

Akt-dependent Pp2a activity is required for epidermal barrier formation during late embryonic development

Ryan F. L. O'Shaughnessy^{1,2,*}, Jonathan C. Welti¹, Katherine Sully¹ and Carolyn Byrne^{1,3}

Acquisition of epidermal barrier function occurs late in mouse gestation. Several days before birth a wave of barrier acquisition sweeps across murine fetal skin, converging on dorsal and ventral midlines. We investigated the molecular pathways active during epidermal barrier formation. Akt signaling increased as the barrier wave crossed epidermis and Jun was transiently dephosphorylated. Inhibitor experiments on embryonic explants showed that the dephosphorylation of Jun was dependent on both Akt and protein phosphatase 2A (Pp2a). Inhibition of Pp2a and Akt signaling also caused defects in epidermal barrier formation. These data are compatible with a model for developmental barrier acquisition mediated by Pp2a regulation of Jun dephosphorylation, downstream of Akt signaling. Support for this model was provided by siRNA-mediated knockdown of Ppp2r2a (Pr55 α or B55 α), a regulatory subunit of Pp2a expressed in an Akt-dependent manner in epidermis during barrier formation. Ppp2r2a reduction caused significant increase in Jun phosphorylation and interfered with the acquisition of barrier function, with barrier acquisition being restored by inhibition of Jun phosphorylation. Our data provide strong evidence that Ppp2r2a is a regulatory subunit of Pp2a that targets this phosphatase to Jun, and that Pp2a action is necessary for barrier formation. We therefore describe a novel Akt-dependent Pp2a activity that acts at least partly through Jun to affect initial barrier formation during late embryonic epidermal development.

KEY WORDS: Epidermis, Barrier function, Akt, Jun, c-jun, Phosphatase, Pp2a, Ppp2r2a, Mouse

INTRODUCTION

The epidermal barrier prevents transepidermal water loss, essential for ex-utero survival. To this end mammalian fetuses acquire barrier function late in gestation (Hardman et al., 1998; Hardman et al., 1999). Formation of the murine epidermal barrier is rapid. Between embryonic day 16.5 (E16.5) and E17.5 a wave of epidermal barrier formation sweeps across murine fetal skin to converge on ventral and dorsal midlines. Barrier formation can be visualized by dye permeability assays (Hardman et al., 1998; Hardman et al., 1999) (see also Fig. 1; timing is strain dependent; see Materials and methods).

The serine/threonine kinase Akt changes expression and activity during fetal barrier acquisition (O'Shaughnessy et al., 2007b) (Fig. 1), and Akt activity is essential for barrier formation. Mice lacking both Akt1 and Akt2 die postnatally, probably due to lack of epidermal barrier function (Peng et al., 2003). However, although *Akt1* nulls have defective cornified envelopes (O'Shaughnessy et al., 2007b), both Akt1 and Akt2 knockouts are viable, suggesting a degree of redundancy between these Akt proteins. Phosphorylated active Akt is found in two regions of the epidermis – the lower suprabasal epidermis and the upper granular layer (Janes et al., 2004; O'Shaughnessy et al., 2007b). Our previous work has determined that this lower suprabasal activity mostly comprises Akt2, whereas the upper granular layer activity mostly comprises Akt1 (O'Shaughnessy et al., 2007a; O'Shaughnessy et al., 2007b).

Akt activity is also present in cultured keratinocytes, probably corresponding to the lower suprabasal activity (Janes et al., 2004; Calautti et al., 2005; Thrash et al., 2006; O'Shaughnessy et al.,

2007b). Multiple roles have been proposed for epidermal Akt, from the initiation of the terminal differentiation program (Janes et al., 2004; Calautti et al., 2005; Thrash et al., 2006; O'Shaughnessy et al., 2007b; Janes et al., 2009) to the correct formation of the cornified envelope during late terminal differentiation, partly by regulating filaggrin processing (O'Shaughnessy et al., 2007a; O'Shaughnessy et al., 2007b).

In vivo, lower suprabasal Akt activity peaks around E17 with expression diminishing around E18, which encompasses the period of barrier acquisition in the mouse embryo (O'Shaughnessy et al., 2007b). However, the relationship between Akt function and developmental barrier acquisition remains to be ascertained. To investigate the relationship between embryonic epidermal development and the initial acquisition of epidermal barrier function we used an approach that co-visualizes protein expression and barrier function.

We found that there was a 'pulse' of high Akt activity coincident with the barrier acquisition front. Dephosphorylation of Jun (also known as c-jun) also coincided with barrier acquisition and was prevented by inhibition of either Akt or protein phosphatase 2A (Pp2a). We show that expression of the Pp2a regulatory subunit Ppp2r2a, also known as B55 α or Pr55 α , was Akt-dependent, and that in organotypic culture reducing Ppp2r2a expression resulted in maintenance of Jun phosphorylation and a transient barrier defect, followed by hyperkeratosis. In this paper we present evidence that a novel Akt-mediated Pp2a function, acting via Jun signaling, is necessary for epidermal barrier function.

MATERIALS AND METHODS

General immunohistochemistry and immunofluorescence

Immunohistochemistry on paraffin and frozen sections was by standard techniques. Keratinocytes were fixed for 10 minutes at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS), then permeabilized in 0.2% Triton X-100 (Sigma, Gillingham, UK) for 30 minutes. Antibodies used were as follows: rabbit anti pSer473 Akt (Cell Signaling Technologies, Danvers, USA) 1/10; rabbit anti Keratin 10 (Covance, Cambridge, UK) 1/500; rabbit anti-filaggrin (Covance) 1/500; rabbit anti-loricrin (Covance)

¹Centre for Cutaneous Research, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, Whitechapel, London E1 2AD, UK.

²Dermatology and Immunobiology, Institute of Child Health, University College London, London WC1N 1EH, UK. ³Cancer Research UK Skin Tumour Laboratory, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry.

*Author for correspondence (r.oshaughnessy@ich.ucl.ac.uk)

1/500; rabbit anti-Jun (Cell Signaling Technologies) 1/10; rabbit anti-pSer63 Jun (Cell Signaling Technologies) 1/10; rabbit anti-pSer2448 mTOR 1/50 (Cell Signaling Technologies); sheep anti-PPP2R2A (Abcam, Cambridge, UK) 1/25; mouse anti-PP2AC (BD Biosciences, Dendermonde, Belgium) 1/50; goat anti-KLFL (KLF4) (Santa Cruz Biotechnologies, Santa Cruz, US) 1/20. Antibody detection was by either the Elite Avidin-Biotin-Complex system and DAB (Vector Laboratories, Burlingame, USA) or Alexa Fluor 488 (green) and 536 (red)-conjugated secondary antibodies (Molecular Probes). Counterstaining was with Hematoxylin for immunohistochemistry and 4',6-diamidino-2-phenylindole (DAPI) for immunofluorescence. Images were taken with a Nikon Eclipse E600 microscope with either $\times 20$ (NA 0.4) or $\times 60$ oil immersion (NA 1.40) objectives, using a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, USA) with Spot RT Software v3.0.

Barrier assays, selection of skin samples flanking the barrier front and co-visualization of barrier activity and protein abundance

The Toluidine Blue barrier assay (dye permeability assay, dye exclusion assay) was performed on E16.5 CD1 embryos or embryonic epidermal explants fixed in 4% paraformaldehyde in PBS as described (Hardman et al., 1998); note that barrier formation will occur later, approximately E17.5, in other strains of mouse, e.g. C57BL/6. Briefly, fixed embryos or tissues were dehydrated with methanol (approximately 1 minute each in 25, 50, 75 and then 100% ethanol) then rehydrated using the reverse procedure, followed by immersion in 1% Toluidine Blue in water, then extensive destaining in PBS.

For selection of epidermal samples flanking the barrier front, we took mouse embryos at E16.5, removed a strip of epidermis from the middle of the body and cut it in half longitudinally. We fixed and stained one half in Toluidine Blue. Lining this up with the adjacent strip allowed us to obtain skin that was crudely pre-barrier formation and post-barrier formation (Fig. 1B). Alternatively, the barrier front was marked by an incision.

For visualizing protein and barrier competence simultaneously, and for visualizing barrier function in organotypic culture, fixed and dehydrated/rehydrated embryos or cultures were dipped for one minute in 1% Hematoxylin in water and washed several times before embedding in paraffin. Subsequent immunohistochemistry was by standard techniques.

Embryonic epidermal explant culture

Explant culture of murine late embryonic skin has been described previously (O'Shaughnessy et al., 2007a). Briefly, skin (epidermis plus dermis) was dissected from the torso region of E15.5 CD1 mice, rinsed in sterile PBS and cultured dermis side down, at the air-liquid interface for 48 hours, covering

the period of barrier formation. Explants were cultured in Williams Medium E supplemented with 10 $\mu\text{g/ml}$ insulin, 10 ng/ml hydrocortisone, 2 mM glutamine, 100 IU/l penicillin, 100 mg/l streptomycin and 25 $\mu\text{g/ml}$ amphotericin (all reagents from Invitrogen, Paisley, UK). For the inhibitor experiments, SH-5 (100 μM ; Enzo Life Sciences, Exeter, UK), okadaic acid (10 nM; Sigma), Fostriecin (20 μM ; Sigma) and fenvalerate (20 μM ; Sigma) in DMSO (Sigma) were added to the medium before culture. Explants were fixed in Bouin's solution (Sigma) for 2 hours before processing for paraffin embedding, or embedded in OCT for cryosectioning.

siRNA constructs, rat epidermal keratinocyte culture and organotypic culture

The SureSilencing shRNA plasmids to rat PPP2R2A (insert sequences siRNA1-GCAGATGATTTGCGAATTAAT, siRNA2-TGACTGGATCC-TACAATAAT in pSuperNeo) were from Tebo-bio (Peterborough, UK). Constructs were transfected into transformed rat epidermal keratinocytes (REK) (Marjukka et al., 2003) cells using lipofectamine plus (Invitrogen, Paisley, UK). Selection in 100 μM G418 (Invitrogen, Paisley, UK) was undertaken for 2 weeks. REK cells were passaged in DMEM +10% fetal calf serum. For immunofluorescence analysis, the cells were subsequently simultaneously fixed and permeabilized in 4% paraformaldehyde/0.2% Triton X-100. Organotypic culture was performed as described by O'Shaughnessy et al. (O'Shaughnessy et al., 2007b), briefly, 2×10^5 rat epidermal keratinocytes were cultured in medium containing 100 mg/ml G418 on de-epidermized dermis made from cadaverous skin (Euro Skin Bank, Beverwijk, Netherlands) in a metal ring until confluent. Subsequently the organotypic cultures were raised to the air-liquid interface and cultured for a further 10 days, unless specified. When used in organotypic culture, SP600125 (Sigma), a Jun kinase inhibitor, was added to the medium at 50 nM. The constructs were processed for paraffin embedding by fixing in Bouin's solution (Sigma) or 4% paraformaldehyde before the Hematoxylin barrier assay.

Western blot analyses

Keratinocytes were lysed by incubation on ice for 10 minutes in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ leupeptin, 1% Nonidet-40). Immunoprecipitation of the lysate was performed with 1 μg of either a total Jun antibody (Abcam) or Ppp2r2a antibody (Abcam), and the complexes were pulled down using protein A conjugated to agarose (Sigma).

Lysates were separated on 7.5-10% SDS/polyacrylamide gels and transferred onto nitrocellulose filters (Hybond C+—Amersham). Primary antibodies and concentrations were as follows: rabbit anti-pSer473 (Cell

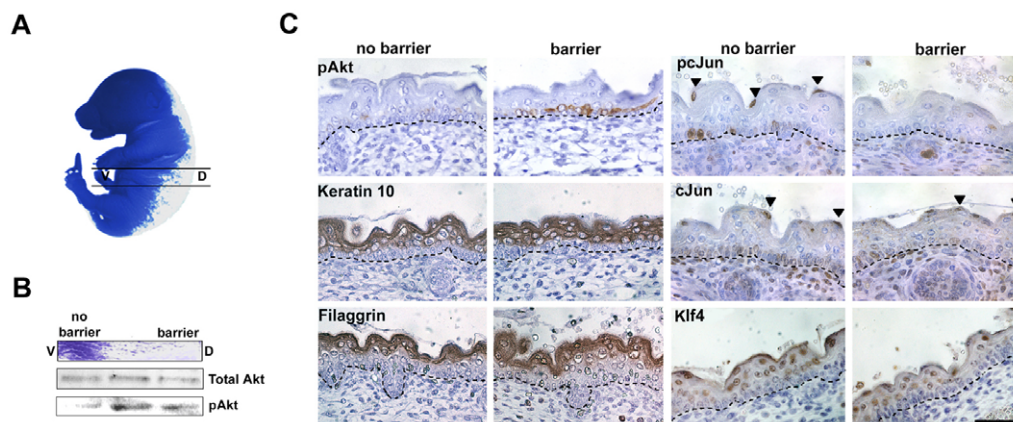


Fig. 1. Akt signaling and Jun dephosphorylation at the barrier front. (A) Typical Toluidine Blue dye penetration (permeability) assay to assess barrier in an E16.5 mouse embryo. The barrier has progressed half way around the animal. The strip indicates the region assessed for protein expression. V, ventral; D, dorsal. (B) Assessment of Akt expression and activity (pAkt) by western blots of lysates from an epidermal strip comprising the barrier front (top panel). (C) Immunohistochemical detection of active Akt (pAkt), keratin 10, filaggrin, phosphorylated Jun (pJun), Jun and Klf4 in skin sections flanking the barrier front. Arrowheads point to expression of Jun and pJun in the upper granular layer. Dashed lines indicate the dermal-epidermal junction. Scale bar: 50 μm .

Signaling Technologies) 1/500; rabbit anti-total Akt (Cell Signaling Technologies) 1/1000; mouse anti-beta actin (Sigma) 1/2000; sheep anti-PPP2R2A (Abcam) 1/500; rabbit anti-Jun (Abcam) 1/100. Primary antibody incubations were in TBST [100 mM Tris HCl, 0.2 M NaCl, 0.1% Tween-20 (v/v)] containing 5% bovine serum albumin (Sigma) either overnight at 4°C or for 1-2 hours at room temperature, whereas secondary antibody incubations were in 5% skimmed milk powder for 1 hour at room temperature. The following concentrations were used: goat anti-rabbit-HRP (Jackson ImmunoResearch) 1:5000; rabbit anti-mouse HRP (DakoCytomation) 1:2000; donkey anti-goat (Jackson ImmunoResearch) 1:3000. Protein was visualized using the ECL Plus kit (Amersham).

RESULTS

Akt signaling increases and pJun expression falls during barrier formation

The formation of the epidermal barrier in the embryonic mouse can be visualized by dye permeability assays (Fig. 1A) (Hardman et al., 1998). We used these assays to select skin samples flanking the barrier formation front (Fig. 1B, see Materials and methods) and examined the expression of total Akt and active Akt, as determined by phosphorylation at serine 473 (pAkt). Total levels of Akt were unchanged during barrier formation, but there was an increase in pAkt (Fig. 1B), showing that Akt activity increased at this crucial point in late epidermal development.

Immunohistochemistry was performed on E16.5 embryonic skin samples flanking the barrier front (Fig. 1C). Consistent with the western data, there was increased expression of pAkt in the lower suprabasal region of the epidermis in the sample taken after barrier acquisition. This must represent the initiation of the pulse of suprabasal Akt activity associated with Akt2 previously reported in late gestation murine skin (O'Shaughnessy et al., 2007b). There was no change in the epidermal differentiation markers keratin 10 or filaggrin. The expression of the transcription factor Klf4, which is strongly implicated in the

formation of the cornified layer (Segre et al., 1999), was present both post- and pre-barrier acquisition, suggesting that this important transcription factor does not play a role at this stage of barrier acquisition, although it clearly has a role in the developmental program that leads to cornified layer formation.

Like pAkt, Jun has been reported in two regions of adult epidermis: in the lower suprabasal layer and in the uppermost granular layer (Mehic et al., 2005; Zenz et al., 2005). We found both unphosphorylated Jun (Jun) and phosphorylated active Jun [pJun, Ser63 phosphorylation (Binetruy et al., 1991; Smeal et al., 1991)] in the basal/lower suprabasal and upper granular layers (the arrowheads in Fig. 1C show the granular layer – note this flattened layer contains fewer nuclei) of embryonic epidermis before barrier formation (Fig. 1C). However, after barrier formation phosphorylation of Jun at Ser63 was undetectable, whereas Jun expression was maintained. These data suggest that during barrier formation, signaling via Jun is deactivated, possibly by phosphatase activity (Fig. 1C).

The dephosphorylation of Jun is transient during epidermal development, with phosphorylation being restored by E18.5 (Fig. 2) and maintained in adult epidermis (data not shown). This dynamic pattern of Jun dephosphorylation associated with the barrier front resembles the pulse of high suprabasal Akt activity in the vicinity of the barrier front (Fig. 1B,C; Fig. 2, compare E17.5 panels) (O'Shaughnessy et al., 2007b).

Co-visualization of barrier formation and protein expression shows a pulse of Akt activity at the barrier front co-incident with Jun dephosphorylation

To examine precisely the dynamics of Akt and Jun phosphorylation as barrier formed, a modification of the standard barrier assay was used (see Materials and methods) whereby the permanent histological dye Hematoxylin was used instead of

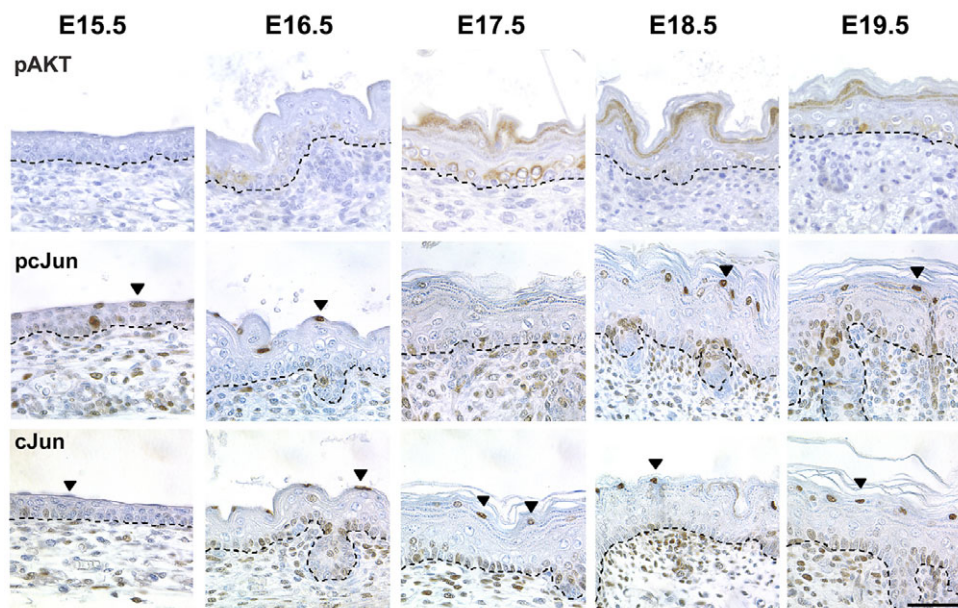


Fig. 2. Reduced Jun phosphorylation correlates with suprabasal Akt activity during development. Immunohistochemical analysis of pAkt, pJun and Jun in E15.5-19.5 dorsal mouse skin. pAkt expression increases from E16.5 in the lower suprabasal epidermis but is lost by E18.5. Upper granular layer pAkt expression starts at E17.5 and persists. Jun expression is maintained throughout the developmental series; however, pJun expression is markedly reduced in E17.5 epidermis. Arrowheads indicate examples of nuclear expression of pJun and Jun in the granular layer. The dashed lines indicate the dermal-epidermal junction. Scale bar: 50 μ m.

Toluidine Blue. Hematoxylin penetrates the embryos and stains the nuclei in the absence of barrier, and this staining persists through tissue embedding and preparation of sections for immunohistochemistry (Fig. 3A,B). Phosphorylated Jun disappeared after the barrier front passed (Fig. 3A, compare insets 1 and 2 with inset 3). The phosphorylation of Akt in lower suprabasal keratinocytes began before the formation of barrier (barrier front shown with an arrowhead in Fig. 3B) with activity diminishing after the barrier front had passed (Fig. 3B, compare insets 1 and 2 with inset 3). These data show precise coincidence of the pulse of suprabasal Akt activation with barrier formation, overlapping and slightly preceding the pulse of Jun dephosphorylation.

Jun is dephosphorylated in keratinocytes during barrier formation by an Akt-dependent mechanism

Explant cultures from developing late embryonic mouse skin recapitulate *in vivo* development and after 2 days in culture form a bone fide differentiated epidermis comparable with E17.5 epidermis with barrier function (O'Shaughnessy et al., 2007a). Akt activity, measured by expression of pAkt, is present in both the lower suprabasal epidermis and the upper granular layer. This experimental system was used to modulate Akt activity using the SH-5 phosphatidyl inositol analog, shown to be a highly specific inhibitor for Akt (Kozikowski et al., 2003). Treatment with SH-5 caused a reduction in pAkt expression in explants detectable by immunohistochemistry (Fig. 4A) and by western blot (see Fig. S1 in the supplementary material). SH5 treatment caused inhibition of stratum corneum formation, compared with DMSO controls (Fig. 4A), consistent with previous reports of epidermal Akt inhibition (Peng et al., 2003; O'Shaughnessy et al., 2007b). Loricrin expression was unchanged, suggesting that the overall program of epidermal terminal differentiation was unaltered. Treatment with SH-5 reduced epidermal pAkt levels and reduced the expression of phosphorylated mTOR, a known downstream target of Akt (Levine et al., 2006), validating the experimental approach. Toluidine Blue barrier permeability assays on the explant cultures showed that Akt inhibition by SH-5 had qualitatively impaired barrier formation (Fig. 4B), another known consequence of inhibiting Akt signaling during development (Peng et al., 2003; O'Shaughnessy et al., 2007a).

SH-5 treated explants failed to downregulate the phosphorylation of Jun (Fig. 4A), which occurred in the DMSO-treated explant controls with intact Akt signaling and is expected developmentally (Figs 1-3). Abundant unphosphorylated Jun was detectable after both treatments. Therefore, taken together we can deduce that dephosphorylation of Jun during barrier formation is likely to be by an Akt-dependent mechanism.

Dephosphorylation of Jun and formation of epidermal barrier function are mediated by a Pp2a phosphatase

Pp2a is a serine threonine protein phosphatase that is known to play an important role in epidermal differentiation, including the dephosphorylation of filaggrin (Kam et al., 1993). As Pp2a can also downregulate the activity of the Jun N-terminal kinase (JNK) (Shanley et al., 2001), we chose this phosphatase for further investigation.

Late embryonic skin explants were treated with the Pp2a inhibitors okadaic acid and Fostriecin to determine whether the dephosphorylation of Jun in epidermis was mediated by a member

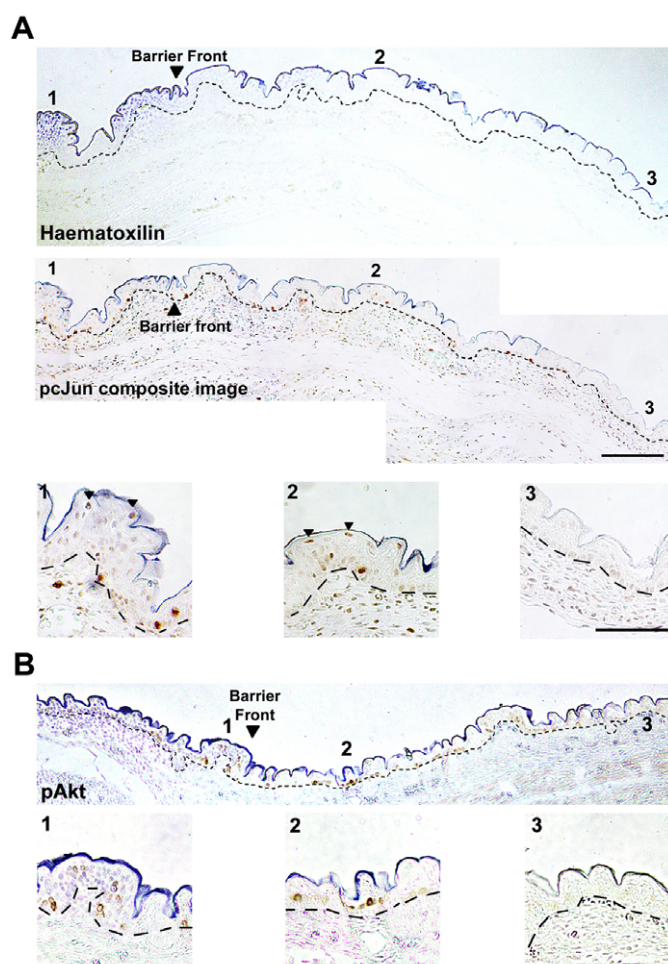


Fig. 3. Co-imaging of the barrier front and protein expression shows that barrier acquisition occurs within the pulse of suprabasal Akt activation and just before the pulse of Jun dephosphorylation. (A) Serial sections comparing the barrier front (arrowhead) in a Hematoxylin-stained mouse fetus with expression of pJun. 1, 2 and 3 correspond to the respective inset figures. The pJun figure is a composite image, allowing for better imaging of pJun expression in the upper granular layer. Small arrowheads in the insets indicate examples of pJun expression in the upper granular layer. (B) Comparison of pAkt expression with the barrier front (arrowhead) in epidermis from a Hematoxylin-stained mouse fetus, with positions 1, 2 and 3 shown as insets. The dashed lines in A and B indicate the dermal-epidermal boundary. Note that the barrier front is moving from right to left in this figure. Scale bars: 100 μ m.

of the Pp2a family, and whether the inhibition of Pp2a led to an epidermal barrier defect (Fig. 5). Fostriecin was included as it has greater specificity for Pp2a than okadaic acid. Fenvalerate was used as a second control, as it inhibits the Pp2b family phosphatases.

In both the okadaic acid- and Fostriecin-treated explants there was maintenance of phosphorylation of Jun, which was not observed in the fenvalerate-treated explants or the DMSO control (Fig. 5A). In all explants tested, expression of total Jun was maintained. Interestingly, pAkt expression levels were increased in the okadaic acid- and Fostriecin-treated explants (Fig. 5B), consistent with a known role for Pp2a in the dephosphorylation of Akt (Ugi et al., 2004) and validating the specificity and activity

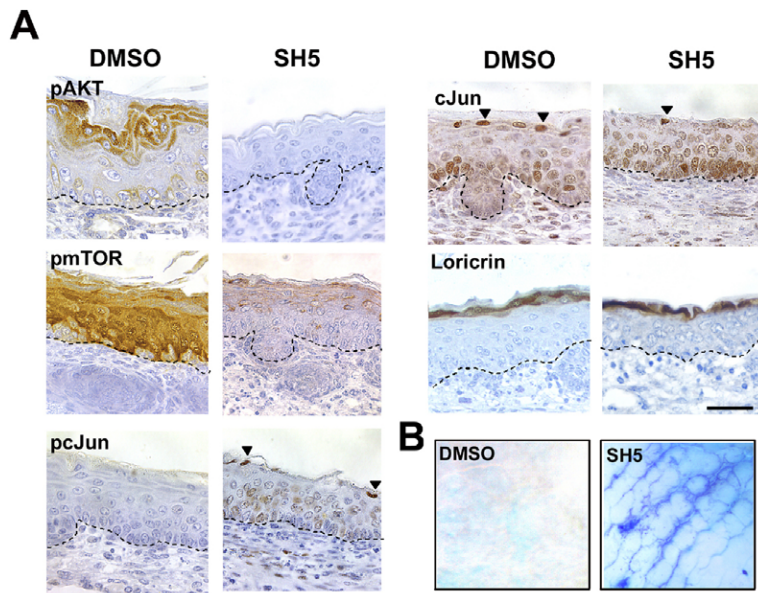


Fig. 4. Dephosphorylation of Jun and epidermal barrier function are Akt dependent. (A) Immunohistochemical analysis of embryonic explants treated with 100 μM SH-5 or vehicle (DMSO). Inhibition of Akt by SH-5 was effective, reducing levels of pAkt and expression of the downstream Akt target pmTOR. Loricrin expression was maintained in both explants, showing that the overall differentiation program remained unchanged. However, inhibition of Akt by SH-5 resulted in maintenance of Jun phosphorylation; note that Jun phosphorylation is developmentally downregulated, as expected, in the DMSO-treated control. (B) Surface view of epidermal explants after Toluidine Blue dye penetration assay. The DMSO-treated control was resistant to dye penetration, indicating barrier acquisition, whereas explants treated with SH5 had impaired barrier function. Arrowheads indicate examples of nuclear expression of Jun and pJun in the granular layer. The dashed lines indicate the dermal-epidermal junction. Scale bar: 50 μm.

of the Pp2a inhibitors in this experiment. pAkt expression levels in the fenvalerate-treated explants was comparable to that in the controls.

We tested the treated explants for barrier function by Toluidine Blue exclusion (Fig. 5C). Both the okadaic acid- and Fostriecin-treated explants had defective barrier function to a lesser degree

compared with the DMSO control. Fenvalerate-treated explants also allowed penetration of Toluidine Blue, although to a lesser degree than those treated with the Pp2a inhibitors. These data show that both the developmental downregulation of Jun phosphorylation and barrier formation could be mediated by Pp2a activity.

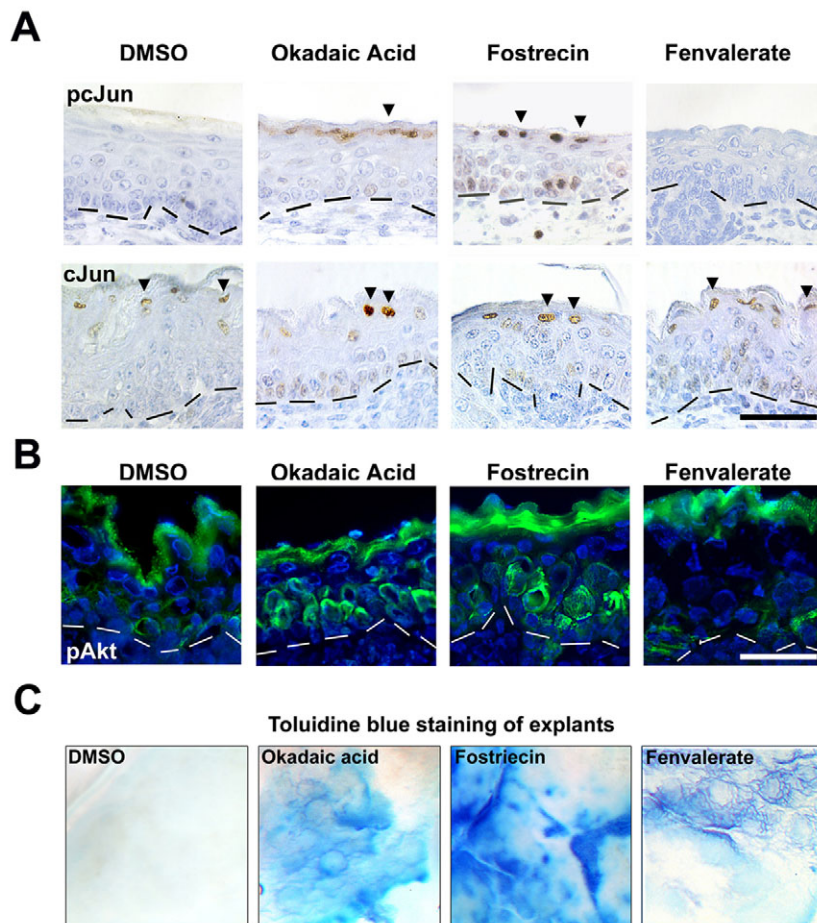


Fig. 5. Dephosphorylation of Jun and epidermal barrier function are Pp2a dependent.

(A) Immunohistochemical analysis of pJun and Jun in embryonic explants treated with the Pp2a inhibitors okadaic acid (10 nM), Fostriecin (20 μM) and fenvalerate (20 μM). Phosphorylation of Jun was maintained in explants in which Pp2a, but not Pp2b, was inhibited. Vertical arrowheads indicate nuclear Jun and pJun in the granular layer. (B) Immunofluorescence analysis of pAkt expression in treated explants. Expression was increased in explants treated with the Pp2a inhibitors. (C) Toluidine Blue dye penetration assay on explants treated with okadaic acid, Fostriecin or fenvalerate. Barrier function was impaired when Pp2a was inhibited, and to a lesser extent when Pp2b was inhibited. Dashed lines indicate the dermal-epidermal junction. Scale bars: 50 μm.

Expression of Ppp2r2a is affected by Akt activity

Recent data functionally link the Ppp2r2 family members to Akt (Kuo et al., 2008). We performed RT-PCR analysis of E15.5–18.5 skin and determined that Ppp2r2a was the most highly expressed family member during barrier acquisition, with expression of Ppp2r2a message peaking at E16.5 (see Fig. S2 in the supplementary material). Immunofluorescence showed increased expression of Ppp2r2a in E17.5 fetal skin (Fig. 6A) and expression in the nuclei of cultured keratinocytes, which coexpress Jun (Fig. 6B), Hence, Ppp2r2a could potentially be the regulatory subunit of Pp2a that targets Jun during barrier formation.

Expression of epidermal Ppp2r2a was markedly reduced in SH-5 treated explants (Fig. 6C), implying that its expression levels were indeed regulated by Akt signaling. The expression of the catalytic subunit of Pp2a, Pp2ac, was unchanged in the SH-5 treated explants, showing that the regulatory subunit expression was specifically affected by Akt signaling. Immunoprecipitation of a rat epidermal keratinocyte lysate with a Jun-specific antibody, unlike the isotype control, was able to pull down Ppp2r2a (Fig. 6D), suggesting a physical interaction between the two proteins.

Ppp2r2a is necessary for both the dephosphorylation of Jun and for the rapid acquisition of barrier function in an organotypic culture model

We transfected rat epidermal keratinocytes with two different shRNA plasmid constructs in order to downregulate the expression of Ppp2r2a by siRNA. The second construct (Ppp2r2a siRNA2) knocked down expression of Ppp2r2a by around 90% in western blots compared with levels in cells transfected with a scrambled oligonucleotide control (scram, Fig. 7A). Expression of Ppp2r2a was markedly reduced in siRNA2-expressing cells compared with scrambled controls (Fig. 7B).

Ppp2r2a knockdown perturbed stratum corneum homeostasis, consistent with barrier dysfunction. Hyperkeratosis was observed in the siRNA-expressing cultures, diagnostic of barrier perturbation, and there were associated changes in the program of terminal differentiation, with a marked increase in both loricrin and involucrin expression, whereas expression of keratin 10 appeared unchanged (Fig. 7C).

Reducing Ppp2r2a expression led to substantially increased Jun phosphorylation, suggesting that Ppp2r2a is a regulatory subunit responsible for the dephosphorylation of Jun (Fig. 7D). In the Ppp2r2a siRNA cultures, pAkt expression was unchanged (not shown).

To link the changes in Jun phosphorylation to developmental barrier acquisition, rat epidermal keratinocyte organotypic cultures were grown on de-epidermized dermis at the air-liquid interface for 5 and 10 days (Baden and Kubilus, 1983; Marjukka et al., 2003; O'Shaughnessy et al., 2007b) to assay differences in initial barrier acquisition. Ppp2r2a siRNA and scrambled control cultures were also treated with the JNK inhibitor SP600125 or vehicle, to test whether inhibiting Jun phosphorylation could restore normal barrier acquisition characteristics. Barrier function was assayed by staining the organotypic cultures whole mount with Hematoxylin then sectioning (Materials and methods) (see Fig. 3). In this assay, barrier-incompetent organotypic cultures will allow Hematoxylin to penetrate, staining the nuclei of the epidermis blue. The scrambled oligonucleotide controls were barrier competent at day 5, whereas the barrier was still defective at day 5 in the siRNA-expressing cultures (Fig. 8A,B). Histological analysis of the culture indicated that the epidermis of the scrambled cultures gradually acquired a cornified layer, whereas from day 5 onwards the siRNA-expressing cultures were hyperkeratotic, showing an increasingly thickened cornified layer at days 5 and 10 (Fig. 8C). The siRNA-expressing culture also showed retention of pJun staining as expected (Fig. 8D). Therefore the knockdown of Ppp2r2a expression caused a defect during epidermal barrier development in culture, which is apparently only ameliorated when the cornified layer is thickened.

Treatment of the cultures with the JNK inhibitor SP600125 had no effect on barrier acquisition in the scrambled siRNA control (data not shown). However, strikingly, barrier acquisition in SP600125-treated siRNA cultures appeared restored to that found in the scrambled control culture (Fig. 8B–D), providing further evidence that Ppp2r2a acts through Jun dephosphorylation to bring about barrier acquisition.

DISCUSSION

We report here that epidermal barrier acquisition in late embryonic development is accompanied by a pulse of Akt activation and a transient decrease in Jun phosphorylation. Experimental inhibition

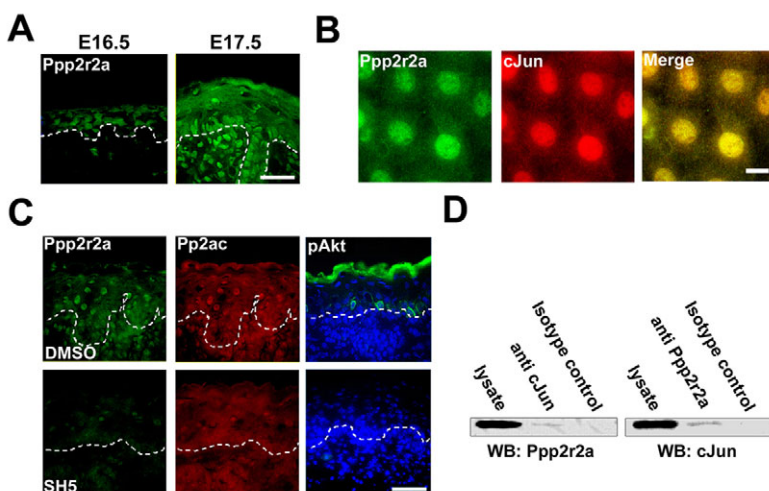


Fig. 6. Ppp2r2a expression is Akt dependent and increases after barrier formation. Ppp2r2a co-localizes with, and can bind, Jun. (A) Immunofluorescence analysis for Ppp2r2a of frozen sections of E16.5 and E17.5 dorsal skin, corresponding to timepoints pre- and post-barrier acquisition. (B) Co-immunofluorescence analysis of Ppp2r2a and Jun in rat epidermal keratinocytes showing widespread co-localization. (C) Co-immunofluorescence analysis of frozen sections from drug- and vehicle-treated epidermal explants for the expression of Ppp2r2a and Pp2ac. Note that although the expression of Ppp2r2a decreased in the SH5-treated explants, the expression of Pp2ac was unchanged. Analysis of pAkt confirms that the SH-5 has inhibited Akt. (D) Immunoprecipitation (IP) western of rat keratinocyte lysate pulled down with an anti-Jun antibody or with an anti-Ppp2r2a antibody and with a respective control isotype (IgG) antibody. Scale bars: 50 μ m in A,C; 10 μ m in B.

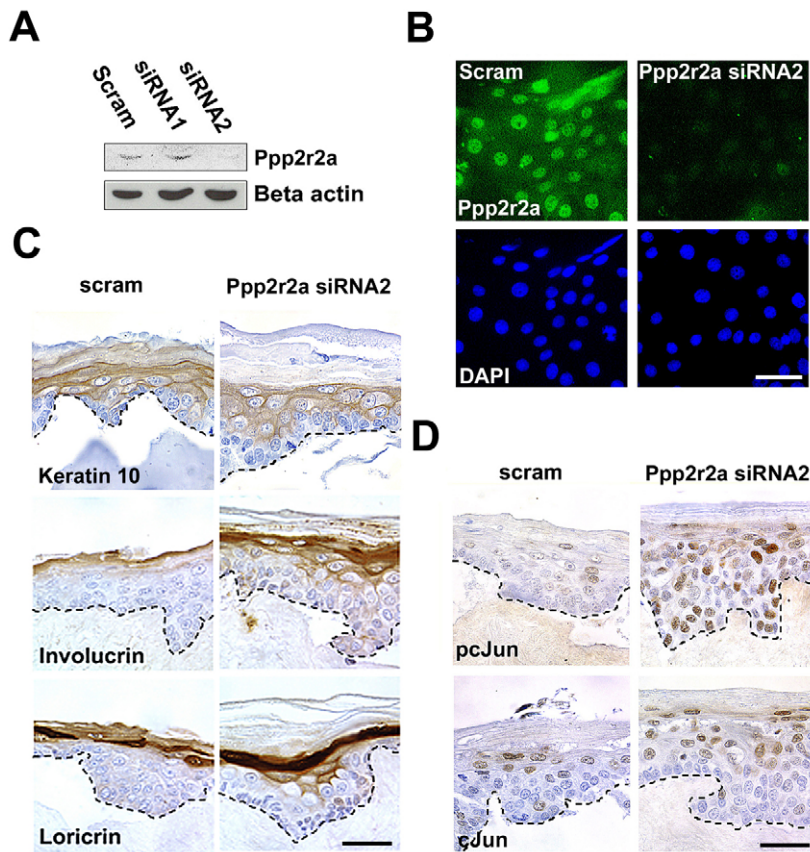


Fig. 7. siRNA knockdown of Ppp2r2a maintains Jun phosphorylation and causes defects in terminal differentiation. (A) Western blot analysis of two lines of rat epidermal keratinocytes (REKs) expressing different Ppp2r2a shRNA-containing plasmids and one expressing a scrambled control for Ppp2r2a, with actin as a loading control. siRNA2 caused substantial knockdown of Ppp2r2a. (B) Immunofluorescence analysis of Ppp2r2a in the scrambled line and siRNA2 line, confirming substantial knockdown. (C) Immunohistochemical analysis of keratin 10, involucrin and loricrin in the same organotypic cultures. (D) Immunohistochemical analysis of pJun and Jun in the scrambled control and siRNA-expressing organotypic cultures. Scale bars: 50 μ m.

of Akt signaling in a developing epidermal model reduces Jun dephosphorylation and prevents barrier acquisition, suggesting that this ‘pulse’ of cessation of Jun phosphorylation is downstream of Akt signaling and is necessary for barrier formation. We used inhibitor studies in the same developing epidermal model to show that dephosphorylation of Jun is mediated by Pp2a, and that expression of a Pp2a regulatory subunit, Ppp2r2a, is regulated by Akt. Hence, we propose that the developmental pulse of Akt activity upregulates Pp2a via Ppp2r2a, causing dephosphorylation of Jun and barrier acquisition (Fig. 9). In support of this proposal we show that reduction of Ppp2r2a expression by siRNA significantly increases phosphorylation of Jun and interferes with epidermal barrier formation, and inhibition of Jun phosphorylation restores barrier function even in the absence of Ppp2r2a.

There are clear links between Akt function and barrier acquisition. In the *Akt1*; *Akt2* double null mouse, barrier function is severely affected, leading to perinatal lethality (Peng et al., 2003), whereas we have reported that mice null for *Akt1* have a mild barrier dysfunction in adulthood (O’Shaughnessy et al., 2007b). Epidermal-specific *Pten* knockouts, which exhibit hyperactivation of Akt, show postnatal hyperplasia and hyperkeratosis (Suzuki et al., 2003; Backman et al., 2004). Barrier acceleration was not reported in these animals, although this may be because Akt activity during embryogenesis is already above the threshold to induce barrier function.

One of the most striking observations was the transient dephosphorylation of Jun at the barrier front. Little is known about epidermal Jun and its role in barrier acquisition and homeostasis. Wound healing is one situation in which Jun expression is upregulated (Yates and Rayner, 2002) and barrier function is reduced to facilitate the closing of the wound by increasing the

migration of the keratinocytes at the wound margin. However, Jun expression is reduced in psoriasis, a hyperproliferative and hyperkeratotic disorder that is associated with a barrier dysfunction (Basset-Seguin et al., 1991), and a psoriasis-like disease was observed in mice with an inducible knockdown of Jun and JunB (Zenz et al., 2005). This suggests that the roles of Jun during normal homeostasis and pathology are different.

There are several reports showing that either pharmacologic inhibition of the PI3 kinase pathway or activation of AKT signaling reduced Jun phosphorylation in diverse human cell lines (Levresse et al., 2000; Suhara et al., 2002; Song and Lee, 2005). However, recent data indicates that *PTEN* null cells, although they have increased AKT phosphorylation, exhibit higher levels of JUN phosphorylation (Vivanco et al., 2007). Therefore, the relationship between AKT signaling and JUN phosphorylation could be context dependent.

Pp2a has a role in Jun dephosphorylation and barrier formation downstream of Akt. However, Pp2a can act as a phosphatase upstream of Akt (Yamada et al., 2001; Ugi et al., 2004; Gao et al., 2005), and this suggests a mechanism whereby the developmental pulse of Akt could occur. However, although Pp2a inhibition increased Akt serine phosphorylation, Ppp2r2a knockdown had no effect on Akt serine phosphorylation. This is because inhibitors target the range of Pp2a holoenzymes, whereas the siRNA is specific for the Ppp2r2a regulatory subunit. Therefore, it is plausible that Ppp2r2a is downstream of Akt and that another Pp2a regulatory subunit controls the subsequent downregulation of Akt. This is consistent with findings in *Caenorhabditis elegans*, in which B56 β and not B55 α (Ppp2r2a) is responsible for dephosphorylation of AKT-1 at the equivalent serine (Padmanabhan et al., 2009).

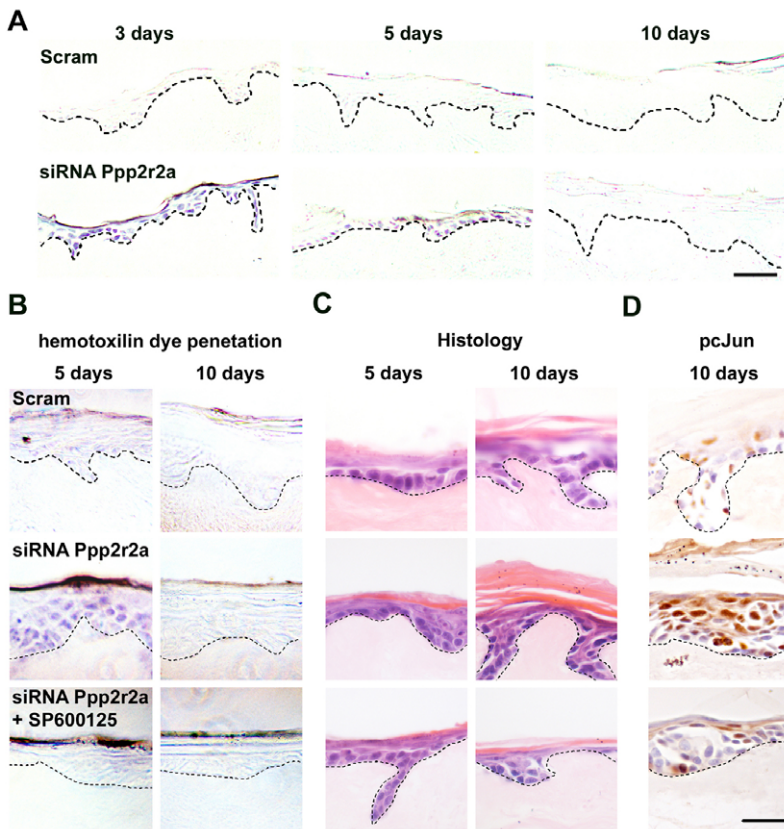


Fig. 8. siRNA knockdown of Ppp2r2a causes a transient barrier defect and hyperkeratosis in organotypic culture, which is restored by JNK inhibition. (A) Hematoxylin barrier assay of organotypic cultures expressing the Ppp2r2a siRNA and scrambled control, grown for 3, 5 and 10 days at the air-liquid interface. Blue nuclei indicate defective barrier function in the siRNA organotypic culture at 3 and 5 days. (B) Hematoxylin barrier assay of organotypic cultures expressing the Ppp2r2a siRNA and scrambled control and the Ppp2r2a siRNA-expressing culture grown in 50 nM SP600125 for 5 and 10 days at the air-liquid interface. (C) Hematoxylin and Eosin staining of the scrambled, siRNA-expressing and SP600125-treated siRNA-expressing cultures showing the hyperkeratotic response in the siRNA-expressing cultures, which is abrogated by addition of the JNK inhibitor SP600125. (D) pJun expression in each of the cultures, confirming reduction of Jun phosphorylation in siRNA-expressing cultures treated with SP600125. Dashed lines indicate the dermal-epidermal junction. Scale bars: 50 μ m.

Ppp2r2a knockdown leads to hyperphosphorylation of Jun, and Ppp2r2a and Jun bind in immunoprecipitation assays, providing evidence that Pp2a holoenzymes containing this regulatory subunit can directly target Jun, in agreement with reports that Pp2a interacts with Jun in bacterial two-hybrid assays (Avdi et al., 2002; Zhao et al., 2008).

Both Pp2a inhibition and Ppp2r2a knockdown led to gross barrier dysfunction, as shown by dye-penetration assays. The only well-characterized function for Pp2a in epidermis is filaggrin dephosphorylation (Kam et al., 1993). Also protein-protein interactions between filaggrin and Hsp27 are necessary for correct cornified layer formation and corneocyte assembly (O'Shaughnessy et al., 2007b), whereas filaggrin mutations are associated with ichthyosis vulgaris, a common barrier dysfunction disorder (Smith et al., 2006). This clearly shows an association, at least via filaggrin, between Pp2a and barrier function. We are proposing here that Pp2a also has important multiple roles in the acquisition of barrier function, including the dephosphorylation of Jun. Further research is warranted on the multiple functions of Pp2a holoenzymes in the epidermis.

In Ppp2r2a knockdown cultures, barrier function is rescued concomitant with premature hyperkeratosis (a thickening of the cornified layers). It is possible, therefore, that the sheer thickness of the cornified layer confers barrier function. This suggests two key ideas. Firstly that initial barrier acquisition precedes the formation of the mature cornified layer in the organotypic model, consistent with observations in mice that barrier acquisition precedes the formation of the mature cornified layer (Hardman et al., 1998). Secondly, these data suggest that hyperkeratosis occurs in response to impaired barrier and is therefore a secondary effect. This is in agreement with the widely held concept that hyperkeratosis is a universal response to barrier impairment (Williams and Elias, 2003). This would also be

consistent with the grafting of transglutaminase 1 null epidermis on nude mice, in which the initial barrier defect in the epidermis is counteracted by hyperkeratosis (Kuramoto et al., 2002).

To conclude, we provide evidence establishing an important role for Akt signaling in the initial acquisition of epidermal barrier function, consistent with known roles of Akt in epidermal barrier

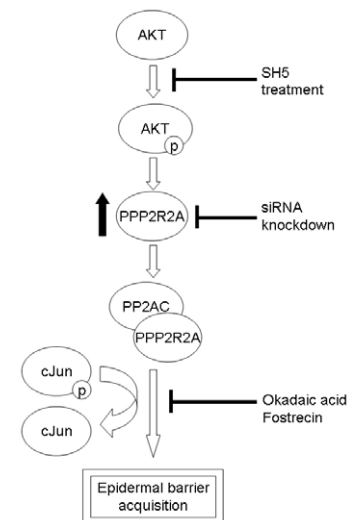


Fig. 9. A model of the mechanism of Jun dephosphorylation by Akt-mediated Pp2a function. Akt activity causes upregulation of Ppp2r2a expression. Inhibition of Akt activity reduces Ppp2r2a expression and leads to barrier defects. Ppp2r2a then forms part of the Pp2a holoenzyme that specifically targets Jun for dephosphorylation, leading to barrier acquisition. Consistent with this, both siRNA knockdown of Ppp2r2a and inhibition of Pp2a activity lead to barrier defects.

function (Peng et al., 2003; O'Shaughnessy et al., 2007b). The discovery of Akt regulation of Ppp2r2a expression and the subsequent control of Jun phosphorylation is yet another important function for Akt signaling in epidermis.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/20/3423/DC1>

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