38 within 4 hours. In contrast, $\alpha3^{-/-}$ MKs were unable to deposit laminin 5 or adhere to the BSA or dephosphorylate p38 for the duration of the assay. Both $\alpha3^{+/+}$ MKs and $\alpha3^{-/-}$ MKs rapidly adhered to exogenous laminin 5 and dephosphorylated P-p38, indicating exogenous laminin 5 was sufficient to rescue the ability of $\alpha3^{-/-}$ MKs to adhere and dephosphorylate p38. Total levels of p38 did not change significantly in either the $\alpha3^{+/+}$ MK or $\alpha3^{-/-}$ MK populations.

sufficient to regulate dephosphorylation P-p38 and this may impact the polarity of the LKs and restricted expression of activation components like P-p38 and ATF3 to the LKs but not the following keratinocytes.

Discussion

We have identified adhesion-dependent changes in the phosphorylation of p38 that regulate protein expression in LKs. These p38-dependent changes in protein expression result from wounds or defects in laminin 5 or integrin β4. First, we used an in vitro epidermal wound model and DNA microarrays to identify mRNAs for ATF3 and other activation components (Fig. 1B). The activation mRNAs are elevated in LKs within 1.5 hours of injury. Both in vitro and in vivo, ATF3 protein was upregulated, translocated to the nucleus of LKs, then downregulated in a transient window of expression between 2 and 12 hours after wounding (Figs 2-4). Phosphorylation of p38 MAPK and ATF2 was required for ATF3 expression in LKs of scrape wounds (Figs 5 and 6). Like scrape wounding, cell suspension was sufficient to phosphorylate p38 and elevate ATF3 protein expression. Significantly, seven of the first 50 mRNA transcripts elevated 1.5 times or more in LKs, are regulated by p38 (Fig. 1B). This indicates that p38 is a major contributor to initial epidermal wound activation in LKs. In vitro, re-adhesion of suspended cells to laminin 5 downregulates both P-p38 and ATF3 independently of cell spreading. Consistently, in vivo adhesion defects in laminin 5 or β4 elevated ATF3. We conclude that adhesion to laminin 5 via α6β4 in vivo has two functions: first, laminin 5 is necessary to anchor quiescent epidermis via α6β4 and prevent blistering; second, adhesion to laminin 5 suppresses P-p38 cell signaling, ATF3 and other activation mRNAs. We suggest that adhesion to laminin 5 may limit excessive migration or proteolytic remodeling of the basement membrane and dermis by LKs. Defects in deposition of laminin 5, or destruction of laminin 5 in cancers may elevate P-p38 to increase activation mRNAs that drive migration, stromal proteolysis and cell invasion. Here, we discuss these findings in relation to laminin 5 in regulating p38, transcription and adhesion in wounds, adhesion defects and cancer.

Function of P-p38 and ATF3 in LKs of wounds

ATF3 is a member of the ATF/Creb family of transcription factors and is upregulated in response to mechanical injury and stress stimuli (Hai and Hartman, 2001; Hai et al., 1999). In vivo, these stimuli include nerve axotomy (Tsujino et al., 2000), ischemia of the heart (Chen et al., 1996) and kidney (Yin et al., 1997), partial hepatectomy and brain seizure (Chen et al., 1996). In vitro, microtubule binding agents including taxol and colchicine (Shtil et al., 1999), genotoxic agents including UV and ionizing radiation, transforming growth factor β, tumor necrosis factor α and serum each stimulate ATF3 expression (Hai and Hartman, 2001; Hai et al., 1999). For example, transforming growth factor β in combination with UV radiation elevates SMAD3 and P-p38, increasing ATF3. Subsequently, ATF3 represses the ID1 transcription factor to inhibit proliferation (Kang et al., 2003). Other target genes for ATF3 repression include gadd153/Chop10, a transcription factor that mediates effects of stress (Wang and Ron, 1996; Wolfgang et al., 1997), and E-selectin, an adhesion receptor activated by TNFα (Nawa et al., 2000). However, the function of ATF3 is still not established: ATF3 is reported to both inhibit (Kawauchi et al., 2002; Zhang et al., 2002) and promote (Nawa et al., 2002) p53-dependent apoptosis. ATF3 has also been reported to promote adhesion on collagen (Ishiguro et al., 2000). Studies here demonstrate that ATF3 is upregulated in LKs at the wound margin, whereas quiescent following cells are unaffected. In principle, the elevation of ATF3 in LKs may repress ID1 in LKs, inhibiting cell cycle progression while contributing to migration (Natarajan et al., 2003; Onuma et al., 2001; Sharma et al., 2003). Consistently, the laminin 5 null MKs with elevated ATF3 (Fig. 9) cannot adhere (Fig. 10) and do not progress through the cell cycle without exogenous ligand (Ryan et al., 1999; Sigle et al., 2004). Alternatively, inhibition of p53 by elevated ATF3 in LKs may contribute to the resistance of LKs to apoptosis in the hostile environment of the wound. Future studies will attempt to understand the

function of ATF3 in LKs when transiently expressed in wounds and chronically expressed in laminin 5 null tissue.

How phosphorylated p38 upregulates ATF3 mRNA and protein remains to be established. Prior studies established that JNK or p38 are activated in response to stress to phosphorylate ATF2 and Jun (Cai et al., 2000; Hai and Hartman, 2001; Hai et al., 1999). P-ATF2 heterodimerizes with P-Jun to upregulate transcription of ATF3. Consistently, SB203580 or actinomycin D inhibits expression of ATF3. Furthermore, P-p38 activates transcription of mRNAs for MMP1 (Xu et al., 2001), MMP9 (Turchi et al., 2003), MMP13 (Ravanti et al., 1999) and collagen α1 and α2 transcripts (Ivaska and Heino, 2000; Ivaska et al., 2002; Ivaska et al., 1999). Alternatively, p38 regulates gene expression post-transcriptionally by stabilizing adenylate/uridylate-rich (AU-rich) mRNAs (reviewed by Clark et al., 2003). Consistent with a role for p38 in contributing to the phenotype of LKs, seven of the first 50 mRNA transcripts elevated at least 1.5-fold in LKs in wound screens (ATF3, TNFαIP3, uPA, GRO1, IL1β, PMAIP1 and SOX9 in Fig. 2), are AU-rich mRNAs previously reported to be transcriptionally or post-transcriptionally stabilized by P-p38 (Frevel et al., 2003). Thus, p38 is a significant contributor to changes in mRNA levels that define the phenotype of LKs in wounds. Conceivably, activated p38 may also contribute to the blistering phenotype resulting from adhesion defects in laminin 5 and α6β4. Conceivably, the role of p38 in invasion of cancer cells (Huang et al., 2000) may relate to decreases in laminin 5 in cancer (Klezovitch et al., 2004; Yu et al., 2004). Defects in laminin 5 expression may impact migration and invasion as follows. Scratch wounding removes most adhesive substrate ligand including laminin 5 at the wound edge but not under the LKs. The initial movement of integrin α3β1 from cell-cell contacts to substrate focal adhesion requires the existing laminin 5 under the LKs. The movement of α3β1 from cell-cell contacts at the outside edge of the wound coincides with the time course for initial expression of ATF3 in nuclei of LKs (see Fig. 2B). However, subsequent movement of the cell over the wound requires expression and deposition of endogenous laminin 5 by LKs (Sigle et al., 2004). Even when there is exogenous collagen or laminin 5 on the substratum, the LKs degrade the ligand and replace it with deposits of endogenous laminin 5 (Frank, 2004). Without the transcription, translation and deposition of laminin 5, polarized migration of LKs into the scrape wounds is impaired because of the degradation of exogenous ligands (Frank, 2004). Expression of P-p38 in the LKs may facilitate the degradation of the exogenous ligands and migration of LKs. Deposition of endogenous laminin 5 by LKs generates a path of laminin 5 over which following cells migrate. The deposited path of laminin 5 downregulates the expression of P-p38 (Fig. 10) and restricts its expression to the LKs and may prevent the following cells from degrading the deposited laminin 5.

Mechanisms that may link re-adhesion to dephosphorylation of P-p38

It is not apparent how re-adhesion of cells to the extracellular substratum signals 'outside-in' for the intracellular dephosphorylation of P-p38. Adhesion to laminin 5 mediated by α6β4 suppresses ATF3 in vivo (Fig. 9). Adhesion-dependent changes in lipid rafts have been implicated in regulation of α6β4 signaling (Gagnoux-Palacios et al., 2003). Inhibition of α6β4 association with lipid rafts by mutations in the palmitylation sites does not inhibit adhesion or hemidesmosome assembly, but does inhibit α6β4-dependent signaling through EGFR to activate ERK. ERK regulates expression of the dual specificity phosphatase MKP-1 that dephosphorylates P-p38 (Kiemer et al., 2002; Kim and Corson, 2000; Sharma et al., 2003). Alternatively, smooth muscle cells stimulated by cyclic mechanical stretching upregulate P-p38 within 10 minutes of stretching, which initiates P-p38-dependent motility (Li et al., 1999). This p38 activation is inhibited by pertussis toxin, a G-protein antagonist, and enhanced by suramin, a growth factor receptor antagonist that suppresses ERK and increases P-p38. As discussed above for α6β4, decreased ERK activity may suppress the dual specificity phosphatase MKP-1, which binds to and dephosphorylates P-p38 (Kiemer et al., 2002; Kim and Corson, 2000; Sharma et al., 2003).

Defects in quiescent anchorage on laminin 5 increase P-p38 and wound response proteins

Adhesion defects in laminin 5 and β4 result in chronic ATF3 expression in the epidermis in vivo (Fig. 9). Laminin 10/11 is present in the basement membrane in laminin 5 null mice (Ryan et al., 1999) and functions as an adhesive ligand for α6β4 and α3β1 in vitro (Kikkawa et al., 2000). Despite this adhesive function in vitro, laminin 10/11 is not sufficient in vivo to compensate for the absence of laminin 5 in generating stable anchorage, assembly of hemidesmosomes (Ryan et al., 1999) or suppression of ATF3 protein. In vitro, adhesion via integrin and non-integrin receptors without cell spreading is sufficient to dephosphorylate P-p38 (Fig. 8A,B). At this time, it is not apparent why adhesion to laminin 5 via α6β4 in vivo is necessary to suppress ATF3 whereas in vitro, adhesion via integrin or non-integrin receptors without cell spreading is sufficient to suppress P-p38 and ATF3 (Fig. 8A,B). Conceivably, adhesion defects in laminin 5 and α6β4 may generate stress in addition to the activation of P-p38 that contributes to the elevation of ATF3.

In vitro, re-adhesion via β1 integrins on dermal ligands also dephosphorylates P-p38 (Fig. 8) consistent with other reports (Frisch et al., 1996; Khwaja and Downward, 1997). However, re-adhesion on collagen has also been reported to phosphorylate p38 required for migration of keratinocytes (Li et al., 2001; Saika et al., 2004; Sharma et al., 2003; Turchi et al., 2002), smooth muscle cells (Mayr et al., 2000) and endothelial cells (Mudgett et al., 2000). Both of these observations may be correct if re-adhesion to laminin 5 dephosphorylates P-p38, whereas re-adhesion via α2β1 integrins on collagen maintains sufficient P-p38 for transcription and migration (Fig. 8C). Adhesion via β1 integrins appears to maintain levels of P-p38 sufficient for motility despite the major decrease in levels of P-p38 on re-adhesion (Fig. 8). This observation is consistent with the report that deposition of laminin 5 over dermal collagen switches integrins from α2β1 on collagen to α3β1 and α6β4 on laminin 5 and switches signaling from Rho-dependent to PI3K- and Rac-dependent (Choma et al., 2004; Gu et al., 2001; Nguyen et al., 2001). Adhesion to laminin 5 provides sufficient anchorage to withstand shear stress on the epidermis without

activating signals through Rho, p38 and ATF3 or urokinase that may promote excessive migration or invasion of stroma (Huang et al., 2000; Montero and Nagamine, 1999). Consistently, active Rho and P-p38 contribute to degradation of substrate collagen (Berdeaux et al., 2004; Turchi et al., 2003). Therefore, deposition of laminin 5 that reduces P-p38 and Rho-dependent adhesion on collagen may also reduce collagen degradation (Frank, 2004) or cell invasion.

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