Differential expression of the _*Hs*Kin17 protein during differentiation of in vitro reconstructed human skin

Denis Biard, Yannick Saintigny, Michel Maratrat, Marie-Catherine Vozenin, Michèle Martin, François Daburon, Jaime F. Angulo

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D. S. F. Biard · Y. Saintigny · M. Maratrat
M.-C. Vozenin · M. Martin · F. Daburon · J. F. Angulo

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Abstract **In eukaryotic cells, various proteins homologous to the *E. coli* RecA protein are involved in the elimination of DNA damage. These proteins contribute to the repair of double-strand breaks and to genetic recombination. The mouse Kin17 protein is recognised by antibodies directed against the RecA protein. Kin17 has a zinc-finger domain allowing binding to curved DNA stretching over illegitimate recombination junctions. In the present study, we identified the human counterpart of the mouse Kin17 protein (named $_{Hs}$Kin17) in skin cells. We employed an in vitro reconstructed skin model composed of an epidermal sheath lying on a dermal matrix with human fibroblasts embedded in rat collagen type I. The maturation programme (proliferation versus differentiation) of keratinocytes was highly dependent on stromal cells. Immunohistochemical staining of frozen sections obtained from skin specimens was monitored by an interactive laser cytometer. In this way we analysed protein levels in both dermal and epidermal compartments. After having characterised the epithelium, we focused our attention on $_{Hs}$Kin17 expression. We detected $_{Hs}$Kin17 in human keratinocytes. $_{Hs}$Kin17 protein levels increased in proliferating epithelial keratinocytes after 7 days of culture. After 2 weeks of culture, epidermal sheaths acquired most of the differentiated features of mature epithelium. At this time, $_{Hs}$Kin17 protein dropped below measurable levels in the stratum corneum, and diminished in nucleated cells. This study showed that $_{Hs}$Kin17 is expressed in human reconstructed epithelium under conditions of hyperproliferation.**

Key words $_{Hs}$Kin17 · $_{Mm}$Kin17 · Human keratinocytes · Reconstructed skin · Differentiation · Laser cytometer

D. S. F. Biard (✉) · Y. Saintigny · J. F. Angulo
Laboratoire de Génétique de la Radiosensibilité,
DSV-DRR, CEA, BP 6, 92265 Fontenay aux Roses, France
Tel. (33) 01 46 54 87 32; Fax (33) 01 46 54 87 34
e-mail BIARD@DSVIDF.CEA.FR

M. Maratrat
Rhône Poulenc-Rorer, Service Histopathologie Moléculaire,
Division Thérapie Génique, CRV Vitry-Alfortville, BP 14,
94403 Vitry sur Seine, cedex, France

M.-C. Vozenin · M. Martin · F. Daburon
Laboratoire de Radiobiologie et d'Etude du Génôme,
DSV-DRR, CEA, 91191 Gif sur Yvette, cedex, France

Introduction

In *E. coli*, RecA protein is essential for genetic recombination and controls numerous genes involved in the cellular response to genotoxic agents, termed the 'SOS response'. Genetic approaches have allowed the detection of RecA-homologous proteins in eukaryotic cells. Various reports emphasise a key role for the Rad family of yeast genes in the repair of DNA double-strand breaks (DSBs) [1]. Of these, Rad51, Rad52, Rad55, and Rad57 have been identified as putative RecA homologs. The Rad51 protein of *S. cerevisiae*, probably together with Rad52 protein, is involved in both mitotic and meiotic recombination and in the repair of DSBs caused by X-rays [2]. In vitro yeast Rad51 protein catalyses the pairing of single-stranded DNA with homologous double-stranded DNA and allows strand exchange between synapsed DNA partners. This strand exchange reaction is dependent upon the presence of ATP. The human Rad51 binds to single- and double-stranded DNA, exhibits DNA-dependent ATPase activity, and unwinds duplex DNA to form helical nucleoprotein [3, 4]. Angulo et al. used an immunological approach to detect mouse proteins recognised by anti-RecA antibodies [5]. One of them was identified and called Kin17 [5]. The cross-reactivity between $_{Mm}$Kin17 protein and anti-RecA antibodies is probably due to a slight sequence homology of 39 amino acids with the *E. coli* RecA protein in a region of Kin17 which may be involved in DNA binding [6]. Kin17 protein is conserved among mammals and contains a zinc-finger motif which is involved in the binding to double-stranded DNA, especially to curved DNA stretching over illegitimate recombination

junctions [7, 8]. A role for the Kin17 protein in controlling gene expression has been proposed based on the fact that the expression of $_{Mm}$Kin17 in *E. coli* represses the bgl operon [9]. Recently, we have detected enhanced levels of Kin17 protein in rat cells irradiated with ionising radiation [10]. Furthermore, the expression of the $_{Mm}$*Kin17* gene is correlated with cell proliferation, and UV irradiation of cultured mouse cells induces $_{Mm}$*Kin17* gene expression (Kannouche, personal communication). We have reported the molecular characterisation of the mouse $_{Mm}$*Kin17* gene and the housekeeping-like nature of its regulatory region [11]. All these results, together with the conservation of *Kin17* gene among mammals, suggest that Kin17 protein participates in DNA metabolism, probably in illegitimate recombination processes (for review see reference 12). However, the precise role of Kin17 remains to be determined.

The participation of *Kin17* gene in the response to physical genotoxic agents and its conservation between mice and humans raises the question of whether Kin17 protein can be detected in human organs, particularly in those such as the skin, which are frequently exposed to physical damage from the environment. In the present study, we detected Kin17 protein in a previously described in vitro reconstructed skin model [13]. Many organotypic culture models have been reported [14], but are hampered by the long culture required to obtain a well-developed epithelium, the difficulty of handling culture specimens, and the technology used for accurate analysis of immunohistochemical staining of frozen sections. Our experiments were designed to obtain small and easily handled culture specimens, with a high degree of spatial and structural organisation after less than 2 weeks of culture. This model mimicked the differentiation programme of human skin keratinocytes [13]. We used an interactive laser cytometer in order to accelerate and simplify the localisation and quantification of specific gene products in frozen sections. We report the localisation of a marker of the basal membrane (collagen type IV) and of differentiation products of keratinocytes (e.g. keratin 10) during the maturation programme of in vitro reconstructed epithelium. We subsequently investigated the expression profile of the human Kin17 protein (named here $_{Hs}$Kin17) during the tuning of gene expression leading to the differentiation of in vitro reconstructed epithelium. We evaluated the degree of differentiation of epithelium as a function of the number of days in culture.

Considering the conservation of *Kin17* gene and the strong sequence identity of mouse and human Kin17 protein (Kannouche, personal communication; [11]), we used antibodies directed against the mouse Kin17 protein to detect $_{Hs}$Kin17 in in vitro reconstructed skin epithelium. Western blot analysis revealed a band of an apparent molecular weight of about 45 kDa, which corresponds to the $_{Hs}$Kin17 protein. By immunohistochemical staining, we detected higher $_{Hs}$Kin17 protein levels in proliferating keratinocytes seeding onto a collagen matrix containing living human fibroblasts, after 7 days of culture, when all cell layers were nucleated. In contrast, $_{Hs}$Kin17 protein levels decreased when the epithelium reached a higher degree of differentiation after 2 weeks of culture. This study revealed that the regulation of the $_{Hs}$Kin17 protein expression is a function of the state of differentiation of human epidermal keratinocytes.

Materials and methods

Cell isolation and culture

Normal human epidermal keratinocytes and fibroblasts were isolated from human mammary skin obtained from a healthy 22-year-old woman during mammoplasty. The fat was removed from the dermis. Large amounts of dermis were removed and small dermis explants were cultured to obtain fibroblasts. At passage 2, frozen stocks of fibroblasts were prepared. These stocks were used at passages 5 to 10. For maintenance and propagation, fibroblasts were maintained as monolayers in Dulbecco's modified essential medium supplemented (DMEM) with 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 50 µg/ml streptomycin).

The remaining skin sheaths were cut and incubated overnight at 4° C in trypsin (0.25%). The epidermis was separated from the dermis with fine forceps and the dermis-epidermis boundary was scraped with a blade to recover basal keratinocytes. Gentle aspiration of the epidermal sheaths released the keratinocytes. Trypsin was inhibited by medium supplemented with 10% FCS, and keratinocytes were passed through a nylon gauze and then stored in liquid nitrogen or immediately plated in the presence of human fibroblasts mitotically inactivated (by γ-irradiation or mitomycin C treatment) as feeder cells [15]. The culture medium was adapted from that of Bohnert et al. [16]; it consisted of a mixture of Ham's F12 (three parts) and DMEM (one part) supplemented with 5% FCS, antibiotics (100 U/ml penicillin, 50 µg/ml streptomycin), 5 µg/ml insulin, 1.8×10^{-4} *M* adenine, 10^{-9} *M* cholera toxin, 0.4 µg/ml hydrocortisone and 10 ng/ml epidermal growth factor (all from Sigma). Keratinocytes from the first passage were used.

In vitro reconstructed skin

Culture specimens were prepared as previously described [13]. Ice-cold collagen type I isolated from rat tail tendons (4 mg/ml) was mixed with Hank's balanced saline solution and neutralized with NaOH. FCS with 10^5 mesenchymal cells was added and cell-populated collagen was poured into each well of 24-well plates (day 1 of culture). Collagen gels were polymerised for 1–2 h in a humid incubator. DMEM 10% FCS was added to each well and culture proceeded for 24 h, after which (day 2) 5×10^5 keratinocytes in suspension in culture medium [Ham's F12 (one part) and DMEM (three parts) supplemented with 10% FCS, 100 U/ml penicillin, 50 µg/ml streptomycin, 10^{-9} *M* cholera toxin, 0.4 µg/ml hydrocortisone and 50 µg/ml ascorbic acid (Sigma)] were plated on the top of each gel and incubated under submerged conditions for 24 h. Culture specimens were transferred to the air-liquid interface 1 day later (day 3) and allowed to grow for an additional period of 7 or 14 days. The medium was changed every 2 days.

More than ten independent experiments were performed in studying the constitutive Kin17 protein level in in vitro reconstructed epidermis. We carried out two independent experiments, with different pools of frozen cells (keratinocytes and fibroblasts), to determine $_{Hs}$Kin17 protein levels as a function of the degree of differentiation of our reconstructed skin.

Indirect immunofluorescence with differentiation marker antibodies

Tissue specimens were mounted in Tissue-Tek (Miles, Elkhart, USA), snapfrozen in liquid nitrogen and stored at –80° C until use. Vertical sections (8 µm thick) were cut in a cryostat at –20° C and

collected on aminoalkylated glass slides. Frozen sections were air dried and then fixed with paraformaldehyde (1%, 30 min) and Triton X-100 (0.1%, 10 min). After extensive washing with PBS, frozen sections were incubated with primary antibodies for 1 h at room temperature. Monoclonal antibodies against keratin K10 and collagen type IV (both diluted to 1:100) were from Sigma. After three extensive washes, fluorescein-conjugated antibodies were applied for 45 min at room temperature, followed by additional washes in PBS. Dichlorotriazinyl aminofluorescein-conjugated antimouse or antirabbit IgG secondary antibodies (diluted to 1:500) were from Immunotech (Marseille, France). Samples were treated with RNase A (5 μg/ml) in the presence of propidium iodide (PI; 5 μg/ml). Sections were mounted and examined with an ACAS 570 (Adherent Cell Analysis and Sorting) interactive laser cytometer (Meridian, Michigan, USA).

Antibodies against Kin17 protein

Polyclonal rabbit antibodies (S61 and S64) against Kin17 and the polyclonal antibody against RecA were obtained as previously described [6, 10, 17]. Antibodies were immunopurified against $_{Mm}$Kin17. Briefly, $_{Mm}$Kin17 protein produced in *E. coli* was separated by gel electrophoresis and transferred onto a nitrocellulose membrane. Antibody solutions were adsorbed onto $_{Mm}$Kin17 protein immobilised on a nitrocellulose filter, washed several times to eliminate other proteins, and the anti-$_{Mm}$Kin17 protein antibodies were recovered by pH shift [6, 10].

Analysis with the interactive laser cytometer

We analysed $_{Hs}$Kin17 protein levels as a function of the degree of differentiation of in vitro reconstructed skin in two independent experiments. For each time-point (day 7 and day 14), three reconstituted skin samples were frozen. For each specific staining with serum directed against $_{Mm}$Kin17, or with the preimmune serum, six different frozen sections were cut, and three culture specimens were examined. Immunohistochemical stainings were monitored by laser cytometry using the ACAS 570 cytometer equipped with a 5 W argon laser light source (Coherent) as described previously [10, 18]. After excitation at 488 nm, green fluorescence was collected through a 530/30 nm interference filter, while the red fluorescence of PI was collected above 605 nm. Fluorescence intensities were represented by a panel of pseudocolour digitised computer images. Each specimen was analysed simultaneously for fluorescein staining and PI. For brightfield examination (phase contrast) of culture specimens, frozen sections were examined by means of the ACAS 570 in the Phase Mergescan Routine, without fluorescein/PI dichroic (575SP) and ON-axis (530/30 BP) filters. For each staining, negative controls without primary antibodies were carried out to test the fluorescein staining alone. The same tuning of the laser cytometer was used for all experiments.

Western blotting analysis

Epithelium was recovered separately from the collagen matrix with fine forceps. Four skin epithelium samples were pooled for each point. Proteins were extracted with 62 m*M* Tris (pH 8), (2%) SDS, (143 m*M*) β-mercaptoethanol and heated at 95° C for 20 min. Cells grown in monolayers were lysed under the same conditions. The recombinant $_{Mm}$Kin17 protein was diluted in Laemmli buffer and heated at 95° C for 10 min. All the samples were loaded onto a 10% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a trans-blot apparatus (Biorad). Blots were blocked with 5% defatted milk, and incubated for 1 h at room temperature with immunospecific anti-Kin17 antibody (diluted to 1:2000). After removal of the primary antibody, blots were incubated for 45 min with peroxidase-conjugated goat antibody antirabbit IgG. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham). We used the same protocol for PCNA detection (Novo Castra; diluted to 1:2000).

Results

Morphological features of reconstructed epidermis

Under our culture conditions, the onset of epidermal differentiation was observed from 4 days after seeding of 10^5 fibroblasts into the collagen matrix. We observed the appearance of different cell layers (stratification) with the characteristics of the stratum basale (SB), the stratum spinosum (SS), the stratum granulosum (SG), and the stratum corneum (SC) (data not shown). Numerous nucleated cells were seen 14 days post-plating at the basal level of the epithelium. Fewer nucleated cells were seen in the upper cell layers (Fig. 1). We observed a regular and continuous epithelium stretching throughout the collagen matrix. We noted a well-developed SC made up of flattened (denucleated) dead cells, stacked to form more than 25% of the total thickness of the epidermis, as in normal skin (Fig. 1) [19]. After a few days in culture (e.g. 7 days post-plating), we usually detected numerous basal keratinocytes undergoing division as evidenced by BrdU staining (data not shown). The proliferation rate (BrdU labelling index) of the basal layer had decreased 14 days post-plating and continued to fall thereafter, indicating a decrease in cell proliferation in favour of cell differentiation, as judged by the detection of specific differentiation markers such as keratin K10 (Fig. 2). We also detected fibroblasts embedded in the type I collagen matrix (Figs. 1 and 2). Staining with antibody directed against BrdU revealed a large number of positive fibroblasts after less than 7 days of culture (data not shown). This number subsequently decreased considerably.

Differentiation markers of basement membrane

We checked whether in vitro reconstructed skin samples displayed protein expression similar to that observed in human skin in vivo. We evaluated the expression of well-characterised differentiation-related structural proteins by immunohistochemical staining and laser cytometry analysis. For each experiment, double stainings were performed with antibodies against a specific differentiation marker of the skin, e.g. collagen type IV (Fig. 2A, B), and with PI. PI allowed determination of the DNA content of keratinocytes.

The production, excretion and polar deposition of collagen type IV, and other components of the basement membrane, were correlated with the formation of an organised and differentiated epithelium. Type IV collagen staining was diffuse 7 days after plating, indicating incomplete secretion (data not shown). Substantial deposition of collagen type IV had occurred at the boundary of the dermal and epidermal sheaths 14 days after plating (Fig. 2A, B). This deposition was homogeneous throughout the dermal-epidermal junction, and was strictly localised beneath basal keratinocytes of the SB.

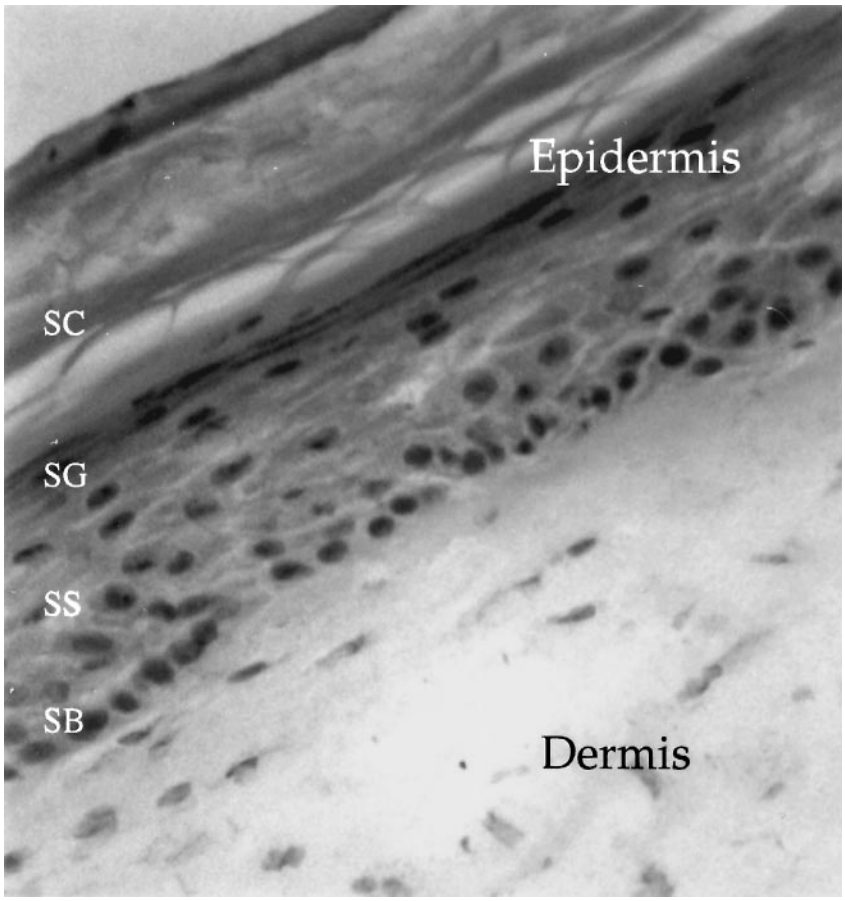

Fig. 1 Main features of culture specimens as seen by normal light microscopy and eosin-haemalum staining. (*SC* stratum corneum, *SG* stratum granulosum, *SS* stratum spinosum, *SB* stratum basale; (× 20)

Differentiation markers of keratinocytes

Keratins are constituents of intermediate filaments and one of the predominant differentiation products of keratinocytes. They are among the earliest biochemical indicators of the onset of epidermal differentiation [20]. In normal skin, a sequence of changes in keratin expression occurs when keratinocytes move from the SB through the SS and SG to the SC. In the presence of mesenchymal cells in the collagen gels, most basal keratinocytes did not express the keratin pair K1/K10, while the suprabasal layers showed marked expression (Fig. 2C, D), as seen in all experiments carried out 7 or 14 days post-plating. We and others have systematically observed this type of staining [21]. It was noteworthy that the K10 protein expression profile in our in vitro reconstructed skin specimen looked like that observed in vivo (Fig. 2C, D).

For each experiment, we analysed the degree of differentiation of our in vitro reconstructed skin using other differentiation markers of the epithelium (involucrin and filaggrin) or of the connective tissue (vimentin) (data not shown).

Characterisation of antibodies directed against the Kin17 protein

Our recent data suggest that both the mouse and human Kin17 proteins show considerable amino acid sequence homology (Kannouche, personal communication). For this reason, we used antibodies directed against the mouse $_{Mm}$Kin17 protein to detect its human counterpart. The antibodies used have been previously described [6, 10]. One of them was directed against the *E. coli* RecA protein; the others were directed against the whole mouse $_{Mm}$Kin17 protein (S61 and S64 antisera). We selected antibodies immunopurified on immobilised recombinant mouse Kin17 protein to increase their specificity.

We characterised our antibodies after transfection of human cells with expression vectors carrying or not carrying the $_{Mm}$*Kin17* cDNA. We employed Epstein-Barr-derived plasmids in which $_{Mm}$*Kin17* cDNA was fitted downstream of the heavy metal inducible mMT-I (mouse metallothionein I) promoter. These plasmids were derived from those reported elsewhere [22]. In this approach, we transfected epithelial human embryonic kidney (HEK) 293 cells as recipients. The preimmune sera failed to detect any band in the human protein extracts. Under the same conditions, both anti-RecA and anti-Kin17 (S64 serum) antibodies recognized a band at about 45 kDa in protein extracts from cells bearing the $_{Mm}$*Kin17* cDNA af-

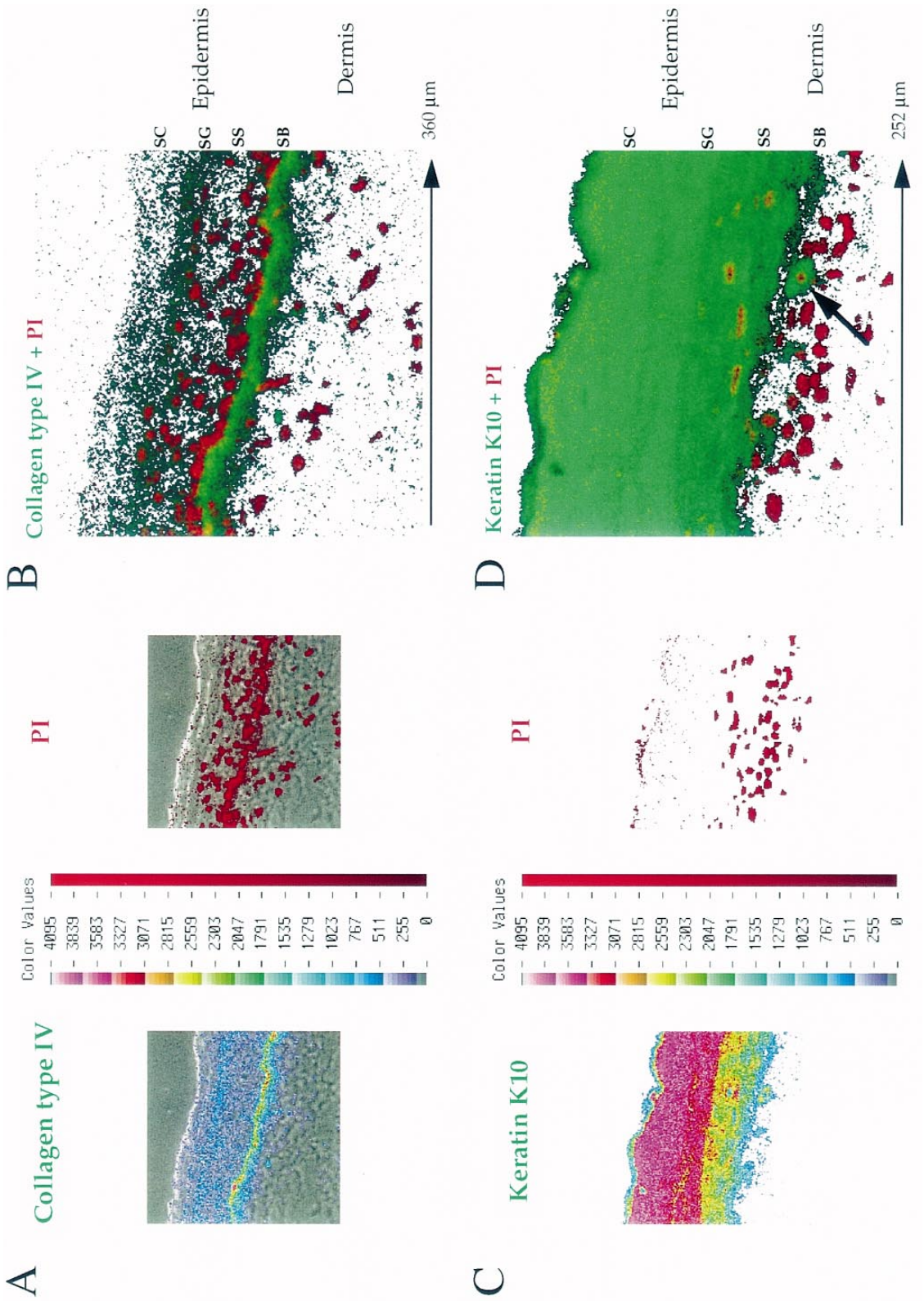

Fig. 2A–D Laser cytometry analysis of in vitro reconstructed human skin epithelium. Specimens were cultured for 14 days, snap-frozen and sectioned. Frozen sections were stained simultaneously with one monoclonal antibody against collagen type IV (or keratin K10) and with propidium iodide (PI). Analysis was performed using an ACAS 570 cytometer (Meridian). (**A, B**) Collagen type IV deposition. **A** Side-by-side stainings. **B** Fluorescein and PI stainings. Each section was analysed at the same time for collagen type IV (*left*) and PI (*right*). Colour scales corresponding to fluorescence intensities are shown with their values expressed on an arbitrary fluorescence scale. Fluorescence intensities specific to collagen type IV staining are represented by a panel of pseudocolour digitised computer images (**A**) or by a green scale (**B**). The PI signal was visualised with a red scale (**A, B**). *SC* stratum corneum, *SG* stratum granulosum, *SS* stratum spinosum, *SB* stratum basale. (**C, D**) Keratin K10 expression. **C** Side-by-side stainings. **D** fluorescein and PI stainings. The *arrow* in **D** represents a basal keratinocyte overexpressing keratin K10

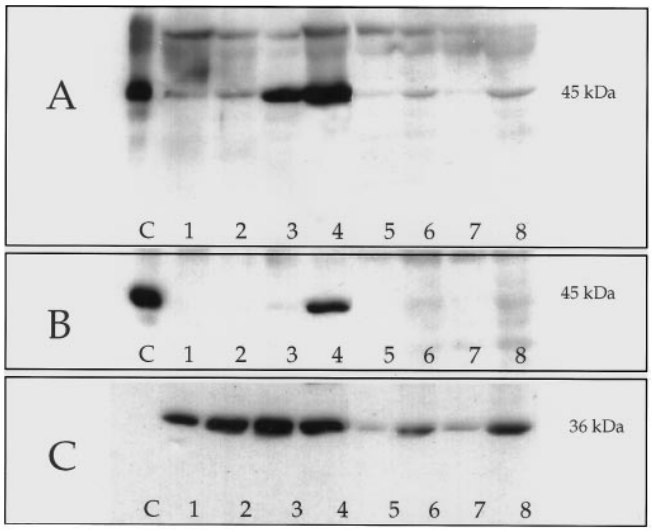

Fig. 3A–C Kin17 detection in human cell lines. Proteins were extracted from human cell lines and analysed by Western blotting as indicated in Materials and methods (*lane 1* 293 cells transfected with a control vector (without heavy metal for 24 h); *lane 2* 293 cells transfected with a control vector (with heavy metal for 24 h); *lane 3* 293 cells transfected with a vector carrying the mMTI-$_{Mm}$Kin17 cartridge (without heavy metal for 24 h); *lane 4* 293 cells transfected with a vector carrying the mMTI-$_{Mm}$Kin17 cartridge (with heavy metal for 24 h); *lane 5* mitomycin-treated human fibroblasts (feeder layer) after 8 days of culture; *lane 6* human keratinocytes cultivated in monolayers after 8 days of culture; *lane 7* mitomycin-treated human fibroblasts (feeder layer) after 15 days of culture; *lane 6* human keratinocytes cultivated in monolayers after 15 days of culture). Kin17 protein was detected with (**A**) the immunospecific rabbit S64 antiserum, or with (**B**) the immunospecific antibody directed against *E. coli* RecA protein (*C* control corresponding to the recombinant $_{Mm}$Kin17 protein). Protein loading was checked with Ponceau S staining and thereafter with an antibody directed against the human PCNA protein (**C**)

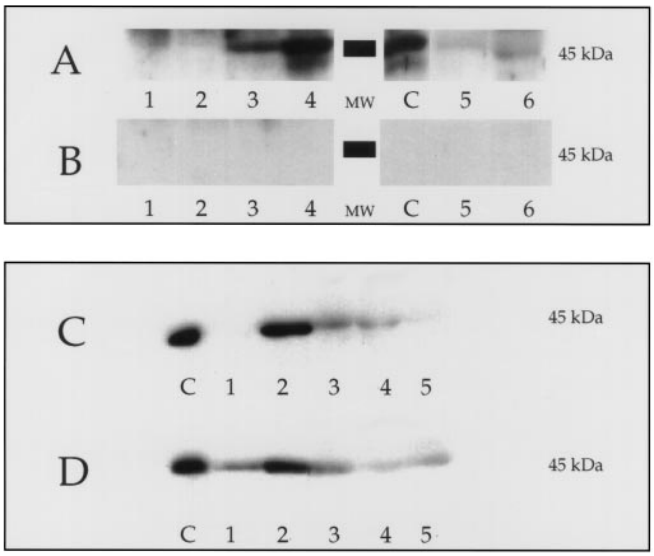

Fig. 4A–C **A, B** Competition with the immobilised recombinant $_{Mm}$Kin17 protein (*lane 1* 293 cells transfected with a control vector (without heavy metal for 24 h); *lane 2* 293 cells transfected with a control vector (with heavy metal for 24 h); *lane 3* 293 cells transfected with a vector carrying the mMTI-$_{Mm}$Kin17 cartridge (without heavy metal for 24 h); *lane 4* 293 cells transfected with a vector carrying the mMTI-$_{Mm}$Kin17 cartridge (with heavy metal for 24 h); *lanes 5, 6* two protein extracts from reconstructed epithelia (*C* recombinant $_{Mm}$Kin17 protein, *MW* molecular weight). **A** Kin17 protein was detected with the non-immunopurified S64 serum; **B** the non-immunopurified S64 serum competed with immobilised recombinant $_{Mm}$Kin17 protein. **C, D** Human $_{Hs}$Kin17 protein detection in in vitro reconstructed epithelium; *lane 1* 293 cells transfected with a vector carrying the mMTI-$_{Mm}$Kin17 cartridge (without heavy metal for 24 h); *lane 2* 293 cells transfected with a vector carrying the mMTI-$_{Mm}$Kin17 cartridge (with heavy metal for 24 h); *lanes 3, 4 and 5* proteins were extracted from reconstructed epithelium after 4, 7 and 10 days of culture, respectively (*C* recombinant $_{Mm}$Kin17 protein). Kin17 protein was detected with (**C**) the immunopurified anti-RecA antibody, or with (**D**) the immunopurified S64 serum

ter metal stimulation (Fig. 3A, B; lane 4). In transfected 293 cells, we also detected leakage of the mMT-I promoter when the culture was carried out without heavy metal (Fig. 3A; lane 3), using immunopurified S64 serum. Using the same antibody, we detected a weak signal at 45 kDa in 293 cells transfected with the control vector without $_{Mm}$*Kin17* cDNA (Fig. 3A; lanes 1, 2). This band had the same molecular weight as the $_{Mm}$Kin17 overexpressed in transfected 293 cells. We conclude that this band corresponded to the endogenous $_{Hs}$Kin17 protein. As a control, we used a PCNA antibody which gave rise to a very strong signal at 36 kDa in transfected 293 cells (Fig. 3C). In another attempt, we incubated the S64 antibody with the mouse recombinant protein immobilised on a nitrocellulose membrane. Our aim was to assess the specificity of the antibodies. As expected, using the non- immunopurified S64 serum, we noted an intense 45 kDa band in the control (recombinant $_{Mm}$Kin17 protein) and in protein extracts from transfected HEK 293 (Fig. 4A; lanes 1–4); this band disappeared after competition with the recombinant mouse $_{Mm}$Kin17 protein (Fig. 4B).

The above results confirmed the specificity of our antibodies in detecting the Kin17 proteins expressed in human cells.

$_{Hs}$Kin17 protein levels in human cells

We analysed the endogenous $_{Hs}$Kin17 protein level in human skin cells. We noted a 45 kDa band in protein extracts from human keratinocytes plated in monolayers after 8 or 15 days in culture using the S64 antiserum (Fig. 3A, lanes 6, 8). This band had the same molecular weight as $_{Mm}$Kin17 protein overexpressed in human cells (Fig. 3A, B; lane 4). We failed to detect a 45 kDa band in the feeder layer composed of human fibroblasts mitotically blocked by mitomycin C after 8 or 15 days of culture (Fig. 3A; lanes 5, 7).

We assessed by Western blot the $_{Hs}$Kin17 protein levels in keratinocytes cultivated on fibroblast-containing collagen gels. We recovered proteins from reconstructed epithelium after 4, 7 and 10 days of culture. We clearly identified a band at 45 kDa which slowly decreased with time of cultivation (Fig. 4C, D). This band disappeared after competition with the recombinant $_{Mm}$Kin17 protein (Fig. 4A; lanes 5, 3).

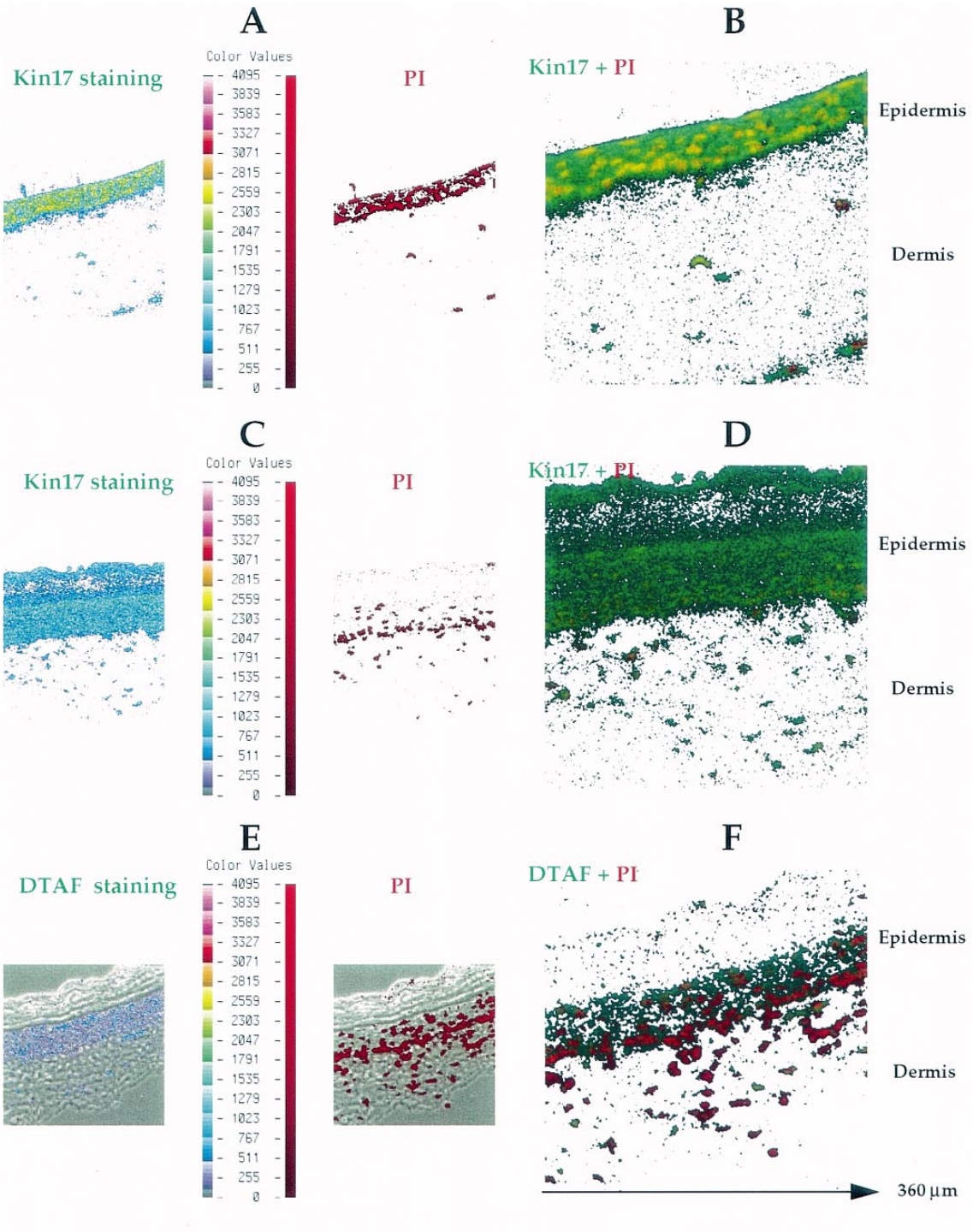

Fig. 5A–F $_{Hs}$Kin17 protein level during maturation of human epithelium. Specimens were cultured for 7 or 14 days and analysed as indicated in the legend to Fig. 2. We used immunopurified rabbit S64 antiserum. **A, C** Side-by-side images of $_{Hs}$Kin17 staining and PI staining of culture specimens after 7 days (**A**) or 14 days (**C**) of culture. **E** Negative staining with fluorescein-conjugated antibody (DTAF) alone. **B, D** Superimposed images of $_{Hs}$Kin17 staining and nuclear localization with PI after 7 days (**B**) or 14 days (**D**) of culture. PI is represented by a red scale while specific staining is represented by a green scale. **F** Negative controls

The marked amino acid sequence identity between mouse and human Kin17 proteins (Kannouche, personal communication), and the ability of our antibodies (especially the S64 serum) specifically to recognise $_{Mm}$Kin17 expressed in human cells, led us to hypothesise that the 45 kDa band observed in human protein extracts was specific to the human Kin17 ($_{Hs}$Kin17) protein. For this reason, we used the S64 serum for immunohistochemical staining.

Laser cytometry analysis of the $_{Hs}$Kin17 protein in in vitro reconstructed skin

Using immunospecific antibodies, we investigated whether $_{Hs}$Kin17 protein was expressed during skin differentiation of in vitro reconstructed skin epithelium. The immunopurified antibody, S64 prepared as described above, was employed to detect $_{Hs}$Kin17 protein in in vitro reconstructed epithelium by immunohistochemical staining and laser cytometry analysis. At 7 or 14 days after seeding of fibroblasts and keratinocytes, in vitro reconstructed skin was snap-frozen and sectioned. Strong immunofluorescence staining was seen in proliferating cells of the epithelium after 7 days of culture (Fig. 5A, B). The fluorescence was detected throughout the epithelium in all nucleated keratinocytes. The observed signal due to anti-Kin17 antibodies decreased considerably in the differentiated epithelium (after 14 days of culture), particularly in cells of the SC and SG (Fig. 5C, D). Slight fluorescence was detected in nucleated keratinocytes of the SB. A Kin17 fluorescence signal was noted a few days after seeding in fibroblasts embedded in the collagen matrix. At this time, most fibroblasts strongly incorporated BrdU (data not shown) and this observation may be related to our recent finding of a relationship between Kin17 and cell proliferation (Kannouche, personal communication). At later times, no staining was detected in the dermal compartment (Fig. 5C, D).

Discussion

Processing of mutagenic DNA damage by the DSBs in eukaryotes most likely occurs via multiple pathways, including homologous recombination. Because Kin17 protein bound to curved (bent) DNAs, a specific DNA conformation associated with illegitimate recombination junctions, it has been postulated that Kin17 could be required for illegitimate recombination [7, 8]. Two compelling lines of evidence have recently indicated a role of Kin17 protein in DNA metabolism: (1) Kin17 protein can efficiently substitute for the H-NS transcription factor and can control gene expression in bacteria [9], and (2) Kin17 protein participates in genetic programmes of differentiation (Kannouche, personal communication; [12]). Kin17 protein has also been detected in particular regions of the rat central nervous system [23].

Many experiments are currently in progress to elucidate the exact functions of this nuclear protein, especially in human cells. The *Kin17* gene has been detected in different eukaryotic organisms emphasising the conservation of this gene among species [11]. Ongoing experiments have highlighted substantial sequence homology between mouse and human *Kin17* cDNAs (Kannouche, personal communication). We report here the immunological detection of the $_{Hs}$Kin17 protein in in vitro reconstituted human skin epithelium. In vitro reconstructed skin was used because it offered the opportunity to study both proliferation and differentiation in somatic cells, and because the skin is an essential barrier against xenobiotic insults. We used an organotypic culture model [13] which yields small skin samples which are easy to handle and amenable to routine work with 100 specimens under various conditions. Fibroblast-populated gels with keratinocytes were lifted on a metal grid to create an air-medium interface. Mesenchymal cells and keratinocytes were nourished from below by diffusion through the gel while the apical side was exposed to the air. We obtained well-differentiated epithelium within 2 weeks of culture. Interactive laser cytometry was used to monitor protein expression in reconstituted skin. The differentiation of human reconstituted skin involves an ordered sequence of defined morphological changes accompanied by sequential expression of specific differentiation products, such as keratin K10, involucrin and filaggrin. Epidermal maturation (proliferation and differentiation) required the presence of fibroblasts. Under these conditions, human keratinocytes rapidly gave rise to a well-differentiated epithelium, about 250 μm thick, after only 14 days of culture. The protein profile expression of various differentiation markers resembled those reported in vivo. In particular, strong and regular deposition of collagen type IV was seen throughout the reconstructed skin, suggesting normalization of tissue homeostasis after more than 1 week in culture.

HsKin17 expression was analysed in human cells of epithelial origin. We first tested our antibodies in HEK 293 after transfection with EBV-derived vectors carrying (or not carrying) the $_{Mm}$*Kin17* cDNA under the control of an inducible promoter. Under these conditions, we clearly determined the specificity of the antibodies used to detect the $_{Mm}$Kin17 protein expressed in human cells. We then analysed by Western blotting proteins extracted from either human keratinocytes cultivated in monolayers or from in vitro reconstructed epithelium. We found a band at 45 kDa, which corresponded to the molecular weight of $_{Hs}$Kin17 protein. Other attempts performed in our laboratory have found a similar band at 45 kDa in melanoma protein extracts (Lataillade, personal communication).

Using these antibodies, we monitored $_{Hs}$Kin17 protein during the differentiation of in vitro reconstructed human epithelium, and found higher levels in rapidly dividing keratinocytes than in their differentiated counterparts, suggesting a participation of $_{Hs}$Kin17 protein in the maturation programme of human skin. It seems likely that $_{Hs}$Kin17 protein is a marker involved in the transition between highly proliferating (basal) keratinocytes to differentiated (suprabasal) keratinocytes in in vitro reconstituted human epithelium.

Our findings provide insight into a new protein involved in DNA metabolism in human skin. Some reports describe the immunohistochemical distribution of proteins required for DNA repair in human skin. For instance, Duguid et al. [24] have reported the cellular and subcellular protein localisation of human apurinic/apyrimidic endonuclease (APE) in human epidermis. While the APE protein level was elevated in all cell layers, the authors noted less staining in the differentiated uppermost cell layers (SG and SC) than in the basal and suprabasal

cell layers. In another study, Wani and D'Ambrosio [25] found a variation in O^6-alkylguanine-DNA alkyltransferase mRNA distribution in neonatal foreskin tissue with an undetectable expression in suprabasal keratinocytes in comparison with keratinocytes of both the basal and granular cell layers. Hence, expression of some DNA repair enzymes seems to be dependent on the differentiation state of keratinocytes, and that raises the question as to whether differentiated cells exhibit a modified potential for repairing DNA.

Our results raise two questions: (1) does $_{Hs}$Kin17 participate in the regulation of genes involved in a disequilibrium between proliferation and differentiation, as observed in wound healing and cutaneous pathologies? and (2) do the expression and location of $_{Hs}$Kin17 change after treatment with DNA-damaging agents? With regard to the second question, Haaf et al. [26] have recently observed a relocalisation of the hsRad51 protein, a human RecA-related protein, after treatment of somatic cells with chemical (methyl methane sulphonate) and physical (UV or ^{137}Cs) genotoxic agents. We now intend to characterize the level and spatial distribution of $_{Hs}$Kin17 in in vitro reconstructed human skin after genotoxic treatments, such as ionising or ultraviolet irradiation. Our approach may provide insights into the understanding of cell type-specific responses in differentiated organs subjected to genotoxic injuries.

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