[54] Use of Conditionally Active Ras Fusion Proteins to Study Epidermal Growth, Differentiation, and Neoplasia

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Abstract

Ras proteins are membrane-bound GTPases that play a central role in transmitting signals from the cell surface to the nucleus and affect a wide array of biological processes. The overall cellular response to Ras activation varies with cell type, experimental conditions, signal strength, and signal duration. Most current studies, however, rely on expression of constitutively active protein to study Ras function and thus ignore temporal variables, as well as signal strength. These experiments may provide contradictory results, as seen in the case of epidermal keratinocytes. In this setting, Ras has been shown to both promote and oppose proliferation and differentiation. By providing control over timing, duration, and signal magnitude, conditional systems allow for more precise investigation of the role of Ras in carcinogenesis, as well as normal cellular physiology. This chapter focuses on use of a ligand-responsive steroid hormone receptor fusion of Ras, ER–Ras, to study aspects of cellular transformation in epidermal keratinocytes.

Introduction

Ras (from rat sarcoma) GTPases were first identified from strains of rat sarcoma viruses and were subsequently shown to have cellular homologs, H-Ras, N-Ras, and K-Ras. Ras proteins are activated by a variety of cell surface receptors in response to an array of extracellular signals including growth factors, extracellular matrix cues, cytokines, hormones, and neurotransmitters. Oncogenic forms of Ras can be created by altering codons 12, 13, 59, or 61. Mutation at these residues inhibits Ras's ability to cycle between its active GTP-bound and inactive GDP-bound forms, generating a constitutively GTP-bound active protein (Campbell *et al.*, 1998). Conversely, amino acid substitution at codon 17 reduces Ras's affinity for GTP and results in a dominant-negative Ras protein. GTP-bound Ras interacts with and activates an ever-growing list of effector molecules to modulate a diverse collection of cellular processes, including proliferation, differentiation, survival, migration, and polarity.

The ultimate outcome of Ras activation varies with cell type, experimental conditions, signal strength, and duration (Ewen, 2000; Shields *et al.*,

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2000). Specifically, Ras signaling has been shown to induce transformation in some experimental settings, whereas it promotes growth arrest in others. Studies of Ras's impact on proliferation and differentiation (Lin and Lowe, 2001; Mainiero *et al*., 1997; Roper *et al*., 2001; Zhu *et al*., 1999), as well as survival and apoptosis, have also yielded contradictory results. Sorting through and attributing true biological significance to these results remains an area of active investigation. Certain of these observations may be a consequence of strong persistent Ras activation in cultured cells and would be clarified by the use of conditional alleles in a more native tissue context.

Normal mammalian epidermis is a self-renewing tissue that maintains homeostasis by precise control over proliferation and growth arrest–associated terminal differentiation. Cells in the basal layer are mitotically active but cease proliferating and begin to express differentiation markers as they migrate progressively outward towards the skin's surface. As in other settings, the role of Ras in this process has been controversial. By combining conditional, constitutive, and dominant interfering approaches both *in vivo* and *in vitro*, a model for Ras effects in epidermal homeostasis has been proposed (Dajee *et al*., 2002; Tarutani *et al*., 2003). This model suggests that Ras is necessary for maintenance of the proliferative, undifferentiated epidermal phenotype. Moreover, Ras is only able to exert these effects in the basal layer of cells directly adherent to the underlying epidermal basement membrane. As a potent regulator of epidermal homeostasis, it is not surprising that Ras has also been implicated in the pathogenesis of epidermal squamous cell carcinoma (SCC). Current estimates place the cumulative mutation frequency in cutaneous SCC for all Ras isoforms at approximately 25% (http://www.sanger.ac.uk/genetics/CGP/cosmic/), although we have demonstrated recently that pathway activation (as measured by Ras-GTP levels) occurs in most SCCs regardless of Ras-mutation status (Dajee *et al*., 2003).

Characterization of Ras effects in cells and tissues benefits from the ability to control the timing and strength of Ras activity. Such control can be achieved in a number of ways, including transcriptional regulation of Ras constructs and by generation of conditionally active Ras fusions. Nearly two decades ago it was first demonstrated that combining the hormone-binding domain (HBD) of steroid receptors with heterologous proteins generated fusions whose activity was dependent on the presence of the cognate steroid hormone (Eilers *et al*., 1989; Picard *et al*., 1988). Since that time, regulated fusions of various oncoproteins, transcription factors, tyrosine kinases, and serine/threonine kinases have been generated, illustrating the broad applicability of the system (reviewed in Picard, 1994). Most likely, regulation occurs because of steric hindrance from interaction of the HBD with an Hsp90 complex. HBD-ligand interactions

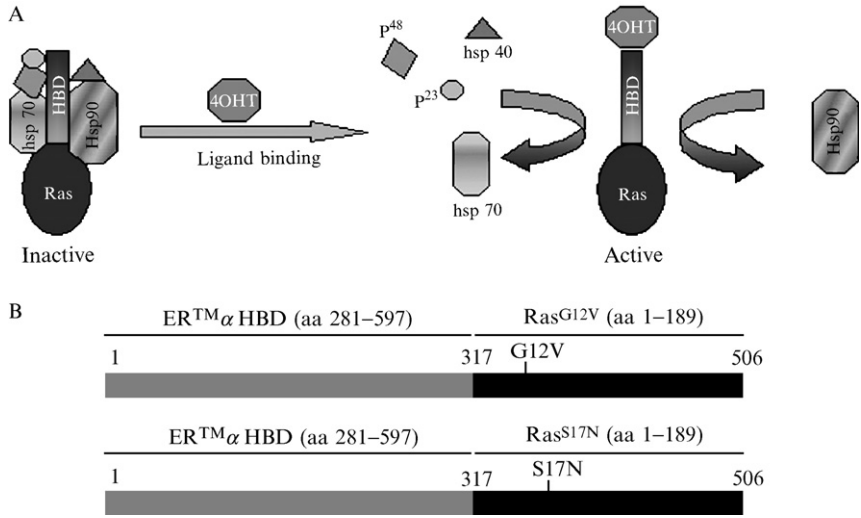

FIG. 1. Generating ER–Ras constructs. (A) Model of steroid fusion regulation. In the absence of ligand, an Hsp90 complex is bound to the HBD and prevents Ras signaling. Addition of 4OHT relieves this inhibition by causing the dissociation of the Hsp90 complex. (B) Schematic of ER–Ras constructs. ER was fused in-frame to the N-terminus of Ras. G12V denotes a glycine to valine amino acid substitution at codon 12 of Ras. This mutation locks Ras in its active conformation. Similarly, S17N denotes a serine to asparagine substitution at codon 17. Ras^{S17N} has reduced affinity for GTP and acts as a dominant-negative.

result in release of the Hsp90 complex and activation of the heterologous moiety (Fig. 1A). Because much of the regulation is posttranscriptional, it is both rapid and reversible. Another attribute of the system is the ability to titrate protein activity by varying ligand concentrations. These characteristics, as well as the potential to combine multiple steroid fusions, make precise assessments of the contributions of individual proteins within complex signaling networks possible. We have used this HBD fusion approach to generate inducible active and dominant-negative H-Ras isoforms.

Protocols and Results

Generating ER–Ras Fusions

ER–Ras proteins were generated as in-frame fusions of the mouse HBD of the estrogen receptor ER^{TM}, (comprised of amino acids 281–597) with either full-length H^{G12V} (constitutively active H-Ras) or $H\text{-}Ras^{S17N}$ (dominant-negative H-Ras) (Fig. 1B). Specifically, this construct was generated by subcloning the *Bam*HI-*Eco*RI fragment of

fragment of ER (Littlewood *et al*., 1995) in frame, upstream of H-Ras^{G12V} or H-Ras^{S17N} in the LZRS retroviral backbone (Kinsella and Nolan, 1996). (ER contains an amino acid substitution at codon 525 (G525R) and is one of several mutants of the estrogen receptor that have altered sensitivity to hormone stimulation. The G525R mutation renders the HBD largely insensitive to 17β-estradiol at concentrations less than 100 n*M* but does not abrogate its responsiveness to the synthetic ligand 4-hydroxytamoxifen (4OHT, Sigma). (ER™ is particularly useful, because it not only reduces concerns of activation by endogenous hormone *in vivo* but also obviates the need to remove steroids from serum used to make culture medium. Furthermore, ER™ is not stimulated by phenol red, a pH indicator added to many culture medias, which has also been shown to activate certain steroid receptors (Berthois *et al*., 1986).

Initially, both N and C-terminal ER fusions of Ras were generated. Although fusion of the HBD to the N-terminus results in 4OHT-regulated Ras activity, C-terminal fusion completely abolishes activity in culture (Tarutani M, unpublished data). Appropriate membrane localization is essential for Ras activity, and the C-terminus of Ras contains multiple signals required for correct membrane targeting, including the CAAX motif (reviewed in Shields *et al*., 2000). Presumably, fusion of ER to this region disrupts the targeting process and thus abrogates Ras function. Successful N- and C-terminal fusions have been made for Myc (Eilers *et al*., 1989) and Raf (Mirza *et al*., 2000; Sewing *et al*., 1997), illustrating that either conformation is feasible. Lessons from E1A indicate that physical proximity of the HBD to the protein domain whose activity is to be regulated is also of critical importance (Picard *et al*., 1988). In general, it is difficult to predict *a priori* the best end to place the HBD; therefore, it is advisable to try multiple strategies when constructing a new fusion protein.

Conditional Ras Activation in Primary Human Keratinocytes in Culture

To study the effects of regulated Ras activation *in vitro,* high-efficiency retroviral transduction was used to introduce ER–Ras into primary keratinocytes.

Protocol: Retroviral Infection of Primary Keratinocytes (Choate et al., *1996; Kinsella and Nolan, 1996; Deng* et al., *1998)*

1. 8–12 h before infection, cells are split such that they will be 10–15% confluent at the time of transduction.
2. Viral supernatant containing polybrene (Sigma) 1 μg/ml in HBSS (GibcoBRL) is then added to cells.
3. Centrifuge at 32°at 1200 rpm (Allegra 6R Beckman centrifuge) for 1 h.

4. Remove supernatant, wash cells with PBS, and replace keratinocyte medium (serum-free medium with keratinocyte supplements, Gibco BRL).

This process can be repeated every 8–12 h to introduce multiple genes (Lazarov *et al*., 2003). By use of this approach (termed multiplex serial gene transfer or MSGT), we routinely achieve transduction rates of greater than 95% for multiple genes in primary cells, circumventing the need for potentially mutagenic drug selection. Efficiency rates during serial infections may decrease, depending on the confluency and health of the keratinocytes. For instance, expression of constitutive Ras causes a senescence-like phenotype in keratinocytes and, thus, will adversely affect the transduction rate of subsequent infections.

Moderate Ras activation in primary keratinocytes induces a basal cell state characterized by lack of differentiation marker expression and increases in proliferation and integrin expression. Constitutive Ras activity affects differentiation and integrin levels as expected but also causes a senescence-like phenotype. Ras-induced senescence is typified by decreases in proliferation, increases in cell size, vacuolization, and expression of senescence-associated β-gal. Unlike keratinocytes expressing constitutive Ras, cells expressing ER–Ras maintain normal morphology before 4OHT treatment (Fig. 2A). Proliferation rates may be slightly increased in culture, but experience *in vivo* (see next section) suggests that this difference is insignificant. On the other hand, introduction of 10 n*M* 4OHT, but not ethanol (vehicle control), into the culture medium for 36–48 h induces a phenotype indistinguishable from that observed in keratinocytes overexpressing constitutively active Ras (Fig. 2A). This phenotype is completely dependent on continued 4OHT stimulation, although morphological reversion requires trypsinizing and replating the cells 48 h after 4OHT withdrawal. Concentrations up to 100 n*M* of 4OHT have been tested and have no adverse effects on viability or morphology of normal primary keratinocytes in culture.

Consistent with data on ΔRaf-1–ER, activation of the MAPK cascade in ER–Ras–expressing cells can be detected biochemically long before morphological changes occur (Samuels *et al*., 1993). Increases in levels of phosphorylated ERK1/2 can be detected by means of Western blot as early as 10 min after 4OHT treatment (Tarutani M, unpublished data).

Experience with ΔRaf-1–ER also indicates downstream induction of Ets-2 target genes like heparin-binding epidermal growth factor occurs within 30–60 min of hormone treatment (McCarthy *et al*., 1995). Furthermore, relaxation of phospho-ERK levels occurs within the first hour after hormone removal in ΔRaf-1–ER expressing cells (Samuels *et al*., 1993).

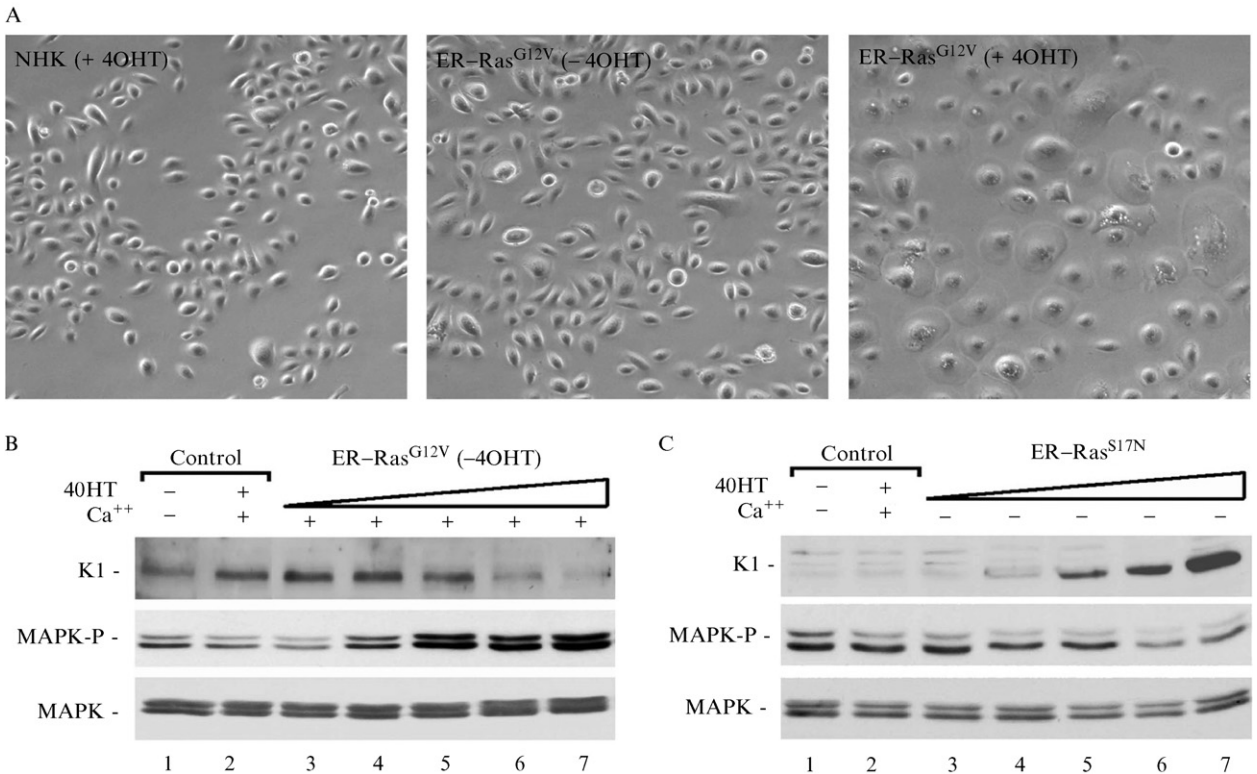

FIG. 2. *In vitro* induction of ER–Ras constructs. (A) Primary human keratinocytes were transduced with a retrovector for ER–Ras^{G12V}. Addition of 10 n*M* 4OHT to the media 24 h after transduction results in senescence-like phenotype within 36–48 h. Normal primary human keratinocytes treated with 4OHT or untreated ER–Ras^{G12V}–expressing cells remain morphologically unaltered. (B) Regulated active Ras inhibits

Given the similarities in pathway stimulation, it is likely the ER–Ras acts in an analogous manner with regard to downstream transcriptional targets and pathway inactivation after 4OHT removal, but this has yet to be formally demonstrated.

Addition of 4OHT in a range of concentrations to cells expressing ER–Ras suggests Ras activation occurs in a dose-dependent manner. Maximal phospho-ERK1/2 levels are reached by 1 n*M* 4OHT (Fig. 2B). Another aspect of Ras activity in keratinocytes is an inhibition of calcium-induced differentiation. Cytokeratin-1 (K1) is normally expressed in differentiating keratinocytes but is greatly diminished on 4OHT treatment in ER–Ras–expressing cells (Fig. 2B). Analogous experiments overexpressing a regulated dominant-negative form of Ras corroborate these results. Induction of ER-fused Ras^{S17N} reduces phospho-ERK1/2 levels and induces K1 differentiation marker expression maximally at 5 n*M* and 25 n*M* 4OHT, respectively (Fig. 2C). These data indicate that altering Ras function in epidermal cells exerts a dominant effect on differentiation. In this context, active Ras prevented differentiation, even in the face of a strong calcium-mediated differentiation stimulus, and Ras inhibition triggered differentiation in the absence of other differentiating stimuli.

Modulating Ras Activity In Vivo

ER–Ras can be activated *in vivo* by daily administration of 4OHT by means of topical application or intraperitoneal (i. p.) injection. For topical treatment, 4OHT is dissolved in ethanol to a final concentration of 10 mg/ml.

Protocol: Preparation of 4OHT for i. p. injection (adapted from Metzger et al., 2001)

1. Dissolve the 4OHT in ethanol (67 mg/ml) by heating to 48° for 10 min.
2. Mix 4OHT solution with heated, sterile corn oil (7.5 mg/ml).
3. Sonicate 20 sec at 5 volts, then place on ice for 1 min (Sonic dismembrator model 100, Fisher Scientific). Repeat three times.
4. Aliquot to avoid freeze/thaw cycles.

4OHT prepared for topical or i. p. administration can be stored at −20° for at least 2 months or 2 weeks, respectively. Before i. p. injection, 4OHT

differentiation protein expression in a dose-dependent manner. Cells were transduced with inducible ER–Ras^{G12V}, and 4OHT was added at 0, 0.5, 1, 5, and 10 n*M* to lanes 1, 2, 3, 4, 5, 6, and 7, respectively. (C) Regulated dominant-negative Ras induces differentiation protein expression in a dose-dependent manner. Cells were transduced with ER–Ras^{S17N}, and 4OHT was added at 0, 0.5, 2, 5, and 25 n*M* to lanes 1, 2, 3, 4, 5, 6, and 7, respectively.

should be sonicated again (3×, 20 sec, at 3 volts) and injected immediately. A 1-mg dose of 4OHT is used for topical delivery, whereas a 750-μg dose is used for i. p. administration. Data from targeted expression of ER–Ras using the keratin-14 promoter in transgenic mice suggests that Ras activation decreases in basal cells with time (Tarutani M, unpublished results). We speculate that this failure to activate Ras is a consequence of lack of 4OHT penetration because of hyperplasia and/or hyperkeratosis. For this reason, i. p. injection is recommended for long-term experiments.

As in culture, Ras activation by 4OHT occurs rapidly *in vivo*. GST-Raf binding domain (RBD) pull-down assays on tissue extracts of K14-ER–Ras transgenic epidermis illustrate increases in GTP-bound ER–Ras within 16 h after topical 4OHT treatment (Fig. 3A).

Protocol: GST-RBD Pull-Down Assay (de Rooij et al., 1997)

1. Incubate 150 μl of *Escherichia coli* GST-RBD lysate with 30 μl of glutathione sepharose beads (Amersham) at room temperature for 30 min with shaking.
2. Wash in R.I.P.A. buffer.
3. Homogenize tissue sample (Tissue Tearor model 398, Biospec Products, Inc.) in 500 μl RIPA buffer on ice.
4. Centrifuge at 13,000 rpm (Biofuge fresca, Sorval) for 10 min at 4° and collect supernatant.
5. Incubate precoupled beads with 500 μg of epidermal tissue extract at 4°for 1 h with shaking.
6. Centrifuge at 6000 rpm for 5 min at 4° and remove supernatant.
7. Wash 3× in R.I.P.A.
8. Resuspend in SDS-PAGE sample buffer.
9. Run 12% SDS-PAGE and perform Western blotting for Ras.

GTP-ER–Ras levels returned to normal within 72 h after 4OHT withdrawal (Tarutani *et al.*, 2003). Endogenous Ras-GTP levels remain unaffected by application of 4OHT (Fig. 3A). Downstream pathway activation, as assessed by phospho-ERK1/2 levels, is also evident 16 h after topical 4OHT treatment (Fig. 3B).

In vivo, Ras induction in epidermis rapidly leads to three major changes: (1) increased proliferation, (2) up-regulated integrin expression, and (3) inhibited differentiation. All of these changes are consistent with an expansion of the undifferentiated, proliferative basal layer epidermal compartment. Within 5 days of treatment, there is obvious epidermal thickening, and by 3 weeks the phenotype is fully manifested. 4OHT-treated, ER–Ras skin displays hyperplasia, with histopathological changes including hyperkeratosis and hypogranulosis (Fig. 3C). Alterations in polarity and differentiation are also evident: strong β1 and β4 integrin

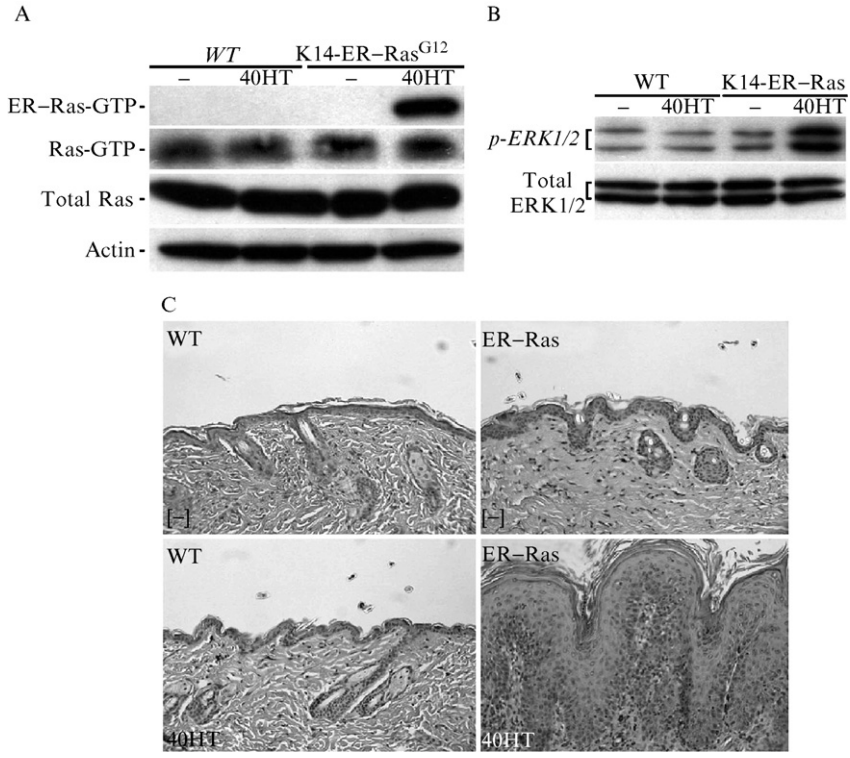

FIG. 3. Expression of inducibly active Ras in transgenic epidermis. (A) Pull-down assay assessment of levels of GTP-bound Ras in wild-type (*WT*) and K14-ER–Ras^{G12V} transgenic epidermal tissue extracts in response to topical 4OHT treatment. Levels of active GTP-bound ER–Ras fusion (ER–Ras-GTP, *top panel*) and endogenous active Ras (Ras-GTP, *second panel from top*) in tissue treated with ethanol vehicle (–) or 4OHT was assessed by immunoblotting 16 h after application. Levels of total Ras and actin loading control are shown in the *bottom two panels*. (B) Western blots demonstrating inducible increases in active phosphorylated ERK1/2 (*p-ERK1/2*) in epidermal tissue from K14-ER–Ras transgenic mice 16 h after treatment with topical 4OHT or vehicle alone (–). Levels of total ERK1/2 are shown in the same samples as a loading control. (C) Histology of adult skin of mice after induction of Ras. K14-ER–Ras^{G12V} (ER–Ras) 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 activation.

expression is induced throughout multiple epidermal layers, and expression of differentiation markers, including involucrin and keratin 10, is lost from the spinous layer (Fig. 4). Interestingly, many of these changes are associated with SCC, although ER–Ras–expressing keratinocytes are not fully malignant, because they fail to invade into the underlying mesenchyme.

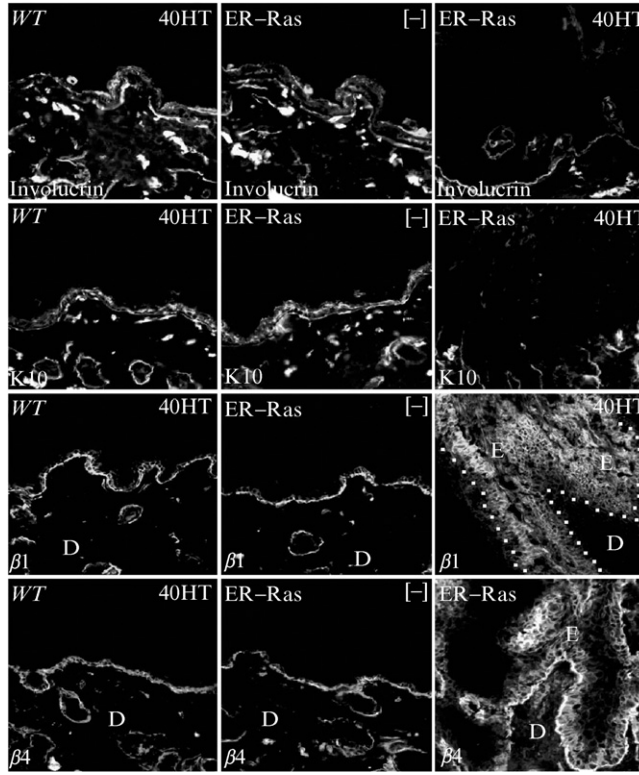

FIG. 4. Expression of marker of differentiation and progenitor cell phenotype. Skin of adult wild-type (*WT*), K14- K14-ER–Ras^{G12V} (*ER–Ras*) mice was analyzed by immunofluorescence after 4 weeks of daily treatment of topical 4OHT or ethanol vehicle (−). The *top two panels* represent double immunostaining for the differentiation markers involucrin and K10 (*both in green*) and nidogen (*orange*) to mark the basement membrane zone. Loss of differentiation marker expression and hyperplasia is observed in treated ER–Ras epidermis. The *bottom two panels* represent single immunostains for the β1 (green) and β4 (orange) integrin subunits. Induction of Ras leads to expression of β1 and β4 integrin subunits extending multiple layers above the basement membrane zone (*white dots*). E, epidermis; D, dermis.

The phenotype described previously is completely reversed after cessation of 4OHT treatment for 1 month (Tarutani *et al.*, 2003).

Concluding Remarks

Most protooncogenes serve as nodes for complex signaling networks. Although much progress has been made by perturbing these systems with constitutive proteins, the intricacy of these pathways requires the use of

more elegant strategies. In the case of Ras, the capability for conditional activation or inhibition in adult tissue allows bypass of potentially problematic effects of protooncogene perturbation during development. Combining classical approaches, like constitutively active and dominant interfering proteins, with newer strategies, such as conditional alleles and RNAi, will greatly facilitate our understanding of how these molecules contribute to normal cellular physiology, as well as to cancer.

Buffers and Antibodies

R.I.P.A buffer: 50 m*M* TRIS pH 8.0, 150 m*M* NaCl, 0.5% DOC, 1% NP40, 0.1% SDS, 0.1 PM aprotinin, 1 PM leupeptin, and 1 m*M* PMSF.

Antibodies: Ras (Santa Cruz), Phospho-ERK1/2 and total-ERK1/2 (Cell Signaling), β-actin (Sigma), keratin-1 (Babco), keratin-10 (Babco), β-1(Chemicon), β4 (Chemicon), and involucrin (Babco).

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