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Inducible Activation of Ras and Raf in Adult Epidermis[1]

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ABSTRACT

Ras effects vary with developmental setting, with oncogenic *RAS* activation implicated in epithelial carcinogenesis. In epidermal cells, previous studies described conflicting Ras impacts on growth and differentiation, with the only *in vivo* studies relying on constitutive alterations of Ras function throughout development. To study Ras effects in developmentally mature adult epidermis, we expressed a 4-hydroxytamoxifen (4OHT)-regulated Ras fusion in transgenic mice using the keratin 14 promoter. Resulting adult K14-ER:Ras mice displayed 4OHT-inducible activation of Ras as well as elements of Raf/mitogen-activated protein kinase (MAPK) but not RalGDS/Ral or phosphatidylinositol 3′-kinase (PI3K)/Akt downstream Ras effector pathways. Ras reversibly induced massive cutaneous hyperplasia and suppressed differentiation. Ras-driven hyperproliferation was accompanied by increases in β1 and β4 integrin epidermal progenitor markers. Epidermal expression of inducible Raf produced similar changes. These findings indicate that activation of Ras in adult epidermis promotes proliferation and inhibits differentiation and that Raf is sufficient to mediate these effects.

INTRODUCTION

Ras GTPases can either promote or oppose cellular growth and differentiation, depending on the tissue context and developmental setting, as well as the signal strength and duration (1, 2). Somatic mutations leading to expression of active Ras mutants have been observed in epidermal carcinogenesis (3, 4) and are believed to contribute to the pathogenesis of a subset of human SCCs.[4] In epidermal keratinocytes, however, conflicting data have been presented suggesting that Ras/Raf/MAPK either promotes proliferation and suppresses differentiation (5–7) or does the opposite (8, 9). Work performed to date *in vivo* has altered Ras function in a constitutive manner throughout epidermal development. These studies have shown that overexpression of constitutively active oncogenic *RAS* leads to epidermal proliferation and inhibits differentiation (7, 10–12), whereas expression of dominant-negative Ras leads to hypoproliferation and premature differentiation (7). These *in vivo* studies, however, suffer from the limitation that they all rely on overexpressing Ras mutants through gestation, making it difficult to separate primary Ras impacts from secondary developmental effects. The results of direct activation of Ras signaling in adult epidermis have not yet been reported.

Ras GTPases act via downstream effector cascades to alter cellular growth and differentiation (13). Of these cascades, those proceeding via Raf/MAPK, PI3K/Akt and RalGDS/Ral are perhaps the best characterized (14). Assigning Ras effector function, however, is complicated by the fact that Ras does not activate all of its known targets in all cell types. For example, although Ras triggers PI3K pathway function readily in fibroblasts, it fails to do so in lymphocytes (15). In epidermal tissue, the effects of activating specific Ras effectors have not been systematically examined. The role of specific effector cascades in epidermal Ras signaling and whether Ras is even capable of activating specific individual downstream cascades in epidermal tissue is also not fully characterized.

Here we demonstrate that activation of Ras in developmentally mature epidermis promotes proliferation and suppresses differentiation. These effects are accompanied by increases in markers associated with epidermal progenitor cells. In epidermal tissue, Ras induction leads to activation of elements of the Raf/MAPK but not PI3K/Akt or RalGDS/Ral downstream signaling cascades. Inducible activation of Raf itself in adult epidermis demonstrates that Raf is sufficient to mediate these Ras effects on growth and differentiation. Our findings thus indicate that activating Ras in adult epidermis promotes the proliferative undifferentiated epidermal cell state characteristic of neoplasia and suggest that these effects can proceed via the Raf downstream effector pathway.

MATERIALS AND METHODS

Transgenic Animals. Sequence encoding the NH_2-terminal ER:Ras (7) and COOH-terminal Raf:ER (16) fusions were subcloned downstream of a 2075-bp human K14 promoter construct, which targets expression to keratinocytes within the basal epidermal layer, and used to produce transgenic mice. Transgene integration and copy numbers were confirmed by PCR followed by Southern blot analysis. The following primers are used in PCR genotyping: 5′-CACCACCAGCTCCACTTCAG CACATT-3′ and 5′-CGCACCAACGTGTAGAAG GCATCCTC-3′ (for K14-ER:Ras); 5′-CGTGC TGGTTATTGTGCTGTCT CATCA-3′ and 5′-GAGGCGGTCGTGGGTCTGGAAA-3′ (for K14-Raf:ER). An *Eco*RI-*Eco*RV fragment of K14-ER:Ras and an *Xho*I-*Eco*RI fragment of Raf:ER were used in Southern analysis. To activate ER:Ras and Raf:ER in the skin of adult transgenic mice, 1 mg of 4OHT (Sigma) per day, dissolved in ethanol (1 mg/0.1 ml), was applied topically to a shaved area of dorsal skin. Genetically matched wild-type littermates were treated in the same way as controls in all of the experiments.

Protein Expression. Skin samples were homogenized in lysis buffer [25 mM HEPES (pH7.5), 150 mM NaCl, 1% NP40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol] with protease inhibitors and were denatured by boiling with ~20 μg of extract loaded per lane. Antibodies were obtained from the following sources: pan-Ras (Oncogene); phospho-ERK1/2, ERK1/2, phospho-Akt1 and Akt1 (Cell Signaling); RalA (Transduction Laboratories); Raf1 (Santa Cruz Biotechnology). Immunoblots were stripped and reprobed with antibodies to β-actin (Santa Cruz Biotechnology) as an additional control for loading and extract quality.

Active Ras and Ral Pull-Down Assay. Pull-down assays to analyze levels of GTP-bound Ras (17) and Ral (18) were performed under nonsaturated conditions as described. Briefly, 150 μl of *Escherichia coli* GST-RBD or GST-RalBD lysate was incubated with 30 μl of glutathione-Sepharose beads (Amersham) at room temperature for 30 min with shaking. After washing, 500 μg of epidermal tissue extract was added at 4°C for 1 h with shaking. After 3 washes, the samples were subjected to 12% SDS PAGE. Levels of active Ras and Ral protein were detected by a pan-Ras antibody (Oncogene) or a monoclonal anti-RalA antibody (Tranduction Laboratories) and quantitated as noted above.

Immunohistochemistry. For immunostaining, 5-μm cryosections were allowed to air-dry for 30 min and were permeabilized with cold acetone for 10 min. Sections were blocked with 10% horse serum for 1 h and treated with the primary antibody for 1 h at room temperature. Slides were then washed three times with PBS and incubated for 30 min with secondary antibodies. After

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[4] The abbreviations used are: SCC, squamous cell carcinoma; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3′-kinase; 4OHT, 4-hydroxytamoxifen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; ER, estrogen receptor; K14, keratin 14; ERK, extracellular signal-regulated protein kinase; MEK, MAP/ERK kinase; GST, glutathione *S*-transferase.

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three washes with PBS, slides were mounted in vectashield (Vector Laboratories) and examined under a Zeiss 100M Axiovert microscope. The following panel of antibodies were used in immunostaining: antimouse involucrin, keratin 10 (Babco); antimouse Ki-67 (Dako); nidogen, anti-integrin β4, β1 (Chemicon); FITC-conjugated goat-antimouse IgG, FITC-conjugated goat-antirabbit IgG, and FITC-conjugated rabbit-antirat IgG (Sigma); Cy3-conjugated goat-antirat IgG (Jackson). Apoptosis was detected in paraffin-embedded sections by the TUNEL assay with the ApopTagR peroxidase *in situ* apoptosis detection kit (Intergen) following the manufacturer's instructions.

RESULTS

To study regulated Ras activation in adult epidermis, we expressed a NH_2-terminal fusion of H-Ras^{G12V} to a 4OHT-responsive mutant ER ligand binding domain (ER:Ras) (7) in transgenic mice using the K14 promoter. Multiple lines of K14-ER:Ras mice were generated, including those with low, medium, and high transgene copy number and expression of the ER:Ras fusion protein in epidermis (Fig. 1*a*). All of the mice were viable and fertile, exhibiting no detectable phenotype. Biochemically, ER:Ras remained inactive in untreated adult transgenic skin; however, topical application of 4OHT induced activation of the ER:Ras fusion (Fig. 1*b*), as assessed by GST-RafBD pull-down assay of epidermal tissue extracts under nonsaturating conditions (17). Endogenous levels of GTP-bound Ras remained stable (Fig. 1*b*). Increased GTP-bound active ER:Ras levels occurred within 16 h of application and returned to normal within 72 h. These studies confirmed the inducibility of ER:Ras in adult murine epidermis *in vivo*.

Constitutively altering Ras function through development is associated with perinatal death (7). To explore the impact of activating Ras in a localized region of epidermis in adult mice, we applied 4OHT topically to the skin of K14-ER:Ras transgenic lines daily for 1 month. Although 4OHT application over this period produced no visible changes in the skin of genetically matched littermate controls, K14-ER:Ras lines underwent significant cutaneous changes (Fig. 2). Hyperkeratosis and skin thickening were observed, with increasing degree of severity in low-, medium-, and high-expressing ER:Ras lines. This phenotype was fully developed by 3 weeks of application and remained stable for the final week, with complete resolution within 1 month after cessation of topical 4OHT (Fig. 2). Therefore, activation of Ras function in adult epidermis leads to reversible

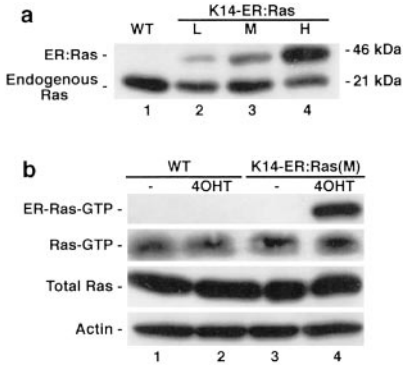

Fig. 1. Expression of inducible Ras in transgenic epidermis. *a*, Western blots demonstrating expression of ER-Ras fusion protein in epidermal tissue extracts from low- (*L*), medium- (*M*), and high- (*H*) expressing transgenic lines. *The upper band*, the constitutively present ER-Ras fusion protein expressed at differing amounts in each line; *the lower band*, endogenous Ras proteins (detected in both cases by pan Ras antibodies). *b*, pull-down assay assessment of levels of GTP-bound Ras in wild-type (*WT*) and medium expressing transgenic [*K14-ER:Ras(M)*] epidermal tissue extracts in response to topical 4OHT. Levels of active GTP-bound ER-Ras fusion (ER-Ras-GTP, *top panel*) and endogenous active Ras (Ras-GTP, *second panel from the top*) in tissue treated with ethanol vehicle (-) or 4OHT were assessed by immunoblotting 16 h after application. Levels of total Ras and actin loading control are shown in the *bottom two panels*.

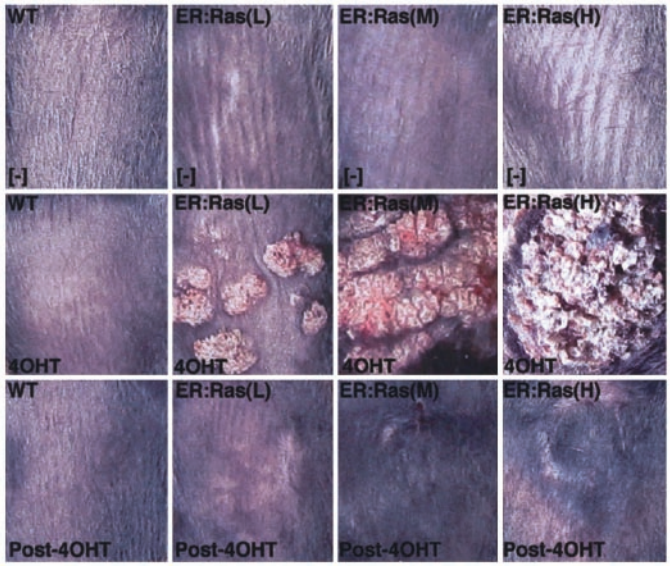

Fig. 2. Appearance of skin from K14-ER:Ras transgenic lines before, during, and after Ras induction with topical 4OHT. Back skin of WT and K14-ER:Ras transgenic mice before (*top row*), at the completion of 4 weeks of topical vehicle alone (-) or 4OHT (*middle row*), and 4 weeks after cessation of treatment (*bottom row*). Photos are shown of the same mouse in each group through the course of the treatment regimen and are representative (n = 5 mice/group/time point).

cutaneous hyperkeratosis and thickening, with progressively greater changes seen with higher transgene expression levels.

Three of the best accepted downstream Ras effector cascades encompass the Raf/MAPK, PI3K/Akt, and RalGDS/Ral proteins (14). To determine whether ER:Ras induction altered the status of these pathways, we examined markers of their activation in epidermal tissue 16, 24, and 48 h after 4OHT application. Epidermal Ras activation increased levels of phosphorylated active ERK1/2 (Fig. 3*a*), consistent with activation of the Raf/MEK/MAPK cascade. In contrast, levels of phosphorylated active Akt1 and GTP-bound RalA were unchanged in epidermal tissue extracts (Fig. 3, *a* and *b*); extracts from keratinocytes expressing constitutively active mutants RalGDS-CAAX and PI3K p110α-CAAX served as positive controls (Fig. 3*c*). These findings indicate that regulated Ras primarily activates the Raf effector pathway; however, they do not exclude induction of PI3K and RalGDS by Ras at alternate time points. In all cases, no changes were observed in genetically matched nontransgenic littermate controls treated with either 4OHT or vehicle alone. These findings indicate that epidermal Ras induction via the ER:Ras fusion selectively activates features of the Raf/MAPK downstream Ras effector pathway.

In addition to Raf, PI3K, and RalGDS, Ras has been shown to influence a number of other effector pathways (14), and it is possible that the observed epidermal Ras effects may proceed via such alternate signaling cascades. To determine the sufficiency of Raf to mediate the observed epidermal effects of Ras activation, we generated transgenic mice with inducible epidermal Raf function. To do this, we expressed a 4OHT-inducibly active Raf1:ER fusion (16) in epidermis using the K14 promoter. K14-Raf:ER mice expressed the fusion protein in epidermis (Fig. 4*a*). As in the case of ER:Ras, three independent lines of viable and fertile mice were generated; however, all of the K14-Raf:ER mice that were generated displayed similar levels of fusion protein expression (data not shown). Inducible Raf activation in response to topical 4OHT was confirmed by the demonstration of increased active ERK1/2 protein levels on tissue immunoblot and immunohistochemistry (Fig. 4, *b* and *c*). Application of 4OHT for 1 month produced the clinical hyperkeratosis and thickening observed in mid-copy K14-ER:Ras mice (data not shown). K14-

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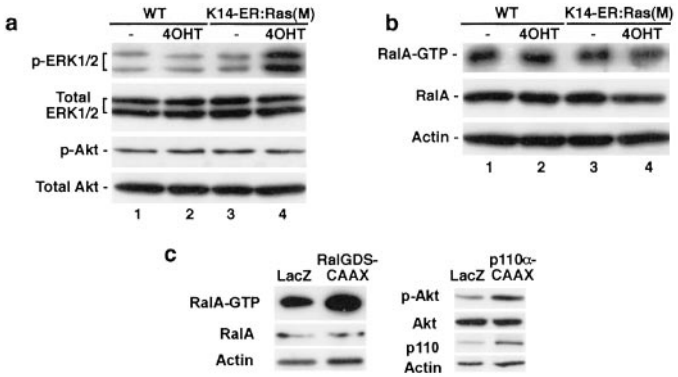

Fig. 3. Induction of downstream Ras effectors. *a*, Western blots demonstrating inducible increases in active phosphorylated ERK1/2 (*p-ERK1/2*) but not Akt1 (*p-Akt*) in epidermal tissue from K14-ER:Ras transgenic mice 16 h after treatment with topical 4OHT or vehicle alone (-). Levels of total ERK1/2 and Akt1 are shown in the same samples. *b*, pull-down assay assessment of levels of active GTP-bound Ral in epidermal tissue 16 h after treatment with topical 4OHT or vehicle alone (-). Levels of total RalA and actin-loading control are shown in the *bottom two panels*. *c*, positive controls for Ral-GTP and phosphorylated Akt1. Ral-GTP pull-down assay/immunoblotting for RalA-GTP (*left panel*) and immunoblotting for active Akt1 (*right panel*) were performed using extracts from primary keratinocytes expressing constitutively active RalGDS-CAAX and p110α-CAAX mutants.

Raf:ER mice thus display evidence of regulated Raf induction and provide the opportunity to study phenotypic resemblance to K14-ER:Ras mice.

Activation of Raf function led to epidermal changes indistinguishable from those induced by Ras. Both Ras and Raf induced epidermal hyperplasia, with histological changes including acanthosis and hypogranulosis (Fig. 4*d*). To further characterize and compare epidermal changes triggered by Ras and Raf in developmentally mature epidermis, we analyzed expression of markers of proliferation, differentiation, and epidermal progenitor cell function. Although untreated K14-ER:Ras and K14-Raf:ER epidermis was indistinguishable from 4OHT and untreated wild-type control in all respects, treated K14-ER:Ras and K14-Raf:ER epidermis displayed similar changes of all features studied (Fig. 5). These changes included an increased mitotic index, decreased expression of differentiation markers and increased expression of β1 and β4 integrin subunits (Fig. 5). In the case of differentiation marker expression, involucrin and keratin 10, which normally appear in the spinous layer, were virtually absent. β1 and β4 integrin subunits, implicated with their partners as increased in epidermal progenitors (6, 19), were expressed strongly and in cells multiple-cell-layers above the basement membrane zone in a pattern that was similar to that observed in epidermal SCC (20). No increases in apoptotic cells were observed, as assessed by *in situ* TUNEL staining (data not shown). These findings indicate that activation of both Ras and Raf in adult epidermis promotes the undifferentiated, proliferative phenotypic characteristics observed in epidermal cancer. They also confirm that Raf induction is sufficient to recapitulate effects of Ras in driving these epidermal changes.

DISCUSSION

Here we have demonstrated that activation of Ras in adult epidermal tissue selectively activates MAPK but not other characterized cascades and leads to reversible epidermal hyperplasia. Increasing epidermal ER:Ras expression in individual lines of mice led to progressively more pronounced visible effects. These changes were accompanied by suppression of epidermal differentiation and enhanced expression of integrins normally constrained to regions of the epidermal basal layer. Ras-driven epidermal alterations were recapitulated by inducible Raf activation, indicating that Raf is sufficient to mediate Ras effects in this setting.

Prior studies of the effects of activating Ras and Raf in postnatal human and murine keratinocytes in culture presented contradictory findings with respect to growth and differentiation (5–9). *In vivo*, however, epidermal overexpression of active Ras mutants constitutively, through development in transgenic mice by a number of groups, led to hyperplasia in all cases (7, 10–12). Signal strength and cellular context have been clearly implicated as contributing to the disparities with *in vitro* findings (7). Nonetheless, the possibility remained that the developmental state of the keratinocytes studied could also play a role. Because adult activation of Ras has been implicated in development of epidermal SCC in UV- and chemically induced carcinogenesis (3, 21, 22), it was of interest to examine the effects of controlled epidermal Ras activation in the adult context. Our data indicate that such activation produces effects similar to those seen with constitutive expression of active oncogenic *Ras* through development in transgenic mice. Moreover, we show here that these epidermal Ras effects are entirely reversible. This suggests that the changes induced by Ras, seen in both

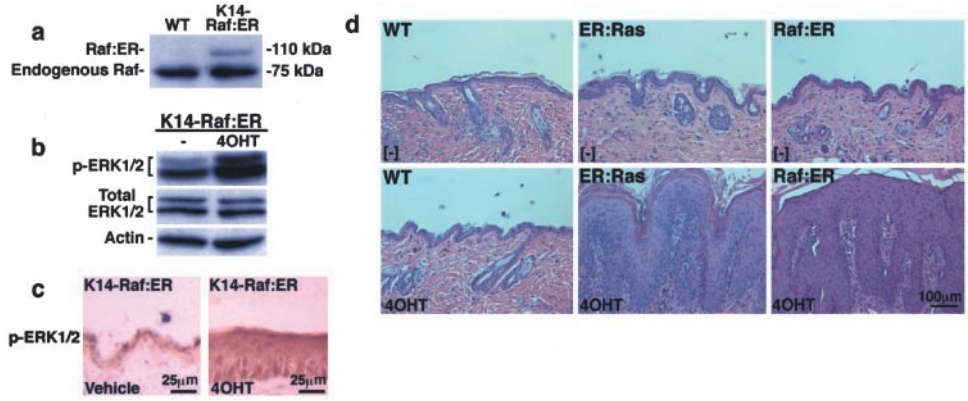

Fig. 4. Expression of inducibly active Raf in transgenic epidermis. *a*, Western blots demonstrating expression of Raf1:ER (Raf:ER) fusion protein in epidermal tissue extracts from wild-type littermate (*WT*) and K14-Raf:ER transgenic mice. The *upper band* denotes the constitutively expressed Raf:ER fusion protein and the *lower band* represents endogenous Raf proteins, as detected in both cases by Raf1 antibodies. *b*, Western blots demonstrating inducible increases in active phosphorylated ERK1/2 (*p-ERK1/2*) in epidermal tissue from K14-Raf:ER transgenic mice 16 h after treatment with topical 4OHT or vehicle alone (-). Levels of total ERK1/2 and actin-loading control are shown in the *bottom two panels*. *c*, epidermal expression of active p-ERK1/2 protein 48 h after treatment with topical 4OHT or vehicle, detected immunohistochemically with antibodies to phosphorylated ERK1/2 (augmented p-ERK1/2 and increased thickness of K14-Raf:ER epidermis can be seen at this time point). *d*, histology of adult skin of mice after induction of Ras and Raf. K14-ER:Ras (ER:Ras) and K14-Raf:ER (Raf:ER) mice were treated daily with topical 4OHT or ethanol vehicle for 2 weeks before assessment. There were marked hyperplasia and diminished granular layer in epidermis subjected to Ras and Raf activation.

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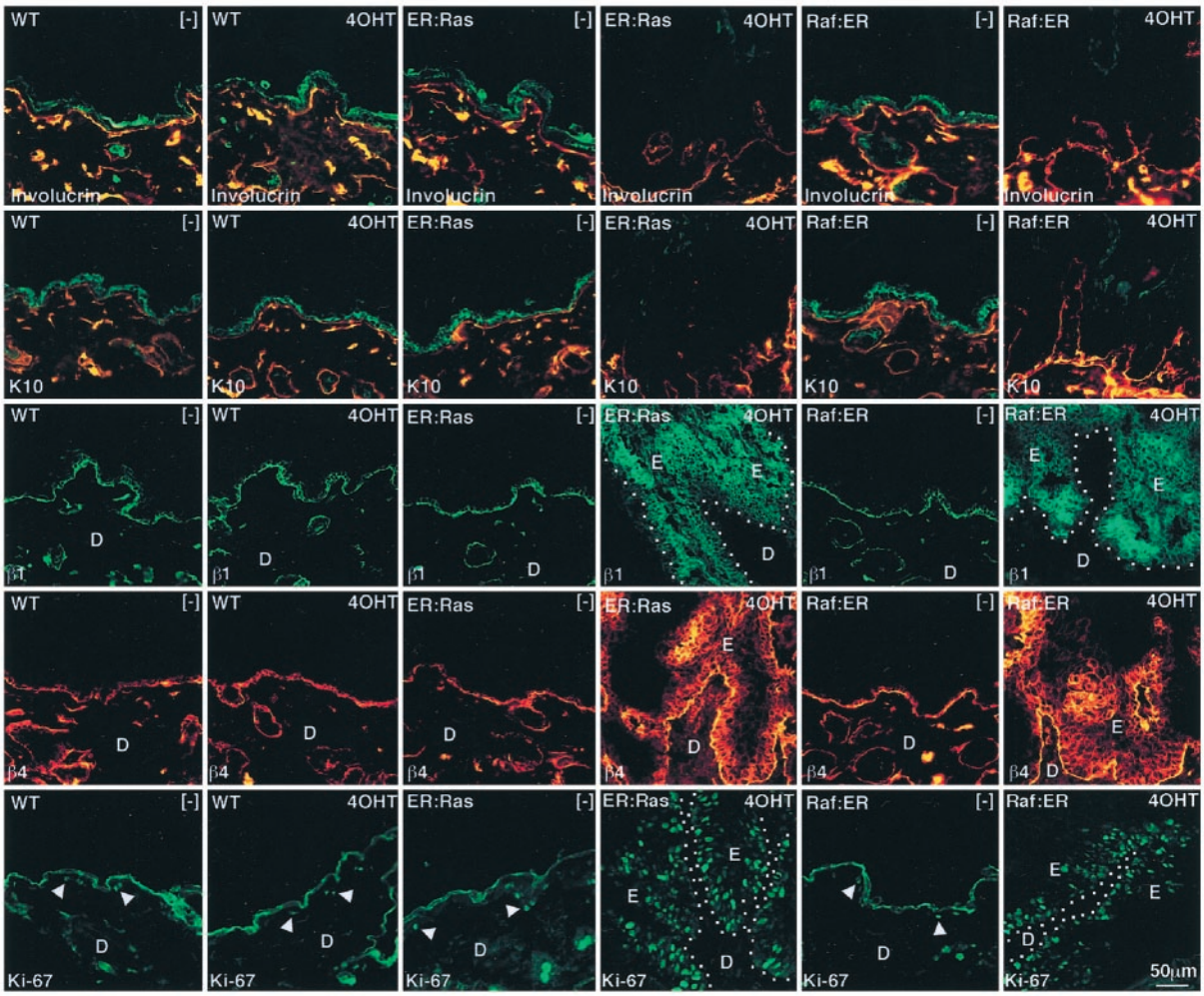

Fig. 5. Expression of markers of differentiation, proliferation, and progenitor cell phenotype. Skin of adult wild-type (*WT*), K14-ER:Ras (*ER:Ras*) and K14-Raf:ER (*Raf:ER*) mice was analyzed by immunofluorescence after 4 weeks of daily treatment with topical 4OHT or ethanol vehicle (-). The *top 2 panels* represent double immunostaining for the differentiation markers involucrin and K10 (*both in green*) and nidogen (*orange*) to mark the basement membrane zone. There is a loss of differentiation marker expression in the hyperplastic epidermis that is seen with Ras and Raf induction. The *bottom 3 panels* represent single immunostains for the β1 (*green*) and β4 (*orange*) integrin subunits as well as the proliferation marker Ki-67 (*green*). Induction of Ras and Raf leads to augmented expression of β1 and β4 integrin subunits extend multiple-cell-layers above the basement membrane zone (*white dots*) along with an increased proportion of actively dividing Ki-67(+) cells. *E*, epidermis; *D*, dermis; *arrowheads*, Ki-67(+) cells in nonhyperplastic epidermis.

development and adulthood, require the presence of active Ras itself and are not a stable compensatory change sustained by other factors. The present study, therefore, supports prior *in vivo* work and indicates that Ras induction in the adult tissue setting is sufficient to drive major changes seen in SCC, including hyperproliferation, suppressed differentiation, and enhanced integrin expression (20, 23).

Although observed in some human epidermal SCC, oncogenic *RAS* mutations have been reported to vary in frequencies between different studies, and an actual pathogenic role of *RAS* mutations in human SCC is not established. Epithelial malignancies with low incidences of detected mutations, however, can display high levels of pathogenically significant Ras protein activation, as recently shown in breast cancer (24). This can occur via mechanisms distinct from primary *RAS* mutation that lead to strong Ras induction, such as overexpression of upstream receptor tyrosine kinases. In this regard, the mice generated here offer resources needed to perform long-term studies to determine whether chronic activation of Ras in adult epidermis leads to the development of invasive neoplasia. Further work will be necessary to study this possibility and to determine the therapeutic potential of strategies to inhibit Ras function in human epidermal cancer.

By generating transgenic mice with inducibly active Raf function, we demonstrated that Raf induction is sufficient to trigger changes in epidermal growth and differentiation similar to those induced by Ras. To our knowledge, this is the first *in vivo* Raf gain-of-function study performed in epidermis. On the basis of the congruency of the present regulated Ras findings with prior constitutive expression efforts, we believe it is likely that constitutive Raf expression through development would produce similar effects. The sufficiency of Raf in mediating Ras-driven changes emblematic of epidermal cancer is significant because it is possible that *RAF* mutations may contribute to a proportion of cutaneous malignancies. In this regard, it has recently been demonstrated that a majority of malignant cutaneous melanomas, whereas known for some time to display evidence of MAPK pathway activation, have recently been shown to express enzymatically active Raf mutants via mutation in the *BRAF* gene (25). Combined with the present work, these findings provide a rationale for future studies of epithelial carcinogenesis designed to assess the control and function of MAPKs, which are known to be up-regulated in epithelial SCC (26). Thus, the present work supports the expansion of prior epidermal cancer studies focused solely on Ras to include additional downstream mediators of the Raf/MEK/MAPK pathway.

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REFERENCES

1. Crespo, P., and Leon, J. Ras proteins in the control of the cell cycle and cell differentiation. Cell. Mol. Life Sci., *57:* 1613–1636, 2000.
2. Marshall, C. J. Small GTPases and cell cycle regulation. Biochem. Soc. Trans., *27:* 363–370, 1999.
3. Pierceall, W. E., Goldberg, L. H., Tainsky, M. A., Mukhopadhyay, T., and Ananthaswamy, H. N. *Ras* gene mutation and amplification in human nonmelanoma skin cancers. Mol. Carcinog., *4:* 196–202, 1991.
4. Yuspa, S. H. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis—thirty-third G. H. A. Clowes Memorial Award Lecture. Cancer Res., *54:* 1178–1189, 1994.
5. Mainiero, F., Murgia, C., Wary, K. K., Curatola, A. M., Pepe, A., Blumemberg, M., Westwick, J. K., Der, C. J., and Giancotti, F. G. The coupling of alpha6beta4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. EMBO J., *16:* 2365–2375, 1997.
6. Zhu, A. J., Haase, I., and Watt, F. M. Signaling via β1 integrins and mitogen-activated protein kinase determines human epidermal stem cell fate *in vitro*. Proc. Natl. Acad. Sci. USA, *96:* 6728–6733, 1999.
7. Dajee, M., Tarutani, M., Deng, H., Cai, T., and Khavari, P. A. Epidermal Ras blockade demonstrates spatially localized Ras promotion of proliferation and inhibition of differentiation. Oncogene, *21:* 1527–1538, 2002.
8. Lin, A. W., and Lowe, S. W. Oncogenic *ras* activates the ARF-p53 pathway to suppress epithelial cell transformation. Proc. Natl. Acad. Sci. USA, *98:* 5025–5030, 2001.
9. Roper, E., Weinberg, W., Watt, F. M., and Land, H. p19ARF-independent induction of p53 and cell cycle arrest by Raf in murine keratinocytes. EMBO Rep., *2:* 145–150, 2001.
10. Brown, K., Strathdee, D., Bryson, S., Lambie, W., and Balmain, A. The malignant capacity of skin tumours induced by expression of a mutant H-*ras* transgene depends on the cell type targeted. Curr. Biol., *8:* 516–524, 1998.
11. Bailleul, B., Surani, M. A., White, S., Barton, S. C., Brown, K., Blessing, M., Jorcano, J., and Balmain, A. Skin hyperkeratosis and papilloma formation in transgenic mice expressing a *ras* oncogene from a suprabasal keratin promoter. Cell, *62:* 697–708, 1990.
12. Greenhalgh, D. A., Rothnagel, J. A., Quintanilla, M. I., Orengo, C. C., Gagne, T. A., Bundman, D. S., Longley, M. A., and Roop, D. R. Induction of epidermal hyperplasia, hyperkeratosis, and papillomas in transgenic mice by a targeted v-Ha-*ras* oncogene. Mol. Carcinog., *7:* 99–110, 1993.
13. Ewen, M. E. Relationship between Ras pathways and cell cycle control. Prog. Cell Cycle Res., *4:* 1–17, 2000.
14. Shields, J. M., Pruitt, K., McFall, A., Shaub, A., and Der, C. J. Understanding Ras: 'it ain't over 'til it's over'. Trends Cell Biol., *10:* 147–154, 2000.
15. Genot, E., Reif, K., Beach, S., Kramer, I., and Cantrell, D. p21ras initiates Rac-1 but not phosphatidyl inositol 3 kinase/PKB. Oncogene, *17:* 1731–1738, 1998.
16. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Mol. Cell. Biol., *17:* 5598–5611, 1997.
17. de Rooij, J., and Bos, J. L. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene, *14:* 23–25, 1997.
18. Wolthuis, R. M., Zwartkruis, F., Moen, T. C., and Bos, J. L. Ras-dependent activation of the small GTPase Ral. Curr. Biol., *8:* 471–474, 1998.
19. Li, A., Simmons, P. J., and Kaur, P. Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc. Natl. Acad. Sci. USA, *95:* 3902–3907, 1998.
20. Savoia, P., Trusolino, L., Pepino, E., and Marchisio, P. C. Expression and topography of integrins and basement membrane proteins in epidermal carcinomas: basal but not squamous cell carcinomas display loss of $\alpha 6\beta 4$ and BM-600/nicein. J. Investig. Dermatol., *101:* 352–358, 1993.
21. Berking, C., Takemoto, R., Binder, R. L., Hartman, S. M., Ruiter, D. J., Gallagher, P. M., Lessin, S. R., and Herlyn, M. Photocarcinogenesis in human adult skin grafts. Carcinogenesis, *23:* 181–187, 2002.
22. Yuspa, S. H., Dlugosz, A. A., Denning, M. F., and Glick, A. B. Multistage carcinogenesis in the skin. J. Investig Dermatol. Symp Proc., *1:* 147–150, 1996.
23. Watanabe, S., Ichikawa, E., Takahashi, H., and Otsuka, F. Changes of cytokeratin and involucrin expression in squamous cell carcinomas of the skin during progression to malignancy. Br. J. Dermatol. *132:* 730–739, 1995.
24. von Lintig, F. C., Dreilinger, A. D., Varki, N. M., Wallace, A. M., Casteel, D. E., and Boss, G. R. Ras activation in human breast cancer. Breast Cancer Res. Treat. *62:* 51–62, 2000.
25. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. Mutations of the *BRAF* gene in human cancer. Nature (Lond.), *417:* 949–954, 2002.
26. Mishima, K., Yamada, E., Masui, K., Shimokawara, T., Takayama, K., Sugimura, M., and Ichijima, K. Overexpression of the ERK/MAP kinases in oral squamous cell carcinoma. Mod. Pathol. *11:* 886–891, 1998.

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