CMLS, Cell. Mol. Life Sci. 61 (2004) 610–617
1420-682X/04/050610-08
DOI 10.1007/s00018-003-3433-9

CMLS Cellular and Molecular Life Sciences

Research Article

Alcohol dehydrogenase 3 transcription associates with proliferation of human oral keratinocytes

J. A. Nilsson [a], J. J. Hedberg [a,b,c], M. Vondracek [a], C. A. Staab [b], A. Hansson [a], J.-O. Höög [b] and R. C. Grafström [a,*]

[a] Institute of Environmental Medicine, Karolinska Institutet, Box 210, 171 77 Stockholm (Sweden),
Fax: +46 8 32 94 02, e-mail: roland.grafstrom@imm.ki.se
[b] Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm (Sweden)
[c] Current address: Amersham Biosciences AB, 75 184 Uppsala (Sweden)

Received 20 November 2003; received after revision 16 December 2003; accepted 22 December 2003

Abstract. Gene expression underlying cellular growth and differentiation is only partly understood. This study analyzed transcript levels of the formaldehyde-metabolizing enzyme alcohol dehydrogenase 3 (ADH3) and various growth and differentiation-related genes in human oral keratinocytes. Culture of confluent cells both with and without fetal bovine serum inhibited colony-forming efficiency and induced a squamous morphology. Confluency alone decreased the transcript levels of ADH3, the proliferation markers cell division cycle 2 (CDC2) and proliferating cell nuclear antigen (PCNA), and the basal cell marker cytokeratin 5 (K5), but increased transcripts for the suprabasal differentiation markers involucrin (INV) and small proline-rich protein 1B (SPR1). These changes were variably influenced by serum, i.e., loss of CDC2 and PCNA was inhibited, loss of K5 promoted, increase of SPR1 transcripts inhibited, and increase of INV promoted. The extent and onset of the effects implied that ADH3 transcription serves as a proliferation marker and that confluency with or without serum exposure can serve to selectively analyze proliferative and differentiated cellular states.

Key words. Oral mucosa; keratinocyte; alcohol dehydrogenase; formaldehyde metabolism; squamous differentiation; proliferation; transcript profiling.

Squamous epithelia, including those of the oral cavity, eliminate cells primarily by desquamation to maintain a homeostatic balance with cells generated by proliferation [1]. During the basal to suprabasal transition, keratinocytes gradually lose their proliferative ability and undergo terminal squamous differentiation (TSD) before the cells or cell remains are shed from the outermost layer [1, 2]. Various genes/proteins signify proliferative and differentiated cellular states. Markers of proliferation include cell division cycle 2 (CDC2), also termed cyclin-dependent kinase 1, which constitutes a key component of the cell cycle control system, and proliferating cell nuclear antigen (PCNA), which is part of a multiprotein complex regulating DNA replication [3, 4]. Expression of the corresponding genes decreases when cells commit to TSD as cells then cease to divide [5–7]. The process of oral keratinocyte TSD is coupled with the expression of numerous structural proteins, including those involved in cornification, e.g., involucrin (INV) and small proline-rich proteins [8]. Among the approximately 20 cytokeratins identified so far, K5 is one of the principal basal keratins in stratified epithelia, yet other keratins, like K13, are associated with the induction of TSD [2, 8, 9].
Culture of keratinocytes and the application of different conditions variably mimic epithelial tissue homeostasis and physiological processes [10, 11]. Gene expression un-

* Corresponding author.

derlying loss of proliferation and onset of TSD is only partly understood. Transfer cultures of oral keratinocytes in serum-free medium are reminiscent of basal cells, and show high proliferative ability, relatively small cell size, intense expression of basal keratins but low expression of TSD markers [12, 13]. Addition of fetal bovine serum (FBS) and/or the transition from a proliferative, confluent to a post-confluent stage, induces irreversible growth arrest and TSD, associated with increased cell surface area and the expression of TSD markers [6, 12, 13]. An opportunity to investigate these processes further is provided by the variable expression of these under different culture protocols [reviewed in ref. 11]. Notably, results from other types of epithelia may not apply to oral epithelium due to tissue specific expression of structural proteins and differential responsiveness to toxic agents [6, 14, 15].

Formaldehyde is a toxic, mutagenic and carcinogenic chemical [16, 17]. Oral inhalation of vapor in tissue fixatives, tobacco smoke, and automotive emissions, usage of certain dental materials as well as the ingestion of fruits and other foods lead to oral exposure to formaldehyde [16]. Atmospheric formaldehyde even below permissible exposure limits causes micronuclei and chromosome breakage in oral buccal and nasal epithelium, implying a need to characterize the enzymatic defense against formaldehyde [18]. Alcohol dehydrogenase 3 (ADH3), also known as glutathione-dependent formaldehyde dehydrogenase, is the primary formaldehyde scavenger [16, 19]. Assessment of ADH3 expression in oral epithelium demonstrated transcripts in the basal and parabasal layers without detectable transcripts in upper layers [20]. In contrast, the ADH3 protein was present throughout the epithelium and, moreover, it remained expressed and metabolically functional in cultured oral keratinocytes under several conditions [20].

Previous analysis of contact-inhibited, differentiated cultured oral keratinocytes showed decreased levels of ADH3 transcripts compared to sparse, proliferative cells, but the coupling to alteration of proliferation and differentiation remained unclear [20]. The current study utilized oral keratinocytes under several growth protocols to analyze the association of ADH3 transcription with markers of proliferation and/or TSD, including those normally expressed at basal or more superficial positions in oral epithelium. The cultures were expanded from a sparse to a confluent state and then maintained at confluency for up to 15 days, with or without the presence of 2 and 10% FBS. Proliferative and differentiated states were initially assessed from changes in colony-forming efficiency (CFE) and induction of morphological changes. Numerical data for the transcript levels relative to the number of β-actin transcripts were then obtained for ADH3, CDC2, PCNA, K5, INV, and small proline-rich protein 1B (SPR1) using a recently established technique for quantitative RT-PCR (StaRT-PCR), shown to be reproducible among different laboratories [21]. Finally, Northern blot analysis was used to quantify the levels of β-actin transcripts and K13 under the different conditions; the latter gene served as a positive control since the level of the corresponding transcript was previously shown to increase upon serum exposure under confluency [6]. The overall results demonstrate several means of inducing TSD markers in oral keratinocytes and, moreover, indicate that ADH3 transcription terminates in association with a loss of proliferation without association with the onset or execution of TSD.

Materials and methods

Cell cultures

Procedures used to culture human oral keratinocytes, including the preparation of the serum-free epithelial medium with high amino acid supplementation (EMHA medium) have been described recently [11]. Buccal tissue was obtained from healthy donors (non-tobacco users) undergoing reconstructive surgery, with the approval of the ethical committee at Karolinska Institutet. Primary cell cultures were initiated from incubation of tissue for 18–24 h at 4°C with 0.17% trypsin in phosphate-buffered saline to form single cells and small aggregates. The mixture was resuspended in EMHA and plated onto fibronectin/collagen-coated dishes at an approximate density of 5×10^3 cells/cm^2. Cultures were transferred at $4–5 \times 10^3$ cells/cm^2 in 60- or 100-mm Petri dishes. The various analyses were made with cells in second passage. Keratinocytes were seeded at 5×10^3 cells/cm^2 to reach confluency (100%) at 6–8 days. The term confluency (100%) was regarded as the stage/moment when the cultures were (first) grown to fully occupy the dish surface area as determined from visual inspection and photographic documentation under a phase contrast microscope. Thereafter, the assessments were based on time, and cultures were analyzed at 5, 10 and 15 days at the post-confluent stage using EMHA with or without FBS (2 or 10%) as culture condition. Medium was exchanged at 2-day intervals.

Statistical analysis

The various results were based on a minimum of three separate experiments and statistical differences were determined by analysis of variance (ANOVA). Dunnett's multiple-comparison post-test was subsequently used for comparison of multiple values to one reference value, and the Tukey-Kramer post-test was used for comparison of all results among multiple values.

Colony-forming efficiency

CFE was assayed at confluency and beyond (0, 5, 10 and 15 days). Briefly, the cells were removed using trypsin

and seeded at a density of 50 cells/cm^2 dish as described elsewhere [11]. The cells were subsequently incubated for 8 days in EMHA until the surviving colonies were clearly detectable under phase contrast microscope. The cultures were then fixed in 10% formalin and stained with 1% aqueous crystal violet. The mean CFE was determined from duplicate dishes and based on microscopic counting of colonies, each containing at least 12 cells.

Standardized quantitative RT-PCR

An established protocol termed 'standardized quantitative RT-PCR' (StaRT-PCR) was utilized [22, 23]. The SV Total RNA Isolation System protocol (Promega, Madison, Wis.) was applied for isolation of total RNA. Reverse transcription of 1 µg RNA to a corresponding amount of cDNA was carried out and the sample was stored at –20 °C. Standardized mixtures of internal standard competitive templates (CTs) for the genes under study (table 1) were purchased from Gene Express (Toledo, Ohio). Briefly, the reagents for the quantitative PCR amplification of cDNA were mixed in a 0.6-ml Eppendorf tube including 0.05 µg of the respective forward and reverse primer (Invitrogen, Glasgow, UK) dissolved at 1 mg/ml in RNA-free water (Sigma-Aldrich, St. Louis, Mo.), 0.5 U Taq polymerase (5 U/µl stock; Promega) 1 µl PCR buffer (10 × stock, Ficoll Dye, 30 mM $MgCl_2$; Idaho Technology, Idaho Falls, Idaho), 0.2 mM dNTPs (2 mM stock; Promega) 5 µl RNAse-free water, 1 µl of the CT mixture, 1 µl of the relevant primers (final concentration 5 ng/µl) and 1 µl of the cDNA sample. The mixture was transferred to a glass capillary and the reactions were amplified (35 cycles) in a RapidCycler (Idaho Technology). Amplified fragments were then subjected to electrophoresis on a 3% NuSieve/1% SeaKem agarose gel (FMC Bioproducts, Rockland, Me.) followed by visualization by ethidium bromide staining. Band intensities were analyzed by densiometry using the Gel Doc 1000 system (Bio Rad, Hemel Hempstead, UK) and NIH Image software [obtained from http://rsb.info.nih.gov/nih-image; for details, see ref. 22] The values for transcript levels at each growth condition were derived from analysis of a minimum of three separate PCR reactions. Depending on the efficiency of the PCR, including aspects related to the primer hybridizations, the sensitivity of the assay was between 1–10 molecules per 10^6 molecules of β-actin [21–23].

Northern blot analysis

The SV Total RNA Isolation System protocol (Promega) was applied for isolation of total RNA, and 25 µg of RNA was then subjected to electrophoresis under denaturing conditions in 1% agarose containing 6.5% formaldehyde. After electrophoresis, the RNA was blotted to Hybond-N nylon membranes (Amersham Biosciences, Amersham, UK) and cross-linked by oven baking or UV exposure according to the manufacturer's recommendations. Keratin 13 (K13) mRNA was probed with an *Eco*RI/*Hind*III fragment from a human K13 cDNA clone and a 2-kb human β-actin fragment was used as control. The probes were labeled with [α-^{32}P]dCTP (megaprime DNA labeling system; Amersham Biosciences) and hybridizations were performed as described elsewhere [24]. Quantification of signals was performed by phosphorImager analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.) and obtained values were correlated to the amount of RNA, determined spectrophotometrically (OD_{260}), loaded on the gel.

Table 1. Primers used for PCR amplification.

Gene	Abbreviation	Accession number	Native (bp)	CT (bp)	Primer sequence	
					forward	reverse
β-actin	β-actin	X0035/ J0074	532	416	5′ GAT TCC TAT GTG GCC GAC GAG 3′	5′ CCA TCT CTT GCT CGA AGT CC 3′
Alkohol dehydrogenase 3	ADH3	M30471/ M29872	408	338	5′ CCA GCT GGT AAC AGG TCG CAC ATG GAA A 3′	5′ GGA GGA GCA TCC AGA AAA CAG GTT CAT G 3′
Proliferating cell nuclear antigen	PCNA	J04718	346	286	5′ GCT CCA GCG GTG TAA ACC TGC A 3′	5′ CGT GCA AAT TCA CCA GAA GGC A 3′
Cell division cycle 2	CDC2	X05360	340	222	5′ GGC CTT GCC AGA GCT TTT GGA ATA CC 3′	5′ AGC CAT TTT CAT CCA AGT TTT TGA CA 3′
Keratin 5	K5	M19723	371	262	5′ CTC AAG GAT GCC AGG AAC AA 3′	5′ ACA CTG AGC CCA CCA CCT AG 3′
Small proline-rich protein 1B	SPR1	M84757	371	285	5′ CCA GCC ACT GTT GCA GCA TGA 3′	5′ AGG CAA ATG GGA CTC ATA CAC 3′
Involucrin	INV	M13902	348	266	5′ CCT TAC TGT GAG TCT GGG TTG A 3′	5′ TGG GTT TTC TGC TTT CTG ATA T 3′

Results

Morphology of confluent and post-confluent mass cultures

Oral keratinocytes were grown from a stage of single, separated cells into a confluent monolayer of pleomorphic, mostly polygonal and epithelial-like cells (fig. 1A). Further culture for 5, 10, and 15 days generated a tightly packed mass of cells that progressively exhibited the typical flattened, squamous phenotype. Confluent cultures showed lack of further expansion due to enlargement of cell surface areas and some cell detachment (not shown). At 15 days, the cultures variably exhibited differences in shape and size, including signs of multi-nucleation, vacuolization and multi-focal growth (fig. 1B, C). The latter phenomenon, including formation of ridges, was more pronounced in cultures grown without FBS (fig. 1B). Cultures maintained with 2% FBS showed an intermediate phenotype to those maintained without serum and with 10% FBS (not shown).

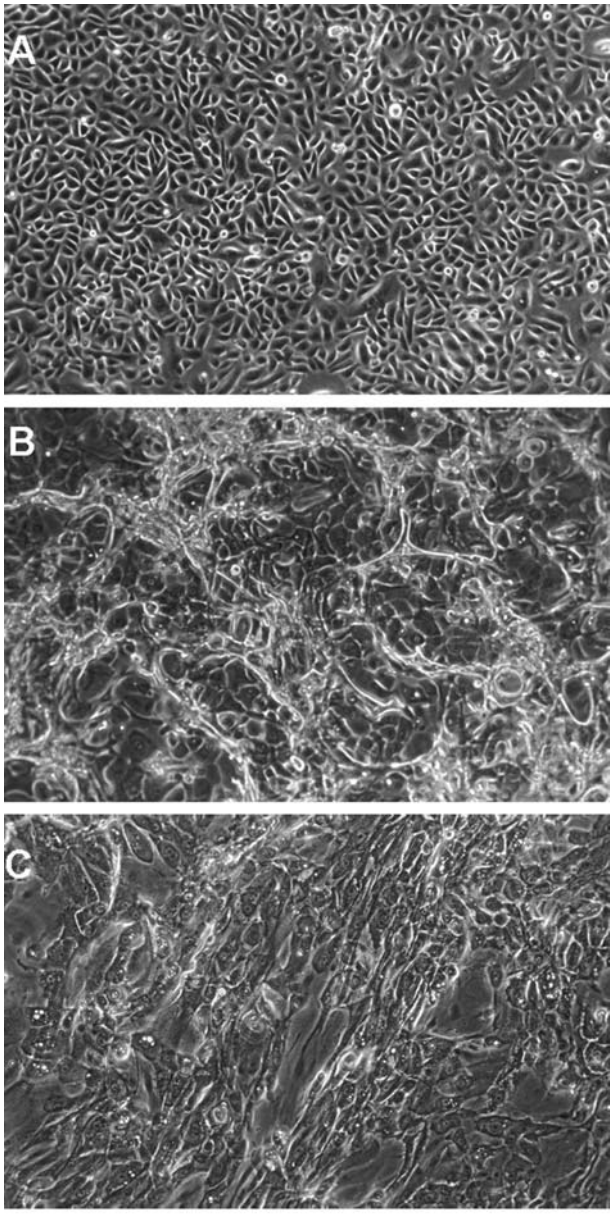

Figure 1. Morphologies of normal human oral keratinocytes grown to a confluent and post-confluent state. The cells were grown to confluency in serum-free EMHA and subsequently maintained for up to 15 days in EMHA alone or in EMHA with FBS as described in Materials and methods. Photographic documentation was made at phase contrast (× 100). (*A*) A culture that has just reached a confluent state. (*B, C*) Confluent cultures maintained for 15 days in EMHA (*B*) or in EMHA with 10% FBS (*C*).

Colony-forming efficiency

Keratinocyte cultures grown from three individuals exhibited a mean CFE of 7% following growth to a stage when they had just reached confluency (fig. 2). Subsequent maintenance at confluency resulted in significant loss of CFE at all time points. Especially apparent at 5 days, the presence of FBS counteracted the loss of CFE in a concentration-dependent manner. At day 15, the ability to form colonies was essentially abolished under all conditions.

Gene expression determined by StaRT-PCR

The mRNA levels for ADH3, CDC2, PCNA, K5, INV, and SPR1 were quantified relative to β-actin; numerical

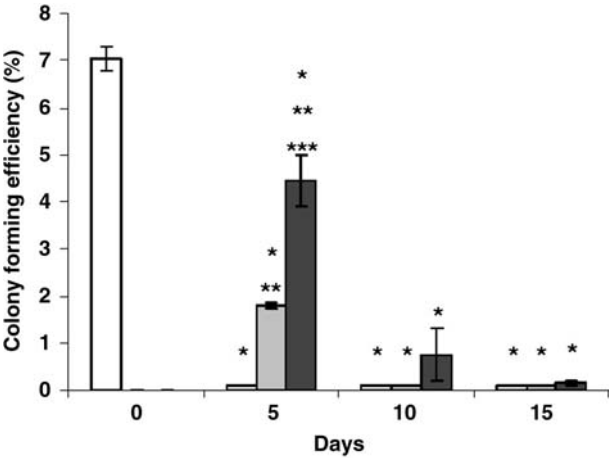

Figure 2. CFE of oral keratinocytes maintained at confluency with or without FBS. The cells were grown to confluency in EMHA and subsequently maintained for 5, 10, and 15 days in EMHA alone or in EMHA with 2 or 10% FBS. The CFE was assessed following passage of cells as described in Materials and methods. Cultures grown to just reach 100% confluency are indicated as day 0 and the respective number of days shown indicate the length of subsequent maintenance of cells at a post-confluent state. The data were obtained from three separate experiments, each with duplicate dishes, and the results are expressed as mean ± SE. EMHA alone (open columns); EMHA with 2% FBS (light-gray columns); EMHA with 10% FBS (dark-gray columns). *, significantly different from the value at day 0 ($p < 0.05$); **, significant differences compared to 0% FCS ($p < 0.05$); ***, significant difference compared to 2% FCS ($p < 0.05$).

data for the relative expression of these genes, including statistical analyses, are shown in table 2. The abundance of transcripts varied greatly among the respective genes and with the conditions, including a range of $<10^2$ to $> 10^6$ per 10^6 β-actin transcripts. Post-confluent culture without FBS decreased the levels of ADH3, at all time points and conditions (fig. 3). The presence of FBS generally had marginal effects on ADH3, except for the increase noted at 15 days in 2% FBS relative to no FBS. The levels of the established proliferation markers CDC2 and PCNA also decreased at confluency, in all conditions (fig. 3). The presence of FBS somewhat counteracted these decreases, including at least one of the markers at each time point. The influences of FBS seemed to be more pronounced at the earlier time points, especially for CDC2.

Transcript levels of the basal keratinocyte marker K5, and the TSD markers INV and SPR1 are depicted in figure 4. Expression of K5 at confluency remained unchanged following 5 days in serum-free medium although it decreased after 10 or 15 days. In contrast, both concentrations of FBS promoted the decrease in K5 at all time points. The transcript levels of INV and SPR1 were increased many-fold under confluency (table 2). Exposure to FBS, especially at 2% supplementation, promoted the increase in INV expression. Interestingly, FBS markedly counteracted the confluency-induced increase in SPR1 transcripts noted without FBS.

Gene expression determined by Northern blot analysis

The level of β-actin mRNA in confluent and post-confluent cultures was determined by Northern blot analysis following spectrophotometric quantitation to ensure equal gel loading. The β-actin mRNA levels did not differ among the samples and growth conditions, including with or without FBS, implying that the relative comparisons based on β-actin was a valid protocol (data not shown). Assessment of ADH3 transcripts by Northern blot analysis (data not shown) agreed with the StaRT-PCR-based analysis (fig. 3), and with previous results [20]. K13 was then assessed relative to β-actin at 15 days as a positive control for FBS-induced TSD. In agreement with published work [6], maintenance at confluency without FBS had marginal effects on the levels of K13 transcripts, whereas FBS increased expression several-fold (data not shown). Finally, the RNA yields were similar among samples at different time points ranging from 40 to 80 µg from the approximately 2.5×10^6 cells collected per 100-mm tissue culture dish.

Discussion

The processes underlying growth and differentiation show variable degrees of tissue specificity, also among different epithelia, and the definition of marker genes for the respective processes aid mechanistic studies [25, 26]. Oral keratinocytes, if regularly transferred in culture without serum, efficiently undergo TSD from confluency and/or serum exposure [11, 27]. Previous efforts to characterize the enzymatic defense against formaldehyde toxicity in oral epithelium demonstrated an absence of ADH3 transcripts in terminally differentiated tissue and keratinocyte cultures although without a similar absence of ADH3 protein [20]. The current study analyzed ADH3 and various other genes at the transcript level using growth conditions that enriched the cell population for

Table 2. Influence of confluency and serum exposure on transcript levels of ADH3 and markers of growth and differentiation in cultured human oral keratinocytes (number of mRNA molecules per 10^6 molecules of β-actin).

Time-exposures	ADH3 ($\times 10^4$)	PCNA ($\times 10^4$)	CDC2 ($\times 10^3$)	K5 ($\times 10^6$)	SPR1 ($\times 10^6$)	INV ($\times 10^3$)
Day 0	6.5 ± 1.3	4.2 ± 0.6	8.9 ± 1.1	2.7 ± 0.5	0.1 ± <0.1	0.1 ± <0.1
+ 5 days						
without FBS	1.6 ± 0.1*	0.9 ± <0.1*	1.8 ± 0.1*	2.9 ± 0.3	3.2 ± 0.7*	2.5 ± 0.3*
2% FBS	2.6 ± 0.4*	1.6 ± 0.1*	6.6 ± 1.0*,**	1.0 ± 0.1*,**	0.2 ± <0.1*,**	5.1 ± 0.5*
10% FBS	1.8 ± 0.2*	2.0 ± 0.8*	5.1 ± 0.8*,**	0.9 ± 0.1*,**	0.2 ± <0.1*,**	6.5 ± 1.9*
+ 10 days						
without FBS	1.4 ± 0.1*	0.3 ± <0.1*	0.2 ± 0.1*	1.6 ± 0.1*	4.0 ± 0.6*	5.1 ± 0.4*
2% FBS	1.3 ± 0.3*	0.3 ± 0.1*	1.9 ± 1.3*	0.9 ± 0.3*	0.1 ± <0.1**	8.8 ± 1.2*,**
10% FBS	1.4 ± 0.1*	0.9 ± 0.1*,**,***	3.4 ± 0.2*	1.2 ± 0.3*	0.2 ± <0.1*,**	4.6 ± 0.3*,***
+ 15 days						
without FBS	1.2 ± 0.4*	1.4 ± 0.1*	0.2 ± 0.1*	1.2 ± 0.3*	2.7 ± 0.8*	2.2 ± 0.6*
2% FBS	3.0 ± 0.3*,**	2.3 ± 0.5*	2.4 ± 0.2*,**	1.1 ± 0.1*	0.5 ± <0.1*,**	28.7 ± 6.0*,**
10% FBS	1.7 ± 0.2*	0.7 ± 0.1*,***	1.4 ± 0.4*	0.6 ± <0.1*	0.1 ± <0.1**	7.1 ± 0.9*,***

The level of the respective transcripts were determined by StaRT-PCR as described in Materials and methods. The cells were grown to a confluent state (denoted as day 0), and then maintained with or without FBS for up to 15 days as indicated. The results are presented as the mean ± SE from a minimum of three separate experiments. *, significantly different relative to day 0, $p < 0.05$; **, significantly different relative to cells maintained without FBS, $p < 0.05$; ***, significantly different relative to 2% FCS, $p < 0.05$.

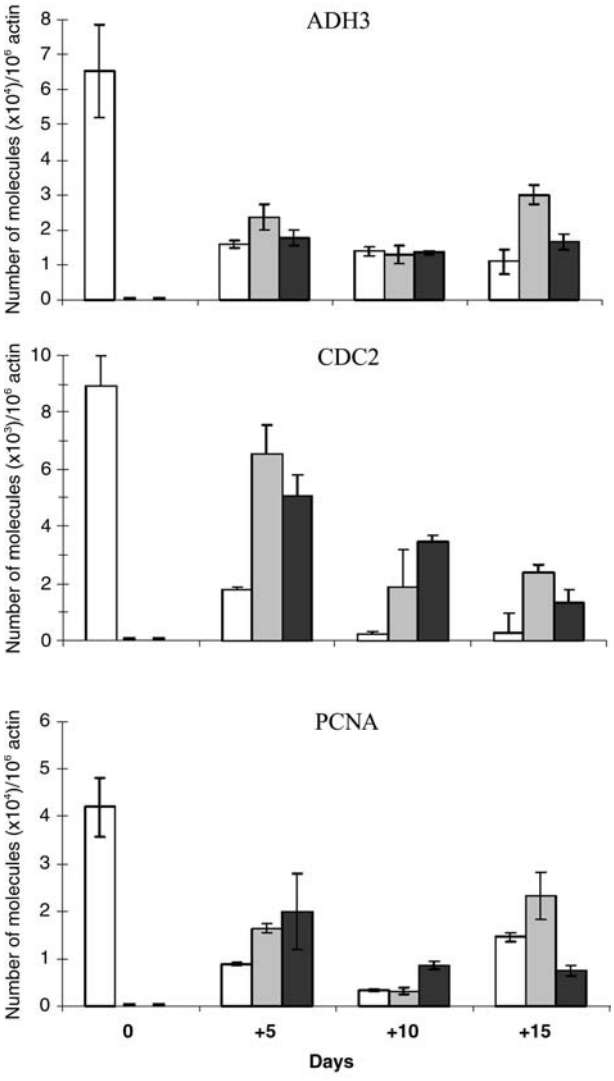

Figure 3. Transcript levels of the ADH3, CDC2, and PCNA genes in oral keratinocytes maintained at confluency with or without FBS. Cells were grown to confluency in EMHA and subsequently maintained for 5, 10, and 15 days in EMHA alone or in EMHA with 2 or 10% FBS. The respective level of mRNA was quantified relative to β-actin by StaRT-PCR and expressed as described in Materials and methods. Cultures grown to just reach 100% confluency are indicated as day 0 and the respective number of days shown indicates the length of subsequent maintenance of cells at a post-confluent state. EMHA alone (open columns); EMHA with 2% FBS (light-gray columns); EMHA with 10% FBS (dark-gray columns).

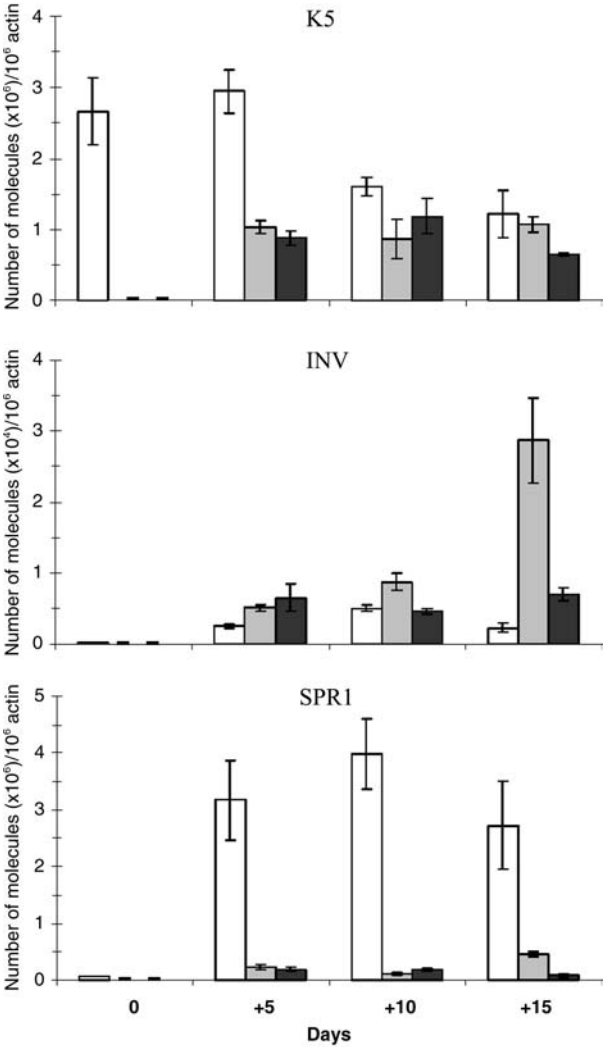

Figure 4. Transcript levels of the K5, INV, and SPR1 genes in oral keratinocytes maintained at confluency with or without FBS. Cells were grown and mRNA quantitated as described in the legend to figure 3. EMHA alone (open columns); EMHA with 2% FBS (light-gray columns); EMHA with 10% FBS (dark-gray columns).

non-proliferative and terminally differentiated states. The overall assessment showed that ADH3 transcription is associated with expression of transcripts for cell proliferation markers, notably without the corresponding (inverse) correlation with transcript levels of TSD markers. Accordingly, ADH3 transcription may serve as a useful proliferation marker in gene expression studies.

The current study utilized a well-established concept for driving proliferative keratinocytes into a state where proliferation is counteracted and TSD induced, i.e., maintenance at confluency without or with FBS [reviewed in ref. 11]. However, the current work expanded previously available protocols by studying different FBS concentrations, a prolonged exposure time, and several time points. The morphological changes confirmed induction of TSD, including increases in cell surface areas, multi-nucleation, multi-focal growth, and formation of ridges and, moreover, measurement of CFE confirmed loss of proliferative ability (cf. figs 1 and 2). Notably, confluency alone without FBS resulted in the most efficient means of inducing the squamous morphology, whereas FBS promoted retention of proliferative cells. In agreement, viable epithelial sheets suitable for grafting are commonly generated under confluency and FBS exposure [reviewed in ref. 11].

The transcript analysis agreed with the efficiency of the culture protocols in arresting proliferation and inducing

TSD (cf. table 2, figs 3, 4). All post-confluency samples showed significant decreases in both CDC2 and PCNA, and significant increases in both INV and SPR1. Expression of K5 remained high at 5 days without FBS but was otherwise significantly decreased at all conditions and time points. A growth-promoting effect of FBS under confluency was indicated by the relatively higher levels of CDC2 and PCNA transcripts noted in several samples. In contrast, the absence or presence of FBS had a profound effect on the TSD markers, i.e., transcripts of SPR1 were found at many-fold higher levels in the absence of FBS, whereas the transcript levels of INV was promoted by FBS, especially at the 2% level. The numerical data for TSD marker transcripts agrees with previous reports on the relative distribution of proliferation- and differentiation-specific proteins in oral epithelium [1, 2, 5–9]. However, the results also show that several culture protocols may be required for expression of the full array of genes/proteins that constitute a terminally differentiated tissue.

ADH3 transcripts may serve as a sensitive marker of keratinocyte proliferation as indicated from a higher degree of correlation with CDC2 and PCNA than the other transcripts, considering both direct or inverse relationships, and at various growth conditions, time points, and amplitudes (cf. figs 3, 4). The association of ADH3 with a basal phenotype, i.e., K5 transcripts, was less apparent, although as indicated for K5, ADH3 transcripts might also be retained for a limited period at confluency [20]. A general association of ADH3 with proliferation would agree with the high transcript levels previously noted in immortalized and malignant keratinocytes, since such lines usually exhibit a higher proliferative potential than normal keratinocytes [11]. The association with proliferation suggests that ADH3 might critically regulate physiological levels of formaldehyde, e.g., for DNA synthesis [16]. Interestingly, the cellular levels of glutathione, the cofactor used by ADH3 and which offers direct protection against formaldehyde, also increase in proliferative states [28, 29]. Since mutability is a hallmark of proliferative cells, the ability to regulate ADH3 transcription might also be essential for protection against formaldehyde exposure and, as recently implied, nitrosative stress, where ADH3 irreversibly metabolizes nitrosoglutathione to prevent nitrosolysation within the cell [30, 31]. Alternatively, once the ADH3 protein is abundant and stable over the expected cellular lifespan, the cell might simply conserve energy by avoiding unnecessary mRNA synthesis.

The current study utilized oral keratinocytes in various growth conditions to explore mechanisms underlying ADH3 transcription. Of significance for the full interpretation of the results, proliferative cultures, which are normally considered to mimic the basal layer, may also be regarded as a disrupted epithelium undergoing regeneration, while a high-density, 'confluent' culture can be regarded as normal unperturbed tissue, and serum exposure is likely to activate functions related to wound healing [11, 32]. Application of the StaRT-PCR technique was instrumental in this study, i.e., well above 10^3 PCR reactions analyzed by gel separation provided a coordinate expression analysis including standardized numerical data for six genes in cells under a total of ten variables (conditions and time points, table 2). Since reagents were not available to analyze K13 expression by StaRT-PCR, the preferential transcription of K13 in serum-exposed confluent oral keratinocytes [6] was confirmed in the current study by Northern blot analysis. The relative lack of exact correlation, direct or inverse, among various markers and the growth assessment in this study may reflect inter-individual-based variations, since quantitatively, but not qualitatively, the responsiveness to stimuli that induce TSD is known to vary considerably among donor cultures [11]. Furthermore, keratinocyte cultures cannot be cultured to fully represent proliferative versus non-proliferative or non-differentiated versus fully differentiated states [11]. For example, inherent to the method of analyzing single-cell cloning with the CFE assay, cell injury from proteolytic removal and dissociation of cells maintained under prolonged confluency may contribute to an overestimated loss of cloning potential (cf. fig. 2).

In conclusion, different culture protocols were used to enrich replicative oral keratinocyte cultures for non-proliferative and terminally differentiated cells in efforts to delineate influences on ADH3 transcription. The overall results demonstrated an association of ADH3 transcripts with transcription of two proliferation markers without consistent positive or negative association with markers of basal and suprabasal differentiation. Therefore, ADH3 transcription associates primarily with a state of cellular proliferation. The overall marker analysis also indicates that subpopulations of oral keratinocytes may be sensitive to growth inhibition and/or induction of TSD, as is the likely scenario in vivo, but that the utilization of one culture protocol is unlikely to allow expression of the fully differentiated phenotype. In contrast, as shown to advantage here, comparison of different growth protocols under confluency and serum may serve to dissociate gene transcription among proliferative/non-proliferative and differentiated/non-differentiated cellular states, and lead to the definition of additional markers useful for the analysis of keratinocyte biology.

1 Squirer C. A. and Kremer M. J. (2001) Biology of oral mucosa and esophagus. J. Natl. Cancer Inst. Monogr. **29:** 7–15
2 Presland R. B. and Jurevic R. J. (2002) Making sense of the epithelial barrier: what molecular biology and genetics tell us about the function of oral mucosal and epidermal tissues. J. Dent. Edu. **66:** 564–574
3 Dorée M. and Hunt T. (2002) From Cdc 2 to Cdk1: when did the cell cycle kinase join its cyclin partner. J. Cell Sci. **115:** 2461–2464

4 Malkas L. H. (1998) DNA replication machinery of the mammalian cell. J. Cell Biochem. Suppl. **30/31:** 18–29
5 Okada N., Miyagawa S., Steinberg M. L. and Yoshikawa K. (1990) Proliferating cell nuclear antigen/cyclin in cultured human keratinocytes. J. Dermatol. **17:** 521–525
6 Brysk M. M., Arany I., Brysk H., Chen S. H., Calhoun K. H. and Tyring S. K. (1995) Gene expression of markers associated with proliferation and differentiation in human keratinocytes cultured from epidermis and from buccal mucosa. Arch. Oral Biol. **40:** 855–862
7 Izumi K., Terashi H., Marcelo C. L. and Feinberg S. E. (2000) Development and characterization of a tissue-engineered human oral mucosa equivalent produced in a serum-free culture system. J. Dent. Res. **79:** 798–805
8 Presland R. B. and Dale B. A. (2000) Epithelial structural proteins of the skin and oral cavity: function in health and disease. Crit. Rev. Oral Biol. Med. **11:** 383–408
9 Morgan P. R. and Su L. (1994) Intermediate filaments in oral neoplasia 1 – oral cancer and epithelial dysplasia. Eur. J. Cancer B. Oral Oncol. **30B:** 159–165
10 Grafström R. C. (1990) Carcinogenesis studies in human epithelial tissues and cells in vitro: emphasis on serum-free culture conditions and transformation studies. Acta Physiol. Scand. **140 (suppl. 592):** 93–133
11 Grafström R. C. (2002) Human oral epithelium. In: Culture of Epithelial Cells, 2nd ed., pp. 195–255, Freshney R. I. and Freshney M. G. (eds), Wiley, London
12 Sundqvist K., Kulkarni P., Hybbinette S. S., Bertolero F., Liu Y. and Grafström R. C. (1991) Serum-free growth and karyotype analyses of cultured normal and tumorous (SqCC/Y1) human buccal epithelial cells. Cancer. Commun. **3:** 331–340
13 Sundqvist K., Liu Y., Arvidson K., Ormstad K., Nilsson L., Toftgård R. and Grafström R. C. (1991) Growth regulation of serum-free cultures of epithelial cells from normal human buccal mucosa. In Vitro Cell. Dev. Biol. **27A:** 562–568
14 Lippens S., Kockx M., Knaapen M., Mortier L., Polakowska R., Verheyen A. et al. (2000) Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing. Cell Death Differ. **7:** 1218–1224
15 Elmore E., Luc T. T., Steele V. E. and Redpath J. L. (2001) Comparative tissue-specific toxicities of 20 cancer preventive agents using cultured cells from 8 different normal human epithelia. In Vitro Mol. Toxicol. **14:** 191–207
16 IARC (1995) Monographs on the Evaluation of Carcinogenic Risks to Humans: Wood Dust and Formaldehyde, Lyon, International Agency for Research on Cancer
17 Nilsson J. A., Zheng X., Sundqvist K., Liu Y., Atzori L., Elfwing Å. et al. (1998) Toxicity of formaldehyde to human oral fibroblasts and epithelial cells: influences of culture conditions and role of thiol status. J. Dent. Res. **77:** 1896–1903
18 Suruda A., Schulte P., Boeniger M., Hayes R. B., Livingston G. K., Steenland K. et al. (1993) Cytogenetic effects of formaldehyde exposure in students of mortuary science. Cancer Epidemiol. Biomarkers Prev. **2:** 453–460
19 Hedberg J. J., Höög J.-O. and Grafström R. C. (2002) Assessments of formaldehyde metabolizing enzymes in human oral mucosa and cultured oral keratinocytes indicate high capacity for detoxification of formaldehyde. In: Crucial Issues in Inhalation Research – Mechanistic, Clinical and Epidemiologic, pp. 103–115, Heinrich U. and Mohr U. (eds), Fraunhofer IRB, Stuttgart
20 Hedberg J. J., Höög J.-O., Nilsson J. A., Zheng X., Elfwing Å. and Grafström R. C. (2000) Expression of alcohol dehydrogenase 3 in tissue and cultured cells from human oral mucosa. Am. J. Pathol. **157:** 1745–1755
21 Crawford E. L., Peters G. J., Noordhuis P., Rots M. G., Vondracek M., Grafström R. C. et al. (2001) Reproducible gene expression measurement among multiple laboratories obtained in a blinded study using standardized RT (StaRT)-PCR. Mol. Diagn. **6:** 217–225
22 Vondracek M., Weaver D. A., Sarang Z., Hedberg J. J., Willey J. C., Wärngård L. et al. (2002) Transcript profiling of enzymes involved in detoxification of xenobiotics and reactive oxygen in human normal and simian virus 40 T antigen-immortalized oral keratinocytes. Int. J. Cancer **99:** 776–782
23 Willey J. C., Erin E. L., Crawford M. S., Knight C., Warner K. A., Motten B. et al. (in press) Use of standardized mixtures of internal standards in quantitative RT-PCR to ensure quality control and develop a standardized gene expression database. In: A-Z of Quantitative RT-PCR, Bustin S. A. (ed.), IUL, La Jolla
24 Estonius M., Svensson S. and Höög J.-O. (1996) Alcohol dehydrogenase in human tissues: localisation of transcripts coding for five classes of the enzyme. FEBS Lett. **397:** 338–342
25 Freshney R. I. (2000) Culture of Animal Cells: a Manual of Basic Technique, 4th ed, Wiley-Liss, New York
26 Freshney R. I. and Freshney M. G. (2002) Culture of Epithelial Cells, 2nd ed., Wiley, London
27 Grafström R. C., Norén U. G., Zheng X., Elfwing Å. and Sundqvist K. (1997) Growth and transformation of human oral epithelium in vitro. In: Recent Results in Cancer Research, pp. 275–306, Müller-Hermelink H. K., Neumann H. G. and Dekant W. (eds), Springer, Berlin
28 Atzori L., Dypbukt J. M., Sundqvist K., Cotgreave I., Edman C. C., Moldéus P. et al. (1990) Growth-associated modifications of low-molecular-weight thiols and protein sulfhydryls in human bronchial fibroblasts. J. Cell. Physiol. **143:** 165–171
29 Atzori L., Dypbukt J. M., Hybbinette S. S., Moldéus P. and Grafström R. C. (1994) Modifications of cellular thiols during growth and squamous differentiation of cultured human bronchial epithelial cells. Exp. Cell Res. **211:** 115–120
30 Conaway C. C., Whysner J., Verna L. K. and Williams G. M. (1996) Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. Pharmacol. Ther. **71:** 29–55
31 Hedberg J. J., Griffiths J. W., Nilsson S. J. F. and Höög J.-O. (2003) Reduction of S-nitrosoglutathione by human alcohol dehydrogenase 3 is an irreversible reaction as analysed by electrospray mass spectrometry. Eur. J. Biochem. **270:** 1249–1256
32 Poumay Y. and Pittelkow M. R. (1995) Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. J. Invest. Dermatol. **104:** 271–276