Forced Expression of Keratin 16 Alters the Adhesion, Differentiation, and Migration of Mouse Skin Keratinocytes

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Injury to the skin results in an induction of keratins K6, K16, and K17 concomitant with activation of keratinocytes for reepithelialization. Forced expression of human K16 in skin epithelia of transgenic mice causes a phenotype that mimics several aspects of keratinocyte activation. Two types of transgenic keratinocytes, with forced expression of either human K16 or a K16-C14 chimeric cDNA, were analyzed in primary culture to assess the impact of K16 expression at a cellular level. High K16-C14-expressing and low K16-expressing transgenic keratinocytes behave similar to wild type in all aspects tested. In contrast, high K16-expressing transgenic keratinocytes show alterations in plating efficiency and calcium-induced differentiation, but proliferate normally. Migration of keratinocytes is reduced in K16 transgenic skin explants compared with controls. Finally, a subset of high K16-expressing transgenic keratinocytes develops major changes in the organization of keratin filaments in a time- and calcium concentration-dependent manner. These changes coincide with alterations in keratin content while the steady-state levels of K16 protein remain stable. We conclude that forced expression of K16 in progenitor skin keratinocytes directly impacts properties such as adhesion, differentiation, and migration, and that these effects depend upon determinants contained within its carboxy terminus.

INTRODUCTION

Following injury to the skin, epidermal keratinocytes located near the wound edge are mobilized to migrate into the wound site and restore the epithelial lining and barrier function. A process termed activation occurs early after injury, and is believed to endow keratinocytes with the elements they need to migrate in a coordinated manner towards the wound (Grinnell, 1992; Clark, 1993; Coulombe, 1997). Among the hallmarks of an activated keratinocyte are cell hypertrophy, formation of cytoplasmic processes in the direction of cell migration, altered cell adhesion, and juxtanuclear reorganization of the keratin intermediate filament network. This last characteristic, remodeling of keratin filaments, is of great interest because it is not understood why or how the normally stable keratin network alters its structure.

Keratin intermediate filaments (IFs) are heteropolymers of type I and type II keratin proteins that occur in the cytoplasm of all epithelial cells. Consistent with this assembly requirement, epithelial cells must coordinate the expression of at least one type I and one type II gene to produce a keratin IF network (Fuchs and Weber, 1994). The expression of many type I and type II keratin genes is regulated in a pairwise and differentiation-specific manner. The keratin genes expressed in "soft" epithelia include the type II K1-K8 and the type I K9-K20 (O'Guin *et al.*, 1990). Given their properties (Ma *et al.*, 1999), abundance, and organization in the cytoplasm, keratin IFs are poised to play an important role of mechanical support in epithelial cells and tissues. Such a role has been shown by transgenic mouse studies and through the discovery of mutations affecting keratin proteins in inherited epithelial fragility disorders (Fuchs and Cleveland, 1998; Takahashi *et al.*, 1998; Irvine and McLean, 1999). In addition to forming a structural scaffolding, IFs fulfill specialized, cell type-specific roles, albeit in a context-dependent manner.

K16 and its type II partner K6 are normally expressed in stratified epithelia including palmar and plantar epidermis, the outer root sheath of hair follicles, nail bed, the oral mucosa, and several others. These keratins do not occur in interfollicular epidermis under normal conditions. However, they are markedly induced, along with K17, in stratified epithelia showing hyperproliferation and aberrant differentiation such as in psoriasis and cancer (O'Guin *et al.*, 1990; McGowan and Coulombe, 1998a). In addition, K6, K16, and K17 are up-regulated in keratinocytes at the wound

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edge within 2–6 h after injury to normal epidermis and other stratified epithelia (Paladini *et al.*, 1996; McGowan and Coulombe, 1998b; Takahashi *et al.*, 1998). This induction correlates temporally and spatially with the onset of keratinocyte activation and, therefore, has brought about the hypothesis that these keratins are involved in this process (Paladini *et al.*, 1996). In support of this, forced expression of human K16 under the control of two distinct promoters in the skin of transgenic mice causes alterations in keratin IF organization and cell–cell adhesion in keratinocytes (Takahashi *et al.*, 1994; Paladini *et al.*, 1996; Paladini and Coulombe, 1998). Moreover, Paramio *et al.* (1999) reported that K16 can stimulate epithelial cell proliferation in a cell-autonomous manner in a transfection-based, cell culture study.

The impact of K16 expression at a cellular level has yet to be addressed in normal skin keratinocytes. Here we take advantage of an existing transgenic mouse model to characterize the effects of K16 expression on the ability of mouse keratinocytes to adhere, proliferate, differentiate, and migrate in a primary culture setting. We also examine the parameters that influence K16's ability to promote a reorganization of keratin IF networks. The findings we report show that K16 expression directly affects several basic properties of keratinocytes, and provide new insights into the mechanisms by which K16 impacts keratin IF organization.

MATERIALS AND METHODS

Isolation of Keratinocytes for Primary Culture

Keratinocytes for primary cultures were isolated as described (Rhouabhia *et al.*, 1992) from wild-type 57Bl6/BalbC3 F1 mice and from transgenic mice with expression of the human K16 cDNA or a chimeric human K16-C14 cDNA under the control of the K14 promoter. Information relative to these transgenic lines, including transgene copy number and genotyping, has been given elsewhere (Paladini and Coulombe, 1998). Final cell pellets were resuspended in media prepared with calcium-free minimum essential medium (BioWhittaker, Walkerville, MD), 8% Chelex-treated fetal bovine serum (Intergen, Purchase, NY), 50 units/l Pen-Strep (MediaTech, Herndon, VA), and 0.2 mM calcium (Hennings *et al.*, 1980; Hennings and Holbrook, 1983). Cells were pooled from animals with identical genotypes and plated at a density of 7–15 $\times$ 10^5/3.5-cm tissue culture dish for ~20 h at 37°C and 5% CO_2 unless otherwise noted. Cells were then washed three times with calcium-free phosphate-buffered saline (PBS) (MediaTech). New medium with calcium levels ranging from 0.05 to 2 mM was added, and cells were grown for up to 96 h.

Plating Efficiency

Plating efficiency was determined by placing 7 $\times$ 10^5 keratinocytes harvested from 0- to 3-d-old mice onto uncoated 3.5-cm tissue culture dishes. Cells were allowed to adhere for 6 h at 37°C and 5% CO_2. Keratinocytes floating in media along with those removed by gentle washes in calcium-free PBS were collected as the nonadherent fraction. Adherent cells were removed by treatment with 0.25% trypsin containing 1 mM EDTA (Life Technologies, Gaithersburg, MD) and collected. Cells in both populations were counted with a hemacytometer. The ratio of adherent cells to total cells was calculated to determine plating efficiency. Cells were then pelleted and resuspended in gel sample buffer (see below) for electrophoretic analyses.

Mitotic Activity and Differentiation

To assess mitotic activity, 7 $\times$ 10^5 keratinocytes from 1-d-old mice were plated onto uncoated glass coverslips in 3.5-cm tissue culture

dishes. After 20 h, fresh low-calcium (0.05 mM) medium was added. At 48 and 72 h after plating, 50 μM bromodeoxyuridine (BrdU) (Sigma Chemical, St. Louis, MO) was added for 2 h. Cells were then fixed (100% methanol at -20°C for 1 min; room temperature for 4 min) and processed for immunofluorescence microscopy. Mitotic activity was quantitated by determining the ratio of BrdU-positive keratinocytes to total (K17-positive) keratinocytes in randomly selected colonies. To assess differentiation, 1.5×10^6 cells from 1-d-old mice were plated into 3.5-cm tissue culture dishes. After 20 h, medium was changed to 0.05 mM calcium. At 72 h after plating, the medium was replaced with 2.0 mM calcium-containing medium. After an additional 24 h, the cultures grown on glass coverslips were fixed (3% paraformaldehyde for 15 min; 100% methanol for 4 min; room temperature) and processed for immunofluorescence microscopy. Alternatively, cultures grown on plastic were pelleted and resuspended in gel sample buffer in preparation for electrophoretic analyses.

Keratin IF Organization and K16 Protein Levels

Keratinocytes (7×10^5) from 1-d-old mice were plated on glass coverslips. After ~20 h, fresh medium with either 0.2 or 0.05 mM calcium was added. Cells were grown for an additional 24–72 h, fixed (100% methanol, 5 min at room temperature), and processed for immunofluorescence microscopy. Percentage of keratin filament reorganization was determined by quantitation of the number of keratinocytes with reorganized keratin filaments compared with total number of cell nuclei as determined by Hoechst staining (Sigma Chemical). To assess transgenic K16 and endogenous keratin protein levels, cells were grown as described but without coverslips. Cultures were rinsed with PBS, scraped, pelleted, and resuspended in gel sample buffer for electrophoretic analyses.

Heat Shock Assay

Keratinocyte from 1-d-old mice were plated at 7 $\times$ 10^5 cells/3.5-cm dish and grown in 0.2 mM calcium medium. After 72 h, cells were placed for up to 90 min at 43°C and 5% CO_2, fixed with 3% paraformaldehyde in PBS followed by methanol at room temperature, and processed for immunofluorescence microscopy.

Explant Culture and Migration Assays

Full thickness skin punch biopsies, 4-mm in diameter, were collected from 1-d-old mice, placed epidermis upward onto an uncoated glass coverslips, and fed with medium. Fresh medium was provided every 3 d. After 8 d in culture, explants were fixed in 3% paraformaldehyde-PBS for 15 min, followed by 4 min in 100% methanol at room temperature. Skin tissue was dissected out with a razor blade and cells that migrated out of the explant were processed for immunostaining. To assess keratinocyte migration, explants were immunostained for K17 (see below) by using a peroxidase-based detection method (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Migration was quantitated by measuring the distance from explant edge to the leading edge at eight equidistant points on each explant. Averages were calculated from a total of 25–30 explants (10 mice; 2–3 explants/mouse) per genotype.

Antibodies

We used rabbit polyclonals directed against K6 or K17 (McGowan and Coulombe, 1998), human K16 (designated 1275; Takahashi *et al.*, 1994), and mouse K16 (RPmK16; Porter *et al.*, 1998). We used mouse monoclonals directed against K10 (K8.60; Sigma Chemical), K14 (LL001; Purkis *et al.*, 1990), K13/K15/K16 (K8.12; Sigma Chemical), and BrdU (Sigma Chemical). We used a human anti-poly(ADP-ribose polymerase) (PARP) (Casciola-Rosen *et al.*, 1995). We produced a chicken polyclonal antiserum against K14 by conjugating a synthetic 16-mer peptide, NH_2-CGKVVSTHEQVLRTKN-COOH, corresponding to the C terminus of human and mouse K14 (Mar-

chuk *et al.*, 1984; Knapp *et al.*, 1987), to maleimide-activated keyhole limpet hemocyanin carrier as described (Pierce Chemical, Rockford, IL). Chickens were immunized according to standard procedures (Covance Research Products, Denver, PA), and the antisera produced were tested as described (McGowan and Coulombe, 1998).

Electrophoretic Analyses

Total keratinocyte protein extracts, boiled in SDS-PAGE sample buffer, were resolved using 8% SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose for Western analysis. Nitrocellulose membranes were blocked with 5% nonfat dried milk in PBS. Subsequent antibody incubations were performed in 0.5% bovine serum albumin (Sigma Chemical) and 0.2% Tween 20 (Sigma Chemical) in PBS. Western blots were revealed using either enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, England) or the alkaline phoshatase methods (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence Microscopy

Keratinocytes grown on coverslips were rinsed with PBS and then fixed. All subsequent steps were done at room temperature. Cultures were blocked with blocking buffer (5% normal goat serum in PBS) for 30 min. Primary antibodies diluted in blocking buffer were added for 45 min. After PBS washes, fluorescein isothiocyanate- or rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Kirkegaard & Perry Laboratories) diluted in blocking buffer were added for 45 min. Cells were washed in PBS, treated with 1 μg/ml Hoechst dye for 5 min, and washed again with PBS. The coverslips were mounted onto glass slides by using a mowiol solution (prepared as described by Osborn and Weber, 1982) supplemented with 4 mM *p*-phenylenediamine (Sigma Chemical), and analyzed via immunofluorescence microscopy.

Electron Microscopy

Cultures were fixed with 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 3 mM $CaCl_2$ at pH 7.4) for 30 min. Cells were rinsed in cacodylate buffer and postfixed in 1% osmium tetroxide in cacodylate buffer for 30 min, rinsed again in buffer, and placed in 2% uranyl acetate in double distilled H_2O for 30 min. Subsequently, cells were dehydrated in graded ethanols, embedded in LX112 epoxy-resin (Ladd Research, Burlington, VT), and cut into ultrathin sections (50–70 nm). Sections were placed on copper grids, counterstained with uranyl acetate and lead citrate, and then visualized by using a transmission electron microscope (EM10A; Carl Zeiss, Thornwood, NY) operated at 60 kV.

RESULTS

We previously reported that ectopic expression of human K16 driven by the K14 gene promoter in transgenic mice causes a delay in the postnatal maturation of skin epithelia (Paladini and Coulombe, 1998). Morphologically, the phenotype includes thickened epidermis and poorly developed hair follicles, and correlates with changes in keratinocyte adhesion, proliferation, differentiation, and in the organization of keratin IFs. These traits are first detectable in 3–4-d-old mice and are most obvious at 7-d post birth. Starting at ~21 d post birth, coincident with the onset of the first hair cycle, the skin begins to return to its normal thickness and a sparse hair coat appears. In the four independent transgenic lines studied (designated #6, 10, 13 and 21), mice that are heterozygous at the single transgene insertion locus appear normal. Three of these lines (#6, 10, 21) develop the skin phenotype described when bred to homozygosity (Paladini

and Coulombe, 1998). Quantitation of K16 protein levels in transgenic epidermis reveals that transgene expression must be at least 60% of endogenous K14 in order to produce a phenotype. These findings suggest a dose-dependent mode of action of K16 coupled with a threshold effect.

Expression of a K16-C14 cDNA to comparable levels in transgenic mouse skin does not produce a detectable phenotype (Paladini and Coulombe, 1998). This cDNA consists of the head and most of the rod domain of K16 (368 amino acids) with the exception that the C-terminal ~105 amino acid residues have been substituted for those of human K14 (Wawersik *et al.*, 1997). The findings in K16-C14 mice imply that the phenotype seen in K16 ectopic mice does not arise from the overexpression of a human keratin in mouse skin keratinocytes, and that the C terminus of K16 plays a role in the induction of the phenotype.

Primary culture provides a suitable context to study the impact of K16 expression on the adhesion, proliferation, differentiation, and migration of individual skin keratinocytes. Culture conditions devised by Hennings *et al.* (1980) (see also Hennings and Holbrook, 1983) allow for manipulation of these basic properties. Growth of keratinocytes in the presence of low calcium concentrations (0.05 to 0.10 mM) results in a proliferative cell monolayer that does not form stable cell–cell junctions. Increasing the calcium concentration up to 2.0 mM permits adhesion and promotes differentiation. Cultures of wild-type, K16 ectopic and K16-C14 ectopic keratinocytes were established using these methods and characterized for their basic properties.

K16 Ectopic Keratinocytes Show a Time-dependent Plating Defect

Assessment of plating efficiency at 6 h after seeding in culture was carried out using cells isolated from 0–3-d-old mice (Figure 1A). Wild-type keratinocytes plate with equal efficiency regardless of the age of the mice from which they are isolated. Keratinocytes from newborn or 1-d-old homozygous K16 mice plate similarly to wild type. When isolated from 2-d-old and especially from 3-d-old homozygous K16 mice, however, keratinocytes show a reduction in plating efficiency. In contrast, heterozygous K16 ectopic keratinocytes isolated from 3-d-old mice plate similarly to wild type (Figure 1A).

Biochemical analyses show that the nonadherent population of wild-type (our unpublished observations) and heterozygous K16 keratinocytes (Figure 1B) contain substantial amounts of the differentiation-related K1 and K10 but relatively low levels of K6, K14, or K16 antigens. The same conclusion applies for day 0 homozygous K16 mice In contrast, nonadherent keratinocytes from 3-d-old homozygous K16 mice, which show a plating defect (Figure 1A), feature increased amounts of K6, K14, and K16 antigens (Figure 1B). The levels of K10 antigen, and of K1 and K10 protein, appear unchanged in transgenic versus wild-type keratinocytes (Figure 1B). This finding is significant, in that it suggests that the pools of control and transgenic cells feature a comparable fraction of suprabasal keratinocytes.

We indirectly tested for apoptosis (Li *et al.*, 1999) under our keratinocyte isolation and plating conditions by examining PARP cleavage through Western analysis (Tewari *et al.*, 1995). A sizable fraction of PARP (116 kDa) is cleaved to an 85-kDa product in extracts prepared from nonadherent
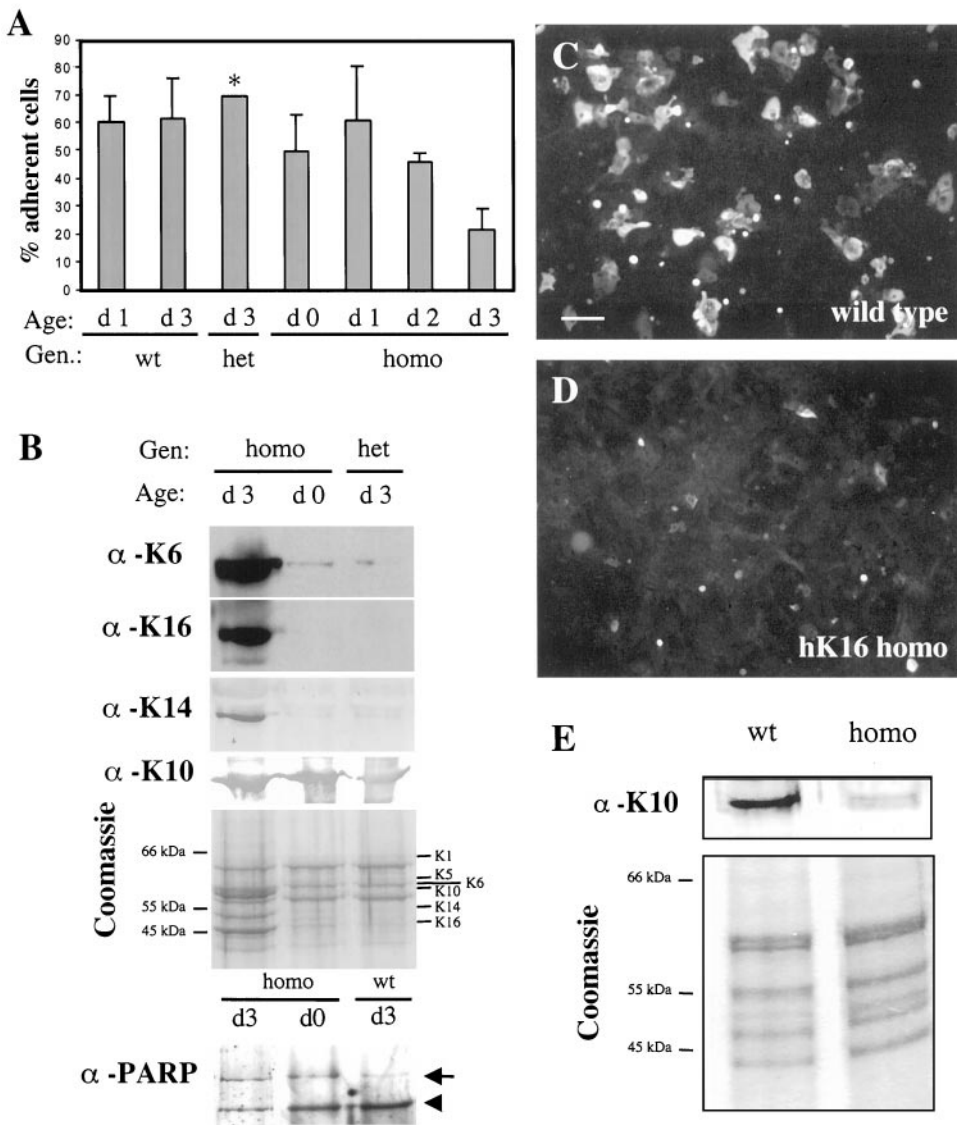
Figure 1. Plating efficiency and differentiation of K16 ectopic keratinocytes. (A) Plating efficiency of 0–3-d old (d 0, d 1, d 2, or d 3) keratinocytes isolated from wild-type (wt), heterozygous (het), or homozygous (homo) K16 ectopic mice was determined after 6 h in standard calcium (0.2 mM) medium conditions. Adherent and nonadherent keratinocytes were collected, counted, and plating efficiency was determined as a ratio (mean ± SE). With the exception of d 3 heterozygous keratinocytes (∗), plating efficiency was quantitated two or more times. (B) Total protein extracts prepared from nonadherent cells were electrophoresed and revealed by Coomassie blue staining or Western blotting. When probed with antibodies for K6, K14, or K16 (1275), extracts prepared from 3-d-old homozygous K16 keratinocytes (d 3, homo) showed increased amounts of these antigens compared with 0-d-old K16 homozygous (d 0; homo) or 3-d-old K16 heterozygous (d 3; het) keratinocytes. Levels of K10 antigen are similar in all three types of keratinocytes. Coomassie staining of duplicate gels show the relative amounts of keratins present in these samples. When reacted with antibodies directed against PARP (α-PARP), d 3 homozygous K16 keratinocyte (d 3; homo) proteins show similar amounts of uncleaved (arrow) and cleaved (arrowhead) antigen, whereas d 0 homozygous and d 3 wild-type keratinocytes display a slight increase in the 85-kDa cleavage product. (C and D) To assess differentiation, equal numbers of keratinocytes were plated and grown to confluency under low-calcium (0.05 mM) conditions for 24 h and then shifted to high calcium (2 mM) for 24 h. Differentiation of wild-type (C) and homozygous K16 keratinocytes (D) was assessed by immunostaining for K10. Bar, 15 μm. (E) Protein extracts from keratinocytes grown under high-calcium conditions were subjected to SDS-PAGE, transfer onto nitrocellulose, and Western blot analysis. There is significantly less K10 antigen in homozygous K16 keratinocytes (homo) compared with wild-type (see Western blot and Coomassie-stained gel), confirming the immunoflorescence data.

wild-type keratinocytes (Figure 1B). Such apoptosis is likely a combined result of the overnight incubation in trypsin-containing solution (see MATERIALS AND METHODS) and the failure of these cells to attach to the substratum after plating. A slightly greater ratio of uncleaved (116-kDa) to cleaved (85-kDa) PARP occurs in nonadherent keratinocytes from day 3 homozygous K16 keratinocytes (Figure 1B). This suggests that an increase in apoptosis does not play a role in the decrease in plating efficiency manifested by these cells (Figure 1A). This trait appears to coincide with the onset of a skin phenotype in vivo and the enrichment of K6, K16, and K14 in the nonadherent keratinocyte population.

Proliferation and Differentiation of K16-expressing Transgenic Keratinocytes

To test the impact of ectopic K16 expression on cell proliferation, BrdU incorporation assays were performed in primary cultures of wild-type and homozygous K16 keratinocytes. Cells from 1-d-old mice were used so that initial cell density would not differ as a result of plating differences. Cultures were grown under 0.05 mM calcium to maximize proliferation. At 48 h after plating, ∼33.7% of wild-type keratinocytes and ∼32.3% of homozygous K16 keratinocytes have incorporated BrdU over a 2-h period. At 72 h after
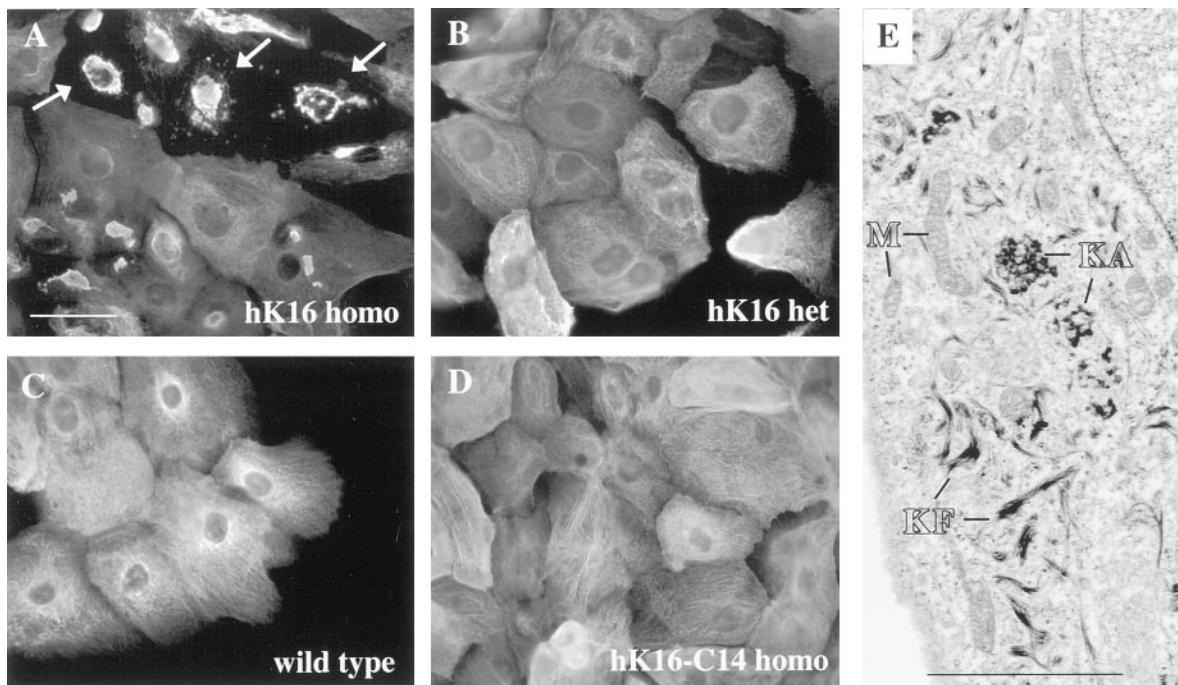
Figure 2. Organization of keratin filaments in primary keratinocyte cultures. (A–D) Keratinocytes from wild-type, homozygous K16-C14 ectopic, and heterozygous and homozygous K16 ectopic keratinocytes were isolated and grown in culture for 72 h in standard calcium (0.2 mM) medium conditions and then immunostained to reveal keratin filament organization. (A) K16 homozygous cultures stained with the anti-K16 (1275) polyclonal antibody show striking alterations in filament organization in a subset of cells (arrows). No filament reorganization is observed in heterozygous K16 (B) cultures immunostained with anti-K16 anti-K16 (1275), or in wild-type (C) and homozygous K16-C14 (D) cultures immunostained for K17. Bar, 20 μm. (E) Similar homozygous K16 cultures were processed for electron microscopy studies. A subset of keratinocytes shows large electron-dense aggregates near the nucleus that are consistent with keratin aggregates (KA). Adjacent to these aggregates are short keratin filaments (KF). The mitochondria (M) and nuclei (N) in these cells are intact. Bar, 2 μm.

plating, ~23.5% of keratinocytes have incorporated BrdU in both wild-type and homozygous K16 cultures. Under these conditions, therefore, ectopic K16 expression does not result in a detectable change in rate of proliferation.

To assess whether K16 expression alters the ability of mouse keratinocytes to undergo epidermal-like differentiation in primary culture, we assessed K10 expression in attached cells after increasing the calcium concentration to 2 mM. By immunofluorescence staining, the number of K10-positive keratinocytes is reduced in homozygous K16 cultures compared with wild type (Figure 1, C and D). This finding is confirmed by Western analysis (Figure 1E). These findings can be interpreted in one of two ways: either increased levels of K16 protein partially inhibit keratinocyte differentiation, or they alter the adhesive properties of differentiating, K10-positive keratinocytes.

Impact of K16 Expression on Keratin IF Organization

The ability of human K16 to impact the organization of the IF network in epithelial cells is a complex and poorly understood phenomenon. Primary culture of transgenic mouse keratinocytes offers a novel context to assess the role of various factors in this phenomenon. Cells were grown for 72 h under standard growth conditions (0.2 mM calcium),

which allow them to divide, form desmosomal cell–cell adhesions, and undergo early steps in differentiation. We find that a subset of homozygous K16 keratinocytes (Figure 2A), but not heterozygous keratinocytes (Figure 2B), shows altered keratin filament organization under these conditions. This phenomenon is seen in cells prepared from the two K16 ectopic mouse lines tested (designated #10 and 21; Paladini and Coulombe, 1998) but not in K16-C14 or wild-type control cultures (Figure 2, C and D). Colocalization of K14 and K16 antigens suggests that the entire keratin IF network is affected in the relevant subset of transgenic keratinocytes (our unpublished observations). That these cells are viable is directly supported by the smooth appearance of their Hoechst-stained nuclei, their ability to incorporate BrdU, and a Western blot analysis for the 85-kDa PARP cleavage product after 72 h in culture (our unpublished observations).

Cultures were processed for electron microscopy analyses to assess the nature of the keratin aggregates and general cell ultrastructure (Figure 2E). Wild-type keratinocytes establish cell–cell adhesions and contain long keratin tonofilaments that extend throughout the cytoplasm (our unpublished observations). Although this is also the case for many homozygous K16 ectopic keratinocytes, a subset of cells shows atypical keratin IF arrays (Figure 2E). These arrays are localized around the nucleus in a manner similar to the keratin aggregates seen through immunofluorescence. They generally
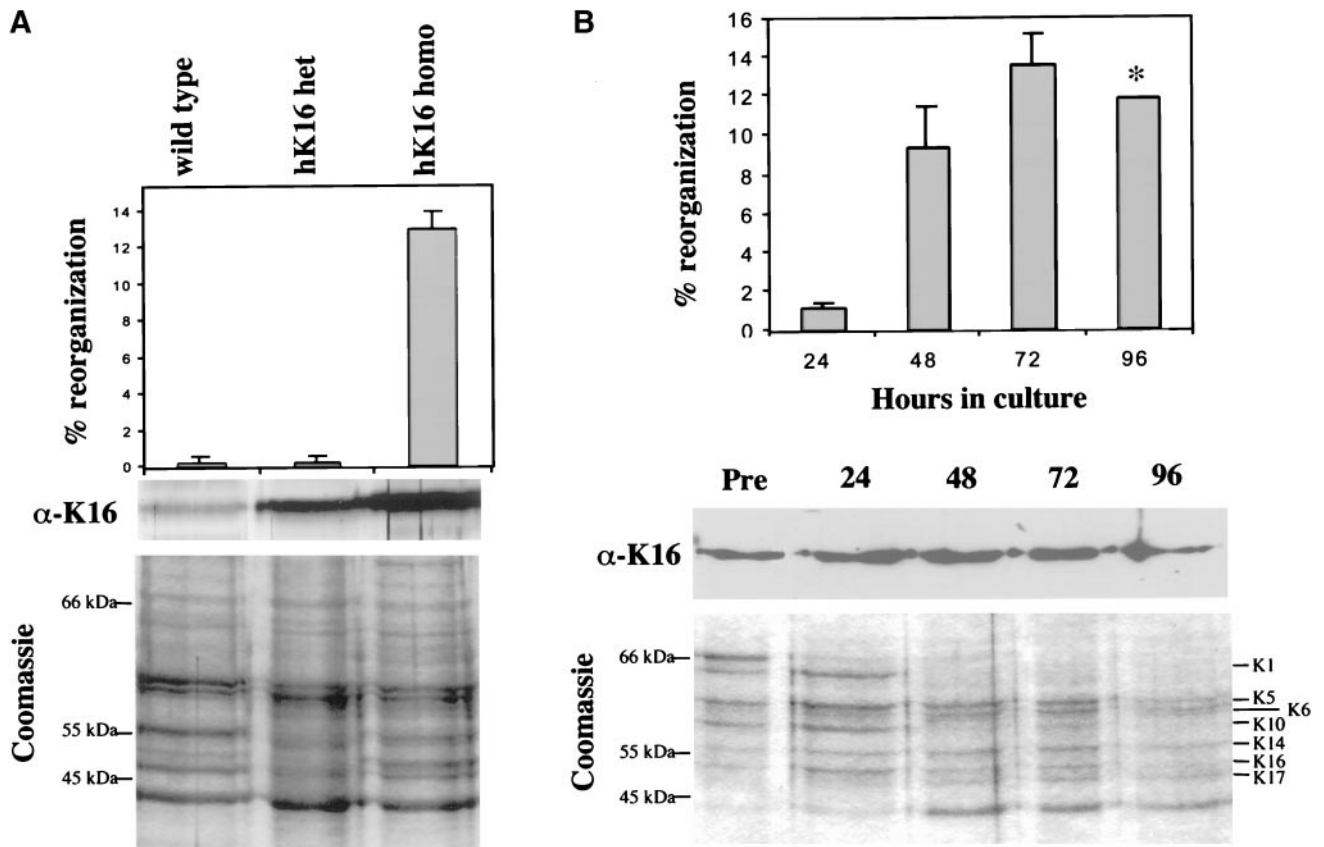
Figure 3. Dose and time dependency of keratin filament reorganization. (A) The percentage of keratinocytes showing severe alterations in keratin filament organization, as assessed by immunostaining, was determined in wild-type, heterozygous K16, and homozygous K16 cultures grown for 72 h in 0.2 mM calcium-containing medium. Bar graph: homozygous K16 keratinocytes show 13 ± 0.9% filament reorganization, whereas wild-type and heterozygous K16 cultures show <1%. To relate the degree of filament reorganization to transgene protein dose, total protein extracts were prepared and analyzed. Quantitation of K16 levels in these extracts through Western blotting indicates that homozygous K16 keratinocytes contain 2-fold more K16 than in heterozygous K16 cells. Levels of endogenous mouse K16 observed in wild-type keratinocyte extracts are much lower in comparison. Coomassie staining shows that equal amounts of protein were loaded from these extracts. (B) Homozygous K16 ectopic cultures were grown in 0.2 mM calcium for 24–96 h and then either immunostained to assess filament reorganization, or processed for protein extraction and analysis. The percentage of keratinocytes showing filament reorganization is 1.3 ± 0.2% at 24 h, increases to 9.4 ± 2.1% after 48 h, peaks at 13 ± 1.6% after 72 h, and is 11.9% after 96 h. With the exception of the 96-h time point (∗), these values were determined through two or more separate experiments. The levels of K16 detected through Western blotting do not vary between total cell extracts before placement in culture (pre) and after 24–96 h in culture (see α-K16 Western). Levels of K16 assessed by Western blotting were normalized to duplicate Coomassie-stained gels (see Coomassie).

consist of short filaments in close proximity to protein aggregates likely made of keratin. Cells that contain these aggregates show intact nuclei and mitochondria (our unpublished observations). This latter finding provides further support for the notion that K16-induced filament aggregation does not affect keratinocyte viability.

Relationship between Transgene Dose and Filament Reorganization

In vivo, the amount of transgenic K16 protein must exceed a given threshold to induce a phenotype in mouse skin (Paladini and Coulombe, 1998). To assess whether this applies to primary culture as well, the number of keratinocytes showing keratin IF reorganization was quantitated and related to keratin protein levels (Figure 3A). After 72 h under standard culture medium conditions, ~13% of homozygous keratin-

ocytes from two independent lines (#10 and 21) show severe alterations in keratin IF organization. In contrast, this occurs in <1% of wild-type and heterozygous K16 keratinocytes. Western analysis reveals a 2-fold increase in K16 protein levels in total protein extracts prepared from homozygous keratinocytes compared with heterozygous cells. Comparable amounts of transgene product are present in extracts prepared from homozygous K16-C14 keratinocytes (our unpublished observations).

We next investigated keratin IF organization and K16 protein levels as a function of time in culture. Homozygous K16 keratinocytes were grown at 0.2 mM calcium for 24–96 h, and IF organization was scored as described above. We find that the number of keratinocytes showing severe filament reorganization increases dramatically between 24 and 48 h, and peaks at 72 h after plating (Figure 3B). The steady-state levels of K16
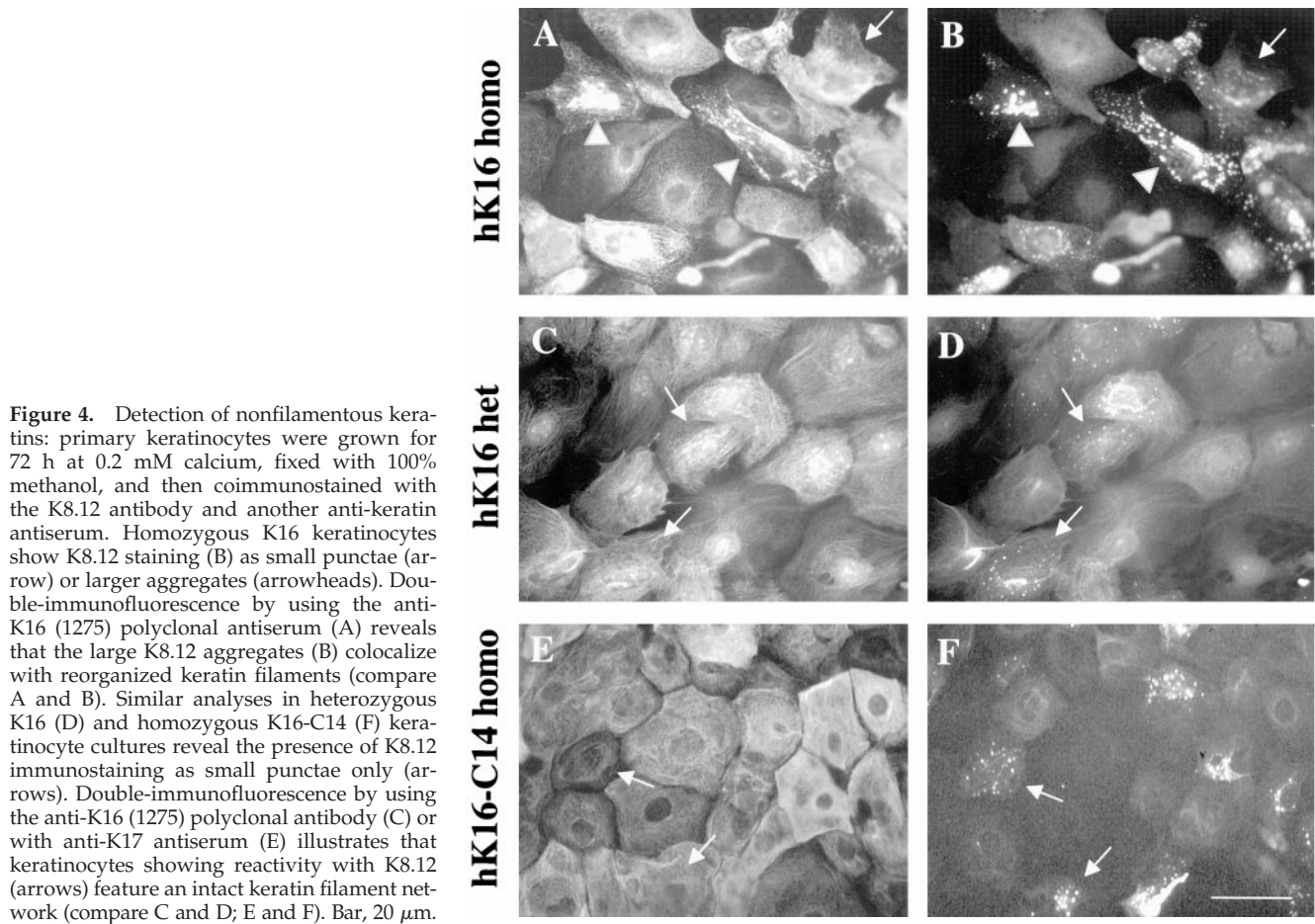
Figure 4. Detection of nonfilamentous keratins: primary keratinocytes were grown for 72 h at 0.2 mM calcium, fixed with 100% methanol, and then coimmunostained with the K8.12 antibody and another anti-keratin antiserum. Homozygous K16 keratinocytes show K8.12 staining (B) as small punctae (arrow) or larger aggregates (arrowheads). Double-immunofluorescence by using the anti-K16 (1275) polyclonal antiserum (A) reveals that the large K8.12 aggregates (B) colocalize with reorganized keratin filaments (compare A and B). Similar analyses in heterozygous K16 (D) and homozygous K16-C14 (F) keratinocyte cultures reveal the presence of K8.12 immunostaining as small punctae only (arrows). Double-immunofluorescence by using the anti-K16 (1275) polyclonal antibody (C) or with anti-K17 antiserum (E) illustrates that keratinocytes showing reactivity with K8.12 (arrows) feature an intact keratin filament network (compare C and D; E and F). Bar, 20 μm.

proteins vary little during this time period (Figure 3B), however, indicating that factors other than dosage contribute to determine K16's impact on keratin IF organization. Interestingly, significant changes in keratin content occur between 24 and 48 h after plating (Figure 3B). Specifically, the levels of K1 and K10 decrease while levels of K6 and K17 increase (Figure 3B). Such an increase in the levels of endogenous K6, K16, and K17 in skin keratinocytes placed in primary culture is to be expected (Roop *et al.*, 1987). These data suggest that a threshold amount of K16 protein is required to elicit changes in keratin filament organization, and that other parameters such as the keratin protein complement are involved as well (Paladini *et al.*, 1999).

Does Keratin Protein Overexpression Play a Role in K16-induced Reorganization?

The monoclonal antibody K8.12 recognizes its epitope, shared by K13, K15, and K16, while these proteins are in a nonfilamentous form (Takahashi *et al.*, 1994). This antibody reacts with both K16 and K16-C14 proteins (our unpublished observations), implying that the epitope is located amino-terminal to residue 368 in human K16. This antibody thus provides a tool to assess the fraction of K16 or K16-C14 protein that has not been incorporated into filaments in transgenic keratinocytes. After 72 h of growth under 0.2 mM calcium conditions, primary cultures were double-stained using the K8.12 antibody and a polyclonal antiserum to either K16 or K17. Wild-type cells do not stain positively with antibody K8.12, even though a subset of them expresses endogenous K16 (see below). In contrast, distinct K8.12-positive punctae are present in all types of transgenic keratinocytes tested, including homozygous K16, heterozygous K16, and homozygous K16-C14 cells (Figure 4, B, D, and F). Coimmunostaining of K8.12 with the polyclonal anti-K16 antiserum shows that cells with severely reorganized keratin IF networks display, in addition to punctae, a sizable number of large K8.12-positive aggregates (Figure 4, A and B). These data suggest that both K16 and K16-C14 transgenic keratinocytes contain nonfilamentous keratin(s) in their cytoplasm, likely a result of the overexpression of a single keratin sequence type. These punctae are not associated with alterations in keratin IF organization unless the transgenic keratin contains the C terminus of human K16 protein. These findings also establish that the K8.12 antibody can be used as a marker for filament reorganization in homozygous K16 keratinocytes.
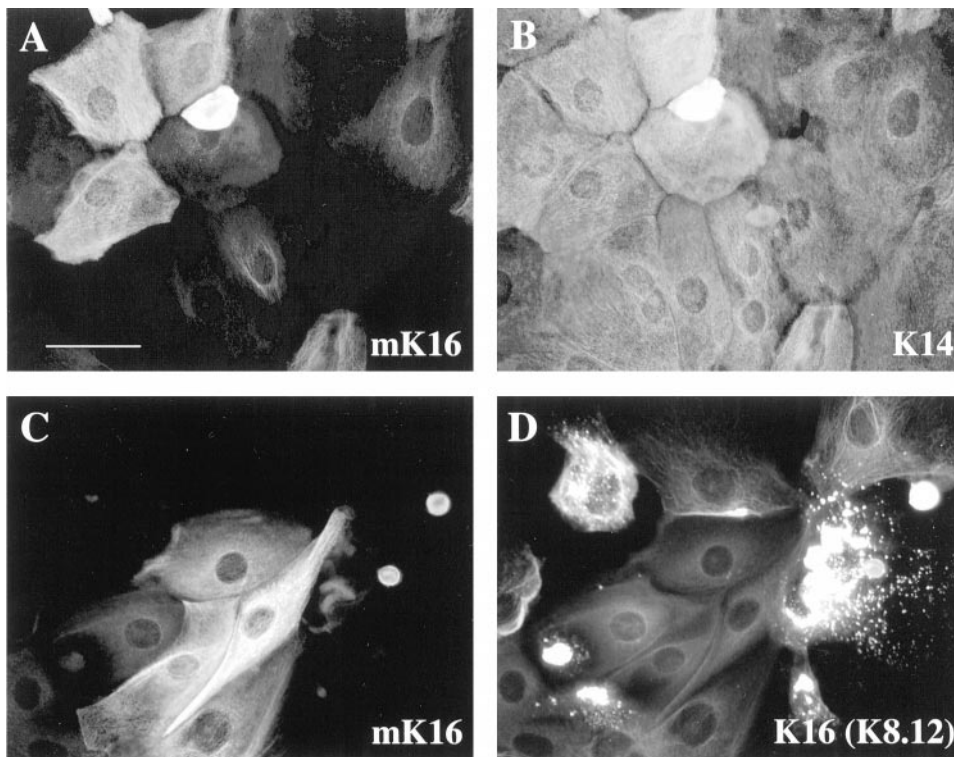
Figure 5. Mutual exclusion of mK16 staining and keratin filament reorganization. Keratinocytes were grown for 72 h in 0.2 mM calcium, fixed, and immunostained. (A and B) Coimmunostaining of wild-type keratinocytes with an anti-mouse K16-specific antibody (A) and a monoclonal anti-K14 (LL001) antibody (B) shows that the mouse K16 antigen is expressed in a subset of keratinocytes in primary cultures. (C and D) Coimmunostaining of homozygous K16 keratinocytes with the anti-mouse K16-specific antibody (C) and monoclonal antibody K8.12 (D) as a marker of keratin aggregates shows that mouse K16 antigen cannot be detected in from K8.12-positive keratinocytes. Bar, 20 μm.

Keratin Filament Reorganization Does Not Occur in Endogenous K16-positive Keratinocytes

Immunostaining of wild-type keratinocyte cultures reveals that expression of endogenous K16 occurs only in a subset of keratinocytes (Figure 5, A and B). Given that filament reorganization is also observed in a subset of homozygous K16 keratinocytes, this creates the possibility that it specifically occurs in the endogenous K16-expressing subpopulation. Under these circumstances endogenous and transgenic K16 might act together to exceed a threshold level of K16 required for induction of filament reorganization. To test this hypothesis, we coimmunostained homozygous K16 keratinocyte cultures with a mouse K16-specific rabbit antiserum (Porter *et al.*, 1998) and monoclonal antibody K8.12. Surprisingly, the mouse K16 antigen is not present in keratinocytes showing K8.12 punctae (Figure 5, C and D). These mouse K16-positive keratinocytes show the flattened morphology and larger size typical of differentiating keratinocytes in submerged cultures. Moreover, all the K10-positive keratinocytes are contained within the pool of mouse K16 antigen-positive cells, and the latter only rarely incorporate BrdU (our unpublished observations). Collectively, these observations establish several key points. First, mouse K16 is primarily expressed at a postmitotic stage in primary keratinocyte cultures. Second, keratin IF reorganization occurs in homozygous K16 transgenic keratinocytes that retain a basal cell character. This observation may be related to the altered differentiation response of these cells in response to increased calcium concentration (Figure 1). Finally, these findings reinforce the notion that alterations in keratin IF organization are not a mere function of total K16 content.

Culture under Low-Calcium or Heat Shock Conditions Increases Keratin Filament Reorganization

Keratin IFs are anchored to the cytoplasmic face of desmosomal adhesion plaques. This connection is frequently severed in keratinocytes whose keratin IF network is reorganized near the nucleus, raising the possibility that loss of stable cell–cell adhesions may contribute to alterations in keratin IF organization in the presence of K16. To test this notion, the number of homozygous K16 cells with reorganized keratin IFs was quantitated under low-calcium (0.05 mM) conditions, which do not allow formation of stable cell–cell adhesions (Hennings *et al.*, 1983). After 48 h in culture, ~9.4% of cells show reorganization in standard calcium (0.2 mM), whereas this increases to ~12% in low calcium (0.05 mM). This difference is even greater after 72 h, where ~13.6 versus ~22.5% of cells have altered keratin networks at 0.2 and 0.05 mM calcium, respectively. Western analysis of cell extracts from homozygous K16 ectopic keratinocytes shows no difference in levels of K16 expression under standard and low-calcium conditions (our unpublished observations). Therefore, low-calcium growth conditions provide yet another context in which keratin IF reorganization increases even though the steady-state levels of K16 protein remain stable.

Heat shock causes the collapse of IFs in cultured fibroblasts (Welch and Suhan, 1985) and in keratinocyte cultures established from epidermolysis bullosa simplex patients (Morley *et al.*, 1995). Based upon these interesting findings we next compared the organization of keratin IFs in wild-

Figure 6. Localization of cells with altered keratin filament networks in heat-shocked and explant cultures. (A–D) Heat–shock experiments. Cultures were grown for 72 h in standard (0.2 mM) calcium medium conditions at 37°C, and then heat shocked for up to 90 min at 43°C, fixed, and analyzed. Wild-type and homozygous K16 cultures were stained with the anti-K17 and anti-K16 (1275) antisera, respectively. (A) Homozygous K16 keratinocyte colony before heat shock. Keratinocytes with reorganized keratin networks are randomly distributed (∗). (B) Homozygous K16 keratinocyte colony at 45 min after heat shock. Several keratinocytes located at the edge show retracted keratin IF networks (arrows). This is not seen in wild-type cultures (our unpublished observations). (C) Wild-type keratinocyte colony at 90 min after heat shock. Many cells located at the edge of colonies in wild-type cultures now show a retraction of keratin filaments toward the nucleus. (D) Homozygous K16 cultures at 90 min after heat shock. Keratin IF retraction is more severe and affect virtually all keratinocytes located at the edge(see arrows). Bar, 25 μm. (E–H) Skin explant cultures. Full-thickness skin punch biopsies of wild-type and homozygous K16 keratinocytes were placed into culture for 7–8 d. Cells migrating out of the explant were fixed and analyzed. (E and F) Homozygous K16 ectopic cultures were immunostained using anti-K16 (1275) antiserum. (E) Filament reorganization, depicted by ∗, occurs specifically at the leading edge of migrating keratinocytes. (F) The cell boxed in E is shown at higher magnification, illustrating filament morphology in leading edge cells. (G and H) Wild-type keratinocyte cultures double-immunostained using the anti-K17 polyclonal antiserum and the LL001 anti-K14 monoclonal antibody. Anti-K17 staining is shown. No keratin punctae or aberrant filament organization is seen in leading edge keratinocytes under low or high magnification. Bar, 25 μm (E and G) or 6.5 μm (F and H).
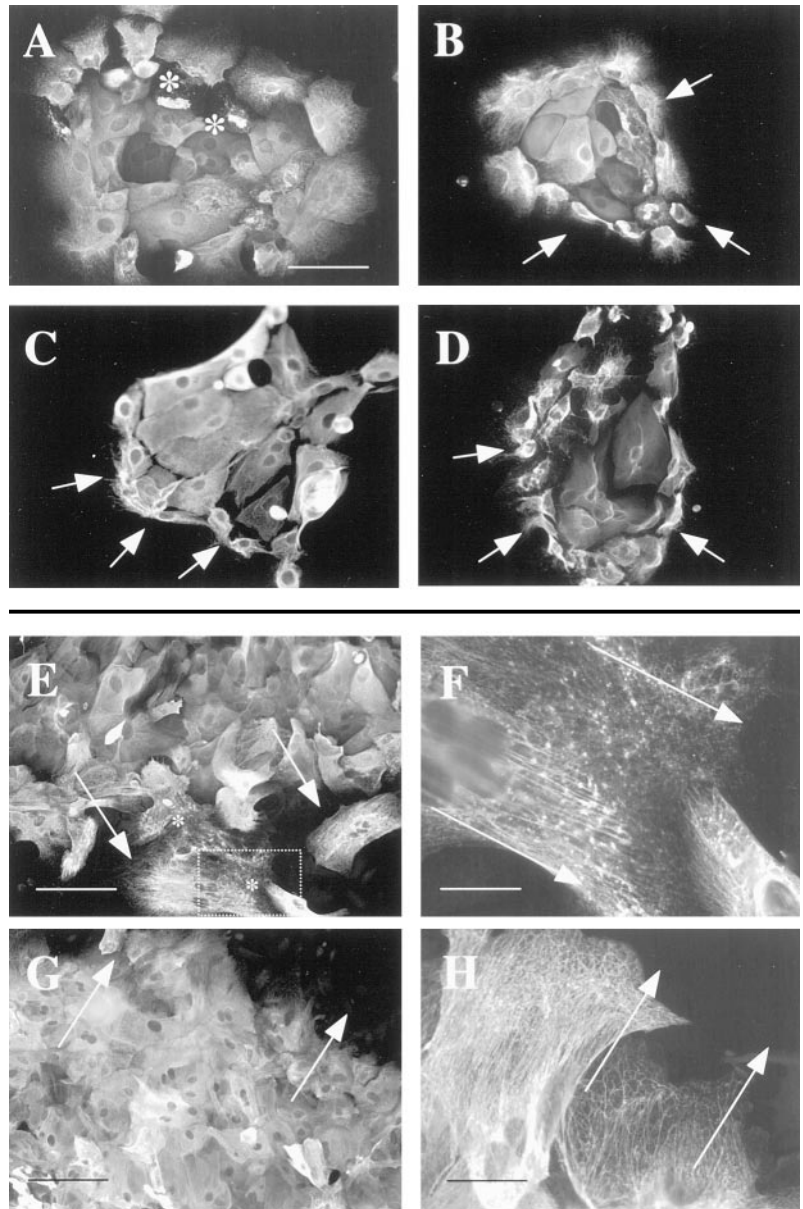
type and homozygous K16 keratinocytes after heat shock treatment. Before heat shock, the subset of homozygous K16 keratinocytes showing reorganized IFs is randomly distributed within colonies (Figure 6A). After 45 min of heat shock, homozygous K16 cultures show a significant degree of IF reorganization at colony edges (Figure 6B), whereas wild-type keratinocyte colonies are relatively unaffected (our unpublished observations). After 90 min of heat shock, keratin IFs in wild-type cells begin to relocalize (Figure 6C), but homozygous K16 keratinocytes are still more severely affected (Figure 6D). These studies show that sustained heat shock promotes keratin IF reorganization in the subset of keratinocytes located at the edge of colonies in wild-type cultures, a novel and interesting finding. This edge effect is enhanced in the presence of increased K16 levels.

Keratinocyte Migration and Keratin Filament Morphology in Explant Cultures

Placement of full-thickness skin punch biopsies into culture creates a "wound-like" situation with cells migrating out of the biopsy (our unpublished data). Depending on their location within the migrating tongue, these cells are exposed to a different environment of cell–matrix and cell–cell interactions. This provides another opportunity to test whether filament reorganization can be associated with a specific keratinocyte context. As shown by immunostaining of homozygous K16 explants, leading-edge keratinocytes selectively feature alterations in keratin IF organization (Figure 6, E–F). This is confirmed by immunostaining with the K8.12
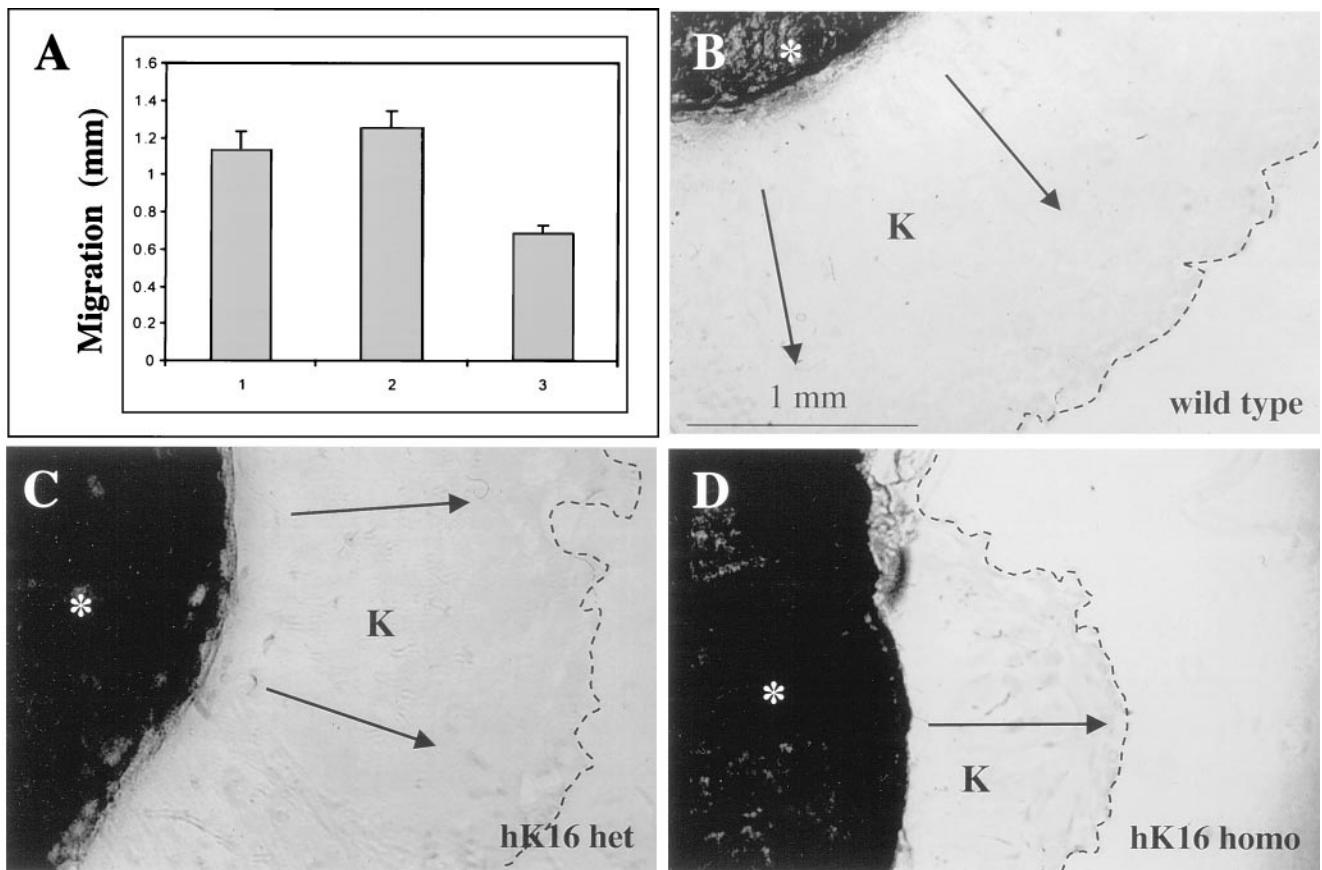
Figure 7. Keratinocyte migration in cultured skin explants. (A) Average distance of keratinocyte migration out of skin explants isolated from wild-type (wt), heterozygous (het), or homozygous (homo) K16 ectopic mice was determined. In each instance, the value reported (mean ± SE) was calculated from a pool of 25–30 explants (see MATERIALS AND METHODS). (B–D) Representative examples of skin explants from wild-type (B), heterozygous K16 (C), and homozygous K16 (D) mice after 8 d of culture. The explants (∗) were immunostained for K17 to reveal migrating keratinocytes (K). The keratinocyte migrating front is depicted by a dashed line.

antibody (our unpublished observations). This edge effect is not seen in wild-type explants (Figure 6, G–H).

We next assessed whether keratinocyte migration is altered in transgenic compared with wild-type biopsies. After 8 d of culture, the sheet of migrating keratinocytes extends 1.13 ± 0.10 mm from the edge of wild-type explants (Figure 7, A and B). Migration occurs to a similar extent in explants harvested from heterozygous K16 mice (Figure 7, A and C). In contrast, explants harvested from homozygous K16 mice (Figure 7, A and D) show an ~40% reduction in the extent of migration. High levels of K16 protein can interfere with keratinocyte migration as tested in this ex vivo explant culture assay.

DISCUSSION

Impact of K16 Expression on the Basic Properties of Skin Keratinocytes

In this study we exploited primary culture to examine the impact that elevated K16 protein level has on a number of basic properties of skin keratinocytes. We found that transgenic mouse skin keratinocytes expressing human K16 at steady-state levels approaching those of endogenous K14 (Paladini and Coulombe, 1998; this study) display defects in cell–substratum adhesion, differentiation, and migration, but not in their ability to proliferate. Although these effects depend in part upon transgene protein dosage, in that a threshold must be exceeded, they are not a simple function of K16 protein levels. These effects are not a consequence of the overexpression of a human keratin in mouse keratinocytes, as shown by findings involving transgenic keratinocytes expressing a chimeric K16-C14 cDNA at comparable levels. This implies that the C-terminal 105 amino acids of K16 play a significant role in its ability to alter these keratinocyte properties.

The keratinocyte plating defect occurs concomitantly with the onset of a phenotype in vivo and correlate with an enrichment of K6, K16, and even K14 in nonadherent cells. The presence of increased amounts of K14 in this fraction is consistent with a greater number of basal-like cells in the unattached cell population. The mechanism(s)

underlying this defect remains to be elucidated. It could reflect alterations in the functional status of integrin receptors at the keratinocyte surface, or alternatively an inability to produce or secrete extracellular matrix components such as laminin-5 (Carter *et al.*, 1990, 1991). The $\alpha 3\beta 1$ and $\alpha 3\beta 4$ integrin receptors are believed to play an important role in the initial attachment of keratinocytes to the substratum under culture conditions (Xia *et al.*, 1996; DiPersio *et al.*, 1997). We previously reported that the distribution of $\alpha 3$ integrin extends into the suprabasal compartment, whereas that of $\alpha 6$ integrin extends into the lateral and apical domain of basal keratinocytes in the epidermis of homozygous K16 ectopic mice (Paladini and Coulombe, 1998). Further characterization of these integrins thus represents a logical starting point toward a mechanistic understanding of the plating properties of these K16 keratinocytes.

The expression of K6 and K16 is often observed in epithelial settings showing enhanced epithelial cell proliferation (O'Guin *et al.*, 1990), leading to the tantalizing notion that these keratins may play a direct role in this phenomenon. Recently, experimental evidence supporting a direct role for human K16 in enhancing cell proliferation has been provided by transfection studies involving human HaCaT keratinocytes and rodent PtK2 cells (Paramio *et al.*, 1999). The significance of these provocative findings in light of other experimental evidence remains unclear. In relevant human epithelia, K16 protein is restricted to postmitotic keratinocytes even in hyperproliferative settings (Stoler *et al.*, 1988; Leigh *et al.*, 1995; Paladini *et al.*, 1996). This implies that at best, K16 would impact on proliferation in a noncell autonomous manner. We previously showed that, as their skin phenotype peaks in intensity, homozygous K16 ectopic mice show hyperproliferation in the epidermis and hypoproliferation in hair follicles. Later on, the proliferation rate normalizes in both compartments (Paladini and Coulombe, 1998). In the present study, we did not find any difference between transgenic and wild-type keratinocyte cultures with regard to their ability to incorporate BrdU. Moreover, we found that K16 expression occurs preferentially in nonmitotically active keratinocytes within wild-type primary cultures. These findings extend previous studies showing that expression of K6 and K16 can be pharmacologically uncoupled from cell proliferation in cultured epidermal and corneal keratinocytes (Schermer *et al.*, 1989; Kopan and Fuchs, 1989; Choi and Fuchs, 1990). Additional studies will be required to solve the differences between these findings and those of Paramio *et al.* (1999).

Indirect evidence also links increased K16 protein levels to a defect or delay in terminal differentiation in the epidermis. For instance, induction of K16 and K17 occurs at the expense of K10 in wound edge keratinocytes after injury to human and mouse skin (Mansbridge and Knapp, 1987; Paladini *et al.*, 1996; McGowan and Coulombe, 1998a). The same phenomenon has been documented in psoriatic epidermis (Weiss *et al.*, 1984; Stoler *et al.*, 1988; Leigh *et al.*, 1995). At another level, forced expression of human K16 under the control of its own promoter causes a dosage-dependent reduction in K10 staining in suprabasal epidermis of transgenic mouse skin (Takahashi *et al.*, 1994). Sun and colleagues proposed that the inverse correlation between K10 and K16

expression in such contexts reflects the adoption of an alternative path of keratinocyte differentiation (Schermer *et al.*, 1989). In support of this, we observe a reduced number of K10-positive cells in primary skin keratinocyte cultures established from homozygous K16 phenotypic mice. This said, we cannot rule out the possibility that the reduced number of K10-positive cells in adherent keratinocytes is a consequence of altered cell–cell adhesion in postmitotic keratinocytes.

Migration of keratinocytes out of skin explants in an ex vivo setting better mimics the corresponding events at the edge of skin wounds than scrape wounding of keratinocyte monolayers (our unpublished results). The studies we report here establish that high levels of K16 protein exert a profound impact on the ability of keratinocytes to migrate in this explant assay. How this finding relates to the alterations seen in keratin IF organization at the leading edge of homozygous K16 explants (Figure 7), and to the plating defects seen in these cells (Figure 1), awaits further study. The physiological relevance of this observation is unclear, because K16 induction is normally restricted to suprabasal keratinocytes at the wound edge. That is, this delay may simply be a consequence of the ectopic expression of K16 in basal cells, a nonphysiological situation. Alternatively, this delay may reflect the real contribution of K16 to migrating keratinocytes at the wound edge. A similar, counterintuitive finding of delayed keratinocyte migration was observed in BPAG1 null mice (Guo *et al.*, 1995). The definitive evidence of K16's contribution at the wound edge must await the production and characterization of K16 null mice. Meanwhile, the data we report here represent the first evidence that K16 can significantly impact on keratinocyte migration in an in vivo-like setting.

Factors Impacting Keratin Filament Organization

We assessed the effects of ectopic K16 expression on the organization of keratin IFs in keratinocytes in a primary culture setting. This phenomenon occurs in a subset of high-expressing (homozygous) K16 keratinocytes. It is not seen in homozygous K16-C14 transgenic keratinocytes, implying a role for determinant(s) located within the C terminus of K16. The reasons for the incomplete penetrance of this trait in homozygous K16 transgenic cultures are not clear. We do not know whether the K14 gene promoter-based expression cassette is equally active in all types of progenitor basal cells within the transgenic skin epithelia. Variability at that level (Wang *et al.*, 1997) could underlie the partial penetrance of this trait. Alternatively, it may reflect the functional heterogeneity that typifies the basal cell compartment in mouse skin epithelia (Kamimura *et al.*, 1997). According to this scenario, a specific subset of transgenic basal cells may be predetermined to develop K16-associated changes once in primary culture. Finally, it may simply reflect the notion that K16-associated filament reorganization is a complicated phenomenon subjected to regulation by several factors. The data we report here extend our previous in vivo analyses (Paladini and Coulombe, 1998; 1999), in showing that the alterations in keratin IF organization are not a simple function of K16 protein dosage.

Based on published studies, we manipulated the primary cultures of skin keratinocytes in various ways in an effort to influence the fraction of cells showing altered IF organiza-

tion and thus gain insights into the mechanisms underlying this phenomenon. We found that K16-associated alterations in keratin IF organization are influenced by time spent in culture, calcium concentration in the medium, and position of the keratinocyte within the colony. These findings suggest that the cellular keratin complement and functional status of cell–cell adhesion play a role in modulating K16's impact at that level. In support of the keratin complement argument, the increase in the frequency of keratin IF alterations that occurs between 24 and 48 h after plating is paralleled by an increase in K6/K17 and a decrease in K1/K10 protein levels. These findings extend previous ones in support of a role for the keratin complement in determining whether K16 impacts on keratin IF organization. Although K16 ectopic mice, which feature normal levels of K14 protein (Paladini and Coulombe, 1998), develop a skin phenotype within a week after birth, breeding them into the K14 null strain background results in distinct tissue and cell alterations that begin at 5 wk after birth (Paladini and Coulombe, 1999). In support of the cell–cell adhesion argument, low-calcium medium conditions increase the fraction of keratinocytes showing filament reorganization in homozygous K16 ectopic cultures. Moreover, filament reorganization typical of K16 overexpression occurs in transgenic keratinocytes located at the edge of colonies following heat shock as well as in the context of explant cultures. Such edge cells show less cell–cell adhesion by virtue of their position within colonies. There are problems with this concept, however, in that low-calcium conditions do not promote reorganization in all keratinocytes, nor is reorganization preferentially observed in keratinocytes at the edge of colonies before heat shock treatment. These inconsistencies are left unexplained, and may reflect the heterogeneous character of the cell population under study.

We also identified factors that do not appear to play a role in K16-associated filament reorganization in skin keratinocytes. This phenomenon is not an artifact resulting from the site of transgene insertion in the host mouse genome because it is seen in keratinocyte cultures established from two independent K16 transgenic lines. It is not an artifact resulting from the overexpression of a human type I keratin because it does not occur in cultures established from K16-C14 chimeric mice. In addition Porter *et al.* (1998) have shown that the mouse and human K16 proteins, which are 82% identical, behave similarly in various types of assays (also our unpublished data). At another level, K16-associated filament reorganization is not associated with cell death because we provide evidence that cells showing disrupted IF networks have intact nuclei and mitochondria and can incorporate BrdU. Moreover, there is no increase in PARP cleavage in K16 transgenic cultures compared with wild type. Finally, we find that filament reorganization is not due to an additive effect of the human K16 transgene and endogenous mouse K16. The mutual exclusion of filament reorganization and endogenous K16 expression is more likely due to differences in intrinsic cell characteristics (e.g., "basal" vs. "postmitotic"). The fact that the K16 transgene is driven off of the basal-layer specific K14 promoter, whereas mouse K16 expression is postmitotic under standard growth conditions, supports this notion.

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