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Research Article

Serum-responsive expression of carbonyl-metabolizing enzymes in normal and transformed human buccal keratinocytes

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Abstract. Gene expression of carbonyl-metabolizing enzymes (CMEs) was investigated in normal buccal keratinocytes (NBK) and the transformed buccal keratinocyte lines SVpgC2a and SqCC/Y1. Studies were performed at a serum concentration known to induce terminal squamous differentiation (TSD) in normal cells. Overall, 39 of 58 evaluated CMEs were found to be expressed at the transcript level. Together the transformed cell lines showed altered transcription of eight CME genes compared to NBK, substantiating earlier results. Serum increased transcript levels of *ALDH1A3*, *DHRS3*, *HPGD* and *AKR1A1*, and decreased those of *ALDH4A1* in NBK; of these, the transformed, TSD-deficient cell lines partly retained regulation of *ALDH1A3* and *DHRS3*. Activity measurements in crude cell lysates, including relevant enzymatic inhibitors, indicated significant capacity for CME-mediated xenobiotic metabolism among the cell lines, notably with an increase in serum-differentiated NBK. The results constitute the first evidence for differential CME gene expression and activity in non-differentiated and differentiated states of epithelial cells.

Keywords. Buccal mucosa, carbonyl-metabolizing enzymes, microarray, quinone metabolism, serum effects, terminal squamous differentiation, xenobiotic metabolism.

Introduction

Terminal squamous differentiation (TSD) represents the major pathway of programmed cell death in stratified epithelia like the buccal mucosa where keratinocytes migrate from a proliferative basal location to a terminally differentiated state in the outmost layer. This process is accompanied by gradually increased expression of genes involved in assembly of the insoluble cell envelope as well as gradually decreased expression of proliferation markers [1, 2].

Normal buccal keratinocytes (NBK) in serum-free culture exhibit a highly proliferative basal-like phenotype [3, 4]. To date, fetal bovine serum (FBS) is the most efficient agent known for driving TSD in cultured NBK [3, 5]. This certainly reflects the involvement of multiple factors in keratinocyte differentiation including various growth factors, vitamins and hormones [6, 7]. Hence, by adding FBS to the culture medium, proliferative (basal-like) as well as

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terminally differentiating (suprabasal-like) buccal keratinocytes can be selectively grown to mimic different layers of an oral mucosa *in vitro*. However, the complex composition of FBS complicates the distinction between TSD-associated and other serum effects. This intricacy can be addressed by the use of TSD-deficient (pre)neoplastic cell lines. SVpgC2a and SqCC/Y1, two independently derived continuous cell lines of buccal origin, are both TSD-deficient and furthermore unique in the aspect that they can be cultured under the same serum-free standardized conditions as NBK [8–10]. Hence, those two cell lines can tentatively serve as negative controls to distinguish between TSD-associated and other serum-related effects; characteristics of both cell lines have been extensively reviewed [11].

The aspect of regulation of xenobiotic metabolism during TSD has received little attention. Alterations in xenobiotic metabolism during buccal TSD are of particular interest in several aspects. First, TSD is commonly circumvented by neoplastic cells, which implies that genes that are differentially expressed during TSD might qualify for tumor markers or drug targets. Second, the buccal mucosa is more permeable than keratinized epithelia and thus an interesting drug delivery site [12, 13]. Third, the supra-basal epithelial cell layers and their defense mechanisms are likely to play an important role in the protection of stem cells in the basal layer from tobacco and food carcinogens.

Previous work assessed expression of cytochrome P450 and conjugation enzymes as well as enzymes involved in the detoxification of reactive oxygen species in NBK. These studies revealed very low expression of cytochrome P450s in NBK, often below the threshold of detection [14, 15]. In contrast, early generation microarray analysis showed expression of carbonyl-metabolizing enzymes (CMEs), including a number of phase I biotransformation enzymes, which among others contribute to the metabolism of oral cancer risk factors and cancer chemotherapeutics [16–20]. Notably, the relationship of epithelial cell differentiation and expression/activity of CMEs has not been addressed previously.

CMEs are involved in the metabolism of aldehydes, ketones and quinones, and can be divided into the following five protein families/superfamilies: zinc-containing alcohol dehydrogenases (ADH), aldehyde dehydrogenases (ALDH), short-chain dehydrogenases/reductases (SDR), aldo-keto reductases (AKR) and NAD(P)H:quinone acceptor oxidoreductases (NQO) [18, 21, 22]. There is some considerable overlap within the CMEs in terms of substrate specificity [17–21]. For instance, various CMEs, including ALDHs, SDRs and AKRs, appear to be involved in the metabolism of lipid peroxidation products as 4-hydroxy-2-nonenal (4-HNE) [18, 23, 24]. Moreover, ζ-crystallin (CRYZ), a member of the ADH family, as well as members of the SDR, AKR and NQO family, can reduce cytotoxic quinones. For instance, menadione is efficiently reduced by carbonyl reductase 1 (CBR1) and NQO1, and 9,10-phenanthrenequinone (9,10-PQ) is efficiently reduced by CBR1, CRYZ and AKRs [24–28]. Menadione and 9,10-PQ are among the best substrates for many of these xenobiotic CMEs and can thus be used to estimate CME-mediated xenobiotic activity under circumstances of limited material, *e.g.*, when using cultured normal cells with a finite life-span [19, 24–27, 29, 30]. Furthermore, the assessment of cofactor specificity and the use of relevant inhibitors enable some discrimination between different CME activities [26, 31]. For instance, NQO1 is unique in that it can use both NADH and NADPH with equal efficiency. Dicoumarol inhibits NQO1, carbonyl reductases (CBRs) of the SDR family and CRYZ, while rutin and related flavonoids inhibit CBRs as well as members of the AKR family [29, 30, 32, 33].

This study was undertaken to elucidate the expression of CMEs in normal, differentiated and transformed cultured oral keratinocytes by examining their differential transcription as well as xenobiotic activities following serum exposure. Transcript analysis was performed using microarray technique and associated enzymatic activities were assessed using menadione and 9,10-PQ in combination with different cofactors and inhibitors.

Materials and methods

Processing of cell cultures. NBK were obtained from healthy, non-smoking donors undergoing maxillofacial surgery (approved by the ethical committees of Karolinska Institutet and Linköping University Hospital) and cultured as previously described [5]. Primary keratinocyte cultures were derived from tissue digestion overnight at 4°C. The mixture was then resuspended in a serum-free medium with high levels of amino acids (EMHA) and plated on dishes pre-coated with fibronectin and collagen. Cultures were transferred onto regular tissue culture plastic at about 75% confluency, and cells in passage 2 were used in the experiments. The immortalized buccal epithelial cell line SVpgC2a and the buccal carcinoma cell line SqCC/Y1 were also cultured and transferred in EMHA. Passages 64–72 and 125–135 for SVpgC2a and SqCC/Y1, respectively, were used in the experiments. For exposure to serum, the cell culture medium EMHA was supplemented with 5% (v/v) FBS (batch 40G3982F, Sigma Aldrich or batch A01122–624, PAA

Laboratories, Pasching, Austria). NBK, SVpgC2a and SqCC/Y1 cells were grown to 100 % confluency followed by serum exposure for three days (i.e., until NBK expressed the typical squamous morphology associated with the onset of TSD). The term 100 % confluency was regarded as the state when the cultures were first grown to fully occupy the dish surface, which was determined under a phase-contrast microscope.

Microarray hybridizations. Total RNA was obtained from six independent cultures of NBK and duplicate cultures of the cell lines SVpgC2a and SqCC/Y1. Altogether, 16 hybridization experiments were carried out including 6 for NBK and 2 for each of the cell lines without serum and likewise for the cells incubated with serum (see Processing of cell cultures). Total RNA was prepared from 3×10^6 cells for each hybridization using the RNeasy Mini Protocol (Qiagen GmbH). The quality of the RNA was verified using Agilent 2100 Bioanalyzer (Agilent Technologies). cRNA was synthesized and hybridized to the Human Genome Focus chip (Affymetrix), which contains ~8400 genes, according to standard Affymetrix protocols (Bioinformatics and Expression Analysis Core Facility, Karolinska Institutet, Sweden).

Microarray data analysis. The data was analyzed using Gene Chip Operating Software (GCOS, http://www.affymetrix.com) as previously described [34, 35]. Approximately 50 % of the 8400 genes were transcribed in each of the three cell lines, similar to previously published data [34]. The data output includes the signal, *i.e.*, the combined and background-adjusted average fluorescence intensity from hybridizations with a number of unique probes for each gene, a measure of the abundance of the transcript. Relative expression values, reported as signal $\log_2$ ratios, were transformed to fold changes, if expression was significantly changed according to the change call (see Statistical Algorithms Description Document, Affymetrix, 2002). All 58 CMEs represented on the Focus Chip were included into the study. An average fold change was calculated for significantly altered transcripts in SVpgC2a and SqCC/Y1 relative to NBK, as well as for altered transcripts all in the serum-exposed cell lines relative to the corresponding unexposed line. A minimum fold change of 1.5, corresponding to a signal $\log_2$ ratio of 0.6, ($p<0.02$) in 11 of 12 pair-wise comparisons for the transformed cell lines relative to NBK, in all 6 comparisons for serum-exposed NBK relative to unexposed NBK, and in all 4 pair-wise comparisons for serum-exposed SVpgC2a/SqCC/Y1 relative to the unexposed lines, was set as a threshold for significant differential expression. The overall quality of the data was verified by correlation analysis and multidimensional scaling plots in R statistical environment using Bioconductor packages (http://www.bioconductor.org). For serum-exposed NBK relative to NBK, all differentially expressed genes were evaluated using unpaired, two-tailed *t*-test as well as statistically verified by Statistical Analysis of Microarray (SAM) [36]. Heat maps were generated in the TIGR Multiexperiment Viewer 4.1 program (http://www.tm4.org) [37].

Lysate preparations and assessment of enzymatic activity. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), scraped into a small volume of PBS and briefly spun down. After removal of the supernatant, the cells were shock-frozen in liquid nitrogen and finally stored at –80°C until use. Cells were resuspended in 10 mM Tris/HCl pH 8.0, 1 mM dithiothreitol and sonicated for a total of 1 min followed by centrifugation at 16 000 *g* for 1 h. For removal of low molecular weight compounds, the resulting lysates were concentrated approximately tenfold using a Nanosep 3K Omega centrifugal Device (Pall) and diluted to the original volume with 10 mM Tris/HCl pH 8.0, 1 mM DTT.

Quinone-reduction activity in crude lysates was measured at 37°C in a Hitachi U-3000 spectrophotometer by monitoring NAD(P)H oxidation at 340 nm in absence and presence of two relevant inhibitors. Reaction rates were calculated using the molar extinction coefficient for NAD(P)H of 6220 $M^{-1}\,cm^{-1}$. All assays were performed in 0.1 M potassium phosphate pH 7.5 with protein concentrations of 0.3–0.5 mg/ml. Substrate, cofactor and inhibitor concentrations in the assays were 80 μM menadione or 9,10-PQ, 200 μM NAD(P)H in presence and absence of 80 μM dicoumarol or rutin. Stock solutions were in water or 0.1 M potassium phosphate buffer, except for the quinones and rutin, which were dissolved in acetonitrile. For inhibition assays including rutin, control activity was assessed adding an equal volume of acetonitrile. Acetonitrile strongly inhibited menadione reduction activity (results not shown). Statistical analysis was carried out using unpaired and two-tailed student's *t*-test. Results are derived from duplicate or triplicate measurements of NBK, SVpgC2a, and SqCC/Y1 lysate preparations and presented as average ± standard deviation (SD). Similar results were obtained for two more independently prepared NBK lysates and one independently prepared lysate of each cell line.

Results

Transcription of CMEs in buccal keratinocytes. Transcription of 58 CMEs was investigated in buccal keratinocytes and of these, 39 were detected in at least one of the assessed cell lines and conditions, including representatives of each of the five major CME families. Most of the present CMEs display average signal values in the range 100–400, and can thus, in comparison to high-abundance transcripts as β-actin and glyceraldehyde-3-phosphate dehydrogenase with signal values between 4000 and 5000, be considered low- to medium-abundant transcripts in the cell types studied (Fig. 1). The majority of the studied transcripts for CMEs were not significantly changed in either the transformed cell lines or following serum exposure. Relative to NBK, transcription of eight genes was significantly altered in at least one of the transformed cell lines (Table 1). Serum exposure of NBK resulted in more than twofold altered expression for five CMEs; however, the transformed cell lines SVpgC2a and SqCC/Y1 completely failed to regulate three of these five in response to serum (Fig. 2, Table 1). Up-regulation of *ALDH1A3* was partially retained in SVpgC2a, while upregulation of *DHRS3* transcription was conserved in both transformed cell lines. *ALDH4A1* transcription, decreased in NBK in response to serum, was decreased in SVpgC2a and SqCC/Y1 relative to NBK irrespective of serum exposure.

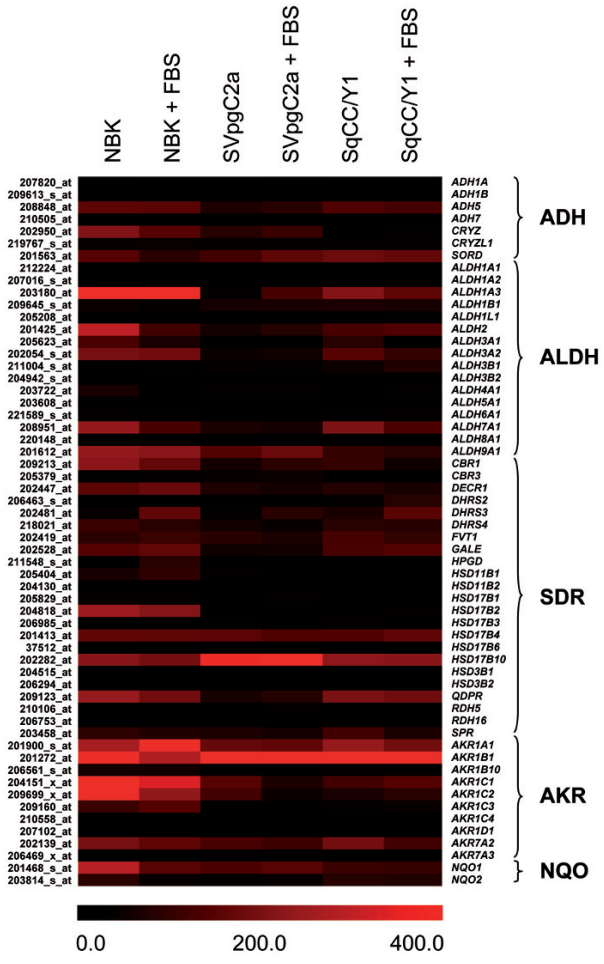

Figure 1. Heat map of gene expression pattern of zinc-containing alcohol dehydrogenases (ADHs), aldehyde dehydrogenases (ALDHs), short-chain dehydrogenases/reductases (SDRs), aldo-keto reductases (AKRs) and NAD(P)H:quinone acceptor oxidoreductases (NQOs) in normal (NBK) and transformed (SVpgC2a and SqCC/Y1) buccal keratinocytes in response to fetal bovine serum (FBS), as detected on Affymetrix Human Genome Focus chip. Affymetrix Probe IDs and approved gene names are given at the left and right, respectively. The color code used to express the average signal intensity, a measure of transcript abundance in solution, is shown below the gene panels. The range 0–400 was chosen to reflect typical carbonyl-metabolizing enzyme (CME) transcript abundance and is unrelated to the dynamic range of the assay. For comparison, high-abundance transcripts, *e.g.*, β-actin and glyceraldehyde-3-phosphate dehydrogenase, uniformly exhibited average signal values between 4000 and 5000. The data show the average signal from six hybridization experiments for NBK ± FBS and two hybridization experiments for SVpgC2a ± FBS and SqCC/Y1 ± FBS.

Transcription of known terminal differentiation and proliferation markers. Transcription of five genes coding for structural proteins well known to be induced during TSD in non-keratinizing epithelia was up-regulated in NBK in response to serum (Fig. 3), but not in the transformed cell lines SVpgC2a and SqCC/Y1. Transcription of the proliferation markers proliferating cell nuclear antigen (*PCNA*) and cell division control protein 2 homolog (*CDC2*) was not significantly changed in any of the cell lines (Fig. 3).

Quinone reduction in cell lysates. Under serum-free conditions, NADPH-dependent menadione reduction activity was significantly increased in SqCC/Y1 relative to NBK (Fig. 4A). NBK, but neither of the transformed cell lines, exhibited increased menadione reduction activity in response to serum (Fig. 4A). These effects were independent of whether NADPH or NADH was used as a cofactor (Fig. 4A, B). Dicoumarol strongly inhibited menadione reduction in NBK and SqCC/Y1, but had no effect on menadione reduction in SVpgC2a (Fig. 4A). In response to serum, the dicoumarol-inhibitable fraction of total activity was considerably increased in NBK, but remained unaltered in SqCC/Y1 (Fig. 4A).

NADPH-dependent 9,10-PQ reduction activity was increased in both transformed cell lines relative to NBK (Fig. 5A). In response to serum, NADPH-dependent 9,10-PQ reduction activity was further increased in NBK and SqCC/Y1, but not in SVpgC2a (Fig. 5A). In contrast, 9,10-PQ reduction activity with

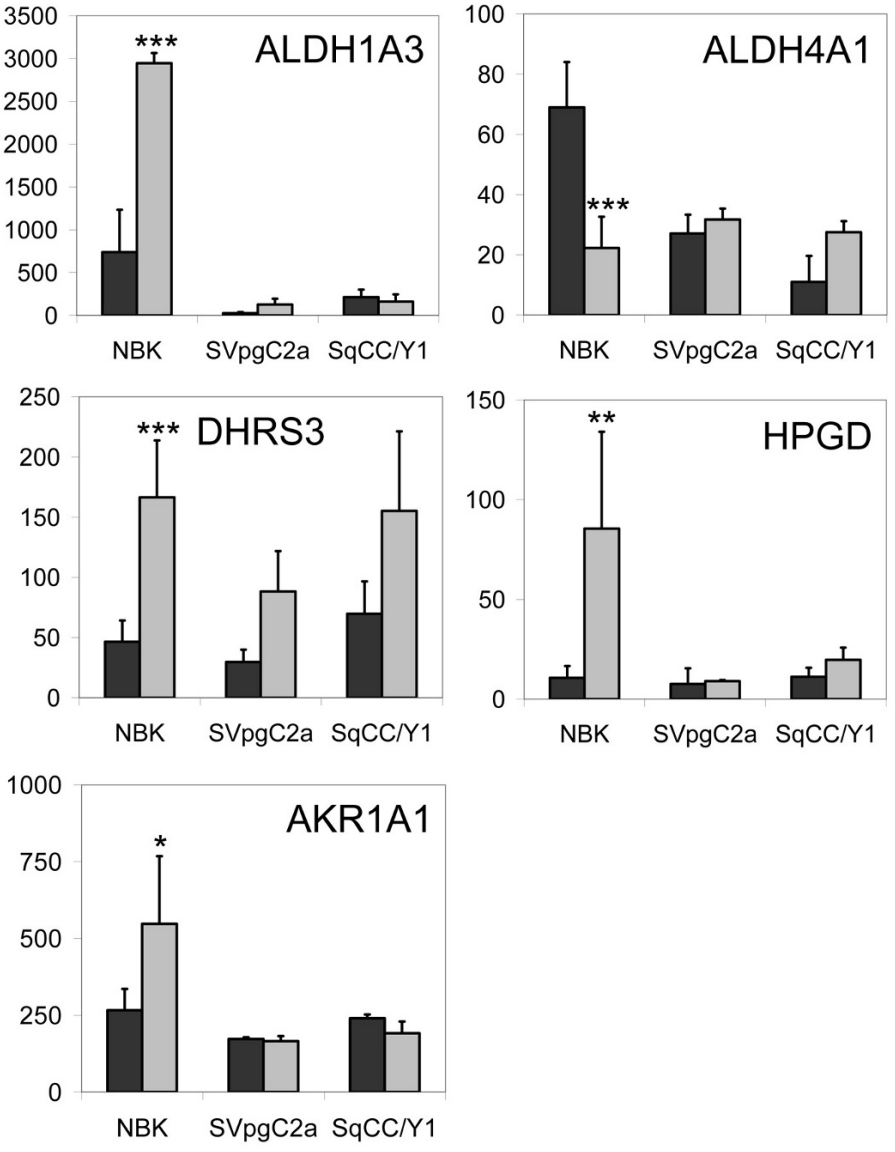

Figure 2. Gene expression of serum-responsive CMEs in unexposed (dark-gray bars) and serum-exposed (light-gray bars) NBK, SVpgC2a and SqCC/Y1. Average signal intensities (y-axis values) are presented with standard deviations (SDs) and are derived from six hybridization experiments for NBK ± FBS and two hybridization experiments for SVpgC2a ± FBS and SqCC/Y1 ± FBS. Statistical significance for NBK ± FBS was determined using unpaired, two-tailed *t*-test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Corresponding fold changes, enzyme names and Affymetrix Probe IDs are given in Table 1.

NADH as cofactor was unaltered in NBK and SqCC/Y1 in response to serum, but modestly increased in SVpgC2a (Fig. 5C). Furthermore, in all cell lysates NADH could be used more efficiently than NADPH, resulting in two- to threefold higher activities. Dicoumarol did not affect NADPH-dependent 9,10-PQ reduction activity in any of the cell lines except in serum-exposed SqCC/Y1 (Fig. 5A). In contrast, rutin inhibited NADPH-dependent 9,10-PQ reduction activity in all three cell lines (Fig. 5B). In response to serum, the rutin-inhibitable fraction of total activity was increased in NBK, remained unaltered in SVpgC2a, and was decreased in SqCC/Y1 (Fig. 5B).

Discussion

Standardized changes of culture conditions permit modeling of basal, non-differentiated, and differentiated epithelial states *in vitro*. This is significant for the characterization of drug metabolism in the buccal mucosa. The current study assessed gene expression of CMEs in normal and transformed human buccal keratinocytes following serum exposure. Serum-induced TSD in NBK, which is associated with increased transcription of *ALDH1A3*, *DHRS3*, *HPGD* and *AKR1A1* and decreased transcription of *ALDH4A1*. *DHRS3* and *ALDH1A3*, was in part similarly regulated in one or both of the transformed cell lines. Dicoumarol- and rutin-inhibitable NADPH-dependent quinone reduction activities were increased in crude cell lysates of NBK in response to serum.

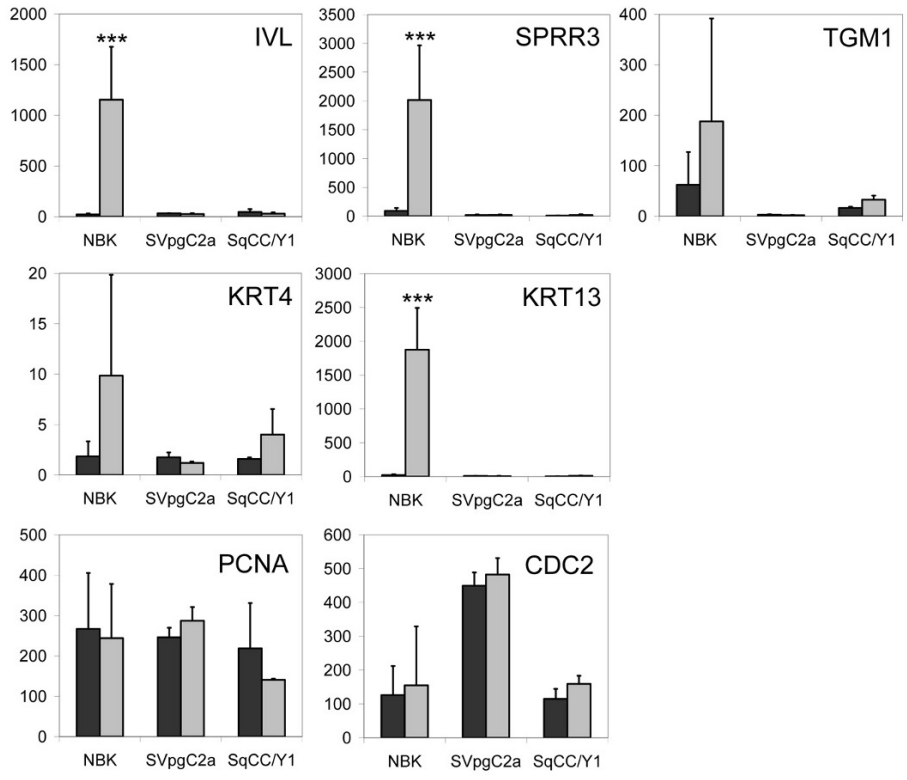

Figure 3. Gene expression of markers of terminal squamous differentiation (TSD) (*IVL*, *SPRR3, TGM1*), suprabasal keratinocyte markers (*KRT4*, *KRT13)*, and proliferation markers (*PCNA*, *CDC2*) in unexposed (dark-gray bars) and serum-exposed (light-gray bars) NBK, SVpgC2a and SqCC/Y1. Average signal intensities (y-axis values) are presented with SDs and are derived from six hybridization experiments for NBK ± FBS and two hybridization experiments for SVpgC2a ± FBS and SqCC/Y1 ± FBS. Statistical significance for NBK ± FBS was determined using unpaired, two-tailed *t*-test (*** $p<0.001$). Corresponding fold changes, protein names and Affymetrix Probe IDs are given in Table 1.

SVpgC2a and SqCC/Y1 demonstrated altered quinone reduction activity relative to NBK, which was considerably less affected by serum. The findings constitute the first evidence that serum-induced TSD in human epithelial cells associates with changes in CME expression and CME-mediated xenobiotic activity.

As proof of concept for the serum-exposure protocol, induction of TSD was judged by expression of well-established markers of TSD and proliferation in NBK and two TSD-deficient buccal cell lines, SVpgC2a and SqCC/Y1 [3, 9]. Increased transcript levels of established markers of buccal TSD in response to serum demonstrated that serum is a potent inducer of TSD in cultured NBK and thus suitable to selectively mimic the phenotypes of basal NBK and suprabasal/superficial NBK in culture (Fig. 3, Table 1). As expected, SVpgC2a and SqCC/Y1, which are less sensitive to the TSD-inducing components of serum, failed to similarly regulate these marker genes [8, 9]. Proliferation markers were largely unaffected, which is in line with a previous study that showed that FBS promotes retention of proliferative cells under confluent conditions [4]. The combined observed effects on markers of early TSD (IVL and SPRR3), late TSD (TGM1) and proliferation markers (PCNA, CDC2) suggests that the culture protocol applied enriched for a proliferative keratinocyte phenotype at the onset of TSD [6]. Regulation of CME genes associated with serum-induced TSD should typically be represented by transcriptional changes, which are observed in NBK upon serum exposure, but not in the TSD-deficient cell lines SVpgC2a and SqCC/Y1. Thus, the results suggest that altered transcription of at least three CME genes, *i.e. ALDH4A1*, *HPGD*, and *AKR1A1* is synchronized with TSD (Fig. 2, Table 1). *ALDH4A1* has been identified as a p53 target gene and codes for a mitochondrial enzyme that catalyzes the NAD^+-dependent second step in the degradation of proline to glutamate, a pathway necessary to interconnect the urea and tricarboxylic acid cycles [38, 39]. *ALDH4A1* transcription is lost not only in serum-differentiated NBK but also in both transformed cell lines, which could be a direct result of cellular p53 status in all three cases: p53 is inactivated in SVpgC2a and absent in SqCC/Y1, and p53 effects appear to be counteracted by p63 during keratinocyte differentiation [7, 9, 40, 41]. *HPGD* codes for 15-(NAD^+)-hydroxyprostaglandin dehydrogenase (HPGD), a COX-2 antagonist that has been suggested to play a role in xenobiotic metabolism, in addition to its function in the inactivation of prostaglandins [42, 43]. *HPGD* is transduced by TGF-β, a TSD-inducing serum component, and considered to fulfill a tumor suppressor function in many cancer types [44–47]. Finally, *AKR1A1* codes for a ubiquitous aldehyde reductase that displays activity for both biogenic and exogenous aldehydes as well as anticancer drugs and

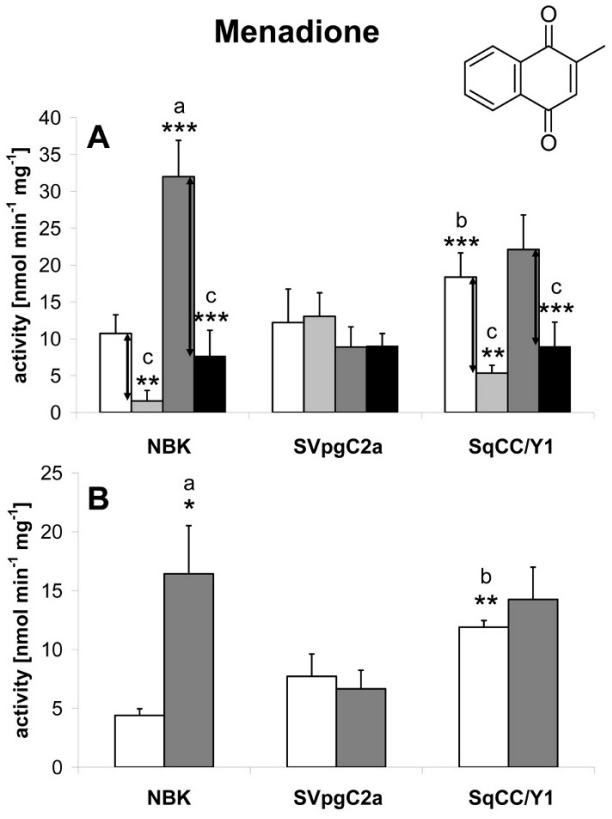

Figure 4. Menadione reduction activities in NBK, SVpgC2a, and SqCC/Y1 cell lysates before and after serum exposure. (*A*) NADPH-dependent menadione reduction activity and its inhibition by dicoumarol before serum exposure (white bars, no dicoumarol; light-gray bars, with dicoumarol) and after serum exposure (dark-gray bars, no dicoumarol, black bars, with dicoumarol). The dicoumarol-inhibitable fractions are indicated by arrows. (*B*) NADH-dependent menadione reduction activity before (white bars) and after serum exposure (dark-gray bars). a, significantly altered relative to unexposed control; b, significantly altered relative to NBK; c, significantly altered relative to control without inhibitor. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (unpaired two-tailed *t*-test).

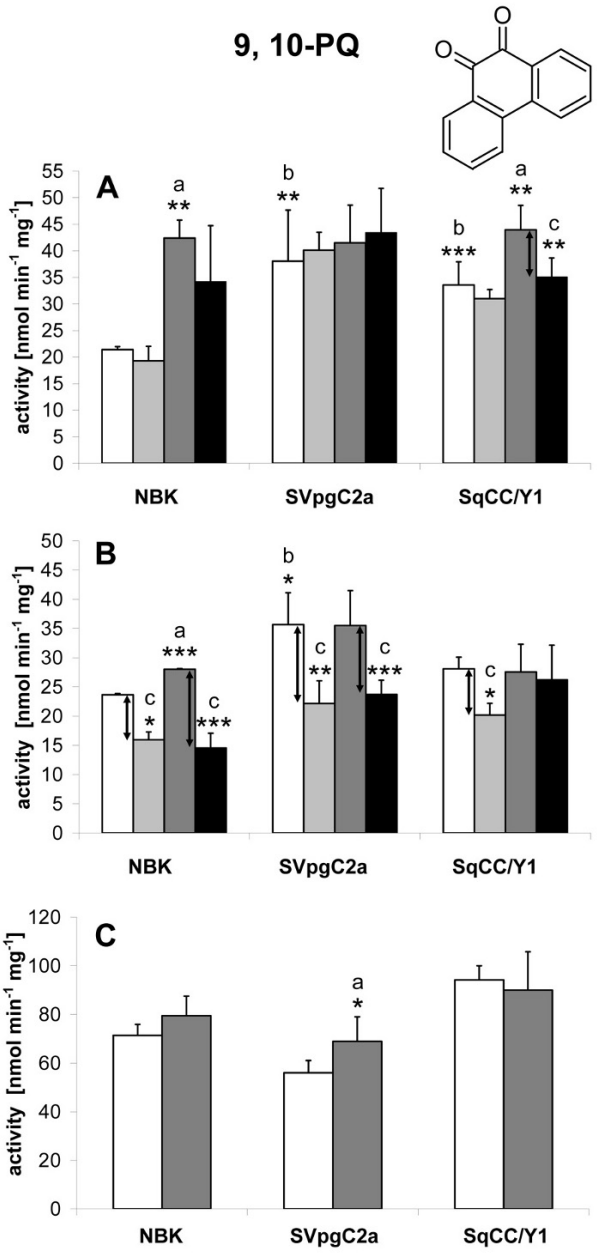

Figure 5. 9,10-Phenanthrenequinone (9,10-PQ) reduction activities in NBK, SVpgC2a, and SqCC/Y1 cell lysates before and after serum exposure. (*A*) NADPH-dependent 9,10-PQ reduction activity and its inhibition by dicoumarol before serum exposure (white bar, no dicoumarol; light-gray bars, with dicoumarol) and after serum exposure (dark-gray bars, no dicoumarol; black bars, with dicoumarol). (*B*) NADPH-dependent 9,10-PQ reduction activity and its inhibition by rutin before serum exposure (white bars, no rutin; light-gray bars, with rutin) and after serum exposure (dark-gray bars, no rutin; black bars, with rutin). As acetonitrile was used as solvent for rutin, an equal volume acetonitrile was added to controls. (*C*) NADH-dependent 9,10-PQ reduction activity before (white bars) and after serum exposure (dark-gray bars). The dicoumarol-inhibitable fractions and the rutin-inhibitable fractions are indicated by arrows. a, significantly altered relative to unexposed control; b, significantly altered relative to NBK; c, significantly altered relative to control without inhibitor. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$ (unpaired two-tailed *t*-test).

quinones [24]. *AKR1A1* gene expression is regulated by ZNF143, factors of the Sp1 family, and CHOP (C/EBP homologous protein) [48]. *In vivo* and *in vitro* evidence suggests that CHOP is up-regulated during early stages of TSD in keratinocytes [49]. Hence, all three changes in CME transcription that are unique to NBK upon serum exposure can tentatively be ascribed to signal transduction pathways with established or plausible function in keratinocyte differentiation.

Increased transcription of two CME genes observed in NBK in response to serum, *ALDH1A3* and *DHRS3*, was partly retained in SVpgC2a and SqCC/Y1. The fact that both cell lines have lost the ability to differentiate highlights the possibility that these genes are increased due to serum effects unrelated to TSD. Yet, both genes code for enzymes involved in the metabolism of retinoids, components of serum and

Table 1. Fold changes of altered transcripts for carbonyl-metabolizing enzymes (CMEs) (upper part) and known terminal squamous differentiation (TSD) and proliferation markers (lower part) in the transformed cell lines SVpgC2a and SqCC/Y1 relative to normal buccal keratinocytes (NBK) and in response to fetal bovine serum (FBS). n.c., not significantly changed.

Gene name	Probe ID	Protein	SVpgC2a vs NBK	SqCC/ Y1 vs NBK	NBK + FBS vs NBK	SVpgC2a + FBS vs SVpgC2a	SqCC/Y1 + FBS vs SqCC/Y1
CRYZ	202950_at	ζ-Crystallin	n.c.	−6.4	n.c.	n.c.	n.c.
ALDH1A3	203180_at	Aldehyde dehydrogenase 1A3	−14.0	n.c.	+ 5.9	+ 4.4	n.c.
ALDH2	201425_at	Aldehyde dehydrogenase 2	−4.8	n.c.	n.c.	n.c.	n.c.
ALDH3A1	205623_at	Aldehyde dehydrogenase 3A1	n.c.	n.c.	n.c.	n.c.	−7.5
ALDH3B1	211004_s_at	Aldehyde dehydrogenase 3B1	+ 2.8	+ 3.3	n.c.	n.c.	n.c.
ALDH4A1	203722_at	Aldehyde dehydrogenase 4A1	−2.4	−5.5	−3.6	n.c.	n.c.
DHRS3	202481_at	Short-chain dehydrogenase/reductase 3	n.c.	n.c.	+ 4.1	+ 2.8	+ 2.8
GALE	202528_at	UDP-galactose-4-epimerase	−2.2	n.c.	n.c.	n.c.	n.c.
HPGD	211548_s_at	15-Hydroxyprostaglandin dehydrogenase [NAD^+]	n.c.	n.c.	+ 10.8	n.c.	n.c.
HSD17B2	204818_at	Estradiol 17-β-dehydrogenase 2	−49	−10.7	n.c.	n.c.	n.c.
QDPR	209123_at	Dihydropteridine reductase	−3.4	n.c.	n.c.	n.c.	n.c.
AKR1A1	201900_s_at	Aldo-keto reductase 1A1 (aldehyde reductase)	n.c.	n.c.	+ 2.1	n.c.	n.c.
IVL	214599_at	Involucrin	n.c.	n.c.	+ 24	n.c.	n.c.
SPRR3	218990_s_at	Small proline-rich protein 3	n.c.	n.c.	+ 21	n.c.	n.c.
TGM1	206008_at	Transglutaminase 1	n.c.	n.c.	n.c.	n.c.	n.c.
KRT4	206969_at	Keratin 4	n.c.	n.c.	n.c.	n.c.	n.c.
KRT13	207935_s_at	Keratin 13	n.c.	n.c.	+ 103	n.c.	n.c.
PCNA	201202_at	Proliferating cell nuclear antigen	n.c.	n.c.	n.c.	n.c.	n.c.
CDC2	203213_at	Cell division control protein 2 homolog	+ 4.3	n.c.	n.c.	n.c.	n.c.

potent modulators of keratinocyte differentiation *in vitro* and *in vivo,* and *DHRS3* expression has been shown to be regulated in a retinoic acid-responsive manner [6, 50–52]. Hence it is possible that the transformed cell lines, despite overall TSD deficiency, have preserved some retinoid-related aspects of TSD. Activities relevant for some of the CMEs displaying differential transcription in response to serum were assessed, but were below detection threshold under our assay conditions (*e.g.* with 1 mM prostaglandin F2α and 3 mM NAD^+ for HPGD or 400 μM 4-HNE and 3 mM $NADP^+$ for AKR1A1, results not shown) [24, 53]. However, assessment of quinone-reduction activities in crude cell lysates revealed an increase in quinone reduction in lysates of serum-differentiated NBK relative to NBK (Figs 4A, 5A). For menadione reduction, the activity increase was characterized by dual NADPH/NADH specificity as well as high dicoumarol sensitivity, both properties of NQO1 (Fig. 4A, B) [27]. Notably, AKRs including AKR1A1 may also exhibit dual NADPH/NADH specificity, but they do not efficiently convert menadione and are less sensitive to inhibition by dicoumarol [54, 55]. Efficient NAD(P)H-dependent menadione-reducing enzymes include CBRs, HSD11B1 and xanthine dehydrogenase, but none of them is characterized by both dual NADH/NADPH specificity and dicoumarol sensitivity [56–58]. Although we cannot with certainty exclude that a mixture of proteins might be responsible for increased menadione reduction in NBK in response to FBS, our data are highly suggestive of NQO1 as the responsible enzyme.

For NADPH-dependent 9,10-PQ reduction, the activity increase came along with rutin sensitivity, indicating increased AKR and/or CBR activity in serum-differentiated NBK (Fig. 5B) [30, 33, 59, 60]. Considering the comparatively low activity of AKR1A1 towards 9,10-PQ, it is unlikely that AKR1A1 alone can account for the rutin-inhibitable fraction observed in NBK, although the observed increase in *AKR1A1* transcription might very well be responsible for the activity increase observed in lysate from serum-exposed NBK [24]. Addition of dicoumarol did not affect 9,10-PQ reduction activity in NBK, indicating that CRYZ does not contribute significantly to quinone metabolism in NBK (Fig. 5A) [29]. Hence, the results imply active roles

for NQO1, AKRs and possibly CBR, but not for CRYZ in buccal xenobiotic metabolism. Both the increases in NQO1-like as well as AKR/CBR-like activities observed in NBK upon serum exposure are not observed in the transformed cell lines, arguing for the TSD-association of these effects. Hence, when extrapolated to the *in vivo* situation, our findings are consistent with CME-mediated xenobiotic activities matching the impact of dietary/inhalatory genotoxic compounds in the buccal mucosa, which is certainly higher in the suprabasal/superficial layers. Notably, the presented results constitute the first evidence of TSD-associated changes in CME gene expression and CME-mediated xenobiotic activity, but further studies including immunodepletion or RNA interference techniques are necessary to establish more specifically which enzymes are responsible for the changes in activity. The results of this study indicate particularly *AKR1A1* and *NQO1* as target genes for such approaches.

As immortalized and tumor-derived cell lines can be cultured relatively effortlessly and reproducibly, there is a great interest in using them in toxicity testing, although their metabolic competence can differ considerably from normal cells [61]. Moreover, such cell lines are commonly established in conditions with serum, where a strong selection for cells resistant to serum-induced TSD can be expected [10]. As to transcription of the 58 CME genes investigated, SVpgC2a and SqCC/Y1 displayed few changes relative to NBK (Table 1), confirming all parts of an earlier generation microarray analysis within a previous study [16]. As to quinone reduction activities in the transformed cell lines, no dicoumarol-inhibitable menadione reduction, *i.e.* NQO1-like activity, was detected in SVpgC2a in comparison to NBK (Fig. 4A). At the same time, AKR/CBR-like activity, *i.e.* rutin-inhibitable 9,10-PQ reduction, was up-regulated; none of these effects was affected by serum (Figs 4A, 5B). Thus, the immortalized cell line SVpgC2a exhibited an altered quinone reductive potential relative to NBK, which was unaffected by serum exposure. In contrast, in SqCC/Y1 relative to NBK, NQO1-like activity was up-regulated but AKR/CBR-like activity unaltered (Figs 4A, 5B). Serum exposure of SqCC/Y1 did not change NQO1-like activity but apparently led to a switch from rutin-inhibitable to dicoumarol-inhibitable activity (Figs 4A; 5A, B). The latter result suggests the involvement of CRYZ. However, this appears unlikely, as according to the presented microarray data, *CRYZ* is not transcribed in SqCC/Y1. Taken together, future toxicity assessments using *in vitro* models should consider that transformed cell lines can have altered quinone reductive potentials, which may be further modulated by serum in the culture medium. Finally, NADH-dependent 9,10-PQ reduction was much more efficient and less variable in all cell lysates (Fig. 4C). A possible explanation for this observation could have been 15-(NAD^+)-hydroxyprostaglandin dehydrogenase (HPGD), for which NADH-dependent 9,10-PQ-reduction activity has been demonstrated [43]. However, this interpretation seems unlikely as no activity was detected with a physiological substrate of HPGD, prostaglandin F2α. AKRs are usually considered to prefer NADPH to NADH as cofactor [62]; however, they should not be ruled out, as cofactor preference has been demonstrated to vary with different substrates [55]. To our knowledge, no other enzyme has been described that efficiently reduces 9,10-PQ under concomitant conversion of NADH.

In summary, using established standardized serum-free culture conditions for various oral keratinocyte lines, the application of serum as inducer of TSD was investigated in an effort to mimic the phenotypes of basal and suprabasal/superficial oral mucosal epithelium. Transcription of five genes encoding CMEs was altered in response to serum, including some involved in xenobiotic metabolism. Of these, *ALDH4A1*, *HPGD* and *AKR1A1* were regulated in NBK, but not in the TSD-deficient lines SVpgC2a and SqCC/Y1, which is strongly suggestive of TSD-associated gene regulation. Taken together, the findings argue for the active involvement of CMEs, particularly of NQO1, AKRs and possibly CBRs in buccal xenobiotic metabolism, but do not support a significant role for CRYZ. The transformed cell lines exhibited altered quinone reductive potential that was variably modulated by serum, a fact that could be important for the application of continuous cell lines in toxicity testing.

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