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REGULAR ARTICLE

Discrimination of epithelium-like and fibroblast-like phenotypes derived from ethanol-treated immortalised human gingival keratinocytes in epithelial equivalents

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Abstract Ethanol treatment of immortalised human gingival keratinocytes (IHGK) yields in an epithelium-like (EPI) and fibroblast-like (FIB) phenotype. With respect to the stratified gingival epithelium, putative structural and molecular differences assigning cells to these phenotypes have not, to date, been analysed in a three-dimensional tissue/epithelial context. Therefore, we generated epithelial equivalents (EEs) in organotypic co-cultures of IHGK, EPI and FIB cells for 1 and 2 weeks and conducted protein and gene expression studies on the EEs for epithelial biomarkers including keratin K14, integrin subunits α6 and β1, E-cadherin, and mesenchymal vimentin. As in the EEs of IHGK and EPI, indirect immunofluorescence revealed continuous expression of β1 integrin in EEs of FIB cells. However, FIB cells exhibited a significant down-regulation in K14 and integrin α6 protein and a loss of E-cadherin at week 2, whereas vimentin was increased. FIB EEs were devoid of transcripts for E-cadherin at both time points, although transcription of the other genes remained constant in all phenotypes. Thus, the FIB phenotype exhibited a poor epithelial structure coinciding with disturbances in the expression of epithelial biomarkers and the persistence of mesenchymal vimentin. Transcription analysis revealed post-transcriptional regulation of vimentin in IHGK and EPI and of K14 and α6 in FIB cells. Our findings indicate that differences in the epithelial integrity and expression of molecules in EEs allow for the discrimination of EPI and FIB cells. This suggests that FIB cells share features of epithelial-mesenchymal transition and reflect a more progressive stage in epithelial cell transformation.

Keywords Immortalised human gingival keratinocytes · Organotypic co-culture · Epithelial–mesenchymal transition · Ethanol treatment · Transcription · Human

Eva Müssig and Thorsten Steinberg contributed equally to this work.

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Introduction

Epithelial structure and tissue function requires the adhesion of basal epithelial cells to their underlying basement membrane (BM) via multimeric adhesion complexes including hemidesmosomes (Weaver et al. 1997). In this context, pivotal molecules include the keratinocyte integrins, which mediate cell-to-cell contacts and adhesion to the extracellular matrix (ECM; for a review, see van der Flier and Sonnenberg 2001); and cadherins also involved in cell-cell contacts (Esteban et al. 2006). In addition, epithelial integrity is based on the expression of certain keratins, indicating the progressive stages of differentiation (Bloor et al. 2003).

For investigating molecules contributing to structural epithelial integrity, organotypic co-culture (OCC) is a suit-

able tool, since it mimics the in vivo situation (Stark et al. 1999; Roesch-Ely et al. 2006). In organotypic cocultures (OCCs), keratinocytes and tissue-corresponding fibroblasts grow separated by a collagen cell culture matrix, hereby counterbalancing the failure of keratinocyte monolayers to form a stratified squamous epithelial equivalent (EE) under in vitro conditions (Boukamp et al. 1990; Roesch-Ely et al. 2006). This interactive cell culture model facilitates in-vivo-related interactions between both cell types but simplifies the in vivo complexities. Therefore, the emerging EEs can be used for studies of the expression of biomarkers that contribute to epithelial homeostasis.

Because of their limited life span, the culturing of primary human keratinocytes in vitro is restricted. To bypass this limitation, primary human keratinocytes can be immortalised by the introduction of the E6 and E7 genes of the human papillomavirus type 16 (HPV16; Halbert et al. 1992). After the immortalisation of keratinocytes derived from oral gingival tissue, the abundance of E6/E7 transcripts in progressive passages indicates that the cells maintain HPV16 gene transcription at a stage at which they can be considered as stable immortalised human gingival keratinocytes (IHGK), i.e. as a cell line (Roesch-Ely et al. 2006). Concurrently, cell immortalisation represents the first feasible step in keratinocyte transformation (Boukamp et al. 1993). In this stage, the whole cell culture acquires an unlimited life span, although it lacks any signs of benign or even malignant tumour growth following subcutaneous injection or transplantation in nude mice (Boukamp et al. 1988). Further keratinocyte transformation requires the activation of oncogenes or the exposure of the cells to chemical agents, such as alcohol (Fusenig and Boukamp 1998). Despite immortalisation, IHGK preserve their tissue-specific properties. This has been extensively proven in monolayers, but also in OCCs derived from these cells. In both culture systems, the expression of hemidesmosomal integrins and early and terminal differentiation markers reflects that of the tissue of origin, whereas in OCCs, the topography of the biomarkers resembles that of native oral gingival epithelium (Roesch-Ely et al. 2006). Alcohol has an impact on IHGK morphology; an ethanol exposure of 9 weeks causes alterations in the morphological phenotype of IHGK, whereupon two phenotypes can be distinguished. The two phenotypes arising in consequence of alcohol treatment exhibit epithelium-like (EPI) and spindle-shape fibroblast-like (FIB) morphology, the latter presenting anchorage-independent growth (AIG; Chamulitrat et al. 2003). The switch from an epithelial phenotype to an anchorage-independent highly motile fibroblastoid or mesenchymal phenotype is called epithelial-mesenchymal transition (EMT). EMT plays an important role in embryogenesis, inflammation and cancer progression (Grunert et al. 2003). On the molecular level, EMT is associated with a remarkably reduced expression or even loss of tissue-specific biomarkers including epithelial intermediate filaments (IFs) and integrins. On the other hand, EMT is accompanied by a gain of markers non-typical for epithelial tissue (Thiery 2002; Grunert et al. 2003; Thiery and Sleeman 2006), e.g. vimentin normally found in cells of mesenchymal origin (Willipinski-Stapelfeld et al. 2005). In previous experiments conducted on monolayers of EPI and FIB cells, the constitutive expression of the IF protein keratin K14, which characterises basal cells of squamous epithelia, has been demonstrated in EPI cells. By contrast, the FIB phenotype is completely devoid of this keratin. An almost *vice versa* situation has been found for vimentin, which can be seen in EPI cells in only a scattered distribution, whereas it is homogeneously distributed in monolayer cultures of FIB cells (Chunglok et al. 2004). These findings point to the acquisition of EMT features by FIB cells.

However, the EMT-related changes have only been observed in conventional monolayers of FIB cells so far, but information concerning their realisation and putative consequences on the epithelial phenotype in a respective three-dimensional tissue context is missing. To address these important issues, we have developed EEs of FIB, EPI and IHGK cells in OCCs and herein compare the epithelial structure and gene expression of the epithelial biomarkers K14, integrins α6 and β1, and E-cadherin (E-cad) together with that of the mesenchymal IF component, vimentin. In this study, we show, for the first time, that EEs clearly discriminate EPI from FIB cells with respect to a stratified epithelium. The differences in gene expression indicate that characteristics of the EMT phenotype are innate to FIB-derived EEs, thereby strongly suggesting that FIB cells reflect a more progressive stage in epithelial cell transformation.

Materials and methods

Cell culture of IHGK, EPI and FIB cells and primary human gingival fibroblasts

EPI and FIB cell populations were established by Chamulitrat and co-workers (2003) by ethanol treatment of IHGK. Whereas parental IHGK were maintained in serum-free KGM medium supplemented with bovine pituitary extract (Promocell, Heidelberg, Germany), EPI and FIB cells were propagated in DME medium (PAA, Pasching, Austria) containing 10% fetal calf serum (FCS; Seromed, Biochrom, Berlin, Germany) for routine cell culture. This also applied to primary human gingival fibroblasts (HGF) required for the generation of epithelial equivalents of the above-mentioned cell populations. Establishment of primary HGF cell cultures

was approved by the local ethics committee (Medical Faculty, University of Heidelberg Germany) according to the Helsinki declaration. For the experiments conducted in this study, cells were used in the following passages: IHGK, 98–102; EPI, 130–135; FIB, 27–32; HGF, 7–12.

Generation of EEs derived from IHGK, EPI and FIB cells

EEs in OCCs were prepared according to previous protocols (Tomakidi et al. 1998). Briefly, HGF were calibrated to a cell number of 1.5×10^5 cells per milliliter of collagen type-I solution (4 mg/ml; Curacyte, Leipzig, Germany) containing FCS and Hanks' buffered salt solution (10×) and the whole mixture was allowed to polymerise at 37°C. Thereafter, IHGK, EPI or FIB cell populations were seeded onto the collagen gel surface at a density of 2.5×10^5cells/ml. For optimal reproducibility at the given periods of 1 and 2 weeks, three OCCs of each cell population were cultured in FAD medium (Ham's F12/DMEM: mixing ratio 1:3; Biochrom, Berlin, Germany) including 5% FCS and containing additionally cholera toxin (8.33 mg/ml), hydrocortisone (0.4 mg/ml), epidermal growth factor (EGF; 0.01 mg/ml) and insulin (5 mg/ml; all additives from Promocell, Heidelberg, Germany). For indirect immunofluorescence (IIF), EEs were embedded in Tissue Tek (Sakura, Zoeterwoude, Netherlands) and frozen in liquid nitrogen vapour.

For reverse transcription/polymerase chain reaction (RT-PCR) analysis, EEs with analogous cell numbers were established in six-well plates with compatible inserts (both Falcon Beckton-Dickinson, Heidelberg, Germany). The use of this culture device facilitated selective RNA isolation from each of the IHGK, EPI and FIB cell populations, devoid of HGF contamination.

IIF analysis

For IIF, frozen sections (10 μm thick) of IHGK, EPI and FIB cell specimens were mounted on adhesive slides (Histobond, Marienfeld, Germany), fixed in ice-cold 80% methanol and acetone (5 min each) and incubated overnight with primary antibodies/monoclonal antibodies directed against keratin K14 (Novocastra, Newcastle, UK; working dilution [wd] 1:50), vimentin and involucrin (abcam, Cambridge, UK; wd 1:50), integrin subunits α6 and β1 (both Chemicon, Temecula, Calif., USA; wd 1:25) and E-cad, laminin-1/10, collagen type-IV and keratin K8 (Progen, Heidelberg, Germany; wd 1:50). The slides were then washed in phosphate-buffered saline (PBS) three times (5 min each), followed by incubation with the relevant secondary fluorochrome-conjugated antibody (Alexa FluorTM 488, MoBiTec Göttingen, Germany; Cy TM3, Dianova, Hamburg, Germany; both IgG [H+L] goat anti-mouse; wd 1:100) for 1 h at room temperature. For total nuclei counterstaining, propidium iodide (Sigma, Deisenhofen, Germany; wd 1:1,000) was added to the secondary antibody. All antibodies were adjusted to their final dilution in PBS containing 0.01% Tween 20 (Sigma, Munich, Germany) and 12% bovine serum albumin (Serva, Heidelberg, Germany). Finally, the sections were embedded in mounting medium (Vectashield, Linaris, Wertheim, Germany) and documented by confocal laser scanning microscopy (Leica TCS/NT, Leica, Bensheim, Germany).

RT-PCR analysis

RNA isolation from EEs was accomplished at 1 and 2 weeks by using the PeqGOLD RNAPure protocol (PEQLAB Biotchnologie, Erlangen, Germany), an optimised guanidine isothiocyanate/phenol method. The quantity (260 nm) and quality (ratio: 260/280 nm) of the RNA were measured by using a TECAN Genios Plus spectrophotometer (Tecan, Crailsheim, Germany). RT was performed according to standard protocols by using random hexanucleotide priming and RevertAid M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) in a total volume of 20 μl. After RT of total RNA (2 μg), 1 μl of the sample was used for PCR. PCRs were performed in a total volume of 30 μl with the primers and conditions listed in Table 1. Following electrophoresis, gel images of RT-PCR products were photographed and the relative densities were measured by using the ImageJ 1.33u program (NIH, USA). Calreticulin/CRP55, as the housekeeping gene (Smith and Koch 1989), allowed the determination of relative mRNA levels (presented as the ratio of the gene of interest to calreticulin/CRP55).

To avoid the use of contaminated PCR cocktail components, the primer pairs under study were tested beforehand without the respective cDNA (water control).

RT-PCR experiments were performed in triplicate for each gene under study (for a summary of the results, see Table 2). Triplicates were obtained from three different cultures, reflecting three independent experiments.

Results

Epithelial structure emerging from EEs of IHGK, EPI and FIB cells

With respect to IHGK, we previously showed that their morphology resembled that of corresponding primary gingival keratinocytes, and that their OCCs yielded stratified EEs displaying the main molecular features of the oral gingival epithelium (Roesch-Ely et al. 2006). Here, we were interested in whether EPI and FIB cells as ethanol-

Table 1 Specific primer sequences (*f* forward, *r* reverse) for the intermediate filaments and adhesion molecules under study (*Vim* vimentin, *Int* integrin) and for the housekeeping gene calreticulin/CRP55

Primer	Oligonucleotide sequences	Product size (bp)	Annealing temperature (°C)
Genes of interest			
K14 (f)	5′-CGATGGCAAGGTGGTGTC-3′	160	58
K14 (r)	5′-GGGTGAAGCAGGGTCCAG-3′		
Vim (f)	5′-TCAGCAATATGAAAGTGTGG-3′	549	54
Vim (r)	5′-TTGATAACCTGTCCATCTCT-3′		
Int α6 (f)	5′-TCCAGAGCCAAGGTCCAG-3′	151	52
Int α6 (r)	5′-CCATCCATATCGTCTTCAATCC-3′		
Int β1 (f)	5′-GAAGCCAGCAACGGACAG-3′	129	54
Int β1 (r)	5′-CTCAGCACAGACACCAAGG-3′		
E-cadherin (f)	5′-GAAGAAGGAGGCGGAGAAG-3′	228	55
E-cadherin (r)	5′-CACGAGCAGAGAATCATAAGG-3′		
Housekeeping gene			
Calreticulin/CRP55 (f)	5′-CTGACCCTGATGCTAAGAA-3′	407	51
Calreticulin/CRP55 (r)	5′-CTCTTTGCGTTTCTTGTCT-3′		

treated descendents of the parental IHGK were capable of exhibiting the above-mentioned epithelial properties. Therefore, we first analysed the epithelial structure in OCCs derived from EPI and FIB cells at week 2, a culture period in which tissue architecture was empirically almost completed (Tomakidi et al. 1997, 1998). As expected, hematoxylin and eosin (H&E)-staining revealed that EEs of IHGK were multilayered and displayed a clear cell flattening in the uppermost cell sheets, concomitant with a frequently observed loss of cell nuclei in the uppermost cell layer (Fig. 1a). This largely also applied to EPI cells, although the EEs appeared to be less organised in the basal and medial parts of the epithelial compartment and the cells were less close-packed. This was substantiated by the respective translucent cellular interspaces inside the epithelial compartment (Fig. 1b). In marked contrast, EEs of FIB cells mostly lacked signs of epithelial organisation, even at earlier culture periods. This was visible in comparisons of OCCs at weeks 1 (Fig. 1c) and 2 (Fig. 1d). At both time points, FIB cells displayed a relaxed sheet conglomerate with cell dispersal into the collagen cell culture matrix; this dispersion was more pronounced at week 2 (Fig. 1d).

Protein presence of IFs, adhesion molecules, and ECM ligands in EEs of IHGK and EPI and FIB cells

Among the IFs, keratins characterise epithelial cells, whereas vimentin is typical for cells of mesenchymal origin (Willipinski-Stapelfeldt et al. 2005). Further, molecules such as E-cad and integrins are indispensable for the maintenance of epithelial structure, because of their crucial role in sustaining epithelial integrity (Ye et al. 2000). The gene expression of vimentin has been reported to occur not only in monolayers of immortalised keratinocytes, but also in primary keratinocytes in vitro (Tomakidi et al. 1997, 1998); in vivo neo-expression has been found at the invasion front of malignant carcinomas (Tomakidi et al. 2003). More recently, vimentin has been characterised as an innate feature of EMT (Grunert et al. 2003; Thiery and Sleeman 2006), thereby assigning it to the multistep process of malignant cell transformation. Keratins are a family of more than 20 polypeptides, which, as couples consisting of a

Table 2 Relative protein presence (indirect immunofluorescence: *IIF*) and gene transcription levels (reverse transcription/polymerase chain reaction: *RT-PCR*) of adhesion molecules and intermediate filaments in immortalised human gingival keratinocytes (*IHGK*), cells with an epithelial-like phenotype (*EPI*) and cells with a fibroblast-like phenotype (*FIB*) as indicated by arbitrary scales (IIF: +++ homogeneous or extensive protein presence, ++ intermediate protein presence, +/− low presence of protein or presence limited to single cells, *Ø* absence of the protein; RT-PCR: +++ strong gene transcription, ++ intermediate gene transcription, *Ø* no gene transcription detectable)

Method	IHGK		EPI		FIB	
	1 week	2 weeks	1 week	2 weeks	1 week	2 weeks
E-cadherin						
IIF	+++	+++	+++	++	+/−	Ø
RT-PCR	+++	+++	+++	+++	Ø	Ø
Integrin α6[a]						
IIF	+++	+++	+++	+++	**Ø**	**Ø**
RT-PCR	+++	+++	+++	+++	**+++**	**+++**
Integrin β1						
IIF	++	+++	+++	+++	+++	+++
RT-PCR	+++	+++	+++	+++	+++	+++
K14[a]						
IIF	+++	+++	++	+/−	**+/−**	**+/−**
RT-PCR	+++	+++	+++	+++	**+++**	**+++**
Vimentin[a]						
IIF	+/−	+/−	+/−	+/−	+++	+++
RT-PCR	**+++**	**++**	**++**	**++**	++	+++

[a] Results of post-transcriptional regulated genes are given in *bold*

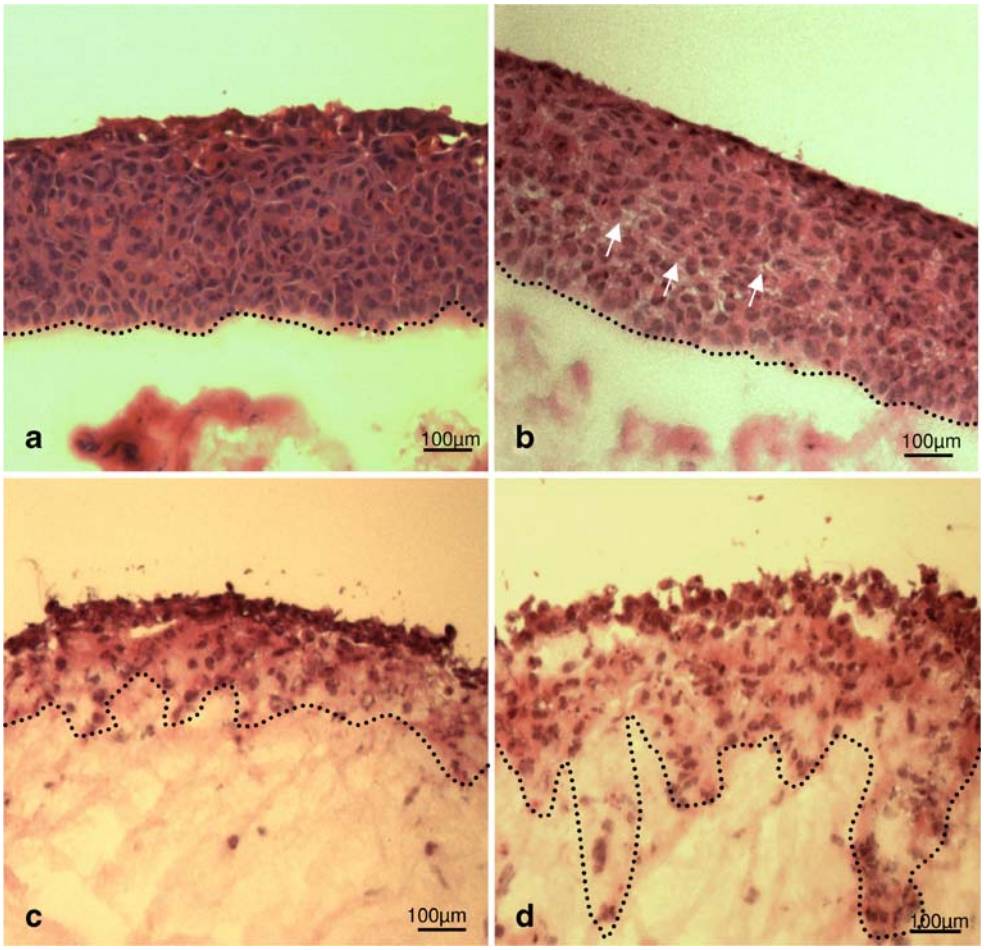

Fig. 1 Hematoxylin and eosin staining of epithelial equivalents (EEs) obtained from organotypic co-cultures of IHGK, EPI and FIB cells. Whereas epithelial structure was compact in EEs of IHGK at week 2 (**a**), EEs of EPI revealed less closely packed cells at this time point, as indicated by intraepithelial translucent intercellular spaces (*arrows* in **b**). The separation of the epithelial and mesenchymal compartments in **a**, **b** was attributable to technical aspects (*dotted lines* epithelial-mesenchymal borders). The compartment formed by FIB cells lacked structural epithelial organisation at early (**c**, week 1) and later (**d**, week 2) culture periods (*dotted lines* epithelial-mesenchymal borders). Note the dispersal of FIB cells into the collagen cell culture matrix (**c**, **d**). *Bars* 100 µm

basic and acidic partner, form an IF. Basal cells of squamous epithelia predominantly exhibit gene expression of K14 and K5 forming the respective IFs (D'Alessandro et al. 2004). Whereas K14 gene expression is restricted to the basal epithelial cell compartment, the protein persists during cell migration up to the uppermost cell layers, thereby rendering K14 an exclusive structural epithelial marker.

In the OCCs under study, IIF revealed a homogeneous protein presence in the EEs of IHGK at weeks 1 (Fig. 2a) and 2 (Fig. 2b). In EEs of EPI cells at weeks 1 (Fig. 2c) and 2 (Fig. 2d), K14-expressing cells were arranged in a patchy pattern. Interestingly, K14-positive cells could also be seen in the collagen gel which may emerge from an apparent dispersion of EPI cells into the cell culture matrix (Fig. 2c, d). Irrespective of the culture period, OCCs derived from FIB cells exhibited the lowest presence of K14 (Fig. 2e,f), thus correlating with the low degree of epithelial organisation as indicated by H&E-staining of the corresponding EEs. The decline in K14 expression, particularly related to the FIB phenotype, raised the question as to whether these cells were capable of undergoing epithelial differentiation. Therefore, we assessed involucrin, a marker related to the commitment of keratinocytes to terminal differentiation and subsequent loss of cells. EEs of IHGK and EPI showed a progressive increase in the presence of involucrin in the suprabasal compartment (see insets in Fig. 2a–d) for weeks 1 and 2. By contrast, FIB cells were devoid of this terminal differentiation marker, even at week 1 (see inset, Fig. 2e), thus pointing to an impaired differentiation capacity. In comparison with K14, an almost *vice versa* situation was observed for the vimentin IF protein (Fig. 3). This was substantiated by the presence of single positive cells at the analysed time points in EEs formed by IHGK (Fig. 3a, b), whereas EEs of EPI displayed vimentin-positive cells preferably at the epithelium/collagen matrix interface (Fig. 3c, d). Unlike IHGK and EPI, extensive vimentin protein abundance was displayed by the EEs of FIB cells at both culture periods (Fig. 3e, week 1; Fig. 3f, week 2). Based on the opposed patterns observed for K14, involucrin and vimentin in EEs derived from the three keratinocyte phenotypes, we were interested to analyse the status of K8 characterising non-squamous simple epithelia. As exemplified by respective insets in Fig. 3 showing OCCs at week 1, K8 was not found in IHGK (see inset, Fig. 3a) and EPI (see inset, Fig. 3c), whereas it was present in FIB (see inset,

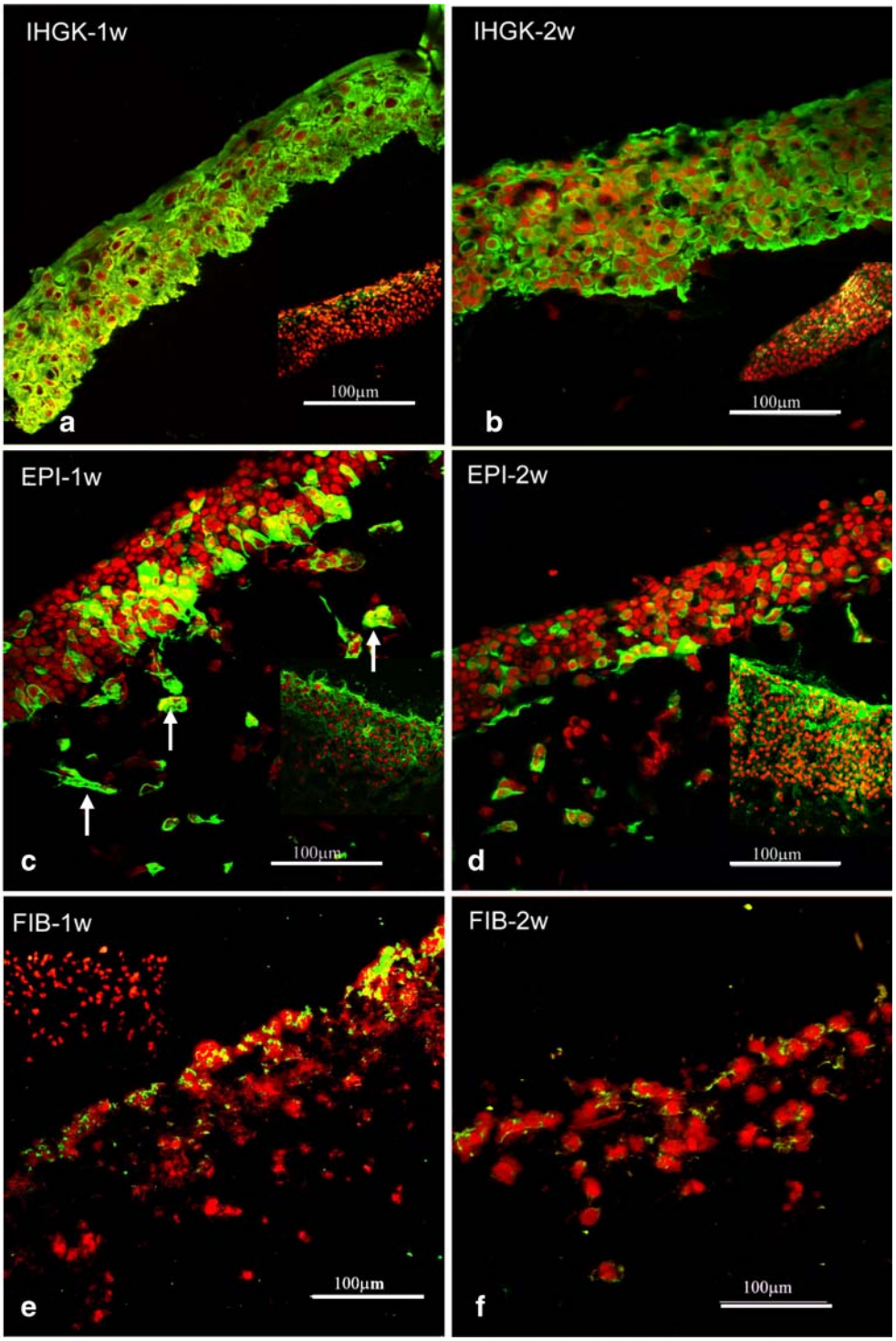

Fig. 2 Detection, by indirect immunofluorescence, of epithelial keratin K14 in frozen sections of the studied cell populations. Homogeneous K14 presence in the epithelial compartment of IHGK at weeks 1 (**a**, *1w*) and 2 (**b**, *2w*). The protein distribution appeared patchy in EPI at weeks 1 (**c**) and 2 (**d**). *Insets* in **a**–**d**: Time-dependent progressive protein presence of the terminal differentiation marker involucrin in IHGK and EPI in the suprabasal compartment from week 1 to week 2. At both time points, K14-positive EPI cells in the cell culture matrix indicated dissemination of epithelial cells in the collagen gel (*arrows* in **c**). EEs of FIB cells exhibited a low protein presence for K14 at both analysed time points (**e**, **f**). *Inset* in **e**: Lack of involucrin in FIB cells. Total nuclei are indicated by propidium iodide counterstaining (*red*); the merging of *green* and *red* fluorescence signals appears as *yellow*. *Bars* 100 µm

Fig. 3e). Together with the aforementioned patterns, the situation found for K8 suggested that FIB cells resembled less differentiated keratinocytes.

With regard to the adhesion molecules, the integrin subunits β1 and α6 can bind to ECM ligands, such as tenascin, fibronectin, laminin-1/10 and laminin-5 (laminin-5 synonyms: epilligrin, kalinin) and collagen type-IV (for a review, see van der Flier and Sonnenberg 2001), the latter three reflecting ECM molecules that constitute epithelium-underlying basement membrane (BM). In addition, the integrin receptor α6β1 can mediate epithelial cell-to-cell contact (Salanova et al. 1995), whereas the β1 subunit can also cooperate with other alpha subunits either to form intercellular contacts (Vitale et al. 1995) or to bind

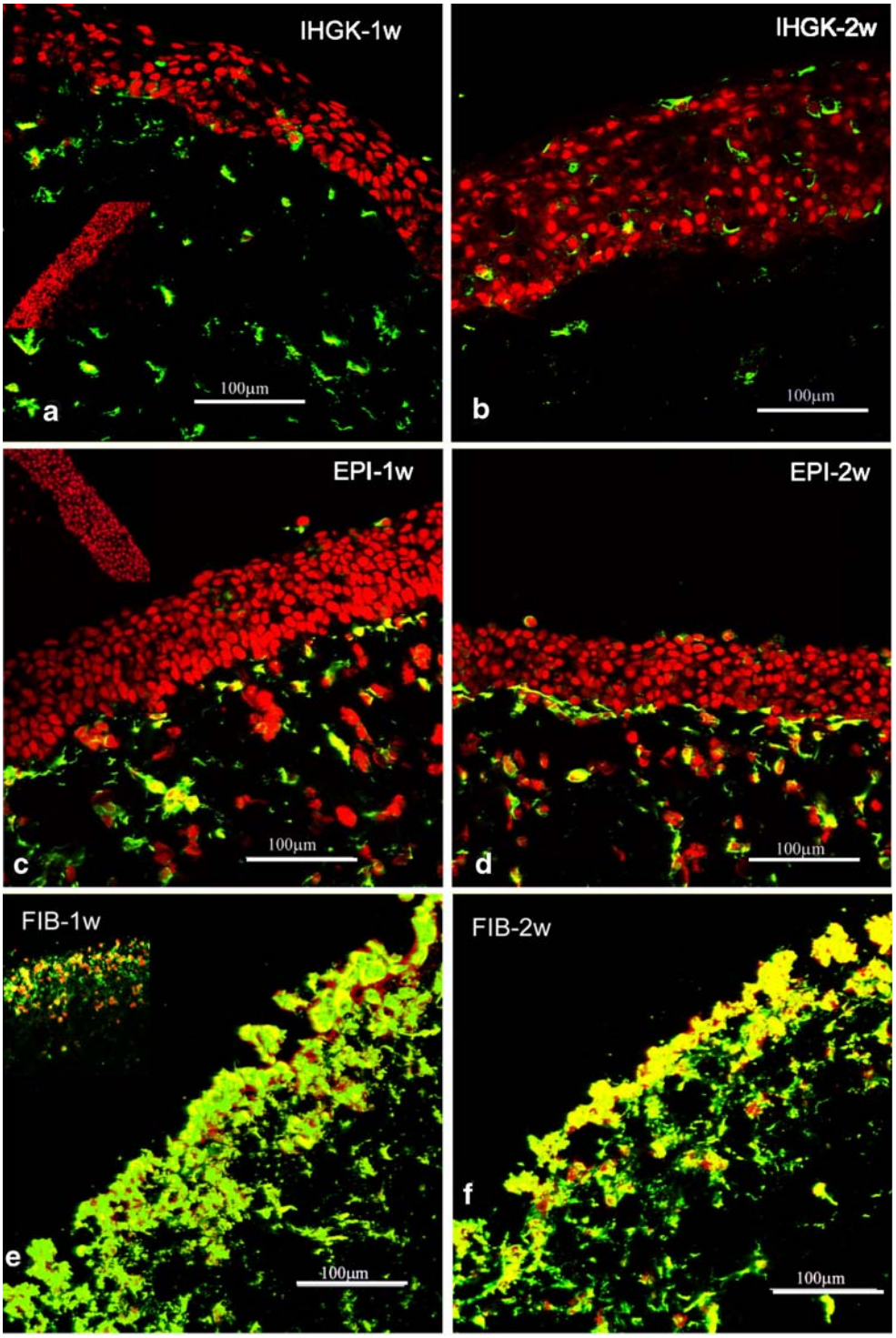

Fig. 3 Presence of the mesenchymal intermediate filament protein vimentin (*green*) is restricted to single cells at weeks 1 and 2 in EEs generated by IHGK (**a**, **b**). This also applies to EPI, although here vimentin-positive cells seem to be preferably localised at the epithelial-mesenchymal border (**c**, **d**). *Insets* in **a**, **c**: EEs of IHGK and EPI cells are devoid of keratin K8, a structural marker of simple epithelia. At both culture periods, EEs of FIB cells display the opposite situation by showing that vimentin is present throughout the FIB compartment (**e**, **f**). *Inset* in **e**: K8 expression in the FIB cell compartment. Total nuclei are indicated by propidium iodide counterstaining (*red*); the merging of *green* and *red* fluorescence signals appears as *yellow*. *Bars* 100 μm

to several extracellular ligands, including laminin-1/10 (van der Flier and Sonnenberg 2001).

IIF demonstrated that integrin β1 was present pericellularly in all epithelial cell layers of the EEs of parental IHGK (Fig. 4a, b) and its ethanol-treated descendents EPI (Fig. 4c, d) and FIB (Fig. 4e, f) but sometimes exhibited a preference for apical layers at week 2 (Fig. 4f). Irrespective of ethanol treatment, the EEs resembled native oral mucosal epithelia in which (unlike the epidermis) the β1 and β4 subunits together with several α subunits, including α6, have a pericellular localisation not only in the basal epithelial cells, but also in the suprabasal cell compartment (for a review, see Thomas et al. 1997). On the other hand, the immunolocalisation of α6 integrin clearly discriminated

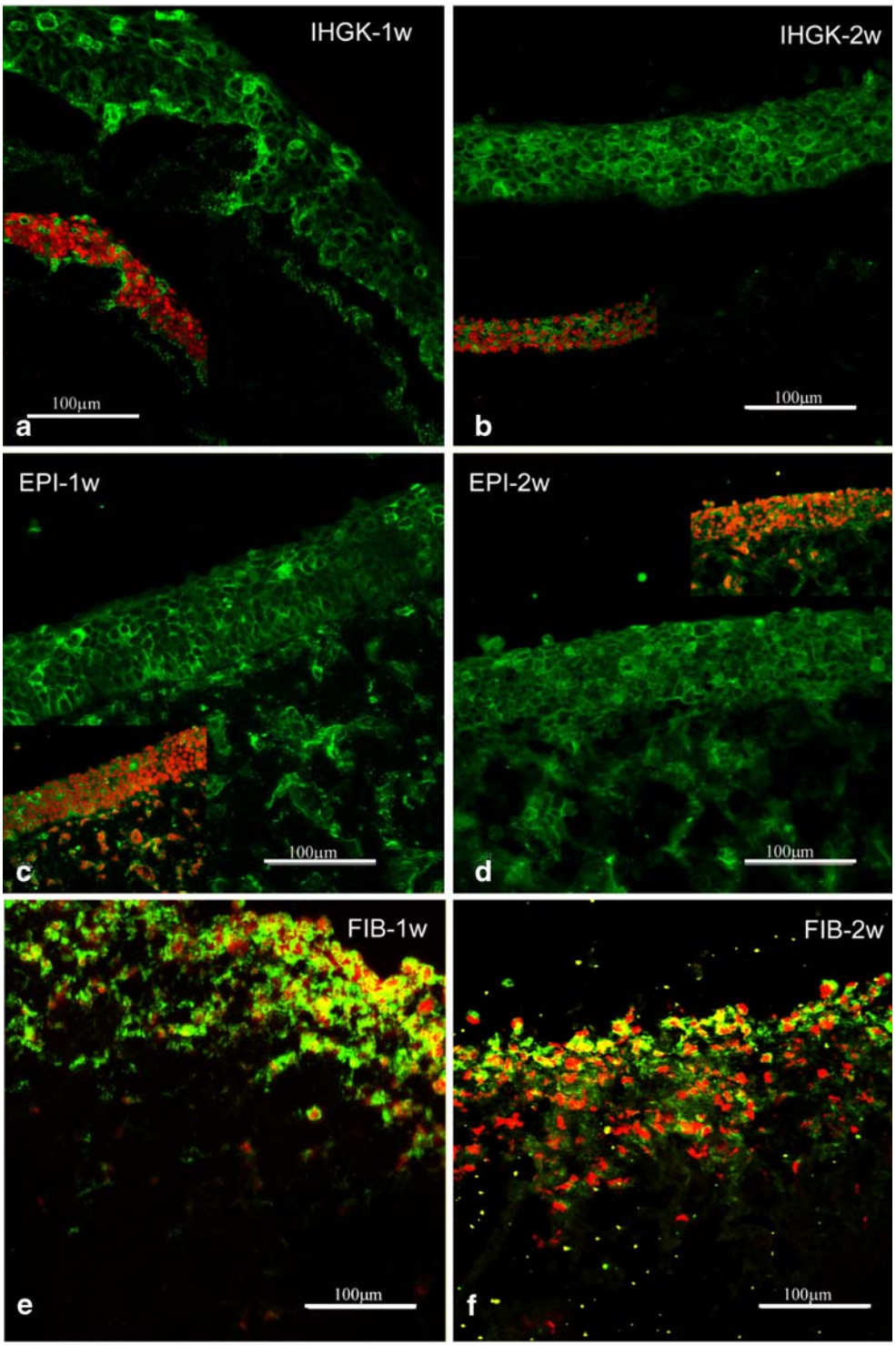

Fig. 4 Extensive protein presence of the integrin β1 subunit in all epithelial cell layers in EEs of IHGK (**a**, **b**), EPI (**c**, **d**) and FIB cells (**e**, **f**) indicating its independence of ethanol treatment or epithelial cell phenotype and culture period. *Green* fluorescence signal in **a–d** visualises the pericellular distribution of intergrin β1 (*yellow* merge of *green* and *red* fluorescence signals). Total nuclei are indicated by *red* propidium iodide counterstaining in *insets* in **a–d** and in images in **e**, **f**. *Bars* 100 µm

EPI from FIB cells, as seen in Fig. 5. Here, EEs generated by EPI cells at weeks 1 (Fig. 5c) and 2 (Fig. 5d) had a homogeneous pericellular α6 presence in almost the entire epithelial compartment comparable with their parental IHGK counterparts (Fig. 5a, b). By contrast, the respective FIB EEs were characterised by only faint remnants of this integrin subunit, which were limited to single cells (Fig. 5e, f). This striking down-regulation in the protein expression of the integrin receptor α6 displayed by FIB cells indicates a possible disturbance in hemidesmosomal ECM/BM attachment or in intercellular contacts in which this receptor participates. For epithelial cadherin/E-cad (Fig. 6), the detected protein expression pattern largely paralleled that of α6 integrin. By analogy, this molecule (involved in the

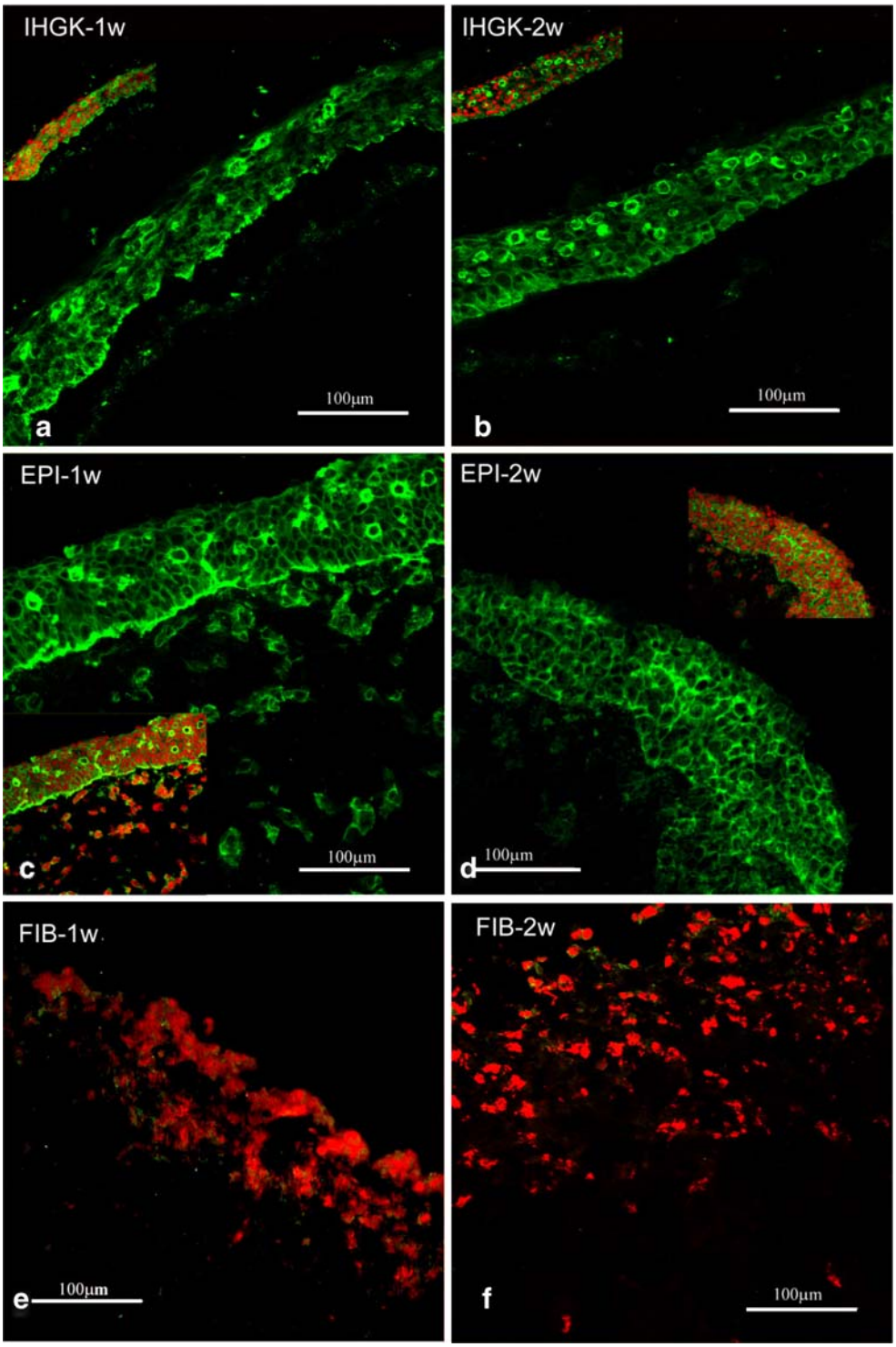

Fig. 5 Both time points demonstrate the homogeneous pericellular distribution of the integrin α6 subunit in the epithelial compartments generated by IHGK (**a**, week 1; **b**, week 2) and EPI cells (**c**, week 1; **d**, week 2). *Green* fluorescence signal in **a–d** visualises the pericellular distribution of intergrin α6. Immunostaining for α6 appears faint in FIB EEs at week 1 (**e**) and is absent at week 2 (**f**). Total nuclei are indicated by *red* propidium iodide counterstaining in *insets* in **a–d** and in images **e**, **f**. *Bars* 100 μm

maintenance of both adherens and desmosomal epithelial junctions) was homogeneously present in the epithelial compartments obtained from EEs of IHGK (Fig. 6a, b) and EPI at week 1 (Fig. 6c), whereas at week 2, it appeared to slightly decline in the basal cell compartment (Fig. 6d). In EEs derived from FIB cells, α6 integrin was faintly visible in single cells at week 1 (Fig. 6e) and was apparently absent at week 2 (Fig. 6f). The relative presence, as measured on an arbitrary scale, of the analysed proteins detected by IIF is summarised in Table 2. Because of the dramatic differences in the expression of the assessed adhesion molecules (particularly the BM contact-forming integrin subunit α6), we analysed the status of the main BM constituents, viz. laminin-1/10, laminin-5, and collagen type-IV, in EEs of

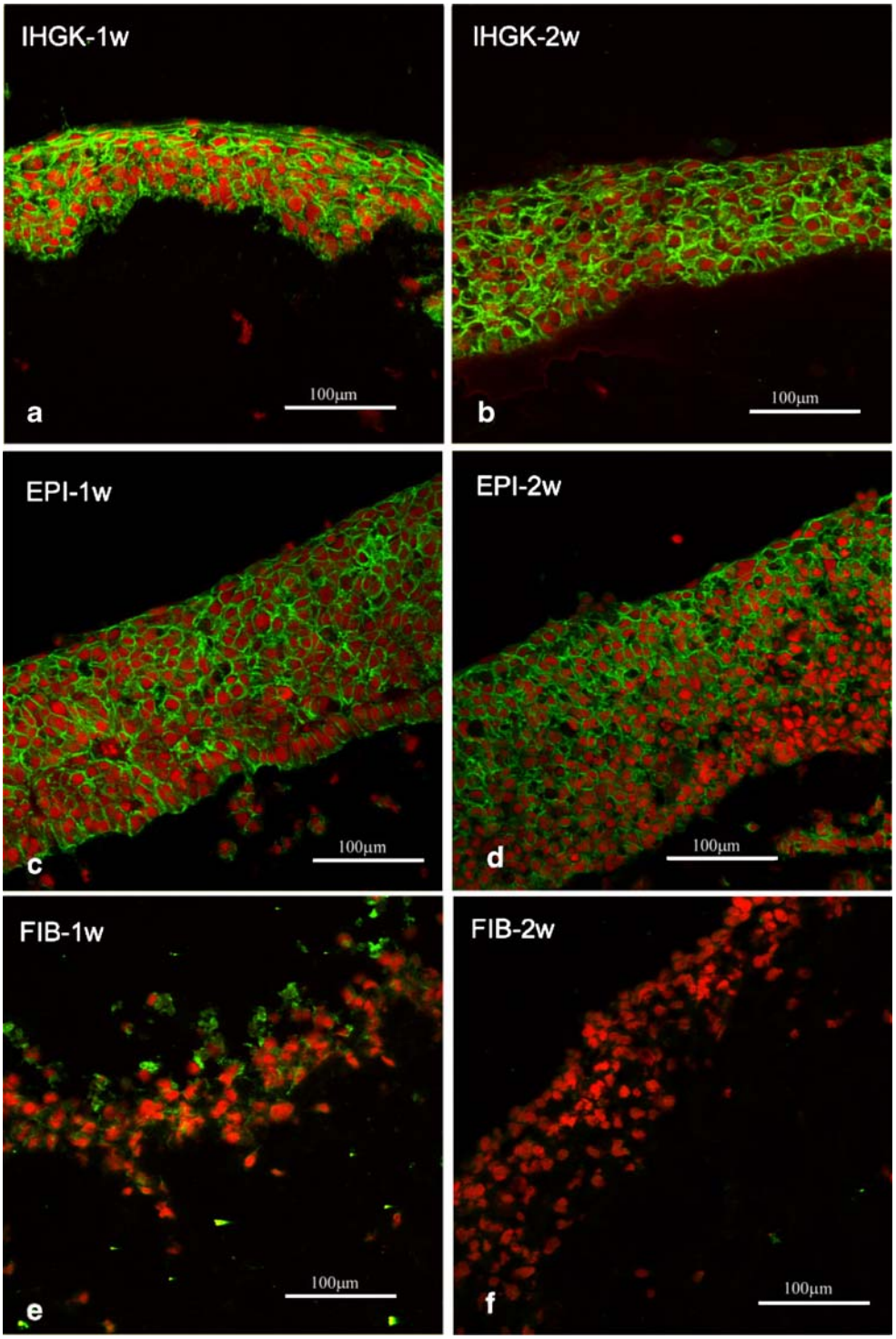

Fig. 6 Presence of E-cadherin at weeks 1 and 2 is constitutive in all epithelial compartments derived from IHGK (**a**, **b**) and the EPI phenotype at week 1 (**c**) but, at week 2, a slight decrease is observed, with preference for the basal cell compartment (**d**). Sporadic cells show the presence of E-cadherin in EEs of FIB cells at week 1 (**e**), whereas the protein is absent at week 2 (**f**). Total nuclei are indicated by *red* propidium iodide counterstaining. *Bars* 100 μm

FIB at week 2, reflecting the more progressive stage of epithelial morphogenesis. Since α6 cooperates with β4 to form the hemidesmosomal integrin α6β4 (mandatory for basal keratinocyte adhesion to the BM), this integrin subunit was also investigated. IIF analysis revealed a residual expression for laminin-1/10 and β4 integrin in the uppermost epithelial layer/layers (Fig. 7a, b), whereas FIB EEs were devoid of laminin-5 and collagen type-IV (Fig. 7c, d). Although the residual β4 expression in conjunction with the α6 results support the assumption of hemidesmosomal disturbances, the striking loss of α6 in the suprabasal compartment indicates failures in intercellular contacts. The constitutive β1 presence in FIB cells may point to occupancy of this integrin receptor by other alpha

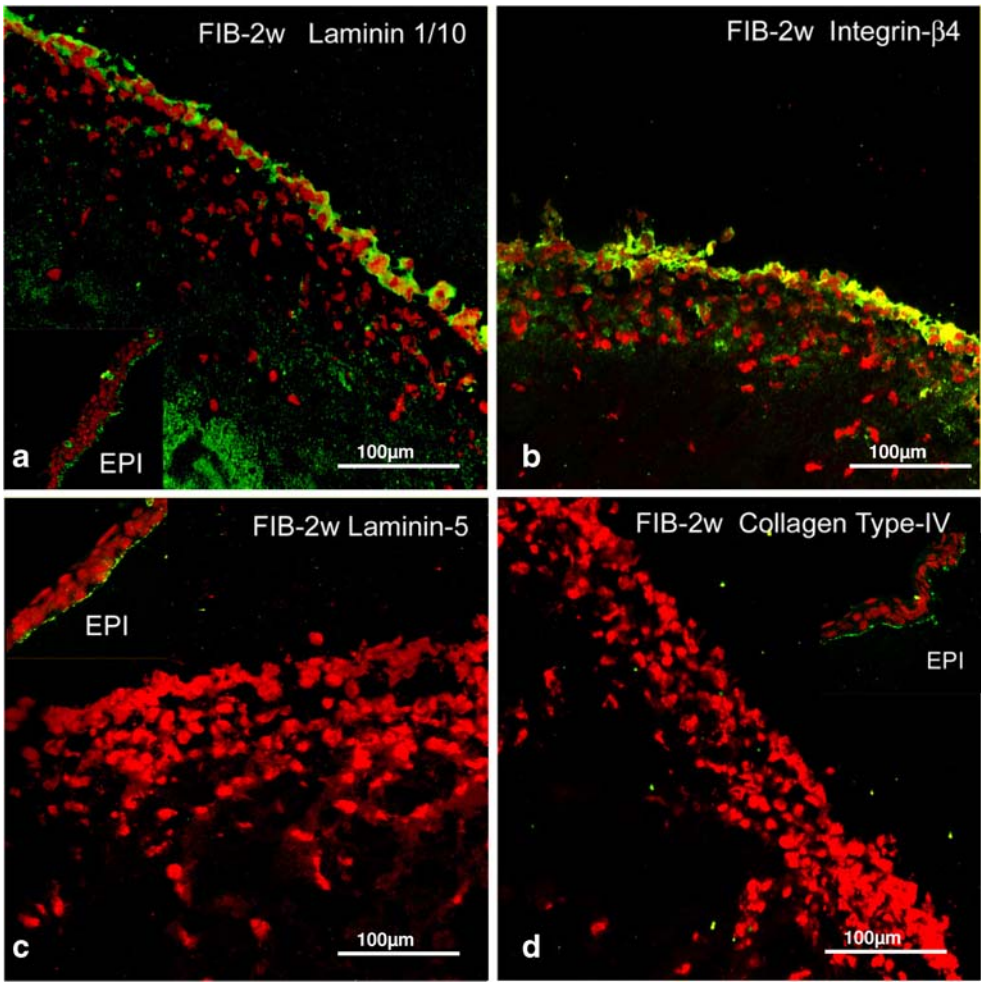

Fig. 7 Analysis of EEs formed by FIB cells after a 2-week culture period shows a residual protein presence in the uppermost cell layer(s) for the basement membrane (BM) constituent laminin-1/10 (**a**) and the hemidesmosomal integrin subunit β4 (**b**), whereas the absence is denoted for the further BM components, laminin-5 (**c**) and collagen-type IV (**d**). *Insets*: Antibody specificity of laminin-1/10 (**a**), laminin-5 (**c**) and collagen-IV (**d**) in EE of EPI cells. Total nuclei are indicated by propidium iodide counterstaining (*red*); the merging of *green* and *red* fluorescence signals appears as *yellow*. *Bars* 100 µm

subunits. This might apply to the alpha subunits 1–3, which can bind a variety of matrix ligands including laminin-1/10, which has been found to be expressed at the apical epithelial layers of FIB cultures, a pattern showing a reasonable coincidence with that observed for β1 in FIB cells at week 2 (cf. Figs. 4f, 7a).

RT-PCR analysis of gene expression of IFs and molecules facilitating cell-to-cell and cell-matrix interactions

Even in non-keratinised regions of the oral epithelia, such as the buccal mucosa, the gene expression of the differentiation-specific keratins is known to be regulated at the post-transcriptional level. This has been shown for keratins K1 and K10, which normally indicate early differentiation, e.g. in the keratinised epidermal skin epithelium. Although the proteins for K1 and K10 are only marginally seen in the differentiated parts of the buccal mucosal epithelium, their mRNAs have been extensively detected in these regions (Bloor et al. 1998).

Based on these differences, we carried out a comparative gene expression analysis for most of the previously described biomarkers to seek putative discrepancies in their abundance at the protein and mRNA levels in EEs derived from IHGK, and EPI and FIB cells. One technical advantage of EEs established in the trans-well-system is that this system retains keratinocyte-fibroblast interactions and facilitates cell-type-selective RNA isolation. Table 2 summarises the relative mRNA level (on an arbitrary scale) of the genes under study exclusively detected in the epithelial compartment derived from IHGK, EPI and FIB cells, as determined by the ratio of the gene of interest to the housekeeping gene calreticulin (data not shown). Generally, for the majority of the tested molecules, the relative mRNA level detected by RT-PCR was high (Table 2), irrespective of ethanol treatment. An exception was the E-cad gene, which was strongly transcribed by IHGK and EPI cells, although its mRNA was not detectable in FIB cells at any time point (Table 2). In this context, the residual E-cad protein seen at week 1 may point to a cessation of the corresponding mRNA synthesis during the first culture week. Whereas the genes for K14 and the integrin subunits β1 and α6 exhibited apparently similarly high mRNA levels in the analysed cell populations at the

chosen time points, vimentin showed a slight time-associated modulation in IHGK and FIB cells (Table 2). In EPI cells, the relative mRNA level for vimentin remained at an intermediate level, regardless of the culture period (Table 2). Interestingly, the results obtained from the transcription analysis indicated discrepancies in the protein presence and gene transcription for vimentin in IHGK and EPI cells and for K14 and α6 integrin in FIB cells, thereby strongly suggesting the regulation of these genes at the post-transcriptional level (Table 2).

Discussion

In the oral cavity, the cell-transformation-bound multistep process of carcinogenesis results in the formation of oral squamous cell carcinoma (OSCC). Recent epidemiological studies have shown that the propensity for OSCC development is correlated to lifestyle risk factors including diet and dental care, tobacco chewing, smoking and alcohol consumption (Subapriya et al. 2007). To elucidate the morphological and molecular changes underlying the multistep process of oral carcinogenesis, but uncoupled from in vivo complexity, several in vitro studies have been conducted by employing cell cultures of ethanol-treated transformed keratinocytes. In recent investigations dealing with the molecular mechanisms of ethanol effects, a marked reduction of important cell cycle inhibitors, such as p16, p18 and p19, has been reported, whereas the expression of cyclin D1, which is important for the G1/S transition, remains unaffected (Hager et al. 2001; Kornfehl et al. 2002). In terminally transformed keratinocytes derived from OSCC, ethanol has been shown to increase cell proliferation, whereas on the other hand, the keratin profile suggests a reduction in cellular differentiation (Kornfehl et al. 1999). Similar to the effects observed for ethanol in OSCC cultures, we have been able to show, in previous studies of monolayers of EPI and FIB cells, that FIB cells display reduced levels of stratified epithelial keratin K14 and neo-expression of K18 normally expressed in less complex epithelia (Chamulitrat et al. 2003). This shift in the keratin profile indicates that the FIB cells have acquired properties that characterise less differentiated keratinocytes.

Maintenance of structural epithelial integrity requires the expression of certain molecules that are indispensable not only for keratinocyte shape, but also for cell polarity. In this context, keratinocyte-intrinsic keratins and integrins serving as cell-matrix receptors (Marchisio et al. 1997) have to be considered, together with E-cad (Wheelock and Jensen 1992; Ye et al. 2000), the latter being a key player in maintaining both adherens and desmosomal epithelial intercellular junctions. Dysregulation in the expression of the above-mentioned molecules leads to the perturbation of epithelial integrity. Causes for this perturbation include pathological tissue situations, such as inflammation in the form of periodontitis (Ye et al. 2000) and tumour progression when malignant transformation of keratinocytes leads to the formation of carcinomas, some having metastatic potential. Although tissue destruction is an innate feature of pathology-related structural epithelial perturbation, dissolution of the epithelial structure also occurs under physiological conditions, i.e. during embryogenesis, when it is required for developmental processes. During embryogenesis, a number of extracellular signals can convert epithelial cells into mesenchymal cells and triggering of this process is termed epithelial-mesenchymal transition (EMT) (for a review, see Thiery and Sleeman 2006). With respect to EMT, a growing body of evidence suggests that it also arises during progressive cell transformation (Huber et al. 2005; Thiery and Sleeman 2006). In our EEs, the IFs under study have revealed a marked down-regulation of the K14 protein in FIB when compared with EPI and parental IHGK, although mRNAs are detectable. Concerning K14, this parallels the above-described situation for the FIB monolayers but points to post-transcriptional K14 regulation. This also holds true for mesenchymal vimentin, which appears less abundant in EPI when compared with FIB (Chunglok et al. 2004). In EEs of FIB cells, the clear detection of vimentin can be seen at both the gene expression and protein levels, whereas EPI and IHGK show sporadic protein presence concomitant with strong gene expression. The discrepancy between the vimentin protein and the gene expression pattern found for EPI and IHGK appears to be attributable to post-transcriptional regulation. This is an interesting finding, since the acquisition or persistence of vimentin in addition to the loss of epithelial-specific or differentiation-specific keratins and neo-expression of non-typical keratins are features of EMT. Regarding the non-typical keratins, this has been corroborated by the detectability of K8 protein in FIB OCCs, whereas this simple epithelial keratin remains undetectable in the EPI and IHGK counterparts. In this context, the presence of K8 in FIB OCCs agrees well with the above-mentioned situation found for these cells in monolayer. The expression of K18, the natural keratin-filament-forming partner of K8 has been described (Chamulitrat et al. 2003). Although not assessed at the IF level, the lack of the terminal differentiation marker involucrin in FIB OCCs at week 1, together with the expression of K8 in OCCs, reinforces the assumption that FIB cells are less differentiated. Among the adhesion molecules analysed, EEs of FIB show no presence of E-cad at the mRNA and protein levels at week 2. Therefore, FIB cells might cease E-cad gene expression during week 1, with the protein that is still detectable at this time point possibly reflecting remnants of this molecule. A comparable situa-

tion has been found for the hemidesmosomal integrin subunit α6 in FIB EEs, for which the protein is detectable in a residual manner. In contrast to E-cad, α6 gene expression is maintained in FIB cells, thereby revealing that post-transcriptional regulation also applies to α6. As previously mentioned for the gain of vimentin, the loss of E-cad further belongs to the criteria of EMT. Our findings that FIB cells share the main EMT characteristics not only in monolayers, but also in EEs that facilitate in-vivo-like cell-to-cell interactions between keratinocytes and fibroblasts suggest that they have acquired EMT-associated cell plasticity by converting to a more motile mesenchymal cell type. This hypothesis is backed by our former finding of reduced levels of a further cell-to-cell-contact molecule, desmoplakin (DP), in monolayers of FIB (Chamulitrat et al. 2003). In addition, in EEs of FIB, the overall appearance of the epithelial structure is poor in relation to that seen for EPI and IHGK at week 2, a culture period during which epithelial histogenesis in oral keratinocyte OCCs is empirically almost completed (Roesch-Ely et al. 2006). Moreover, although not directly addressed by an experiment in the present study, an improved motility of FIB cells can be indirectly concluded from our previous experiments conducted on monolayers; we have demonstrated that FIB cells have the ability for AIG, which is not seen in cultures of EPI (Chamulitrat et al. 2003). AIG has been recently reported for mammary epithelial cells as being a valid criterion for epithelial cells switching to a fibroblastoid cell type during EMT (Jenndahl et al. 2006). In view of the molecular pattern for FIB cells elaborated so far, the gain of vimentin in conjunction with the insufficient levels of DP and E-cad and the failure of the proper expression of α6 are probably molecular contributors for the observed poor epithelial structure. This is mostly attributable to the biological functions of the mentioned molecules, with DP and E-cad being involved in cell-to-cell and cell-matrix contacts, and α6 being important for basal keratinocyte adhesion to the epithelial BM. In this context, the main ECM-BM constituents, collagen type-IV and laminin-1/10, are non-detectable or only residually detectable. This also applies to the β4 integrin subunit, which, together with α6, forms the hemidesmosomal integrin, and to laminin-5, the major component of the anchoring filaments, all of which are pivotal for the integration of α6β4 integrin into the BM. These serious disorders found for the described adhesion molecules and for BM components strongly suggest an incompetence of FIB cells with regard to form proper intra-epithelial and hemidesmosome-based ECM contacts, which are indispensable for epithelial integrity and structure. This hypothesis is supported by the finding that EPI cells, in contrast to FIB cells, express the hemidesmosomal integrin subunit α6 plus laminin-5 and collagen type-IV (see insets in Fig. 7c, d). With emphasis on the hemidesmosome, laminin-5 possesses the extracellular ligand for α6, thereby facilitating keratinocyte adhesion to the BM, the latter comprising laminin-1/10 in the *lamina lucida* and collagen type-IV in the *lamina densa* zone. With respect to the intra-epithelial contacts, the proposed incompetence of FIB cells is further supported by the observation that the α6 integrin subunit, which is also capable of being involved in intercellular contacts (Salanova et al. 1995), displays a loss in almost the entire epithelium. However, we cannot exclude that incompetence in contact formation leads to the progressive decline in K14 expression, since contact of the basal cells to the BM-zone is crucial for epithelial cell polarity (Marchisio et al. 1997; Fukumoto et al. 2006). Concerning K14, single positive cells have been found in the collagen gel of EPI OCCs. These may emerge from an apparent dispersal of cells into the cell culture matrix. In conjunction with the patchy pattern observed for K14, the small decrease of E-cad in EPI OCCs at week 2, and the slight translucencies between the cells seen after H&E-staining, these features may account for the first feasible alterations of the parental IHGK in response to the preceding ethanol treatment.

A further hallmark of EMT is the constitutive presence or even up-regulation of the β1 integrin subunit. This has been concluded from the increased β1 expression observed during the conversion of avian lens epithelium to mesenchyme in collagen gels (Zuk and Hay 1994). More recent reports have shown that transforming growth factor-β (TGF-β) signalling mediated through MAP kinases (MAPK) including p38 and ERK (Lei et al. 2007) is required for EMT and is dependent on β1 integrin (Bhowmick et al. 2001). In our EEs, the integrin β1 subunit displays continuous expression at both the mRNA and protein levels. This maintenance in β1 expression leads to the assumption that TGF-β signalling may also be of relevance in the fibroblastoid conversion of IHGK to FIB cells in response to ethanol. Intriguingly, FIB cells exhibit only protein remnants of hemidesmosomal integrins α6 and β4, whereas intact β1 is present. This suggests that FIB cells have a lower affinity for the formation of stable hemidesmosomal contacts but prefer less stable or focal ECM contacts, which are mediated by the broad panel of β1 integrin heterodimers. This assumption is corroborated by the previously discussed residual or lack of expression of the hemidesmosomal integrin ECM/BM ligands, viz. laminin-5, laminin-1/10 and collagen type-IV, in EEs of FIB.

Among the basic cellular and molecular processes governing EMT, three major signalling pathways have so far been identified, one of them comprising the above-mentioned TGF-β1 pathway via integrin β1. The other two pathways involve E-cad via Wnt/β-catenin signalling, which can cooperate with TGF-β/Smad signalling, and

Raf/MAPK signalling via constitutively active ras or receptor-tyrosin kinases, e.g. the EGF-receptor, the latter pathway being assumed to be the main driver of EMT-related dedifferentiation and cell motility (for a review, see Huber et al. 2005). Based on our findings concerning the cessation of E-cad expression in conjunction with the preservation of β1 in EEs of FIB, Wnt and TGF-β signalling appears possible. Indirect evidence that MAPK-based signalling may also occur in FIB cells is provided by our former studies of FIB monolayers, which have revealed the coincidence of AIG with higher levels of the phosphorylated (activated) MAPK ERK1 and 2, i.e. p42/44 (Chamulitrat et al. 2003).

Taken together, we show here for the first time that EEs allow the discrimination of two epithelial phenotypes derived from IHGK following ethanol treatment in a three-dimensional epithelial tissue context. The molecular changes observed between the EPI and FIB phenotypes include epithelial-specific K14, vimentin, and the adhesion molecules integrin α6 and E-cad, with some of these proteins being regulated at the post-transcriptional level. Intriguingly, the failure of FIB cells properly to express K14, E-cad and integrin α6, while vimentin and β1 integrin persist, is correlated with poor epithelial structure and integrity. The molecular differences discriminating EPI from FIB cells provide valid biomarkers of EMT. Thus, these differences indicate that ethanol can induce EMT-like properties in keratinocytes derived from oral gingiva. In conclusion, the acquisition of EMT properties shows that, in comparison with parental IHGK and EPI cells, FIB cells reflect a more progressive stage in epithelial cell transformation towards carcinogenic tumour cells.

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