Impact of Bcl-2 and Ha-ras on Keratinocytes in Organotypic Culture

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In this study, the role of specific molecular alterations associated with multistep skin carcinogenesis was assessed using *in vitro* organotypic cultures of the spontaneously immortalized, nontumorigenic HaCaT keratinocyte cell line. HaCaT vector control clones and clones expressing bcl-2, activated Ha-ras, or both genes were generated. Clones were induced to stratify and differentiate by culturing on dermal equivalents for 2 wk at the air–medium interface. In parental and vector control HaCaT rafts the expression and distribution of cytokeratin K1, K14, involucrin, proliferating cell nuclear antigen, and $p21^{cip1/waf1}$ were assessed using immunohistochemistry and immunoblotting and were similar to normal epidermis. Apoptosis was also examined using the TUNEL technique. HaCaT-bcl-2 rafts were similar to control rafts but exhibited lower spontaneous rates of apoptosis and a moderate increase in the rate of proliferation. Differentiation was significantly inhibited in HaCaT-ras organotypic cultures and was associated with high rates of proliferation and lower rates of spontaneous apoptosis. Additionally, HaCaT-ras rafts exhibited significantly higher rates of apoptosis following ultraviolet irradiation compared with vector control or HaCaT-bcl-2 rafts. Bcl-2 was able to largely restore normal differentiation, proliferation, and apoptosis in HaCaT-ras/bcl-2 organotypic cultures. Bcl-2 also abrogated apoptosis induction following ultraviolet irradiation in HaCaT-ras/bcl-2 organotypic cultures. Organotypic keratinocyte culture represents a valuable *in vitro* system to evaluate the impact of individual molecular genetic alterations on the coordinate regulation of cell proliferation, differentiation, and cell death. ***Key words: apoptosis/cancer/differentiation/HaCaT/oncogene. J Invest Dermatol 116:366–373, 2001***

The histogenesis of the epidermis is a dynamic process that requires a balance between proliferation and cell death in order to maintain tissue homeostasis (Fuchs, 1993). Epidermal keratinocytes undergo a series of specific and synchronized morphologic and biochemical modifications that ultimately result in the generation of terminally differentiated cells (Fuchs, 1993; Eckert *et al*, 1997). Terminal keratinocyte differentiation results in the generation of anucleated nonviable cells and has been considered a specialized form of apoptosis (McCall and Cohen, 1991; Haake and Polakowska, 1993; Polakowska *et al*, 1994; Maruoka *et al*, 1997). The epidermis therefore provides an excellent system for examining the coordinate regulation of cell proliferation, differentiation, and cell death.

In normal epidermis, the antiapoptotic bcl-2 gene product is confined to the mitotically active basal keratinocyte layer and is undetectable in suprabasal layers (Hockenbery *et al*, 1991; Rodriguez-Villanueva *et al*, 1995; Delehedde *et al*, 1999). Bcl-2 protein is expressed in basal cell carcinomas (Cerroni and Kerl, 1994; Nakagawa *et al*, 1994; Morales-Ducret *et al*, 1995; Rodriguez-Villanueva *et al*, 1995; Delehedde *et al*, 1999). Other proteins of the bcl-2 gene family are also differentially expressed in the epidermis and in nonmelanoma skin cancers (Delehedde *et al*, 1999). Direct evidence for the contribution of bcl-2 (Rodriguez-Villanueva *et al*, 1998), bcl-x (Pena *et al*, 1997), and bax (Cho *et al*, submitted) to multistep skin carcinogenesis has recently been provided using genetically engineered strains of mice. Additionally, activating mutations involving members of the ras gene family occur commonly in human and murine nonmelanoma skin cancers (Quintanilla *et al*, 1991; Pierceall *et al*, 1992). Targeted expression of an activated Ha-ras oncogene in the epidermis results in hyperkeratosis and the formation of papillomas is insufficient for malignant transformation (Greenhalgh *et al*, 1993).

Organotypic cultures (Asselineau *et al*, 1985; Blanton *et al*, 1991; Hurlin *et al*, 1995a; 1995b) of the human nontumorigenic immortalized keratinocyte cell line, HaCaT (Boukamp *et al*, 1988), represent a useful and established model system to study keratinocyte and epidermal biologic processes. These raft cultures were used to evaluate the impact of bcl-2 and/or ras gene deregulation on keratinocyte proliferation, apoptosis, differentiation, and response to ultraviolet irradiation. Functional interactions between bcl-2 and Ha-ras were assessed in clones expressing both of these proteins. The findings provide insight into the potential functional interactions and cooperation of bcl-2 and ras during multistep skin carcinogenesis.

MATERIALS AND METHODS

Cells and culture conditions The immortalized human HaCaT keratinocytes (generously provided by Dr. N. Fusenig, Germany) were

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Abbreviations: AI, apoptotic index; APAAP, alkaline phosphatase antialkaline phosphatase; PCNA, proliferating cell nuclear antigen; PI, proliferative index.

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cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and the antibiotics penicillin (1000 U per ml) and streptomycin (1 mg per ml) (Gibco-BRL, Rockville, MD) (Boukamp *et al*, 1988, 1999). Cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. NIH 3T3 fibroblasts were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS, 100 mM HEPES, and the antibiotics penicillin (1000 U per ml) and streptomycin (1 mg per ml) (Gibco-BRL). Culture medium was changed every 2–3 d.

Transfections were performed with lipofectamine (Gibco-BRL). Plasmids (pEJ, pSV2-neo, or hygro) were used as described previously (Fernandez *et al*, 1994; 1995). Briefly, 10 μg of DNA, pSV2-neo-bcl-2, hygro-ras, or both, was added to the cells with the carrier. Twenty-four hours later, cells from each dish were trypsinized and transferred to new dishes. After another 12 h, G418, hygromycin, or both antibiotics was added to the medium at a concentration of 400 μg per ml and 200 μg per ml, respectively. The cells were fed with fresh medium containing antibiotics every 2 d. After cloning, bcl-2 expressing cells, ras expressing cells, or cells expressing both oncogenes were expanded for further analysis.

Organotypic culture Organotypic cultures were prepared as previously described (Asselineau *et al*, 1985; Blanton *et al*, 1991). To prepare a dermal equivalent, 3×10^5 per ml 3T3 fibroblasts were mixed at 4°C with type I collagen matrix reconstituted according to the manufacturer's specifications (Beckton Dickinson, San Jose, CA). The mixture (600 μl per well) was plated onto 24-well plates and placed at 37°C for 10–30 min (gelation). The dermal equivalents were then covered with medium and allowed to equilibrate overnight (contraction). The transfected clones were seeded on the dermal equivalent and kept submerged until confluent, and then raised on stainless steel grids. They were kept at the air-liquid interface for 2 wk to enable keratinocyte stratification and differentiation. The rafts were rinsed in phosphate-buffered saline (PBS), fixed in formalin, embedded in paraffin, sectioned, and processed for light microscopy. Alternatively, they were washed with PBS and lyzed in a buffer containing 0.3% sodium dodecyl sulphate (SDS) and 1% β-mercaptomethanol for immunoblotting.

In some experiments, raft cultures were ultraviolet irradiated (4 kJ per m^2) using a Westinghouse FS40 sun lamp. After irradiation, fresh medium was added back to the cultures up to the level of the grid and they were put back in the incubator for 24 h. After formalin fixation, they were then processed for light microscopy.

Immunohistochemistry Paraffin sections, 6 μm thick, were deparaffinized in xylene and rehydrated in a graded series of ethanol and water. For immunohistochemical analysis, the primary antibodies used in this work included anti-proliferating cell nuclear antigen (anti-PCNA) (clone PC10, Dako, Carpinteria, CA), rabbit anti-K1 and rabbit anti-K14 (Babco, Berkeley, CA), mouse anti-involucrin (Sigma, St. Louis, MO), and mouse $p21^{cip1/waf1}$ (Calbiochem, San Diego, CA). After rehydration, sections were incubated with the primary antibody for 1 h in a moist chamber. After washing in Tris-buffered saline (TBS), they were incubated with rabbit antimouse antibody, diluted 1:25 in TBS, for 30 min at room temperature or goat antirabbit antibody (Dako) at 1:50 for 20 min at room temperature. The slides were rinsed in TBS. For involucrin staining the slides were rinsed in TBS and then incubated with alkaline phosphatase antialkaline phosphatase (APAAP) at 1:50 for 20 min. To increase the intensity of the staining, the antimouse antibody and APAAP steps were repeated for three cycles with 10 min incubations each. Alkaline phosphatase substrate, Fushine (Dako), was added and incubated for 10 min at room temperature and in the dark, and then rinsed in water. After rinsing in water, slides were counterstained with Mayer's hematoxylin and coverslipped. For all other proteins the slides were rinsed with PBS and covered with a 1:100 dilution of peroxidase-conjugated goat antirabbit IgG antibody in PBS containing 1% goat serum for 45 min at room temperature. Finally, after an additional 30 min with Vectastain ABC horseradish peroxidase, the staining was revealed with the chromogen, diaminobenzadine, and counterstained with hematoxylin. Negative controls consisted of sections in which the primary antibody was omitted.

For PCNA immunodetection, the primary antibody was diluted in PBS containing 0.5% Tween-20 as described previously (Delehedde *et al*, 1995). Negative controls were incubated with PBS without antibody. After washing in PBS, the slides were incubated with secondary horseradish-peroxidase-labeled anti-IgG antibodies (Amersham Life Science, Arlington Heights, IL) diluted 1:100 with 1% goat serum in PBS for 30 min at room temperature. Sections were then incubated with Vectastain ABC horseradish peroxidase for 30 min followed by the addition of substrate for a period of 5–15 min (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and coverslipped. The proliferative index (PI) was calculated as the percentage of PCNA-positive keratinocyte nuclei by scoring 2000 cells in each experiment.

TUNEL method The TdT-mediated dUTP nick end-labeling (TUNEL) reaction was performed on formalin-fixed paraffin-embedded sections. After rehydration, sections were pretreated in 10 mg per ml proteinase K (Trevigen, Gaithersburg, MD) for 30 min at 31°C, washed with PBS, and incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and dUTP (Boehringer Mannheim, Germany) for 1 h at 31°C. Positive controls were performed by treating the slides with DNAse I grade II (Boehringer Mannheim) at 100 ng per ml at 37°C for 30 min. In negative controls, the TdT enzyme was omitted. The apoptotic index (AI) was calculated as the percentage of TUNEL-positive keratinocyte nuclei by scoring 2000 cells in each experiment.

Gel electrophoresis and immunoblotting Aliquots of protein extracts were electrophoresed on 12.5% SDS polyacrylamide gels (SDS-PAGE). After transfer of the proteins to nitrocellulose, filters were blocked overnight at 4°C in blotting solution (PBS, 5% Carnation nonfat dry milk, 0.05% Tween-20). Incubation with the primary antibody diluted at 1:500 was carried out for 1 h at room temperature in the blotting solution. After five washes with 0.05% Tween-20 in PBS, the nitrocellulose was further incubated with secondary peroxidase-labeled anti-IgG antibodies, diluted 1:500 in the blotting solution. After several washes with 0.05% Tween-20 in PBS, immunoreactive proteins were detected with ECL-Western chemiluminescence detection on Hyperfilm (Amersham Life Science). Antibodies included hamster anti-bcl-2 (Pharmingen, San Diego, CA), rabbit anti-K1 (Babco), mouse anti-K14 (Research Diagnostic, Flanders, NJ), mouse anti-involucrin (Sigma), mouse anti-p21 (Calbiochem), rabbit anticyclin D1 and rabbit anti-ras (Santa Cruz, Santa Cruz, CA), mouse anti-PCNA (Dako), and mouse antiactin (Amersham Pharmacia Biotech, U.K.).

RESULTS

Generation of HaCaT bcl-2 and Ha-ras transfectant clones The HaCaT cell line was established from a spontaneously immortalized human keratinocyte (Boukamp *et al*, 1988). Although aneuploid, HaCaT cells are nontumorigenic and possess the capacity to differentiate into normal epidermis when transplanted onto nude mice (Boukamp *et al*, 1988). HaCaT cells overexpressing bcl-2, Ha-ras, or both oncogenes were generated using standard gene transfer techniques. Control clones were transfected with an empty expression plasmid. Appropriate transgene expression was regularly confirmed by immunoblotting using a bcl-2 monoclonal antibody and an antibody specific for the val-12 mutation of Ha-ras (**Fig 1**). In monolayer culture, HaCaT-ras cells had the fastest growth rate, consistent with previously published observations (Boukamp *et al*, 1990), and HaCaT-ras/bcl-2 clones grew slightly faster than the HaCaT-bcl-2 or the HaCaT parental cells (data not shown).

Impact of Ha-ras and bcl-2 on HaCaT epidermal histogenesis *in vitro* Human keratinocytes grown on a "dermal equivalent" at the liquid–air interface have been shown to reconstitute a histologically normal appearing epidermis *in vitro* (Asselineau *et al*, 1985; 1986; Schoop *et al*, 1999). These organotypic raft cultures provide a useful *in vitro* model system to assess the impact of inappropriate expression of individual genes on preneoplastic keratinocyte homeostasis. This *in vitro* raft culture model system enabled the assessment of the impact of enforced Ha-ras and bcl-2 expression on HaCaT differentiation and histogenesis. Raft cultures were formalin-fixed and paraffin-embedded for light microscopy analysis after 2 wk of growth at the liquid–air interface. The HaCaT cells produced a stratified epithelium six to eight layers in thickness (**Fig 2*A***). They developed a cuboidal basal cell layer and superbasal layer of squamous cells exhibiting histologic evidence of differentiation including an intermediate layer of cells possessing keratohyaline granules and a superficial squamous cell layer. A well-formed stratum corneum, as previously noted (Schoop *et al*, 1999), was not observed. Stratification or evidence of differentiation was not observed when the HaCaT keratinocytes were grown on the dermal equivalent submerged in the culture medium (data not shown).

Histologically, HaCaT-bcl-2 cells were indistinguishable from HaCaT control keratinocytes (**Fig 2*B***). HaCaT-ras keratinocytes also generated a stratified epithelium with subtle, yet consistent, differences from control HaCaT cells (**Fig 2*C***). Unlike HaCaT cells, the suprabasal layer of Ha-ras transfected keratinocytes tended to maintain a cuboidal appearance. A superficial squamous layer was observed in HaCaT-ras cells but, unlike HaCaT cells, was discontinuous. Additionally, the nuclear:cytoplasmic ratio in Ha-ras keratinocytes was increased compared with either HaCaT-bcl-2 or HaCaT-ras/bcl-2 keratinocytes. Raft cultures of HaCaT-ras/bcl-2 keratinocytes exhibited histologic evidence of enhanced differentiation (**Fig 2*D***). They failed to exhibit a well-defined layer of polygonal basal keratinocytes. Additionally, suprabasal cells rapidly acquired a squamous morphology. Many of these cells possessed prominent keratohyaline granules. The hematoxylin and eosin stained sections shown in **Fig 2** were representative of the morphology exhibited by control, ras, bcl-2, and ras/bcl-2 clones grown in organotypic cultures in independent experiments.

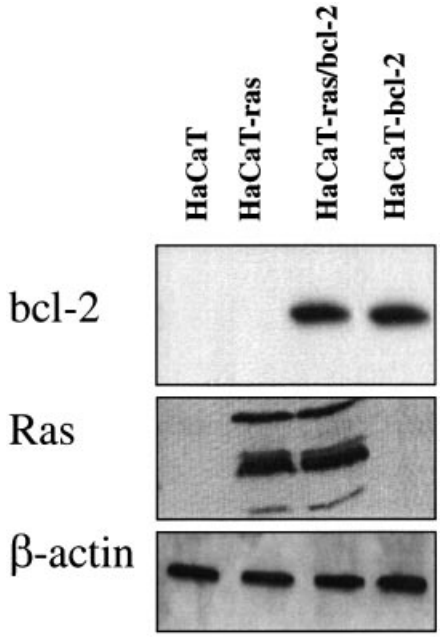


Figure 1. Immunoblot analysis of Ha-ras and bcl-2 protein in representative HaCaT transfectant clones. Total protein extracts were electrophoresed on 12.5% SDS-PAGE, electroblotted, and hybridized with an anti-Ha-ras antibody specific for the valine 12 mutation and antibcl-2 antibodies as described in *Materials and Methods*. Actin served as a control for equal loading.

The impact of Ha-ras and bcl-2 on differentiation of HaCaT keratinocytes in organotypic culture On the basis of the light microscopy observations it was of interest to assess the expression of proteins associated with keratinocyte differentiation using immunohistochemical and immunoblotting techniques. The expression of involucrin is normally limited to the suprabasal layers in the epidermis. Immunohistochemical evaluation of HaCaT and HaCaT-bcl-2 raft cultures demonstrated that involucrin was uniformly expressed in suprabasal cells (**Fig 3*A*, *B***). The basal cell layer exhibited no detectable involucrin expression. The expression of involucrin was also limited to suprabasal cells in HaCaT-ras raft cultures (**Fig 3*C***). Unlike HaCaT and HaCaT-bcl-2 rafts, however, the suprabasal cells in HaCaT-ras cultures exhibited only focal immunoreactivity for involucrin. Rafts generated from HaCaT-ras/bcl-2 keratinocytes exhibited uniform expression of involucrin in all the layers of epithelium (**Fig 3*D***).

The expression of cytokeratin 1 (K1), usually limited to upper layers of the epidermis, and cytokeratin 14 (K14), expressed preferentially in basal keratinocytes, was also examined in raft cultures using immunohistochemistry. In HaCaT and HaCaT-bcl-2 raft cultures K1 was undetectable in the basal cell layers and uniformly expressed in the suprabasal cells (**Fig 4*A*, *B***). In general, the suprabasal keratinocytes in HaCaT-ras rafts expressed lower levels of K1 compared with those derived from HaCaT or HaCaT-bcl-2 clones (**Fig 4*C***). Scattered suprabasal HaCaT-ras cells were more intensely immunoreactive for K1. The distribution of K1 in HaCaT-ras/bcl-2 rafts was heterogeneous, with scattered small clusters of cells exhibiting undetectable levels (**Fig 4*D***). Additionally, K1 expression was focally positive in all layers in extent including basal layer keratinocytes in rafts generated from HaCaT-ras/bcl-2 cells.

K14 was localized to the basal two to three cell layers in both HaCaT and HaCaT-bcl-2 raft cultures (**Fig 4*E*, *F***). More superficial keratinocyte layers did not express detectable levels of K14. In general, K14 was less intensely immunoreactive in HaCaT-ras rafts compared with both HaCaT and HaCaT-bcl-2 rafts (**Fig 4*G***). Additionally, K14 immunoreactivity was present at approximately equivalent levels in superficial keratinocyte layers and basal keratinocytes in HaCaT-ras raft cultures. The distribution of K14 in rafts derived from HaCaT-ras/bcl-2 cells was heterogeneous with basal keratinocytes, in general, being immunoreactive

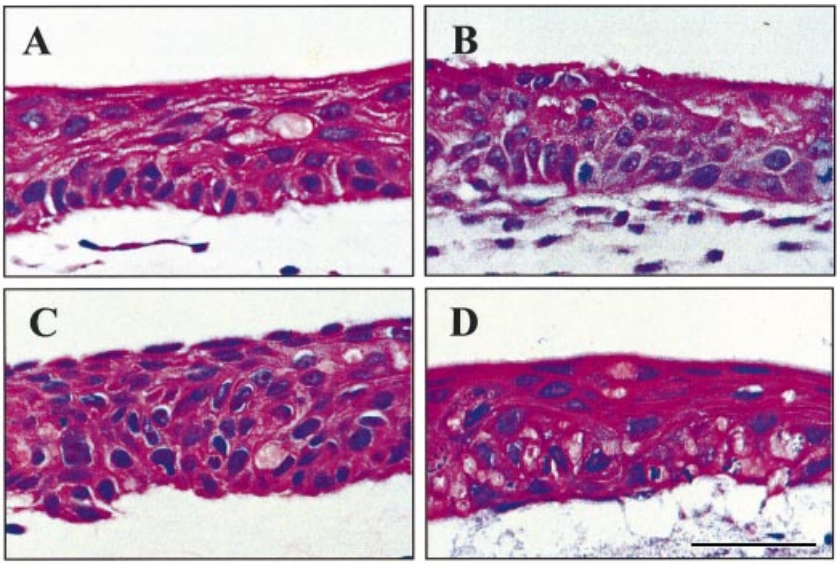


Figure 2. Light microscopy of HaCaT cells grown in organotypic culture. Light microscopy appearance of hematoxylin and eosin stained sections of organotypic cultures generated from HaCaT control cells (*A*), HaCaT-bcl-2 transfectants (*B*), HaCaT-ras transfectants (*C*), and HaCaT-ras/bcl-2 transfectants (*D*); *scale bar*: 50 μm. These sections are representative of rafts made from the control cells and from a total of 11 clones of bcl-2, ras, and ras/bcl-2 transfected cell lines.

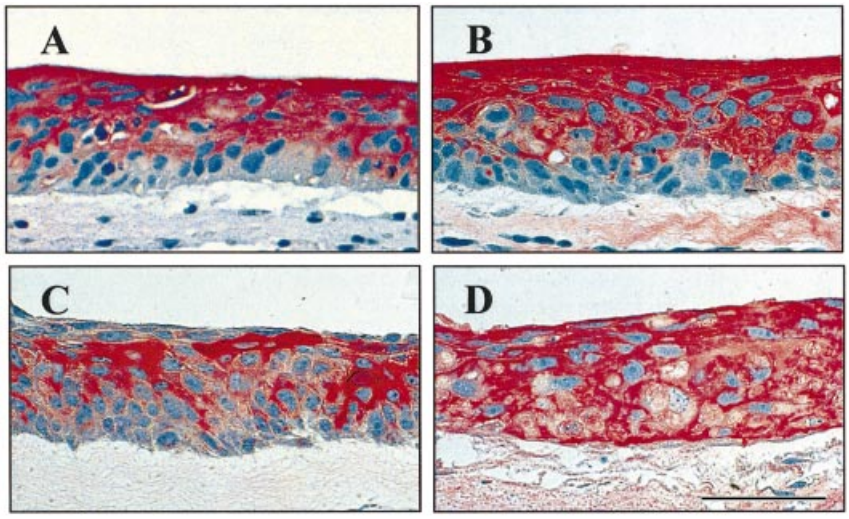


Figure 3. Immunohistochemical evaluation of involucrin expression in organotypic cultures. Two week organotypic cultures of HaCaT control cells (*A*), HaCaT-bcl-2 transfectants (*B*), HaCaT-ras transfectants (*C*), and HaCaT-ras/bcl-2 transfectants (*D*) were stained using an APAAP technique and an anti-involucrin antibody as described in *Materials and Methods*. *Scale bar*: 50 μm.

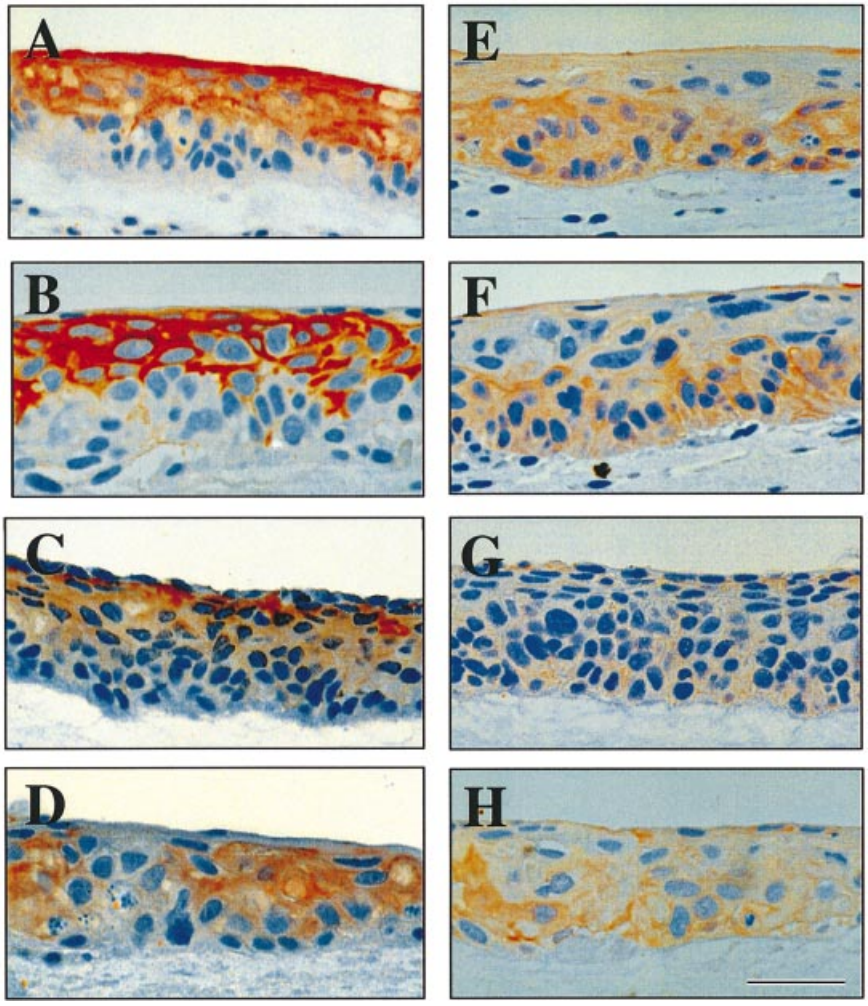


Figure 4. Immunohistochemical evaluation of cytokeratin 1 and cytokeratin 14 expression in organotypic cultures. Two week organotypic cultures of HaCaT control cells (*A*, *E*), HaCaT-bcl-2 transfectants (*B*, *F*), HaCaT-ras transfectants (*C*, *G*), and HaCaT-ras/bcl-2 transfectants (*D*, *H*) were stained using horseradish peroxidase diaminobenzidine reaction and anti-K1 (*A–D*) and cytokeratin 14 (*E–H*) antibodies as described in *Materials and Methods*. *Scale bar*: 50 μm.

(**Fig 4*H***). Scattered K14 positive suprabasal cells were also observed in HaCaT-ras/bcl-2 rafts.

Immunoblot analysis of K1, K14, and involucrin expression in raft cultures was consistent with results obtained using immunohistochemical techniques (**Fig 5**). Approximately equivalent levels of both involucrin and K1 were observed in HaCaT control, HaCaT-bcl-2, and HaCaT-ras/bcl-2 keratinocytes grown in raft cultures. In contrast, the levels of involucrin and K1 were reduced in HaCaT-ras cells (**Fig 5**). The level of K14 protein was similar in all cell lines (**Fig 5**).

The impact of Ha-ras and bcl-2 on HaCaT keratinocyte proliferation in organotypic culture It was of interest to quantitate the extent of proliferation and distribution of proliferative cells in the organotypic raft cultures. An immunohistochemical and immunoblot analysis of PCNA and the cyclin-

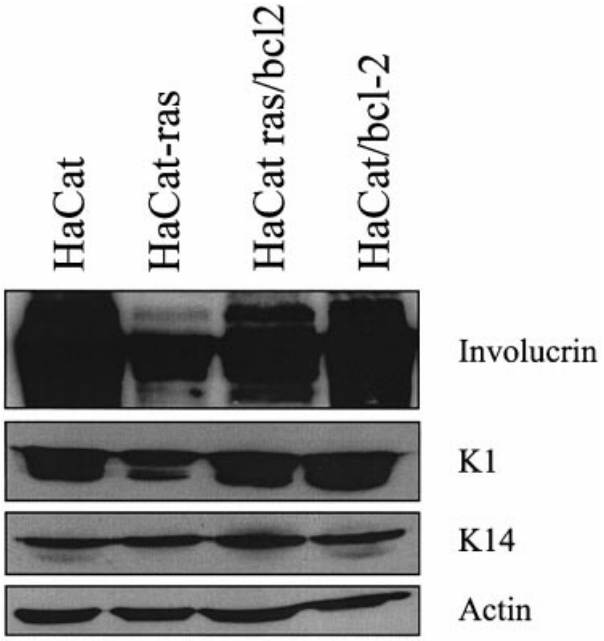


Figure 5. Immunoblot analysis of K1, K14, and involucrin protein in HaCaT organotypic cultures. Total protein extracts were electrophoresed on 12.5% SDS-PAGE, electroblotted, and hybridized with antibodies (involucrin, K1, and K14) as described in *Materials and Methods*. Actin was used as a control for loading.

dependent kinase inhibitor $p21^{cip1/waf1}$ was therefore undertaken. Similar to normal epidermis, PCNA-positive HaCaT cells were, with rare exceptions, limited to the basal layer in raft cultures (**Fig 6*A***). In HaCaT-bcl-2 rafts PCNA-positive cells were not strictly confined to the basal cell layer but included the majority of keratinocytes in the first two to three layers of suprabasal cells (**Fig 6*B***). In contrast, in HaCaT-ras organotypic rafts PCNA-positive cells were present throughout all cell layers (**Fig 6*C***). PCNA-negative cells remained, in general, in the suprabasal layer in HaCaT-ras cultures. Similar to HaCaT rafts, however, the more superficial keratinocyte layers showed only rare PCNA-positive cells. Interestingly, PCNA-positive keratinocytes were limited to the basal cell layer in organotypic cultures generated from HaCaT-ras/bcl-2 coexpressing cells (**Fig 6*D***). The expression of $p21^{cip1/waf1}$ was, in general, limited to the superficial keratinocyte layers in organotypic cultures derived from HaCaT cells (**Fig 6*E***). Scattered suprabasal $p21^{cip1/waf1}$ cells were also present in HaCaT-bcl-2 (**Fig 6*F***) and HaCaT-ras (**Fig 6*G***) rafts, although fewer in number and less intensely stained than in HaCaT rafts. The distribution of $p21^{cip1/waf1}$ in HaCaT-ras/bcl-2 coexpressing rafts was similar to that observed for rafts derived from HaCaT control cells except that occasional basal keratinocytes were also positive (**Fig 6*H***).

The extent of proliferation in the organotypic cultures was quantitated by scoring the number of PCNA-positive nuclei in at least 2000 cells per raft for each of four rafts (**Fig 7*A***). The PI was expressed as the percentage of the total cells that were PCNA positive. The PI were consistent with the results obtained using immunohistochemistry. The PI for HaCaT rafts was $15.43\% \pm 6.71\%$ and was significantly lower than the PI of $75.85\% \pm 8.01\%$ for HaCaT-ras rafts and the PI of $53.73\% \pm 3.12\%$ for HaCaT-bcl-2 rafts ($p < 0.001$). The PI of HaCaT-ras/bcl-2 raft cultures was $15.25\% \pm 5.56\%$ and similar to that observed for control HaCaT cultures and significantly less than the PI of both HaCaT-ras and HaCaT-bcl-2 rafts ($p < 0.001$).

The results of immunoblot analysis of PCNA and $p21^{cip1/waf1}$ protein levels was consistent overall with the observations using immunohistochemistry (**Fig 7*B***). The level of PCNA was increased and $p21^{cip1/waf1}$ decreased in HaCaT-ras and HaCaT-bcl-2 rafts compared to HaCaT control cells. The level of both PCNA and $p21^{cip1/waf1}$ was similar in HaCaT-ras/bcl-2 rafts and HaCaT rafts. Consistent variation in the level of cyclin D protein was not observed.

The impact of Ha-ras and bcl-2 on HaCaT keratinocyte apoptosis and response to ultraviolet irradiation in organotypic raft culture *In situ* labeling of apoptotic cells was accomplished using the TUNEL technique to identify cells possessing fragmented DNA. Apoptosis was quantified by scoring the number of TUNEL-positive cells in at least 2000 cells per raft for each of four rafts. The AI was expressed as the percentage of TUNEL-positive cells (**Fig 8**). The spontaneous AI for HaCaT organotypic rafts was $6.3\% \pm 1.68\%$. The spontaneous AI for HaCaT-ras and HaCaT-bcl-2 rafts were $0.75\% \pm 0.27\%$ and $2.14\% \pm 0.40\%$, respectively, and both were significantly lower than that of HaCaT rafts ($p < 0.028$). Interestingly, the spontaneous AI for HaCaT-ras/bcl-2 coexpressing rafts was $12.75\% \pm 1.12\%$, which was significantly greater than for rafts derived from HaCaT-ras or HaCaT-bcl-2 clones ($p < 0.001$).

The AI was also assessed 24 h following ultraviolet irradiation (**Fig 8**). The AI of HaCaT control cells increased significantly from the spontaneous level to $12.21\% \pm 2.77\%$ ($p < 0.012$). The AI of HaCaT-ras rafts increased approximately 25-fold to $20.37\% \pm 1.47\%$ following ultraviolet irradiation ($p < 0.001$). The AI in HaCaT-bcl-2 rafts also increased significantly ($p < 0.003$) to $7.50\% \pm 2.21\%$ following irradiation but remained significantly less than that observed in HaCaT rafts ($p < 0.038$). The AI of $10.07\% \pm 3.22\%$ in HaCaT-ras/bcl-2 rafts was not significantly different from that for the corresponding unirradiated rafts, but was significantly less than that observed in HaCaT-ras rafts ($p \leqslant 0.001$). Apoptotic cells were found primarily in the suprabasal layers, and were absent in the 3T3 cells in the dermal equivalent (data not shown).

DISCUSSION

The epidermis is a complex epithelium and the maintenance of homeostatic balance is dependent on the coordinate regulation of cell proliferation, differentiation, and cell death. Within the epidermis these physiologic processes are normally limited within specific cytoarchitectural layers. We examined the impact of bcl-2 and Ha-ras gene deregulation on keratinocyte homeostasis using an established *in vitro* organotypic culture model (Asselineau *et al*, 1985, 1987; Blanton *et al*, 1991; Hurlin *et al*, 1995a; Schoop *et al*, 1999). The HaCaT keratinocyte cell line was induced to stratify and differentiate under organotypic raft culture conditions. Histologically, the differentiated HaCaT raft cultures resembled normal epidermis but lacked a superficial stratum corneum. This stratification was accompanied by the appearance, and normal distribution, of biochemical markers of keratinocyte differentiation. Involucrin and K1 were limited to the more superficial squamous keratinocyte layers and K14 was limited to basal cell layers. Additionally, the proliferative cells, as assessed by PCNA immunoreactivity, were limited to the basal cell layer and expression of $p21^{cip1/waf1}$ was limited to the more differentiated strata in HaCaT organotypic cultures similar to normal epidermis.

Activating mutations in the Ha-ras gene occur commonly in the development of nonmelanoma skin cancer (Quintanilla *et al*, 1991; Pierceall *et al*, 1992) and can contribute to multistep skin carcinogenesis (Greenhalgh *et al*, 1993). HaCaT-ras cells retained the ability to stratify under organotypic culture conditions but exhibited a reduction in the ability to form superficial layers with squamous morphology. These histologic alterations were accompanied by a reduction in the expression of involucrin and the maintenance of K14 expression in the more superficial keratinocyte layers. The PI was significantly greater in HaCaT-ras rafts compared to HaCaT rafts, and suprabasal PCNA-positive keratinocytes were frequently observed. This enhanced proliferation was also accompanied by a decrease in the expression of $p21^{cip1/waf1}$. Additionally, the extent of spontaneous apoptosis in HaCaT-ras

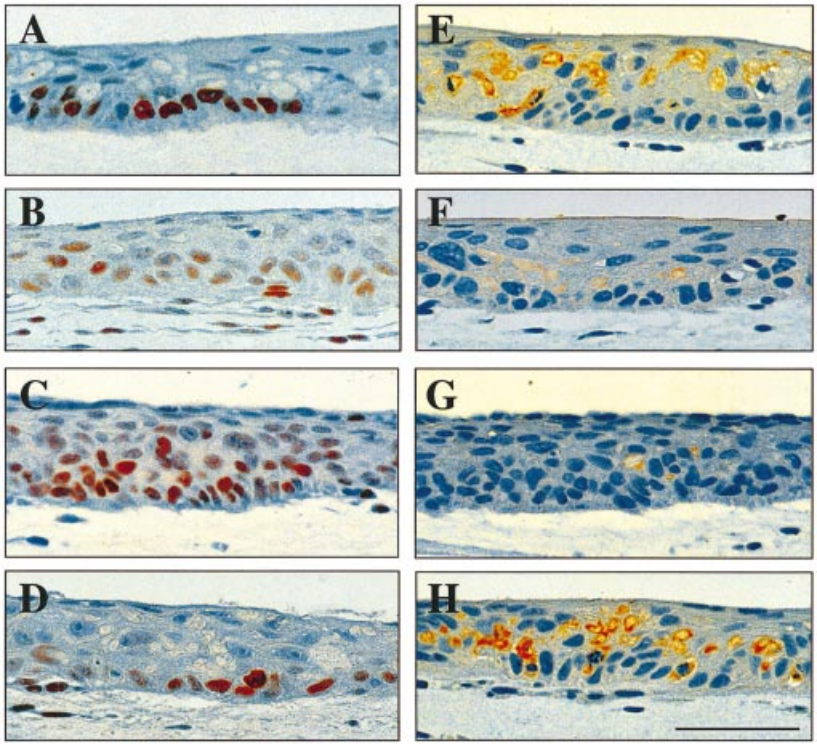


Figure 6. Immunohistochemical evaluation of PCNA and p21 expression in organotypic cultures. Two week organotypic cultures of HaCaT control cells (*A*, *E*), HaCaT-bcl-2 transfectants (*B*, *F*), HaCaT-ras transfectants (*C*, *G*), and HaCaT-ras/bcl-2 transfectants (*D*, *H*) were stained using a horseradish peroxidase technique and anti-PCNA (*A–D*) and p21 (*E–H*) antibodies as described in *Materials and Methods*. *Scale bar*: 50 μm.

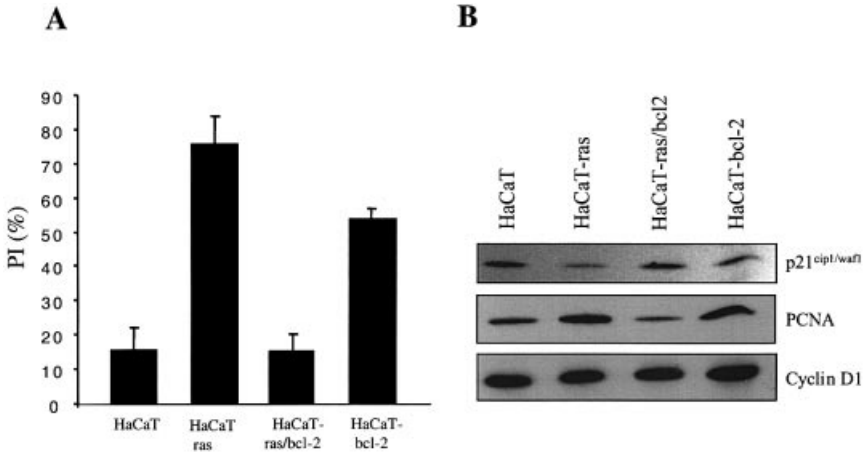


Figure 7. Comparison of proliferative rates and of the expression of the cell cycle proteins in organotypic cultures. (*A*) The PI was calculated as the percentage of PCNA-positive cells by scoring at least 2000 cells in each of four 2 wk organotypic cultures for each genotype. The data represent the mean ± the standard deviation. Statistical comparisons were calculated using Student's *t* test. (*B*) Immunoblot analysis of PCNA, $p21^{cip1/waf1}$, and cyclin D protein in HaCaT organotypic cultures. Total protein extracts were electrophoresed on 12.5% SDS-PAGE, electroblotted, and hybridized with antibodies for PCNA, $p21^{cip1/waf1}$, and cyclin D as described in *Materials and Methods*.

rafts was significantly less than that of HaCaT keratinocytes under organotypic culture conditions. These findings are in general agreement on the impact of Ha-ras activation in keratinocytes with those of others (Ryle *et al*, 1989; Boukamp *et al*, 1990; Breitkreutz *et al*, 1991; Dlugosz *et al*, 1994).

An increase in the level of bcl-2 protein is characteristic of some forms of nonmelanoma skin cancer (Rodriguez-Villanueva *et al*, 1995; Delehedde *et al*, 1999). It has also been recently shown that bcl-2 can contribute to multistep skin carcinogenesis in transgenic mice (Rodriguez-Villanueva *et al*, 1998). The level and distribution of K1, K14, and involucrin were similar in HaCaT-bcl-2 and HaCaT raft cultures. This is also in general agreement with observations of epidermal differentiation in HK1-bcl-2 transgenic mice (Rodriguez-Villanueva *et al*, 1998). The PI in HaCaT-bcl-2 keratinocytes grown in organotypic culture was significantly higher and the spontaneous AI significantly lower than that observed in HaCaT rafts. This was associated with a reduction in the level of $p21^{cip1/waf1}$ and an increase in PCNA protein.

Simultaneous deregulation of bcl-2 and Ha-ras in HaCaT-ras/bcl-2 organotypic cultures resulted in an apparent enhancement in differentiation. This was associated with a rapid acquisition of squamous morphology. The expression of involucrin was no longer limited to the superficial keratinocyte layers; rather it was present in all layers of the organotypic culture. Additionally, in marked contrast to either HaCaT-ras or HaCaT-bcl-2 rafts, proliferating cells were confined to the basal cell layer and levels of $p21^{cip1/waf1}$ approximated that observed in HaCaT rafts. Additionally, the spontaneous AI was significantly higher in HaCaT-ras/bcl-2 rafts compared with those generated from either HaCaT-ras or HaCaT-bcl-2.

These findings suggest that enforced Ha-ras expression disrupts normal keratinocyte homeostasis in organotypic culture, effecting a reduction in differentiation and apoptosis, and augmenting cellular proliferation. Further, it appears that simultaneous expression of bcl-2 with Ha-ras is able, in large measure, to restore keratinocyte homeostasis in that the normal pattern of differentiation and proliferation was observed as well as an augmentation in the rate of apoptosis. In this experimental system, the AI correlated with the extent of keratinocyte differentiation, as assessed by both immunohistochemistry and immunoblot analysis, whereas the PI

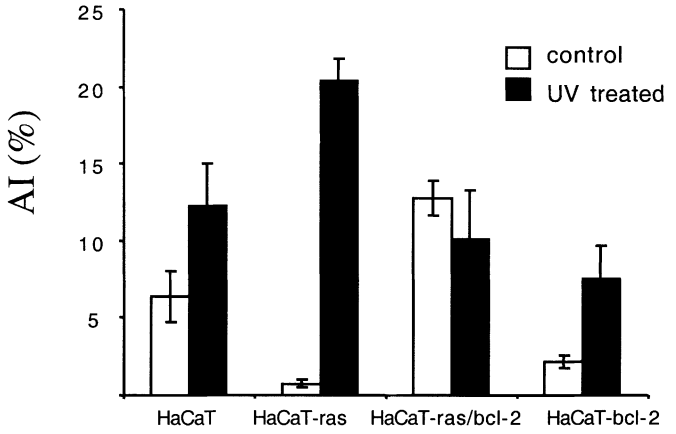


Figure 8. Comparison of apoptotic rates in organotypic cultures before and following ultraviolet irradiation. The AI was calculated as the percentage of TUNEL-positive cells by scoring at least 2000 cells in each of four 2 wk organotypic cultures for each genotype before and 24 h after ultraviolet irradiation (4 kJ per m^2). The data represent the mean ± the standard deviation. Statistical comparisons were calculated using Student's *t* test.

was inversely, in general, correlated with the extent of differentiation. The mechanistic basis of the cooperation of Ha-ras and bcl-2 in this context is unknown. In this regard, it could be anticipated that at least 30% of some types of nonmelanoma skin cancers would be predicted to express high levels of bcl-2 protein (Rodriguez-Villanueva *et al*, 1995; Delehedde *et al*, 1999) and possess activating Ha-ras mutations (Pierceall *et al*, 1992). Additional studies will assess the potential cooperation of Ha-ras and bcl-2 in the epidermis *in vivo* using established transgenic mouse models (Greenhalgh *et al*, 1993; Rodriguez-Villanueva *et al*, 1998).

Signaling events mediated by the ras gene family are complex and may positively and negatively regulate cell death as well as proliferation (Frame and Balmain, 2000). HaCaT-ras cells grown in organotypic culture exhibited significantly lower spontaneous rates of apoptosis than control HaCaT cells. HaCaT-ras keratinocytes were significantly more sensitive to apoptosis induction by ultraviolet irradiation, however. Consistent with previous observations using mouse models, bcl-2 provided significant protection against ultraviolet irradiation in HaCaT-bcl-2 keratinocytes grown in organotypic culture (Rodriguez-Villanueva *et al*, 1998; Muller-Rover *et al*, 2000; Cho *et al*, submitted). The observation that bcl-2 could abrogate apoptosis induction in HaCaT-ras organotypic cultures following ultraviolet irradiation may be relevant for the complementation of these gene products during multistep carcinogenesis.

In general, it was also apparent that the PI and spontaneous AI were inversely correlated ($r = 0.74$). Although a morphometric quantification of absolute cell number in the organotypic cultures was not undertaken in this study, this finding implies that mechanisms other than apoptosis, and its associated DNA fragmentation, may potentially be important for determining the thickness of stratified squamous epithelia. These results are consistent with those of others that terminal keratinocyte differentiation and apoptosis are distinct processes and may be independently regulated (Gandarillas *et al*, 1999).

In several cell types, enforced expression of bcl-2 has been associated with delaying cell cycle progression (Borner, 1996; Linette *et al*, 1996; Huang *et al*, 1997). Most of these experimental systems document the ability of bcl-2 to delay the G_1-S transition in cells stimulated to reenter the cell cycle. Thus, our finding that enforced bcl-2 expression enhanced steady-state levels of proliferation in *in vitro* organotypic cultures of keratinocytes is derived from a cell type and experimental system different from those of other investigators. It should also be recognized, in this regard, that the distribution of bcl-2 protein corresponds to the proliferative compartment within the normal epidermis, i.e., the stratum basale (Hockenbery *et al*, 1991; Rodriguez-Villanueva *et al*, 1995; Delehedde *et al*, 1999).

The level and distribution of $p21^{cip1/waf1}$ protein were correlated with differentiation and inversely correlated with proliferation as assessed by immunohistochemistry and immunoblotting in organotypic cultures of HaCaT keratinocytes. The role of $p21^{cip1/waf1}$ in the regulation of cell cycle progression is well established (El-Deiry *et al*, 1993; Harper *et al*, 1993). Upregulation of $p21^{cip1/waf1}$ is also observed during keratinocyte differentiation in human epidermis (Tron *et al*, 1996). It is known that $p21^{cip1/waf1}$ may be transcriptionally regulated by the p53 tumor suppressor protein. The upregulation of $p21^{cip1/waf1}$ observed in organotypic cultures of HaCaT cells is p53-independent, however, in that this cell line has undergone p53 gene inactivation (Lehman *et al*, 1993). The Sp3 transcription factor has been shown to transcriptionally activate the $p21^{cip1/waf1}$ promoter and appears to be responsible for $p21^{cip1/waf1}$ upregulation during keratinocyte differentiation (Prowse *et al*, 1997). It has recently been shown that the cell cycle inhibitory effects of $p21^{cip1/waf1}$ may be dissociated from its ability to modulate differentiation in keratinocytes (Di Cunto *et al*, 1998).

In summary, the findings provide insight into the cooperation of bcl-2 and Ha-ras gene deregulation in human keratinocytes. It will be necessary to extend these observations to other *in vitro* models of keratinocyte differentiation (Allen-Hoffman *et al*, 2000) and transgenic mouse models (Greenhalgh *et al*, 1993; Rodriguez-Villanueva *et al*, 1998) to ascertain their general significance in the maintenance of epidermal homeostasis and multistep skin carcinogenesis.

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