Targeting of Ras-mediated FGF signaling suppresses Pten-deficient skin tumor

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Deficiency in PTEN (phosphatase and tensin homolog deleted on chromosome 10) is the underlying cause of PTEN hamartoma tumor syndrome and a wide variety of human cancers. In skin epidermis, we have previously identified an autocrine FGF signaling induced by loss of Pten in keratinocytes. In this study, we demonstrate that skin hyperplasia requires FGF receptor adaptor protein Frs2α and tyrosine phosphatase Shp2, two upstream regulators of Ras signaling. Although the PI3-kinase regulatory subunits p85α and p85β are dispensable, the PI3-kinase catalytic subunit p110α requires interaction with Ras to promote hyperplasia in Pten-deficient skin, thus demonstrating an important cross-talk between Ras and PI3K pathways. Furthermore, genetic and pharmacological inhibition of Ras–MAPK pathway impeded epidermal hyperplasia in *Pten* animals. These results reveal a positive feedback loop connecting Pten and Ras pathways and suggest that FGF-activated Ras–MAPK pathway is an effective therapeutic target for preventing skin tumor induced by aberrant Pten signaling.

FGF | skin | Ras | Pten | Erk

As a commonly mutated tumor suppressor gene, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) plays critical roles in tissue homeostasis and cancer development (1). In PTEN hamartoma tumor syndrome (PHTS), germline mutations of *PTEN* cause hyperplastic changes in the skin, which manifest into acral keratosis and papilloma (2). Although somatic mutation of *PTEN* is rare in skin lesions, the majority of human actinic keratosis and squamous cell carcinoma (SCC) of the skin exhibited reduced levels of PTEN, suggesting that epigenetic or posttranscriptional down-regulation of PTEN is an important risk factor in skin tumorigenesis (3–5). Consistent with this, genetic ablation of *Pten* in mouse models readily leads to skin hyperkeratosis, papilloma, and eventually SCC (6–8).

FGF signaling has both pro- and antitumorigenic functions in the skin. Aged animals lacking *Fgfr2b* in the epidermis displayed thickening in the skin and heightened sensitivity to a chemical carcinogen, whereas overexpression of Fgf7 or Fgf10, two cognate ligands of Fgfr2b, resulted in epidermal hyperplasia and tumor formation (9, 10). Paracrine FGFR2 signaling has also been found to be important in mediating the oncogenic activity of *p63*, which is frequently amplified in SCC (11). We recently identified a critical link between Pten and FGF signaling, showing that elevated PI3K signaling in *Pten*-deficient epidermis led to a mammalian target of rapamycin (mTOR)-regulated increase in Fgf10 translation (4). The resulting Fgf10–Fgfr2 signaling in the epidermis was both necessary and sufficient to promote skin tumorigenesis. Interestingly, activating mutations in *FGFR3* and *PIK3CA*, which encode the catalytic subunit of PI3 kinase, have been identified in epidermal nevi and seborrheic keratosis (12, 13). Although these skin lesions have different pathologies from papillomas and SCCs, all of them share the epidermal hyperplasia phenotype. These observations suggest that FGFR and PI3K signaling are intimately connected in human skin tumorigenesis.

How FGF signaling promotes skin tumorigenesis is not well understood (14). A myriad of cytoplasmic proteins have been implicated in mediating FGF downstream signaling (15). The most important among them is adaptor protein Frs2, which can activate Ras signaling by recruiting Grb2 and tyrosine phosphatases Shp2. In addition, Gab1 protein can stimulate PI3K signaling by direct binding to a PI3K regulatory subunit, and PLCγ proteins can induce PKC signaling. Adding to the complexity of the FGF signaling network, there is extensive cross-talk among its downstream pathways. For example, Ras protein can directly bind and activate PIK3CA (16). Conversely, some studies indicate that PI3K-activated AKT may also phosphorylate Ras downstream effector Raf to suppress its activity (17, 18). Interestingly, it has been observed that 7,12-Dimethylbenz[a]anthracene (DMBA)-12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin carcinomas in *Pten* heterozygous mice exhibit either *H-ras* activation or a complete loss of *Pten* alleles, but not both (19). The underlying cause for such mutual exclusivity between *Ras* and *Pten* mutations in skin cancer remains unsolved.

In this study, we took a genetic approach to dissect FGF downstream pathways in skin tumorigenesis. Our results show that *Pten*-deficient skin requires Frs2 and Shp2, suggesting that Frs2/Shp2-activated Ras signaling may be the critical downstream

Significance

***PTEN* (phosphatase and tensin homolog deleted on chromosome 10) deficiency causes skin lesions in *PTEN* hamartoma tumor syndrome patients, and has been implicated in hyperkeratosis and squamous cell carcinoma in the general population. In this study, we show that FGF-induced Ras signaling acts in a feedback mechanism to enhance PI3K activity and promotes epidermal hyperplasia through the MAPK pathway. Genetic and pharmacological targeting of the FGF–Ras–MAPK pathway elicits a strong antiproliferative response. Our finding suggests that FGF–Ras signaling can be explored for therapeutic intervention to treat epidermal lesions in *PTEN*-related hamartoma and skin cancer.**

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The authors declare no conflict of interest.

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effector of FGF signaling. In support of this, although deletion of the p85 regulatory subunit of PI3K had no effect, disruption of the Ras interaction with the p110α catalytic subunit of PI3K suppressed skin hyperplasia. Furthermore, genetic ablation of the Raf–Mek–Erk signaling cascade blocked tumorigenesis in *Pten*-deficient epidermis. These results show that Ras signaling both enhances PI3K activity to cooperate with Pten deletion and also promotes skin hyperplasia by activating the MAPK pathway. Finally, we found that pharmacological inhibition of Erk was sufficient to prevent and even to reverse epidermal hyperplasia induced by Pten deletion. These results demonstrate that targeting of MAPK signaling is an effective approach to treat Pten-deficient skin lesion.

Results

Frs2α and Shp2 Convey FGF Signaling in *Pten*-Deficient Epidermis. Previous studies have established that epidermal deletion of *Pten* in mouse results in skin lesions that closely mimic human PTHS and the onset of SCC (6–8). Using the *Le-Cre* driver to target keratinocytes in the cheek and the eyelid, we observed similar epidermal hyperplasia in 1-mo-old *Le-Cre;Pten*$^{flox/flox}$ (*Pten*^{CKO}) animals (Fig. 1*A*). Analysis of this mouse model demonstrated that loss of Pten led to increasing expression of Fgf10 in keratinocytes, which activated Fgfr2 in an autocrine loop to promote skin tumorigenesis (4). To further determine the mediators of FGF signaling in *Pten*-deficient skin tumor, we genetically ablated the adapter protein *Frs2α*, the predominantly expressed Frs2 family member, in skin epidermis (Fig. S1*A*) (20). Histological analysis showed that deletion of *Frs2α* alone did not cause overt skin abnormality, but the epidermal hyperplasia phenotype was completely suppressed in *Pten*^{CKO};*Frs2α*^{CKO} animals (Fig. 1*A* and Fig. S1 *B* and *C*). Unlike *Pten*^{CKO} animals that exhibit significant expansions in both the K14^{+} basal layer and the K10^{+} suprabasal layer in the epidermis, these two keratinocyte layers in *Pten*^{CKO};*Frs2α*^{CKO} remained unchanged compared with those of control animals. Consistent with abrogation of the hyperplasia phenotype, the proliferation marker K_i-67 and basal epidermis marker ΔNp63 in *Pten*^{CKO};*Frs2α*^{CKO} skin also reverted to the wild-type levels. By immunostaining and Western blot analysis, however, we showed that pAKT, pS6, and p4EBP1 levels remained elevated, reflecting the up-regulation of PI3K signaling induced by Pten deletion (Fig. 1 and Fig. S1*D*). As a result, we still observed increased Fgf10 expression in *Pten*^{CKO};*Frs2α*^{CKO} skin epidermis (Fig. 1 and Fig. S1*E*). We recently showed that FGF signaling requires cooperation between Frs2α and Shp2 to activate Ras signaling in eye development (21, 22). This genetic pathway is apparently conserved in skin lesion, as *Pten*^{CKO};*Shp2*^{CKO} also lacks the epidermal hyperplasia phenotype despite elevated levels of PI3K activity and Fgf10 expression (Fig. 1 and Fig. S1*D*). These results suggested that Frs2α and Shp2 are essential components of the Pten–FGF signaling axis in skin hyperplasia.

Ras Binding to p110α Enhances PI3K Signaling. Ras is the downstream target of Frs2α and Shp2, and it is also known to interact with PI3 kinase (23). The PI3 kinase consists of two subunits: a regulatory subunit p85, which can be directly stimulated by receptor tyrosine kinases, and a catalytic subunit p110, responsible for lipid phosphorylation. Importantly, the p110α subunit also contains a Ras-binding domain (RBD), which has been shown to be required for Ras-induced transformation in multiple types of cancers (23). To examine PI3K signaling in the epidermis, we generated a conditional knockout of p85α and p85β, the two major regulatory subunits of PI3 kinase (24). Surprisingly, we still observed significant facial swelling in adult *Le-Cre;Pten*$^{flox/flox}$;*p85α*$^{flox/flox}$;*p85β*$^{KO/KO}$ (*Pten*^{CKO};*p85*^{CKO}) animals (Fig. S2). This is confirmed by histological analysis, which showed similar expansion of the epidermal layers in *Pten*^{CKO} and *Pten*^{CKO};*p85*^{CKO} skin.

Because p85α and p85β subunits of PI3 kinase were dispensable for *Pten*-depleted skin lesion, we next considered whether

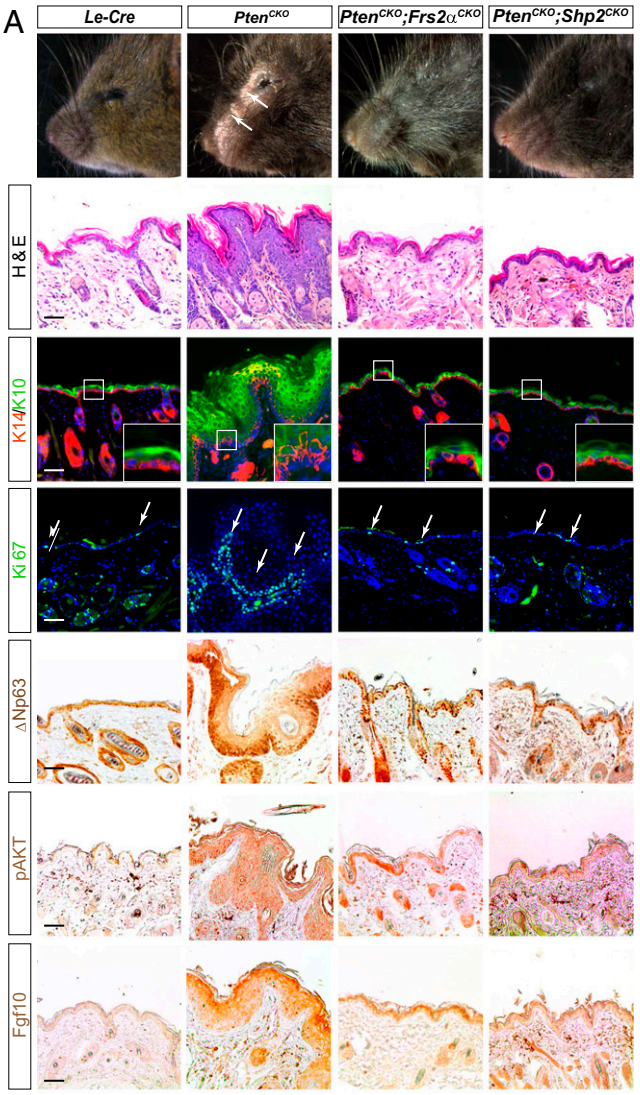

Fig. 1. *Frs2α* and *Shp2* mediate FGF signaling in *Pten*-null epidermis. (*A*) Phenotypic comparison of facial skin in *Le-Cre*, *Pten*^{CKO}, *Pten*^{CKO};*Frs2α* ^{CKO}, and *Pten*^{CKO};*Shp2* ^{CKO} mice. Rescue of the hyperplasia phenotype was evident in *Pten*^{CKO};*Frs2α* ^{CKO} and *Pten*^{CKO};*Shp2* ^{CKO} mice, as shown by H&E and immunostaining for K14/K10, K_i-67, ΔNp63, and Fgf10 on transverse sections of facial skin. Arrows point to K_i-67^{+} cells in the epidermis. Note that pAKT and Fgf10 remained elevated in both mutants. (Scale bars, 50 μm.) (Magnification, *Insets*: 3×.) (*B*) Immunoblotting analyses showed that expression of Fgf10 and PI3K downstream targets pAKT and pS6 was unaffected by ablation of *Frs2α*, but pErk was down-regulated.

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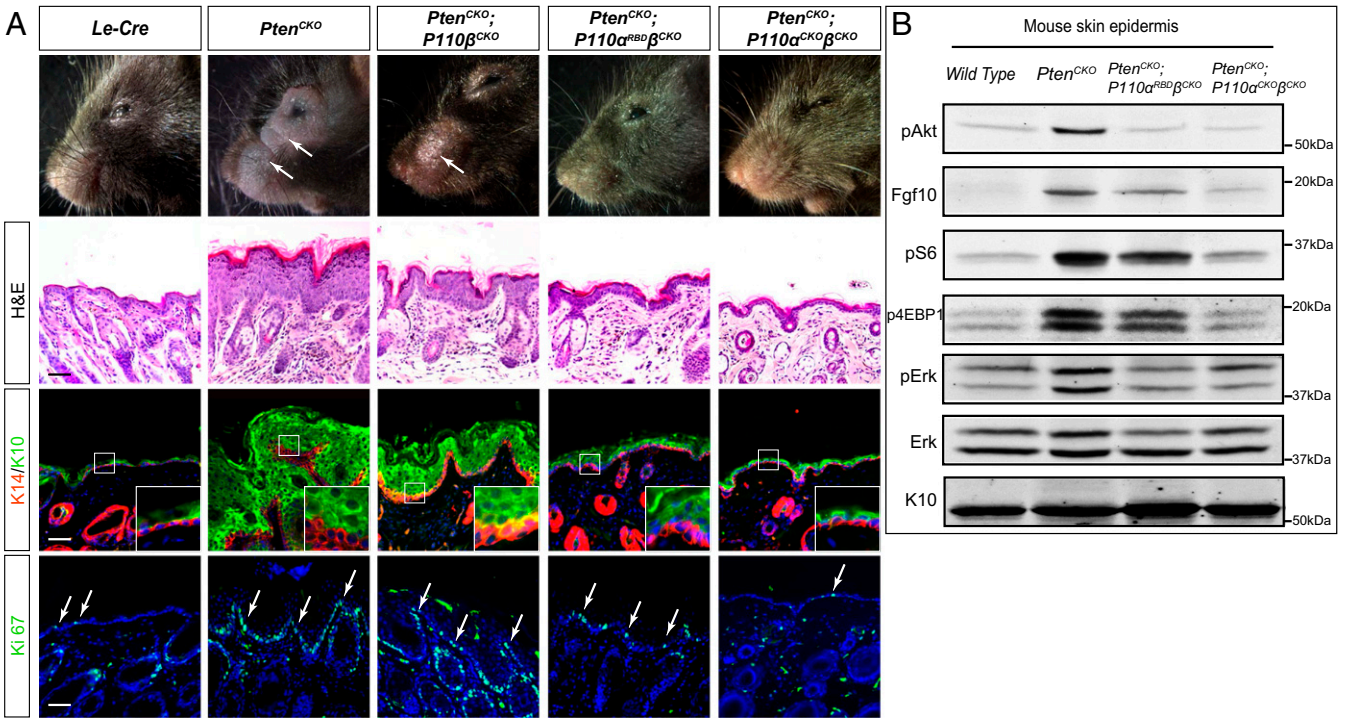

Fig. 2. Ras signaling enhances PI3K-AKT pathway. (*A*) Four-week-old $Pten^{CKO}$ and $Pten^{CKO}$;$p110\beta^{CKO}$ mice displayed hyperplasia in the epidermis (arrows). H&E, K14/K10, and K_i-67 staining in the epidermis showed considerable attenuation of hyperplasia phenotype in $Pten^{CKO}$;$p110\alpha^{RBD}\beta^{CKO}$ and $Pten^{CKO}$;$p110\alpha^{CKO}$;β^{CKO} mice. (Scale bars, 50 μm.) (Magnification, *Insets*: 3×.) (*B*) Immunoblots of mouse facial skin epidermal layer showed that Fgf10, AKT, and ERK signaling were reduced in $Pten^{CKO}$;$p110\alpha^{RBD}\beta^{CKO}$ and $Pten^{CKO}$;$p110\alpha^{CKO}$;β^{CKO} skin epidermis.

PI3 kinase may be directly stimulated by the Ras–p110α interaction. In *Pten*-deficient skin, the epidermal hyperplasia phenotype requires two PI3K catalytic subunits, p110α and p110β (25). Combined deletion of both *p110α* and *p110β* was shown to completely block skin lesions in the absence of *Pten*. Indeed, we observed that skin hyperplasia was partially reduced in *Le-Cre;*$Pten^{flox/flox}$;$p110\beta^{flox/flox}$ ($Pten^{CKO}$;$p110\beta^{CKO}$) mice and abolished in *Le-Cre;*$Pten^{flox/flox}$;$p110\alpha^{flox/flox}$;$p110\beta^{flox/flox}$ ($Pten^{CKO}$;$p110\alpha^{CKO}\beta^{CKO}$) animals (Fig. 2*A* and Fig. S3 *A* and *B*). Taking advantage of a *p110α* allele ($p110\alpha^{RBD}$) that contains two point mutations to disrupt its Ras-binding activity (23), we generated *Le-Cre;* $Pten^{flox/flox}$;$p110\alpha^{RBD/flox}$;$p110\beta^{flox/flox}$ ($Pten^{CKO}$;$p110\alpha^{RBD}\beta^{CKO}$) animals. Compared with $Pten^{CKO}$ mice, the facial skin in $Pten^{CKO}$;$p110\alpha^{RBD}\beta^{CKO}$ mutants was drastically reduced in thickness, although it was still moderately thicker than $Pten^{CKO}$;$p110\alpha^{CKO}\beta^{CKO}$ mice (Fig. 2*A* and Fig. S3 *A* and *B*). Although $Pten^{CKO}$;$p110\alpha^{RBD}\beta^{CKO}$ skin epidermis still showed elevated levels of pS6 and p4EBP1, phosphorylation of Erk was reduced to the wild-type level (Fig. 2*B* and Fig. S3 *A* and *C*). Interestingly, we observed that *Spry4* and *Dusp7*, two repressors of MAPK signaling, were induced in $p110\alpha^{RBD/flox}$;$p110\beta^{flox/flox}$ skin keratinocytes treated with Pten inhibitor and Cre-expressing adenovirus (Fig. S3 *D* and *E*). This suggests that Ras-independent activation of p110α may negatively regulate Erk signaling via *Spry4* and *Dusp7*. Finally, analysis of differentiation and proliferation markers K14/K10, K_i-67, and ΔNp63 further confirmed that $Pten^{CKO}$;$p110\alpha^{CKO}\beta^{CKO}$ mice were resistant to skin hyperplasia induced by Pten loss. Immunohistochemistry and Western blot analysis of $Pten^{CKO}$;$p110\alpha^{CKO}\beta^{CKO}$ skin epidermis showed that pAKT, pS6, and p4EBP1 were reduced compared with those of $Pten^{CKO}$ samples (Fig. 2*B* and Fig. S3*A*). Taken together, these results suggested that Ras signaling directly activates p110α to enhance PI3K signaling, synergizing with Pten deletion in epidermal hyperplasia.

The MAPK Pathway Is Vital for Skin Hyperplasia Induced by Pten Loss. Loss of Pten in keratinocytes has been reported to induce phosphorylation of Erk, the canonical target of Ras–MAPK signaling (6, 8). We similarly observed a consistent increase in the pErk level in $Pten^{CKO}$ skin epidermis, which was abrogated by deletion of p110α and p110β (Fig. 2*B*). This raises the possibility that MAPK signaling may be functionally important in *Pten*-null skin tumor. Ras activates the MAPK cascade by initiating the sequential phosphorylation of Raf, Mek, and Erk kinases, which ultimately phosphorylate a diverse array of nuclear and cytoplasmic substrates (26). Among the three *Raf* kinase genes in the mouse genome, Araf is ubiquitously expressed, but it also has the lowest kinase activity toward Mek (27). On the other hand, Braf and Craf can form homo- and heterodimers in a Ras-dependent fashion, which accounts for the majority of Raf kinase activities (26). In fact, previous studies have shown that ablation of *Craf* alone was sufficient to suppress Ras-dependent tumors in the lung and skin (28, 29). We thus generated *Le-Cre;*$Pten^{flox/flox}$;$Braf^{flox/flox}$;$Craf^{flox/flox}$ ($Pten^{CKO}$;$Brff/Crff^{CKO}$) animals to ablate *Braf* and *Craf* in the epidermis. Surprisingly, despite of the moderate reduction of pErk in the epidermis, the skin hyperplasia phenotype was only modestly mitigated in $Pten^{CKO}$;$Brff/Crff^{CKO}$ mice (Fig. 3*A* and Fig. S4 *A*–*C*), suggesting that *Braf* and *Craf* were not essential for Pten-deficient skin hyperplasia.

Studies in mammals have clearly demonstrated that in certain contexts, Raf could transmit Ras signaling through effectors other than Mek. Conversely, Mek may also function independently of Raf kinases and Erk independently of Mek activity (30–32). This has been supported by phenotypes of various murine Raf, Mek, and Erk kinase knockouts, which resemble each other in some but not all developmental processes. To test if the remaining cascade of the MAPK signaling is still required for skin lesion, we next deleted Mek and Erk kinases in *Le-Cre;*$Pten^{flox/flox}$;$Mek1^{flox/flox}$;$Mek2^{KO/KO}$ ($Pten^{CKO}Mek1/2^{CKO}$) and *Le-Cre;*$Pten^{flox/flox}$;$Erk1^{KO/KO}$;$Erk2^{flox/flox}$

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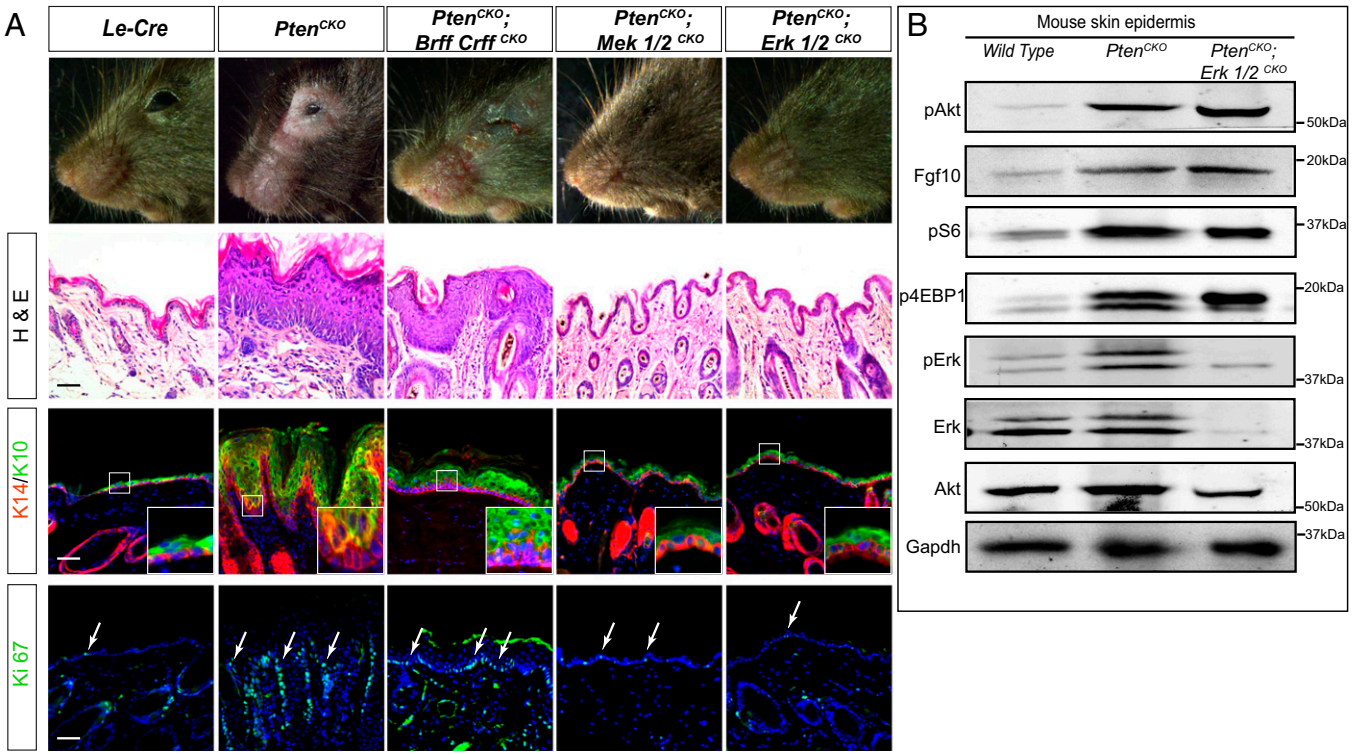

Fig. 3. MAPK pathway activated by Pten–Fgf10 signaling axis is essential for skin hyperplasia. (*A*) In comparison with $Pten^{CKO}$ mice, the epidermal hyperplasia phenotype was moderately reduced in $Pten^{CKO};BrffCrff^{CKO}$ and completely abolished in $Pten^{CKO};Mek1/2^{CKO}$ and $Pten^{CKO};Erk1/2^{CKO}$ mice, as shown by H&E of facial epidermis and immunostaining analyses of K14/K10 and K_i-67. (Scale bars, 50 μm.) (Magnification, *Insets*: 3×.) (*B*) Immunoblotting analyses of the epidermal skin lysate.

($Pten^{CKO}Erk1/2^{CKO}$) mice, respectively. Staining for K14/K10 showed a drastic shrinkage in epidermal layers, and K_i-67 and ΔNp63 indices were indistinguishable from those of WT control mice (Fig. 3*A* and Fig. S4 *A* and *B*). Notably, deletion of Mek and Erk did not induce abnormal apoptosis or abrogate the increase in Fgf10, pS6, and p4EBP1 induced by Pten deletion (Fig. 3 and Fig. S4*D*). Together, these genetic evidence demonstrate that Ras–MAPK effectors Mek and Erk are required for mediating the Pten–Fgf10–Fgfr signaling axis in skin hyperplasia.

Erk Is a Pharmacological Target Critical for *Pten*-Null Skin Hyperplaisa. The striking rescue of the skin hyperplasia phenotype by *Erk* deletion prompted us to investigate whether pharmacological targeting of Erk protein in $Pten^{CKO}$ mice would prevent skin lesion. Wild-type and $Pten^{CKO}$ mice starting at 3 wk of age were dosed twice daily by intraperitoneal injection with Erk inhibitor SCH772984 (25 mg/kg) or vehicle (33). During 2 wk of treatment, whereas vehicle-treated mice showed rapid emergence of hyperplasia, inhibitor-treated animals remained free of lesions (Fig. 4 *A*–*C* and Fig. S1*B*). There was no significant change in body weight between dosed and vehicle-treated mice, but histological analysis showed a substantial decrease in skin thickness in treated mice (Fig. 4 *A* and *B*). K14/K10 staining in skin sections from SCH772984-treated mice also confirmed significant suppression of epidermal expansion. Compared with vehicle-treated $Pten^{CKO}$ mice, drug treatment resulted in a substantial reduction in hyperproliferative cells as shown by K_i-67 and ΔNp63 staining (Fig. 4 *A* and *B*). Lastly, we treated $Pten^{CKO}$ mice at 5 wk when hyperplasia was already apparent. After 2 wk of treatment, the skin epidermis was drastically reduced in thickness, accompanied by diminished expression of K14/K10, K_i-67, and ΔNp63 (Fig. S5 *A*–*C*). This effect is likely cell autonomous, as we observed that hyperproliferation induced by Pten inhibition was similarly suppressed by addition of Erk inhibitor in mouse keratinocyte cultures (Fig. S5*D*). Hence, pharmacological inhibition of Erk signaling could not only inhibit but also reverse lesion formation in *Pten*-deficient epidermis.

Discussion

PTEN deficiency causes skin lesions in PHTS patients and promotes hyperkeratosis and SCC (3–5). Based on our previous finding that loss of *Pten* induced FGF signaling in keratinocytes, we investigated the genetic cascade that led to skin hyperplasia. Our results showed that epidermal FGF signaling is transmitted by Frs2α and Shp2, which are required for Ras activation. In support of the key role of Ras in *Pten*-deficient lesion, genetic disruption of the Ras–PI3K interaction or Ras–MAPK pathway diminished the hyperproliferative effect of Pten loss. Pharmacological inhibition of Erk also blocked lesion formation in Pten-depleted skin (Fig. 4*D*). These results suggest that FGF signaling activates the Ras pathway to cooperate with Pten deletion in skin lesion.

Activation of Ras signaling by the Pten–FGF axis explains a long-standing conundrum in skin cancer—namely, the mutually exclusive nature of *Ras* and *Pten* mutations in a mouse model of SCC. In the wild-type skin, DMBA–TPA treatment is known to predominantly induce *H-ras* mutations (19). The same treatment on the skin of $Pten^{+/-}$ mice, however, produced carcinoma that carried either loss-of-heterozygosity mutations in *Pten* or activation mutations in *H-ras*, but not both. Consistent with this, transgenic overexpression of activated *H-ras* did not significantly enhance malignant transformation of skin tumor generated by genetic ablation of *Pten* (8). According to our model of the Pten–FGF–Ras signaling cascade, deletion of Pten itself is sufficient to induce Ras signaling in skin epidermis, suggesting that there is little additional benefit to induce mutation in the *H-ras* locus. In

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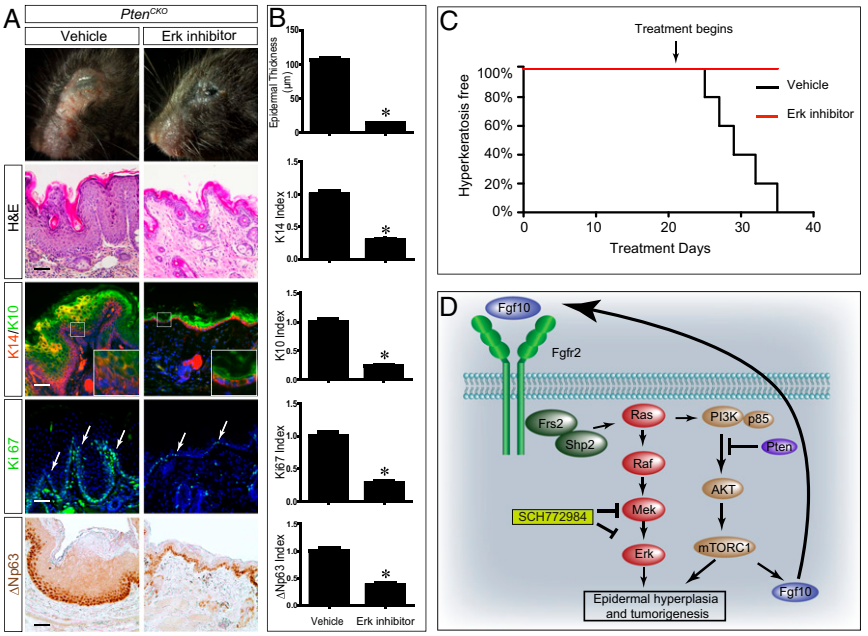

Fig. 4. Pharmacological targeting of Erk prevents *Pten*-deficient skin lesion. (*A* and *B*) Phenotypic comparison of hyperplasia in $Pten^{CKO}$ mice after pharmacological inhibition of Erk. Administration of Erk inhibitor SCH772984 or vehicle control in $Pten^{CKO}$ mice started at 3 wk of age and continued to 5 wk of age. SCH772984-treated $Pten^{CKO}$ mice showed significant reduction in skin thickness, keratinocyte proliferation, and differentiation as observed by K14/K10, K_i-67, and ΔNp63 staining. (Student *t* test: $*P < 0.001$; $n = 9$). (*C*) Kaplan–Meier curve for $Pten^{CKO}$ mice treated with inhibitor (red, $n = 5$) or vehicle (black, $n = 5$) only [log rank (Mantel–Cox) test: $*P < 0.001$; $n = 5$]. The appearance of hyperplasia in each animal was considered as an event toward skin lesion. (Scale bar, 50 μm.) (Magnification, *Insets*: 3×.) (*D*) Model of Ras-mediated FGF signaling in promoting epidermal hyperplasia and tumorigenesis in Pten-deficient skin. Loss of Pten in keratinocytes results in mTORC1-mediated induction of FGf10 to activate Fgfr2. This leads to an Frs2–Shp2–Ras signaling cascade that enhances PI3K signaling and stimulates the Ras–Mek–Erk pathway, both of which are critical for epidermal hyperplasia and eventual tumorigenesis. These skin lesions can be effectively inhibited by Erk inhibitor SCH772984, suggesting a therapeutic approach for PHTS.

contrast, further activation of H-ras in *Pten*-null skin may even be counterproductive, as the overall Ras signaling is pushed too high that it could trigger oncogene-mediated senescence.

Our study suggests that Ras signaling also stimulates PI3K signaling in skin keratinocytes. In Pten-deficient skin lesions, activity of the PI3K pathway is greatly elevated. Although this originated from the removal of Pten as the negative regulator of PI3K signaling, it may also involve a positive regulation of PI3K itself to activate its enzymatic activity (24). The most common mechanism to stimulate PI3K is through its p85 regulatory subunits, whose SH2 domains bind a wide variety of cell-surface receptors or adaptor proteins. In *Pten* mutant skin, however, our results show that deletion of PI3K regulatory subunits *p85α/β* failed to suppress epidermal hyperplasia. Instead, mutation in the RBD of *p110α* strongly reduced the level of pAKT, resulting in significant mitigation of the hyperplasia phenotype. This is consistent with a previous study in mouse embryonic fibroblast cells, which suggests that direct binding of Ras to the RBD motif of p110α is required for FGF2 signaling to AKT (23). Furthermore, blocking the Ras binding region of the p110 alpha subunit also interfered with skin tumor formation induced by H-Ras and lung adenomas driven by Kras (34). Notably, deletion of *p110β* alone also moderately attenuated the *Pten* epidermal phenotype (25). Although p110β does not directly interact with Ras, it can be stimulated by Rac1 and Cdc42, two small Rho GTPases downstream to both receptor tyrosine kinase and G protein-coupled receptor (GPCR) signaling (35). Given this evidence, our work suggests that FGF-mediated positive feedback to the PI3K–Akt pathway is an important oncogenic mechanism in *Pten*-deficient skin.

Perturbations in the RAS–MAPK pathway are common in skin tumors (36). Consistent with previous studies, we observed that the level of pErk was also elevated in Pten-deficient keratinocytes (6, 8). Contrary to previous studies that indicated a crucial role of *Craf* in Ras-driven lung and skin cancer, we found that deletion of both *Braf* and *Craf* only resulted in modest reduction of epidermal hyperplasia (28, 29). Nevertheless, ablation of either Mek or Erk completely suppressed hyperplasia in Pten-deficient skin. Importantly, we showed that SCH772984, a small molecule inhibitor of ERK, was not only effective in preventing epidermal hyperplasia induced by Pten loss; it could also reverse the hyperplasia phenotype after skin lesions were already established. Significant efforts are under way to develop pharmacological inhibitors against the RAS–MAPK pathway, some of which are already approved for clinical use. Our results suggest that FGF–Ras signaling can be explored for therapeutic intervention to treat skin lesions in PHTS and other PTEN-related conditions.

Methods

Mice. Mice carrying $Frs2\alpha^{flox}$, $Shp2^{flox}$, $p110\alpha^{flox}$, $p110\beta^{flox}$, $p110\alpha^{RBD}$ (JAX strain name - B6.129S7(Cg)-$Pik3ca^{tm1Jdo}$/J), $Erk1^{KO}$, $Erk2^{flox}$, $Mek1^{flox}$, $Mek2^{KO}$, $Braf^{flox}$, and $Craf^{flox}$ alleles were bred and genotyped as described (23, 37–42). From Lewis Cantley's laboratory, Weill Cornell Medical College, New York, we obtained the floxed allele of *p85α* (*Pik3r1*) and a knockout allele of *p85β* (*Pik3r2*), two genes that encode the main regulatory subunits of class IA PI3K, p85α/p55α/p50α and p85β, respectively (43, 44). *Le-Cre* mice were kindly provided by Ruth Ashery-Padan, Tel Aviv University, Tel Aviv, Israel (45). $Pten^{flox}$ mice were obtained from Jackson Laboratory (46). All animal experiments were performed according to institutional guidelines and approved by the Columbia University Institutional Animal Care and Use Committee (IACUC).

Keratinocyte Culture. Mouse keratinocytes were isolated and cultured from P0 pups following protocol as described (4). The cells were maintained in keratinocyte-specific basal media from CELLnTEC. A total of 1×10^5 wild-type keratinocytes per well were plated in six-well culture plates and treated with vehicle, Pten inhibitor (VO-OHpic trihydrate; 400 nM), Erk inhibitor (U0126; 10 μM), or both. Total cell count posttreatment was recorded with an automated cell counter at day 2, 4, 6, and 8 using the Trypan blue dye exclusion test.

Histology and Immunohistochemistry. Briefly, after the mice were euthanized, skin from the cheek continuing through and around the eyelids were removed and placed in 4% (wt/vol) PFA overnight. The skin samples collected were either paraffin- or cryo-embedded and sectioned at 8–10-μm thickness. H&E staining was performed using a Leica Automatic Stainer and immunohistochemistry following antigen retrieval in sodium citrate buffer as described (47). Antibodies used are K_i-67 (BD Pharmingen; Clone B56; 1:100), ΔNp63 (Biolegend; Poly6190; 1:500), Keratin 10 (Covance; PRB-159P; 1:200), Keratin 14 (Thermo Scientific; LL002; 1:100), phospho-4E-BP1(Cell Signaling Technology; 236B4; 1:500), phospho-S6 (Cell Signaling Technology; D57.2.2E; 1:500), phospho-Akt^{S473} (Cell Signaling Technology; D9E; 1:100), Fgf10 (Millipore; ABN44; 1:100), pErk (Cell Signaling Technology; E10; 1:1000), and Erk (Santa Cruz; H-72; 1:3000).

K10, K14, K_i-67, and ΔNp63 indices were calculated by counting the number of positively stained nuclei within 100-μm-long skin tissue sections and compared with wild-type controls; one-way ANOVA was performed to determine *P* value.

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Western Blot. Immediately after mice were euthanized, hair around the face was removed using Nair hair removal cream. Skin around the cheek and eyelid area was dissected out in one piece, snap-frozen, and stored at –80 °C until use. The dissected skin samples were then carefully scraped with a curette to selectively remove only the epidermal layer. Scrapings were then lysed in RIPA buffer containing protease inhibitors. Protein concentrations in the lysates were determined using the BCA kit. Equal amounts of the lysates were loaded and separated on 10–12% (wt/vol) SDS/PAGE gels before being transferred onto a PDVF membrane. The blots were stained with primary antibodies overnight and visualized using IRDye-linked secondary antibody.

Quantitative Real-Time PCR. Total RNA was extracted from skin and keratinocyte samples and converted to cDNA using SuperScriptIII RT kit as described (48). Primers used for quantitative PCR analysis are as follows: *Spry2*, CAATGGCAGGCAAATGTATG and GGAGGAAGTGAGCAGAGGTG; *Spry4*, GCAGCGTCCCTGTGAATCC and TCTGGTCAATGGGTAAGATGGT; *Dusp6*, CGTGTGGACCTTGGTGG and ACACGGACAGAACGGATCTC; *Dusp7*, GTGCTGCTCTACGACGAGG and TGAAACCACCTTGGAGGTAGT; and *Gapdh*, AGGTCGGTGTGAACGGATTTG and TGTAGACCATGTAGTTGAGGTCA.

Inhibitor Treatment. $Pten^{CKO}$ mice were injected intraperitoneally with SCH772984 (Chemitek) at a 25-mg/kg dosage twice daily or with vehicle control for a period of 14 d as described (33, 49). A SCH772984 stock solution of 10 mg/mL was prepared by dissolving 11.9 mg SCH772984 in 986 μL 20% hydroxy propyl β cyclodextrin and 14.3 μL 2N NaOH, followed by continuous vortexing/sonication for 5 min and pH adjustment to 4.5. Before dosing, SCH772984 solution was brought to room temperature (~30 min). The mice were monitored daily for changes in hyperplasia phenotype, and facial epidermal tissue was harvested and used for histological analysis after the trial period.

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